Fak56 functions downstream of integrin alphaPS3betanu and suppresses MAPK activation in neuromuscular junction growth

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters.

Citation
Tsai, Pei-I, Hsiu-Hua Kao, Caroline Grabbe, Yu-Tao Lee, Aurnab Ghose, Tzu-Ting Lai, Kuan-Po Peng, et al. 2008. Fak56 functions downstream of integrin alphaPS3betanu and suppresses MAPK activation in neuromuscular junction growth. Neural Development 3:26.

Published Version
doi:10.1186/1749-8104-3-26

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:4875876

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Fak56 functions downstream of integrin alphaPS3beta and suppresses MAPK activation in neuromuscular junction growth

Pei-I Tsai1,2, Hsiu-Hua Kao3, Caroline Grabbe4, Yu-Tao Lee3, Aurnab Ghose5,7, Tzu-Ting Lai1, Kuan-Po Peng1, David Van Vactor5, Ruth H Palmer4, Ruey-Hwa Chen2,6, Shih-Rung Yeh3 and Cheng-Ting Chien*1,2

Address: 1Institute of Molecular Biology, Academia Sinica, Taipei 115, Taiwan, 2Institute of Molecular Medicine, National Taiwan University, Taipei 106, Taiwan, 3Institute of Molecular Medicine, National Tsing Hua University, Hsinchu 300, Taiwan, 4Umeå Center for Molecular Pathogenesis, Umeå University, Umeå, S-901 87, Sweden, 5Department of Cell Biology and Program in Neuroscience, Harvard Medical School, Boston, Massachusetts 02115, USA, 6Institute of Biological Chemistry, Academia Sinica, Taipei 115, Taiwan and 7Indian Institute of Science Education and Research, 900, NCL Innovation Park, Homi Bhabha Road, Pune 411008, India

Email: Pei-I Tsai - newchris@gate.sinica.edu.tw; Hsiu-Hua Kao - g9580501@oz.nthu.edu.tw; Caroline Grabbe - grabbe@biochem2.de; Yu-Tao Lee - lee.yutaot@gmail.com; Aurnab Ghose - aurnab@iiserpune.ac.in; Tzu-Ting Lai - bestmind@gate.sinica.edu.tw; Kuan-Po Peng - ckkboy@imb.sinica.edu.tw; David Van Vactor - davie_vanvactor@hms.harvard.edu; Ruth H Palmer - ruth.palmer@ucmp.umu.se; Ruey-Hwa Chen - rchen@gate.sinica.edu.tw; Shih-Rung Yeh - sryeh@life.nthu.edu.tw; Cheng-Ting Chien* - ctchien@gate.sinica.edu.tw

* Corresponding author

Abstract

Background: Focal adhesion kinase (FAK) functions in cell migration and signaling through activation of the mitogen-activated protein kinase (MAPK) signaling cascade. Neuronal function of FAK has been suggested to control axonal branching; however, the underlying mechanism in this process is not clear.

Results: We have generated mutants for the Drosophila FAK gene, Fak56. Null Fak56 mutants display overgrowth of larval neuromuscular junctions (NMJs). Localization of phospho-FAK and rescue experiments suggest that Fak56 is required in presynapses to restrict NMJ growth. Genetic analyses imply that FAK mediates the signaling pathway of the integrin αPS3βν heterodimer and functions redundantly with Src. At NMJs, Fak56 downregulates ERK activity, as shown by diphospho-ERK accumulation in Fak56 mutants, and suppression of Fak56 mutant NMJ phenotypes by reducing ERK activity.

Conclusion: We conclude that Fak56 is required to restrict NMJ growth during NMJ development. Fak56 mediates an extracellular signal through the integrin receptor. Unlike its conventional role in activating MAPK/ERK, Fak56 suppresses ERK activation in this process. These results suggest that Fak56 mediates a specific neuronal signaling pathway distinct from that in other cellular processes.
Background

Formation and stabilization of neuronal synapses demands communication between pre- and post-synaptic partners, as well as signals from the extracellular matrix (ECM). These signals can reorganize local cytoskeletal structures or be transduced into the nucleus to regulate transcription, thereby modulating neuronal plasticity [1-3]. One major receptor family for ECM signals comprises the transmembrane protein integrins, which have been shown to play critical roles in sequential steps of neuronal wiring, such as in neurite outgrowth, axon guidance, and synaptic formation and maturation [4-7]. In *Drosophila*, various integrin subunits have been shown to function in motor axon pathfinding and target recognition, and synaptic morphogenesis at neuromuscular junctions (NMJs) [8-10]. Mutant analyses for the integrin subunits αPS3 and βPS indicate that integrin signaling is involved in synaptic growth and arborization of larval NMJs [8-10]. Although specific ECM signals for these integrin receptors are not clear, dynamic NMJ growth is regulated by heparan sulfate proteoglycans [11]. Also, the N-glycosaminoglycan-binding protein MTG (encoded by *mind the gap*), a pre-synaptic secreted ECM molecule, has been shown to shape the synaptic cleft and modulate post-synaptic differentiation [12].

Integrin signaling activities in cell adhesion, spreading and migration can be mediated by the non-receptor tyrosine kinase focal adhesion kinase (FAK) [13-15]. In these processes, FAK becomes activated when phosphorylated at tyrosine 397 (Y397) and associates with Src to form a dual kinase complex [14,16]. Activated Src phosphorylates FAK thereby creating a signaling cascade through Ras and mitogen-activated protein kinase (MAPK)/ERK [17-19]. Activated ERK can modulate focal contact dynamics during cell migration, as well as promote cell proliferation and survival. In *Drosophila* larval NMJ growth regulation, ERK is specifically activated by Ras and its activation downregulates the protein levels of the cell adhesion molecule Fasciclin II (FasII) at NMJs [20].

