Oncogene (2008) 27, 1439–1448

www.nature.com/onc

ORIGINAL ARTICLE

Distinct FAK-Src activation events promote α5β1 and α4β1 integrin-stimulated neuroblastoma cell motility

L Wu[1,5,6], JA Bernard-Trifilo[1,5,7], Y Lim[1], S-T Lim[1,6], SK Mitra[1], S Uryu[1,6], M Chen[2], CJ Pallen[2], N-KV Cheung[3], D Mikolon[4], A Mielgo[4], DG Stupack[4] and DD Schlaepfer[1,6]

[1]Department of Immunology, The Scripps Research Institute, La Jolla, CA, USA; [2]Department of Pediatrics and the Child and Family Research Institute, University of British Columbia, Vancouver, Canada; [3]Department of Pediatric Oncology, Memorial Sloan-Kettering Cancer Center, New York, NY, USA and [4]Department of Pathology, Moores UCSD Cancer Center, University of California, San Diego, CA, USA

Signals from fibronectin-binding integrins promote neural crest cell motility during development in part through protein-tyrosine kinase (PTK) activation. Neuroblastoma (NB) is a neural crest malignancy with high metastatic potential. We find that α4 and α5 integrins are present in late-stage NB tumors and cell lines derived thereof. To determine the signaling connections promoting either α4β1- or α5β1-initiated NB cell motility, pharmacological, dominant negative and short-hairpin RNA (shRNA) inhibitory approaches were undertaken. shRNA knockdown revealed that α5β1-stimulated NB motility is dependent upon focal adhesion kinase (FAK) PTK, Src PTK and p130Cas adapter protein expression. Cell reconstitution showed that FAK catalytic activity is required for α5β1-stimulated Src activation in part through direct FAK phosphorylation of Src at Tyr-418. Alternatively, α4β1-stimulated NB cell motility is dependent upon Src and p130Cas but FAK is not essential. Catalytically inactive receptor protein-tyrosine phosphatase-α overexpression inhibited α4β1-stimulated NB motility and Src activation consistent with α4-regulated Src activity occurring through Src Tyr-529 dephosphorylation. In α4 shRNA-expressing NB cells, α4β1-stimulated Src activation and NB cell motility were rescued by wild type but not cytoplasmic domain-truncated α4 re-expression. These studies, supported by results using reconstituted fibroblasts, reveal that α4β1-mediated Src activation is mechanistically distinct from FAK-mediated Src activation during α5β1-mediated NB migration and support the evaluation of inhibitors to α4, Src and FAK in the control of NB tumor progression.
Oncogene (2008) **27,** 1439–1448; doi:10.1038/sj.onc.1210770; published online 10 September 2007

Keywords: Src; FAK; α4 integrin; α5 integrin; motility; neuroblastoma

Correspondence: Dr DD Schlaepfer, Moores UCSD Cancer Center, Department of Reproductive Medicine, 0803 3855 Health Sciences Dr, La Jolla, CA 92093-0803, USA.
E-mail: dschlaepfer@ucsd.edu
[5]These authors contributed equally to this study.
[6]Current Address: Moores UCSD Cancer Center, Department of Reproductive Medicine, La Jolla, CA, USA.
[7]Current Address: Chemicon-Millipore, Temecula, CA, USA.
Received 6 March 2007; revised 20 July 2007; accepted 1 August 2007; published online 10 September 2007

Introduction

Neuroblastoma (NB) represents 8–10% of childhood cancers (Brodeur, 2003) and originates from precursor cells of the peripheral sympathetic nervous system. NB metastatic spread is a major obstacle to clinical treatment. Integrins mediate cell–extracellular matrix interactions that modulate cell adhesion, migration, survival and growth (Guo and Giancotti, 2004). Unligated integrins negatively effect NB survival and metastasis (Stupack *et al*., 2006) whereas integrin-stimulated signaling cascades controlling NB cell motility remain largely undefined.