The significance of FAK in development has been revealed by *fak* knockout mice that are embryonic lethal at embryonic day 8.5 during gastrulation, consistent with its role in cell adhesion and migration [21]. FAK proteins are highly enriched in developing nervous systems, in particular in axonal tracks and growth cones [22-25]. Neuronal-specific depletion of *fak* leads to cortical abnormalities, revealing the requirement of FAK in neural development [26]. At the cellular level, ablation of *fak* in Purkinje cells induces axonal branching and synapse formation, and this FAK activity is suggested to be partially mediated through p190RhoGEF, which modulates cytoskeletal structure [27]. Inactivation of the only *Drosophila* FAK gene, *Fak56*, however, permits normal development and transduction of integrin signaling pathways [28]. A requirement for Fak56 in glial cells of the optic stalk has recently been reported, suggesting for the first time a role for FAK family kinase activity in *Drosophila* [29].

We have generated *Fak56* mutants and identified a role for Fak56 in restricting NMJ growth. Analyses of genetic interactions suggest that Fak56 plays a conventional role in cooperation with Src to transduce integrin signaling. Fak56 is activated at NMJs, as shown by immunostaining for its phosphorylated form and this activation depends on the presence of the integrin βv subunit. ERK activation and FasII protein downregulation were observed at *Fak56* mutant NMJs. The NMJ overgrowth phenotype and FasII downregulation in *Fak56* mutants can be suppressed by reducing ERK activity. The physiological output of the enlarged NMJ in *Fak56* mutant displays increased synaptic response by nerve stimulation. These results suggest that Fak56 negatively regulates ERK activity and modulates synaptic plasticity at NMJs.

Results

**Larval NMJ overgrowth in *Drosophila* Fak56 null mutants**

The Fak56 protein is highly expressed in the ventral nerve cord during embryonic stages [22,25]. To examine whether *Fak56* has a role in NMJ formation, we dissected late third instar larvae from a transheterozygous *Fak56*^{N30/k24} mutant that deletes the *Fak56* gene and lacks *Fak56* mRNA expression (Additional file 1A, B, and Additional file 1 legend for the generation of *Fak56* mutants). This *Fak56* null mutant was immunostained with horseradish peroxidase (HRP) in order to label axonal processes [30], and phalloidin (Pha) to label muscle fibers. No abnormality of motor axonal tracts could be detected, and the pattern and size of muscles were normal, in agreement with earlier observations [28]. However, a more detailed examination revealed that *Fak56* null mutant NMJs were over elaborated in comparison to wild-type ones (Figure 1A, B). NMJs innervating muscles 6 and 7 (NMJ 6/7s) of abdominal segment 3 (A3) were analyzed by immunostaining for HRP and synaptotagmin (Syt) to label presynaptic boutons [31]. Altered branching patterns and ectopic synaptic boutons were observed, with increases in both Ib and Is boutons (arrows and arrowheads, respectively, in Figure 1C, D). Quantitatively, the number of synaptic boutons was increased by 44% and the total branch length increased by 22% when normalized to the total area of muscles 6 and 7 (quantified in Figure 1E). The *Fak56* activity was not limited to NMJ 6/7s since NMJ 4s also displayed overgrowth phenotypes in both total branch length (62% increase) and bouton number (101% increase) (Figure 1F–H). Furthermore, previous reported *Fak56*^{CG1} null mutants [28] also displayed a significant NMJ overgrowth phenotype when compared to wild-type control (Additional file 1C–E).
Figure 1 (see legend on next page)
When scored for NMJ 6/7s, the altered branching pattern in Fak56N30/K24 mutants showed secondary branch reduction by 21% but increases in higher-order branches (73% for tertiary branches and 424% for beyond tertiary; Figure 1I). The increase in higher-order branches was not caused by extension of multiple branches from single boutons, since a normal bifurcating pattern was observed.

To confirm that NMJ overgrowth phenotypes in the Fak56N30/K24 mutant are due to the absence of Fak56 activity, a UAS-Fak56 transgene [25] was introduced. We found that neuronal expression of Fak56 with elav-GAL4 in the Fak56N30/K24 mutant completely suppressed the NMJ phenotypes, as shown in assays for total branch length and bouton number of NMJ 6/7s. In contrast, Fak56 expression with the muscle-specific MHC-GAL4 failed to rescue Fak56 mutant phenotypes (Figure 1E). Taken together, these results suggest that Fak56 is specifically required in presynaptic neurons but not postsynaptic muscles to restrict NMJ growth. The exuberant NMJs in Fak56 null mutants were constructed normally, since molecular markers for various synaptic structures were expressed in a wild-type pattern (Additional file 2). Synaptic ultrastructure analyzed by transmission electron microscope revealed no significant alternations in pre- and post-synaptic structures (Additional file 3).