Fibronectin (FN) signals can facilitate tumor development (Ruoslahti, 1999). α5β1 is a classical FN receptor with binding through FN repeats III-9 and III-10 (Pankov and Yamada, 2002). Upon cell binding and spreading on FN, focal adhesion kinase (FAK) is recruited to sites of α5β1 clustering through FAK C-terminal domain interactions with β1-integrin-binding proteins, such as talin and paxillin (Parsons, 2003). FN-stimulated FAK activation increases FAK Tyr-397 phosphorylation (pY397) and promotes the binding of Src-family protein-tyrosine kinases (PTKs) to FAK, potentially leading to conformational Src activation (Mitra and Schlaepfer, 2006). Maximal Src activation requires Tyr-418 phosphorylation within the kinase domain (Roskoski, 2005). FAK-Src activation leading to p130Cas or paxillin phosphorylation is associated with cell motility (Mitra *et al*., 2005), but the molecular controls regulating these events remain loosely defined.

Cellular FN (cFN) contains an alternately spliced region termed connecting segment 1 (CS-1) that binds α4β1 and α4β7 (Pankov and Yamada, 2002). α4β1 also binds to vascular cell adhesion molecular 1 (VCAM-1) expressed on activated endothelium during inflammation (Rose *et al*., 2002). Studies with chimeric α4 integrin subunits showed that the α4 cytoplasmic domain can confer enhanced migratory properties to cells (Chan *et al*., 1992) and that α4β1 may promote motility through different molecular mechanisms than α5β1 (Mostafavi-Pour *et al*., 2003). In mouse fibroblasts, we previously showed that human α4 expression can create a functional α4β1 pair and promote cell motility through

Src activation (Hsia *et al*., 2005). However, it remains unclear whether endogenous α4 motility-promoting signals occur through similar or distinct mechanisms (Huttenlocher, 2005).

Here, we show that α4 integrin is expressed in late-stage NB tumors and on a variety of human NB cells. We evaluate NB motility and PTK activation to cFN and to specific recombinant FN ligands for α4β1 or α5β1. We find that α4β1-stimulated NB cell motility requires Src but not FAK whereas FAK expression and activity are required to promote α5β1-stimulated NB motility. As recombinant FAK can phosphorylate Tyr-418 within the Src kinase domain, our studies also provide a novel mechanism of direct FAK-mediated Src activation.

Results

α4 and α5 integrins are expressed in late-stage tumors and promote NB cell motility

NB is derived from neural crest cell progenitors and the α4-subunit enhances neural crest motility and survival (Kil *et al*., 1998; Testaz and Duband, 2001). Staining of