**Synaptic transmission is affected in the Fak56 null mutant**

To examine whether the enlarged NMJ in Fak56 null mutants is associated with functional changes in transmitter release, postsynaptic currents were recorded. In the null Fak56N30/K24 mutant, no alteration was observed in the amplitude of spontaneous release of neurotransmitter or miniature junctional potentials (mEJPs) at a low Ca2+ concentration (0.2 mM), as shown in the cumulative frequency plot (Figure 1I). Similar skews of distributions were measured for wild type and Fak56N30/K24 (1.5 ± 0.1 in wild type and 1.7 ± 0.2 in Fak56N30/K24, 0.25 < p < 0.5 by Kruskal-Wallis h test). The variance/mean of mEJP amplitudes were also similar (0.28 ± 0.04 in wild type and 0.25 ± 0.04 in Fak56N30/K24, 0.25 < p < 0.5 by Kruskal-Wallis H test). The frequency of mEJP was not changed significantly (1.2 ± 0.2 Hz in wild type and 1.8 ± 0.3 Hz in Fak56N30/K24, p = 0.16, Student’s t-test). Resting membrane potentials were similar in these measurements (-69.1 ± 1.9 mV in wild-type and -66.6 ± 1.5 mV in Fak56N30/K24, p = 0.32 by Student’s t-test). However, the mean amplitude of nerve-evoked EJPs was significantly enhanced at Fak56 mutant NMJs compared to wild type (p = 0.026 by Student’s t-test, Figure 1K; measurements were also performed at 1 mM [Ca2+]; Additional file 4). These data demonstrate a role of Fak56 in modulating the electrophysiological behavior of Drosophila NMJs.

**Involvement of integrin subunits αPS3 and βν in Fak56-regulated NMJ growth**

We then tested whether Fak56 mediates specific integrin activities at NMJs by genetic analysis. Integrin receptors are composed of heterodimeric α and β subunits [32]. In the Drosophila genome, there are five α subunits: αPS1 (encoded by multiple edematous wings, mew), αPS2 (inflated, if), αPS3 (Vol or scb), αPS4 and αPS5 (both αPS4 and αPS5 uncharacterized), and two β subunits (βPS (myospheroid, mys) and βν(βν)) [33-38]. We tested for possible genetic interactions between the available mutant alleles of integrin subunits and Fak56. In Fak56N30/KG hypomorphic animals, expression of Fak56 mRNA was reduced, but the NMJ appeared phenotypically normal (Additional file 1A, B; Figure 2A). However, when single mutant alleles of scb and βν were introduced into the Fak56N30/KG background, significant NMJ overgrowth was induced (Figure 2B, C). This overgrowth phenotype was not detected when mew, if, and mys were introduced (quantified in Figure 2G). As controls, larvae that were...
Figure 2
Genetic interactions between Fak56 and integrin signaling pathway components during neuromuscular junction (NMJ) growth. (A-F) Images of NMJ 6/7 are shown as described for Figure 1C, D. Hypomorphic Fak56N30/KG mutants showed a normal morphology (A), but one allele of scb-2 (B), βν(C) or LanA9–32 (E) in Fak56N30/KG induced dramatic NMJ growth. Overelaborated NMJs in transheterozygotes βν+/2 (D) and βν+/+;LanA9–32/+ (F) mutants are shown. (G) Quantification of NMJ 6/7 phenotypes for Fak56N30/KG, mew1/+, Fak56N30/KG, ifk27e/+, Fak56N30/KG, mys1/+, Fak56N30/KG, Fak56N30/KG, LanA9–32/+, Fak56N30/KG, LanA9–32216, Fak56N30/KG;LanA+/+, LanA9–32/216 and βν+/;LanA9–32/+ mutants. Asterisks indicate significant difference by Student’s t test (p < 0.05) and error bars represent the standard error of the mean (SEM).
heterozygous for the sbc2 or βv4 mutant alleles displayed normal NMJ bouton number and length (quantified in Figure 2G). These results suggest that compromised αPS3 or βv integrin signaling demands the full-strength of Fak56 activity to constrain NMJ growth. Since NMJ overgrowth has been observed for αPS3 but not βv mutants [9], we examined NMJ phenotypes in the viable βv3,12 mutant. Strikingly, significant increases in both branch length and bouton number were detected, similar to those observed in Fak56 null mutant larvae (Figure 2D). In summary, these genetic analyses suggest that αPS3 and βv are the main integrin subunits in regulating Fak56 activity during NMJ growth.

The laminins are ECM components composed of heterotrimers of α, β, and γ subunits, and are major signals for integrin receptors [39]. In Drosophila, LanA and wing blister (wb) encode two different α chains. We performed genetic interaction for both α chain mutants to test their involvement in Fak56 activity. Introducing one mutant allele of LanA9–32 but not wb41718 into the Fak56N30/KG hypomorphic background promoted a significant increase in the number of synaptic boutons (Figure 2E, G). The total NMJ length was also increased, although it was not significant (p = 0.37). While the hypomorphic LanA9–32/16 mutant displayed normal NMJ phenotypes, transheterozygous βv4/16; LanA9–32/16 mutant displayed strong overgrowth phenotypes, with 61% increase in the bouton number and 32% increase in the total length compared to wild-type NMJs (Figure 2F, G). These results are consistent with a role for the α subunit LanA as a component of laminins to signal integrins during NMJ growth.

**Participation of Src in Fak56-regulated NMJ growth**

Activated FAK forms a complex with Src, and the dual FAK-Src kinase complex induces downstream signaling [40]. To test whether Src is involved in Fak56-regulated NMJ growth, we performed genetic interactions between Fak56 and the Drosophila Src genes Src42A and Src64B. Reducing one gene dosage of either Src42A (Src42AE1) or Src64B (Src64BP) in the Fak56N30/KG background displayed significant NMJ overgrowth, as scored for total branch length and bouton number (Figure 3A, B, E). Controls of Src42AE1/+, Src64B+/+ and Src42AE1/+, Src64BP/+ in a wild-type background displayed no significant NMJ overgrowth (quantified in Figure 3E), suggesting that the efficiency of Src signaling at NMJs is dependent upon Fak56 activity in a dose-dependent manner. These results are consistent with a role for a FAK-Src complex in the restriction of NMJ growth.

We then tested whether severe Src mutants display NMJ growth defects. In the viable Src42AE1/+, Src64BP+/+ mutant that generates the least Src activity [41], the number of boutons was significantly increased and the total branch length was slightly enhanced (Figure 3C, E). To test whether Src has any contribution in the complete absence of Fak56 activity, we generated the combinatorial mutant Src42AE1/+Fak56N30/K24; Src64BP+/+ and found that reducing the gene dosage of Src further increased the number of boutons in the Fak56 null mutant by 21%. In comparison to wild-type animal controls, Src42AE1/+; Fak56N30/K24; Src64BP+/+ mutants displayed an 80% increase in the bouton number and 25% increase in total branch length (Figure 3D). In summary, these genetic analyses suggest that Fak56 and Src have overlapping and distinct contributions in inhibiting NMJ growth.

**Activation of Fak56 at NMJs**

In mammals, activation of FAK and the FAK homolog Pyk2 proceeds with an auto-phosphorylation step at the conserved Y397 of FAK and Y402 of Pyk2 [16,40,42], which corresponds to Y430 in Fak56 [22,25,43]. To examine the activation of Fak56 at NMJs, we immunostained larval NMJs with the anti-FAK [pY397] antibody, which detects Fak56 activation at muscle attachment sites [28]. As shown for NMJ 12/13, phospho-FAK (pFAK) was expressed strongly in Ib boutons (white arrows in Figure 4A1) and weakly in Is boutons (white arrowheads). Expression at NMJ 4 was also prominent (Figure 4B1). In co-staining for HRP, the pFAK signals could be found within boutons and inter-bouton tracks (Figure 4A1, B1), suggesting a presynaptic activation of Fak56. Strong pFAK expression was also detected within the incoming axons that were co-labeled by HRP (yellow arrowhead and inset image in Figure 4B1). Cytosolic punctate staining was also present in muscles. In Fak56N30/K24 null mutants, pFAK signals in axons, presynapses and muscles were completely absent (Figure 4C1, C2), confirming the specificity of the anti-pFAK antibody in detecting Fak56 activation signals.
Fak56 suppresses MAPK/ERK activation at NMJs

To further investigate the role of Fak56 at the presynapse, we generated an RNAi transgene to deplete Fak56 expression (see Materials and methods and Additional file 1F). Expression of the Fak56RNAi transgene in presynapses (elav>Fak56RNAi) resulted in an increase in both total branch length and bouton number of NMJs compared to the elav-LacZ control (Figure 5E, G). In contrast, Fak56 depletion in muscles using MHC-GAL4 retained normal NMJ phenotypes (not shown).

It has been shown that presynaptic ERK activation promotes larval NMJ growth [20]. We then tested whether Fak56 had an effect on ERK activation at NMJs, which can be monitored by immunostaining for diphospho-ERK (dpERK) [44]. The expression of dpERK was detected in punctate patterns in some but not all boutons (Figure 5A1, A2) [20].

We then examined whether dpERK expression at NMJs was altered by presynaptic depletion of Fak56 using RNA interference (RNAi). In elav>Fak56RNAi, dpERK expression was highly enriched in almost all boutons at the NMJ.
**Figure 4** (see legend on next page)
enlarged NMJ (Figure 5B, B1). To quantify the difference among wild-type and Fak56 mutants, the level of dpERK immuno-reactivity within the presynaptic region was normalized to that of co-stained HRP. We found that in elav>Fak56RNAi the ratio was increased by 3.3-fold when compared to that in elav>lacZ. Consistently, neuronal expression of the dominant-negative Fak56Y430F also resulted in strongly enhanced dpERK expression to 3.1-fold (Figure 5C). The enhancement in dpERK expression levels in both approaches to block Fak56 function suggests that Fak56 activation suppresses ERK signaling in presynaptic boutons.

To test whether NMJ overgrowth phenotypes in Fak56 mutants were caused by the increased ERK activity, one wild-type allele of the ERK gene rolled (rl) [45] was replaced with the null allele r\textsuperscript{EMS698} [46] in elav>Fak56RNAi larvae. The control heterozygous r\textsuperscript{EMS698/+} larvae displayed normal NMJ phenotypes. However, reduction of ERK gene dosage by 50% completely suppressed the NMJ overgrowth phenotypes observed in elav>Fak56RNAi (Figure 5D–F). The BMP/Gbb signaling pathway also promotes NMJ growth [47]. We then tested whether the BMP/Gbb pathway would have a similar regulation in Fak56 mutant NMJs. Three mutants in the BMP/Gbb signaling pathway components were tested for potential genetic interactions with Fak56 but failed to significantly modify NMJ phenotypes in elav>Fak56RNAi larvae (Additional file 5). Taken together, these results suggest that Fak56 specifically downregulates the growth-promoting ERK signaling during NMJ growth.