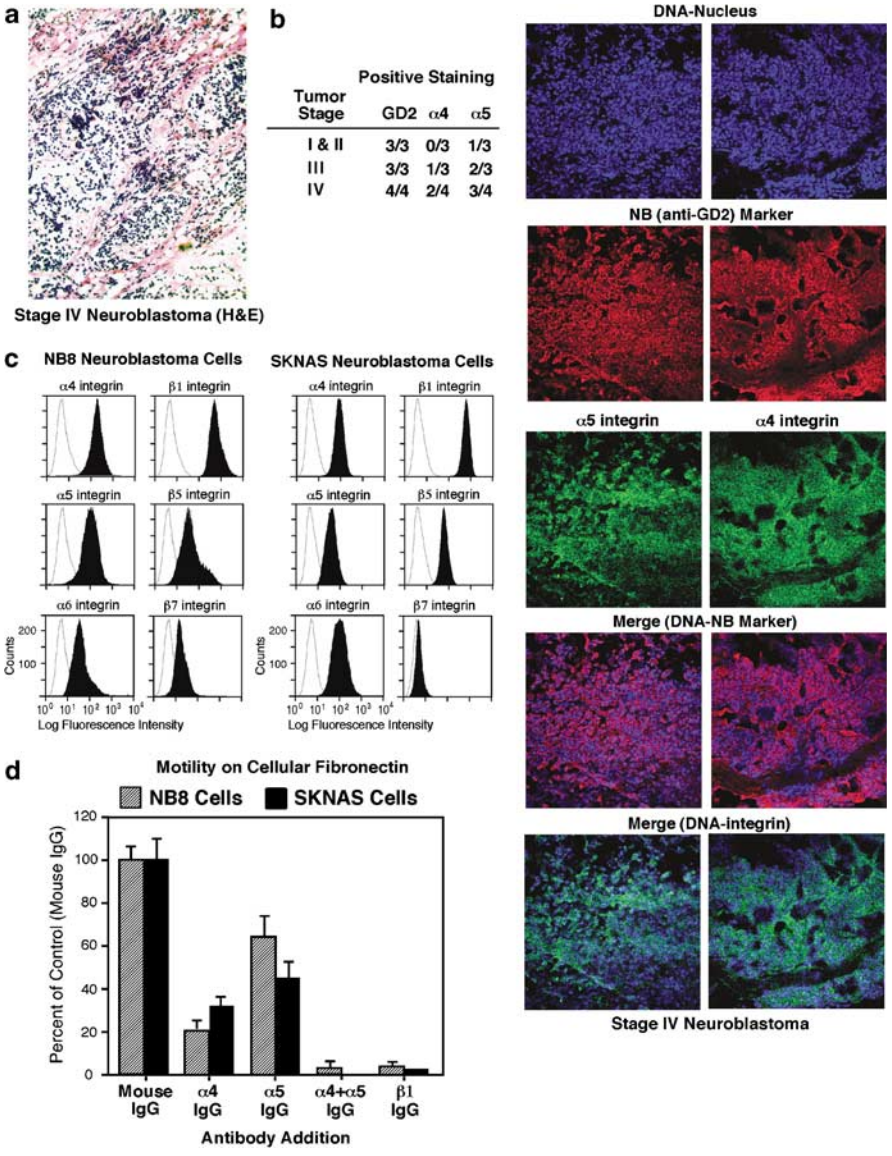


Figure 1 α4 and α5 integrins are expressed in late-stage neuroblastoma (NB) and function to promote NB cell motility *in vitro*. (**a**) Representative H&E staining of stage IV NB tumor sections reveals clusters of tumor cells throughout the stroma. Magnification × 200. (**b**) Chart summarizing GD2, α4 and α5 integrin staining results with stage I–IV tumor samples. Data are presented as positive staining per tumor. Representative co-staining of stage IV NB tumors with a DNA marker (blue), antibody to GD2 (red) and antibodies to either α5 or α4 (green) reveal the enrichment of integrin staining with the NB cells as shown by the merged images of DNA and NB marker (blue–red) and DNA-integrin (blue–green). Magnification × 200. (**c**) Flow cytometry analyses of endogenous α4, α5, α6, β1, β5, β7 expressions in NB8 and SKNAS cells (shaded peaks). Staining with control mAb (open peaks). (**d**)* NB haptotaxis motility on cFN in the presence of anti-α4 mAb (HP2/1, 10 μg ml^{-1}), anti-α5 mAb (P1D6, 10 μg ml^{-1}) and anti-β1 mAb (P4C10, 10 μg ml^{-1}). *Values are means ± s.d. of triplicates from two separate experiments.

Table 1 Neuroblastoma α4 integrin expression

Neuroblastoma cells	*Integrin α4 expression*
SKNJC2	+ + + +
NB8	+ + +
SKNLP	+ + +
SKNJD	+ + +
LA1-66N	+ + +
SKNJC1	+ + +
SKNAS	+ +
IMR6	+ +
SKNER	+ +
IMR32	+ +
NB1691	+ +
BE(2)N	+ +
BE(2)S	+ +
NB16	+ +
NB10	+ +
NB7	+ +
SKNMM	+
LAN-1	+
SH-SY5Y	+
NMB7	+
LA1-15N	+
LA1-5S	+/−
BE(2)C	+/−
BE(1)N	+/−
BE(2)M17	+/−
SKNCH	+/−
LA1-55N	+/−
LA1-6S	+/−
NB5	−
SKNHM	−
CB-JMN	−

Abbreviation: FACS, fluorescence activated cell sorting; MFI, mean fluorescence intensity. Integrin α4 expression on neuroblastoma cell lines was determined by FACS analysis using monoclonal antibody specific to the α4 subunit. Levels of α4 expression in different cell lines were defined according MFI values and designated as + + + +, extremely high; + + +, very high; + +, high; +, low; +/−, poor; −, not detected by FACS.