**Fak56 modulates IgCAM FasII levels at NMJs**

It has been shown that ERK signaling regulates NMJ growth through the modulation of the protein levels of cell-adhesion protein FasII [20]. At NMJs, FasII protein levels are inversely correlated with ERK activation. In elav>Fak56RNAi mutants, the NMJ FasII level was reduced (Figure 6B1). Using the elav>lacZ as the reference, a 33.5% reduction in the ratio of the FasII level to the HRP level was detected (Figure 6A1, D). Comparison of FasII expression between wild type and Fak56\textsuperscript{Y397K24} also revealed a 26.6% reduction in the Fak56 mutant (images not shown). Analyses of these two mutants suggest that Fak56 activity in presynapses is required for the full expression of FasII at NMJs. To examine whether Fak56-regulated FasII expression is mediated through ERK, the FasII protein level was examined in elav>Fak56RNAi\textsuperscript{rlEMS698/+}. We found that the FasII protein level at NMJs of elav>Fak56RNAi was significantly restored by introducing the rlEMS698 allele, with only 14.9% reduction compared to elav>lacZ (Figure 6C1, D). These results suggest that Fak56 regulation of FasII expression at NMJs is at least partially mediated by ERK.

**Discussion**

Growth of the stereotypical NMJs during larval stages is tightly regulated by signaling pathways that either promote or inhibit terminal branching, bouton addition and active zone formation [20,47-50]. In this study, we have identified an inhibitory role of the non-receptor tyrosine kinase FAK in the regulation of NMJ growth. The *Drosophila* FAK is required in presynaptic boutons for the growth process, where it functions in concert with the non-receptor tyrosine kinase Src. As evidenced by our genetic analysis, Fak56 plays a conventional role in promoting terminal branching, bouton addition and active zone formation. In support of this context-specificity of FAK activity, we have noted no gross changes in the dynamic patterns of ERK activation during Fak56 mutant embryogenesis (Additional file 6). Our data suggest that Fak56 activity inhibits ERK signaling in restricting synapse growth (Figure 7).

The importance of FAK in regulating axonal branching of motor neurons in *Drosophila* is revealed in this study and has been shown in Purkinje cells [27]. FAK activity in Purkinje cells has been attributed partially to the recruit-
Figure 5 (see legend on next page)
ment of p190RhoGEF during axonal branching and growth. In integrin-mediated cell adhesion, Rho activity is initially downregulated and followed by sustained activation, leading to actin reorganization [51]. In response to integrin signaling, the initial downregulation of Rho activity requires the activation of p190RhoGAP by tyrosine phosphorylation and association with SH2 domain-containing p120RasGAP, thus providing an alternative link between FAK and the Ras-MAPK pathway. Future studies on the characterization of the p190RhoGAP:p120RasGAP complex in NMJ development should illuminate how FAK regulates synaptic growth and plasticity.

ERK signaling regulates the protein levels of the cell adhesion molecule FasII at NMJs [20]. Homophilic interaction of FasII-like IgCAMs regulates axon pathfinding, target recognition, and synapse formation and remodeling [52-57]. At Drosophila NMJs, FasII is involved in synaptic formation and maintenance [52,53,56,57]. Different levels of FasII play different roles in NMJ formation. While the basal level is essential to form the synaptic structure, a higher-level of FasII protein restricts NMJ growth. We found that Fak56 regulates the high level of FasII at NMJs and this regulation could be accounted for by a suppression of ERK activity. Therefore, in NMJ growth regulation, the cell-matrix interaction mediated by integrin signaling cross-talks with FasII-dependent cell-cell adhesion between pre- and post-synaptic partners (Figure 7).

Previous analysis of the activity of the Drosophila integrin αPS3 in the viable Vol allele suggested that αPS3 regulates NMJ elaboration, synaptic transmission and plasticity [9]. Lack of αPS3 induces moderate NMJ overgrowth with increases in higher-order branches and boutons, similar to what were observed in Fak56 mutants. In our analysis, βν genetically interacts with the Fak56 mutant and the βν mutant NMJ displays an overgrowth phenotype as well, suggesting that βν may be the major β subunit forming integrin heterodimers with αPS3 to restrict NMJ growth. The integrin subunits αPS1, αPS2 and βPS are also expressed at NMJs, and alteration of βPS activity affects NMJ morphology [10]; it is thus foreseeable that multiple modes of integrin signaling pathways regulate NMJ growth.

Laminins are the major component of the ECM and are involved in NMJ synaptic formation and maintenance [58]. Functional laminins are heterotrimers composed of α, β and γ chains, and different chain combinations contribute to laminin diversity. Laminins 4, 9 and 11 are composed of the same β2 and γ1 chain but differ in the α chain (α2, α4 and α5, respectively) and have been shown to localize in synaptic clefts of the mammalian neuromuscular system [59]. In an in vitro culture system, laminin 11 with the α5 subunit serves as a stop signal in motor axon outgrowth [59]. In Drosophila, LanA is most homologous to mammalian α3 and α5 subunits. LanA genetically interacts with Fak56 and βν mutants and may serve as the conserved component of the stop signal to restrict NMJ elaboration.

Conclusion

FAK activation by integrins regulates various cellular processes, and in many cases can be accounted for by an activation of Ras through the recruitment of the GRB2-SOS complex [14]. In our study, Fak56 activity restricts NMJ synaptic elaboration by inhibiting the ERK signaling cascade. This noncanonical link between FAK activity and ERK signaling might be cell-context specific, such as in neurons, or even subcellular site-specific, such as at synapses. Vol (αPS3) functions in the process of learning and memory [35], and can act as the FAK upstream regulator with the same regulatory link proposed here (Figure 7). FAK has been suggested as a putative therapeutic target for its role in tumor cell invasion and metastasis [13,15,60-62]. The neuronal-specific nonconventional link between FAK and ERK proposed in this study may have implications in cancer biology and therapy.

Materials and methods

Fly stocks

Flies were reared at 25°C except where specifically indicated. Wild-type flies used in this study were the w1118 strain. Mutant alleles Fak56KG00304, mew1, if277e, scb2, mys1,
Src42AE, Src64BPH and rlEM698 were obtained from the Bloomington stock center. βν1, βν2 [34], LanA9-32, LanA216 [63] and wb71b8 [64] have been previously described. The various Fak56 alleles used in this study are described in detail in Additional file 1. The transgenic lines elav-GAL4 (X) (used in neuronal Fak56 knockdown and overexpression), elav-GAL4 (III) (used in neuronal Fak56 rescue), and UAS-LacZ were obtained from the Bloomington stock center. UAS-Fak56 [28] and MHC-GAL4 [65] have been described previously. The pUAST-Fak56RNAi construct was generated by subcloning two inverted Fak56 cDNA fragments (base pairs 629–1177) into the pUAST vector and the knockdown effect was examined (Additional file 1F). pUAST-Fak56Y430F flies were generated from pUAST-

Figure 6
Modulation of Fasciclin II (FasII) levels by Fak56. (A–C) Expression of FasII (green) at neuromuscular junction (NMJ) 6/7 in elav>LacZ (A1), elav>Fak56RNAi (B1), and elav>Fak56RNAi; rlEMS698/+ (C1). Co-stained horseradish peroxidase (HRP) is in magenta. (A2–C2) Only FasII staining is shown. Images in (A–C) come from a single section of the Z-stack confocal image. (D) Quantification of FasII levels relative to HRP immunoreactivity shown in (A1–C1). Note that elav>Fak56RNAi had a 33.5% reduction compared to elav>LacZ, which was restored significantly by removing one copy of rl in elav>Fak56RNAi; rlEMS698/+. Asterisks indicate significant difference by Student’s t test (p < 0.05) and error bars represent the standard error of the mean (SEM).

![Confocal images of FasII and HRP expression](image-url)
Model to depict Fak56 and ERK signaling and Fasciclin II (FasII) protein levels at neuromuscular junctions (NMJs).

In restricting NMJ growth, the extracellular matrix signal laminin including the α subunit LanA is received by integrin receptors, including αPS3 and βν subunits. This signal is transduced through the association between Fak56 and Src, and in this process phosphorylation of Y430 Fak56 is essential. Activated Fak56 mediates signaling through suppressing ERK activation at NMJs and consequently upregulates the FasII protein level at NMJs, leading to the inhibition of NMJ growth. Those molecules (shown in grey) were not tested in this study.

Figure 7
Model to depict Fak56 and ERK signaling and Fasciclin II (FasII) protein levels at neuromuscular junctions (NMJs). In restricting NMJ growth, the extracellular matrix signal laminin including the α subunit LanA is received by integrin receptors, including αPS3 and βν subunits. This signal is transduced through the association between Fak56 and Src, and in this process phosphorylation of Y430 Fak56 is essential. Activated Fak56 mediates signaling through suppressing ERK activation at NMJs and consequently upregulates the FasII protein level at NMJs, leading to the inhibition of NMJ growth. Those molecules (shown in grey) were not tested in this study.
Fak56 by PCR based site-directed mutagenesis. To enhance the Fak56RNAi transgene expression, embryos from the elav-GAL4 (X) and pUAST-Fak56RNAi cross were collected for 6 hours, kept at 25°C for 45 hours and shifted to 30°C until late third instar.

Immunostaining
In all experiments, wandering late third instar larvae were dissected for analysis of NMJ phenotypes. After dissection, tissues were incubated in fixative solution (4% formaldehyde in 1× phosphate-buffered saline) for 20 minutes. For immunostaining, primary antibodies used were against synaptotagmin (mouse, 1:25; DHSB, Iowa City, IA, USA), HRP conjugated with TRITC (rabbit, 1:100; Jackson ImmunoResearch, West Grove, PA, USA), FAK [pY397] (rabbit, 1:50; Biosource-Invitrogen, Carlsbad, CA, USA), FasII (1D4, 1:100; DHSB) and dp-ERK-1/2 (mouse, 1:20; Sigma-Aldrich, St. Louis, MO, USA). Alexa 488-, Cy3- and Cy5-conjugated secondary antibodies and TRITC-phalloidin were used (Jackson ImmunoResearch).

Image processing and presentation
Confocal images were acquired using a Zeiss LSM 510 Meta and processed using Adobe Photoshop CS. Images for quantification of NMJ branch length and bouton number were from a projection of 10 z-sections of 6.5–8 μm in total. To quantify the NMJ length and muscle area, the images were analyzed by a measurement tool in Zeiss LSM Image Examiner. For quantification of signal intensity at NMJs, images were acquired under the same scanning parameters. NMJs were outlined and the signal intensity was calculated by histogram analysis in Adobe Photoshop CS.

Electrophysiological recording
For sample preparation, dissected larval body walls (including the central nervous system and motor axons) were exposed in cold (4°C) HL3.1 Ca2+ free saline (70 mM NaCl, 5 mM KCl, 4 mM MgCl2, 10 mM NaHCO3, 5 mM trehalose, 115 mM sucrose, 5 mM HEPES pH 7.2) [66]. Experiments were performed on muscle 6 of segmental nerve was stimulated through the cut end with a suction electrode with 0.1 ms of pulse duration at 2 times the threshold voltage. Once the threshold voltage was reached, the size of EJPs remained unchanged despite the increase in stimulating voltage. Signals were digitized at 64 KHz by a PCI-6221 data-acquisition card (National Instrument, Austin, Texas, USA), and saved on an IBM compatible PC for analysis.