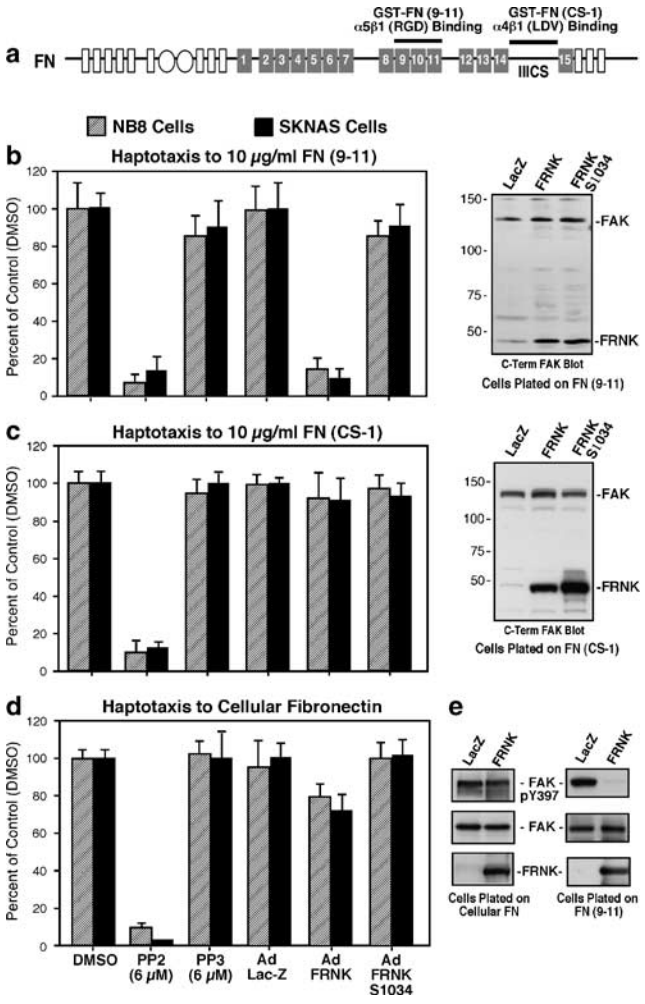


Figure 2 Importance of Src-family PTK but not focal adhesion kinase (FAK) activity for α4β1 NB cell motility. (**a**) Illustration of fibronectin (FN) (type III repeats number 1–15) and recombinant glutathione-*S*-transferase (GST)-FN fusion proteins. GST-FN-(9–11) encompasses repeats 9–11 and contains α5β1-binding motifs. GST-FN-(connecting segment-1 (CS-1)) encompasses the IIICS region and contains the α4β1-binding motif. (**b**–**d**)* Neuroblastoma (NB) cell haptotaxis motility on FN-(9–11) (**b**, left panel), FN-(CS-1) (**c**, left panel) and cFN (**d**, left panel). Transient FRNK expression levels compared to endogenous FAK determined by anti-FAK (5592, C terminus) blotting (**b**–**c**, right panel). (**e**) FAK IPs (5904, N terminus) were made from Ad-LacZ and Ad-FRNK expressing NB8 cells plated on cFN or FN-(9–11) and analysed by anti-pY397 phospho-specific and anti-FAK blotting. *See Figure 1.