Abbreviations
dpERK: diphospho-ERK; ECM: extracellular matrix; FAK: focal adhesion kinase; FasII: Fasciclin II; HRP: horseradish peroxidase; MAPK: mitogen-activated protein kinase; mEJP: miniature junctional potential; NMJ: neuromuscular junction; pFAK: phospho-FAK; RNAi: RNA interference.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
PIT designed the study, wrote the manuscript, and performed and participated in all experiments. HHK, YTL and SRY participated in the electrophysiological experiments and analysis. AG and RHP designed, manufactured, and supplied the Fak56 point mutation and Fak56RNAi constructs, performed dpERK embryonic staining, and contributed to manuscript revisions. AG and DVV helped to characterize the NMJ phenotype of Fak56CG1. TTL and KPP helped to analyze the synaptic markers at NMJ of Fak56 mutants. RHC helped revise the manuscript and provided suggestions with regard to signal transduction and intellectual input for the study. CTC participated in the overall design and coordination of the study and helped to write the manuscript. All authors have read and approved the final manuscript.

Additional material

Additional file 1
Fak56 mutant alleles and expression. This file describes (A) the Fak56 locus and the generation of N30 and K24 deletions from KG00304 P-element insertion, (B) the expression of Fak56 mRNA in different Fak56 allele combinations, (C, D) HRP staining for wild-type and Fak56CG1 NMJs, (E) quantifications for NMJ phenotypes, and (F) the knock-down effect of Fak56RNAi.
Click here for file [http://www.biomedcentral.com/content/supplementary/1749-8104-3-26-S1.pdf]

Additional file 2
Expressions of NMJ proteins in Fak56null. This file describes identical expressions of Dlg (A, B), Brp (C, D), dPak (E, F), GluIA (G, H) and Futsch (I, J) at wild-type and Fak56CG1 NMJs.
Click here for file [http://www.biomedcentral.com/content/supplementary/1749-8104-3-26-S2.pdf]
Additional file 3
Ultrastructures of Fak56<sup>30/162K24</sup> synapses. Electron micrographs of cross-sections through a type-I bouton of muscle 6/7 in wild-type (A) and Fak56<sup>30/162K24</sup> (B) larvae. Quantitative analyses reveal no difference for synaptic ultrastructures (C).
Click here for file
[http://www.biomedcentral.com/content supplementary/1749-8104-3-26-S3.pdf]

Additional file 4
Electrophysiological recording of postsynaptic currents from wild-type and Fak56<sup>30/162K24</sup> in 1 mM Ca<sup>2+</sup>. (A) Cumulative frequency plot reveals a significant shift in the distribution of mEIP amplitudes. (B) Representative traces and mean amplitudes of EIPs in wild-type and Fak56<sup>30/162K24</sup>.
Click here for file
[http://www.biomedcentral.com/content supplementary/1749-8104-3-26-S4.pdf]

Additional file 5
BMP/Gbb signaling-independent mechanism of Fak56 in NMJ growth. No alternations of NMJ phenotypes were detected by introducing mutant alleles (pax6, wit<sup>1112</sup> and med<sup>11</sup>) for BMP signaling components into elav>Fak56RNAI.
Click here for file
[http://www.biomedcentral.com/content supplementary/1749-8104-3-26-S5.pdf]

Additional file 6
ERK phosphorylation in Fak56<sup>30/241</sup> mutant embryos. Expressions of phospho-ERK appear grossly normal during Drosophila embryogenesis.
Click here for file
[http://www.biomedcentral.com/content supplementary/1749-8104-3-26-S6.pdf]

Acknowledgements
We thank S-P Lee of IMB TEM core facility for technical supports, members of the CT Chien and RH Chen labs for discussion and comments on the manuscript, and NH Brown and N Harden for providing reagents. CTC is supported by a National Science Council Frontier Research Grant and an Academia Sinica Sn-Gn Research Grant of Taiwan.