NB tumors with anti-α4 or anti-α5 antibodies revealed their presence in late but not early-stage NB tumors whereas an NB marker (anti-disialoganglioside, GD2) was detected in all tumor samples (Figures 1a and b). Flow cytometry analyses were performed to determine whether α4 expression is maintained in cell lines derived from various tumors (Table 1). α4 surface expression was variable with high expression found in NB cells isolated from metastatic tumor sites and low or no α4 expression found in cells from local–regional or more differentiated tumors (Nai-Kong Cheung, unpublished results). NB8 cells with high α4, α5 and β1 expression, and SKNAS cells with high levels of β1, moderate levels of α4 and α5 expression were selected for further investigation (Figure 1c).

Haptotaxis motility assays of NB8 and SKNAS cells revealed that neutralizing antibodies to α4 or α5 inhibited cell migration 70–80 and 40–60% respectively, whereas a blocking antibody to β1 completely inhibited cFN-stimulated motility. Combined addition of anti-α4 and anti-α5 antibodies blocked cell migration (Figure 1d) supporting the notion that α4β1 and α5β1 are the primary mediators of NB8 and SKNAS motility on cFN. Glutathione-*S*-transferase (GST) fusion proteins encompassing FN-(9–11) or FN-(CS-1) (Figure 2a) can serve to selectively activate either α5β1 or α4β1, respectively (Hsia *et al*., 2005). NB8 and SKNAS motility on FN-(9–11) was blocked by addition of anti-α5 or anti-β1, but not anti-α4 antibodies (Supplementary Figures S1a–d), whereas cell motility on FN-(CS-1) was blocked by addition of anti-α4 or anti-β1, but not anti-α5 antibodies (Supplementary Figures S1a-c, e).

α5β1-stimulated NB motility on FN-(9–11) was reduced by an inhibitor to Src (PP2) and by expression of FRNK (Figure 2b), a dominant-negative inhibitor of FAK (Parsons, 2003). No effects were observed with

treatment of cells with the Src-inactive PP3 compound or by equal expression of FRNK S1034 (Hsia *et al*., 2005), which does not bind paxillin nor co-localize with integrins. Notably, FN-(CS-1) NB motility was inhibited by PP2 but not by FRNK expression (Figure 2c). cFN-stimulated NB cell motility was inhibited by PP2 and only slightly reduced by FRNK expression (Figure 2d). This lack of an effect of FRNK on cFN motility was associated with only minor inhibitory effects on FAK tyrosine phosphorylation (Figure 2e, left panel). In contrast, FRNK potently blocked FN-(9–11)-stimulated FAK tyrosine phosphorylation (Figure 2e, right panel), consistent with FRNK inhibition of FN-(9–11) motility. These results support the importance of Src but not necessarily FAK activity for α4β1-initiated NB cell motility and FAK-Src signaling for α5β1-mediated NB cell migration.

α4β1 promotes Src-dependent p130Cas tyrosine phosphorylation

To elucidate signaling changes associated with α4β1-mediated NB cell motility, NB8 cells were plated onto FN-(CS-1) and analysed by anti-phosphotyrosine (pY) blotting (Figure 3a). Strong phosphorylation of 120–130 kDa proteins was detected and this was blocked by PP2 addition but not by FRNK expression. Analysis of FAK showed no Tyr-397 phosphorylation differences between FRNK-expressing and control cells plated onto FN-(CS-1) (Figure 3a). PP2 treatment inhibited Src activation as measured by phospho-specific blotting to Y418 and PP2 treatment also potently inhibited p130Cas adapter protein tyrosine phosphorylation (Figure 3b). FRNK overexpression did not effect Src pY418 or p130Cas tyrosine phosphorylation upon NB8 plating on FN-(CS-1). These results are consistent with α4β1-stimulated Src activation promoting p130Cas phosphorylation and motility in a FAK-independent manner.