References
1. Nishimune H, Sanes JR, Carlson SS: A synaptic laminin-calcium channel interaction organizes active zones in motor nerve terminals. Nature 2004, 432:580-587.
2. Dityatev A, Schachner M: The extracellular matrix and synapses. Cell Tissue Res 2006, 326:647-654.
3. Dityatev A, Schachner M: Extracellular matrix molecules and synaptic plasticity. Nat Rev Neurosci 2003, 4:456-468.
4. Huber AB, Kolodkin AL, Ginty DD, Cloutier JF: Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. Annu Rev Neurosci 2003, 26:509-563.
5. Clegg DO: Novel roles for integrins in the nervous system. Mol Cell Biol Rev Commun 2000, 3:1-7.
6. Nakamoto T, Kain KH, Ginsberg MH: Neurobiology: New connections between integrins and axon guidance.Curr Biol 2004, 14:R121-123.
7. Milner R, Campbell IL: The integrin family of cell adhesion molecules has multiple functions within the CNS. J Neurosci Res 2002, 69:286-291.
8. Hoang B, Chiba A: Genetic analysis on the role of integrin during axon guidance in Drosophila. J Neurosci 1998, 18:7847-7855.
9. Rohrbough J, Grotewiel MS, Davis RL, Broadie K: Integrin-mediated regulation of synaptic morphology, transmission, and plasticity. Neuron 2000, 28:669-687.
10. Beumer KJ, Rohrbough J, Prokop A, Broadie K: A role for PS integrins in morphological growth and synaptic function at the postembryonic neuromuscular junction of Drosophila. Development 1999, 126:5833-5846.
11. Johnson KG, Tenney AP, Ghose A, Duckworth AM, Higashi ME, Parfitt K, Maruo O, Heslip TR, Marsh JL, Schwarz TL, et al.: The HSPGs Syndecan and Dallylike bind the receptor phosphatase LAR and exert distinct effects on synaptic development. Neuron 2006, 49:517-531.
12. Rohrbough J, Rushton E, Woodruff E, Fergestad T, Vigneswaran K, Broadie K: Presynaptic establishment of the synaptic cleft extracellular matrix is required for post-synaptic differentiation. Genes Dev 2007, 21:2607-2628.
13. Cohen LA, Guan JL: Mechanisms of focal adhesion kinase regulation. Curr Cancer Drug Targets 2005, 5:656-68.
14. Mitra SK, Hanson DA, Sladek DD: Focal adhesion kinase: in command and control of cell motility. Nat Rev Mol Cell Biol 2005, 6:56-68.
15. Sladek DD, Mitra SK, Ilic D: Control of motile and invasive cell phenotypes by focal adhesion kinase. Biochim Biophys Acta 2004, 1692:77-102.
16. Avraham H, Park SY, Schinkmann K, Avraham S: RAFTK/Pyk2-mediated cellular signaling. Cell Signal 2000, 12:123-133.
17. Sladek DD, Hanks SK, Hunter T, Geer P van der: Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature 1994, 372:786-791.
18. Sladek DD, Hunter T: Focal adhesion kinase overexpression enhances ras-dependent integral signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. J Biol Chem 1997, 272:13189-13195.
19. Sladek DD, Jones KC, Hunter T: Multiple Grb2-mediated integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. Mol Cell Biol 1998, 18:2371-2385.
20. Koh YH, Ruiz-Canada C, Gorceyczka M, Budnik V: The Rasl/mitogen-activated protein kinase signal transduction pathway regulates synaptic plasticity through fasciclin II-mediated cell adhesion. J Neurosci 2002, 22:2496-2504.
21. Ilic D, Furuta Y, Karazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T: Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature 1995, 377:539-544.
22. Fox GL, Rebay I, Hynes RO: Expression of DFak56, a Drosophila homolog of vertebrate focal adhesion kinase, supports a role in cell migration in vivo. Proc Natl Acad Sci USA 1999, 96:14978-14983.
23. Menegon A, Burgaya F, Baudet P, Dunlap DD, Girault JA, Valtorta F: FAK+ and PYK2/CACKbeta, two related tyrosine kinases highly expressed in the central nervous system: similarities and differences in the expression pattern. Eur J Neurosci 1999, 11:3777-3788.
24. Contestabile A, Banonami D, Burgaya F, Girault JA, Valtorta F: Localization of focal adhesion kinase isoforms in cells of the central nervous system. Int J Dev Neurosci 2003, 21:83-93.
25. Palmer RH, Fessler LJ, Edeen PT, Edeen PT, Madigan SJ, McKeown M, Hunter T: DFak56 is a novel Drosophila melanogaster focal adhesion kinase. J Biol Chem 1999, 274:35621-35629.
26. Beegs HE, Schahin-Reed D, Zang K, Goebel R, Nave KA, Gorski J, Jones KR, Sretavan D, Reichardt LF: FAK deficiency in cells contributing to the basal lamina results in cortical abnormalities resembling congenital muscular dystrophies. Neuroscience 2003, 40501-5014.
27. Rico B, Beegs HE, Schahin-Reed D, Kimes N, Schmidt A, Reichardt LF: Control of axonal branching and synapse formation by focal adhesion kinase. Nat Neurosci 2004, 7:1059-1069.
28. Grabbe C, Zervas CG, Hunter T, Brown NH, Palmer RH: Focal adhesion kinase is not required for integrin function or viability in Drosophila. Development 2004, 131:5795-5805.
29. Murakami S, Umetsu D, Maeyama Y, Sato M, Yoshida S, Tabata T: Focal adhesion kinase controls morphogenesis of the Drosophila optic stalk. Development 2007, 134:1539-1548.

http://www.neuraldevelopment.com/content/3/11/26

Page 14 of 15 (page number not for citation purposes)
Gabay L, Seger R, Shilo BZ: Expression of synaptotagmin in Drosophila reveals transport and localization of synaptic vesicles to the synapse. Development 1993, 118:1077-1088.

Biggs WH 3rd, Brown NH: Morphogenesis in the absence of integrin: mutation of both Drosophila beta subunits prevents midgut migration. Development 2004, 131:5405-5415.

Grotewiel MS, Beck CD, Wu KH, Zhu XR, Davis RL: Integrin-mediated short-term memory in Drosophila. Nature 1998, 391:455-460.

Fessler JH, Fessler LI: Drosophila extracellular matrix. Annu Rev Cell Biol 1989, 5:309-339.

Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR, Parsons JT: Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. Mol Cell Biol 1994, 14:6800-1689.

Takahashi M, Takahashi F, Ui-Tei K, Kojima T, Saigo K: Increased and decreased MAP kinase required in the sevenless signal transduction pathway. J Cell Biol 2005, 170:391-402.

Fukumoto J, Sawamoto K, Okabe M, Takagi Y, Tezuka T, Yoshikawa S: Expression of synaptotagmin in Drosophila reveals transport and localization of synaptic vesicles to the synapse. J Neurosci 2008, 3:26 http://www.neuraldevelopment.com/content/3/1/26


does not for citation purposes

Publish with BioMed Central and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime." Sir Paul Nurse, Cancer Research UK

Your research papers can be:
- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp

(page number not for citation purposes)