To evaluate the signaling roles of FAK, Src, p130Cas and paxillin in α4β1-stimulated NB cell migration, lentiviral short-hairpin RNA (shRNA) was used to stably knockdown these signaling proteins in NB8 cells (Figure 4a). Greater than 95% reduction in FAK and paxillin expression and ~80% reduction in p130Cas and Src expression was achieved in pooled populations of cells with no significant changes in cell shape (Figure 4b). Whereas no compensatory increase in Pyk2 was detected in FAK shRNA NB8 cells, elevated levels of Hic5 were found in paxillin shRNA NB8 cells (Figure 4c). No differences in either HEF1 or Fyn expression were detected in p130Cas or Src shRNA-expressing cells, respectively (data not shown). Notably, NB8 motility to FN-(CS-1) was inhibited by p130Cas and Src shRNA, while FAK or paxillin shRNA had no impact (Figure 4d). NB8 motility to FN-(9–11) was inhibited by p130Cas, Src and FAK shRNA expression (Figure 4e).

To verify the importance of p130Cas (Cas) in α4β1-stimulated cell migration, studies were performed with Cas−/− and reconstituted mouse embryonic fibroblasts (MEFs) expressing equivalent levels of α5 and β1 but no endogenous α4 (Supplementary Figures S2a and b). Adenoviral (Ad) human α4 infection resulted in high α4 surface expression (Supplementary Figure S2b) and facilitated MEF binding to FN-(CS-1) (Supplementary Figure S2c). At 60 min, Cas-reconstituted MEFs spread to a greater extent than Cas−/− MEFs (100±22%

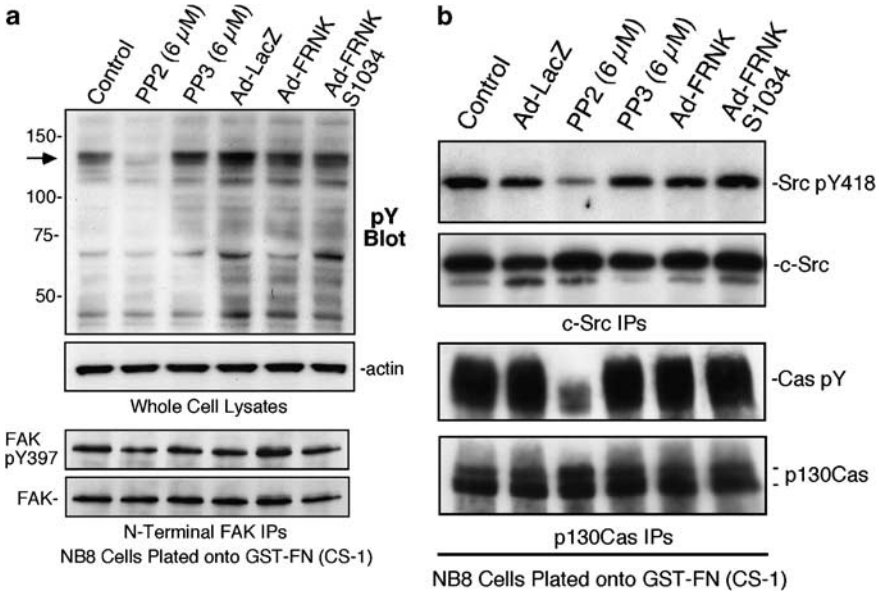


Figure 3 Src inhibition reduces α4β1-stimulated p130Cas tyrosine phosphorylation. (**a**) Anti-phosphotyrosine (pY) blotting of whole cell lysates (WCLs) from NB8 cells plated onto fibronectin (FN)-(connecting segment-1 (CS-1)) for 30 min. Actin blotting verified equal protein loading. Anti-N-terminal domain focal adhesion kinase (FAK) IPs from lysates of NB8 cells plated onto FN-(CS-1) analysed by anti-pY397 FAK and total FAK blotting showed no inhibition by FRNK expression. (**b**) Anti-phosphotyrosine (pY) blotting of Anti-Src immunoprecipitations (IPs) and anti-p130Cas IPs from lysates of NB8 cells plated onto FN-(CS-1). Equal levels of Src and p130Cas in the IPs were verified by anti-Src and anti-p130Cas blotting, respectively.

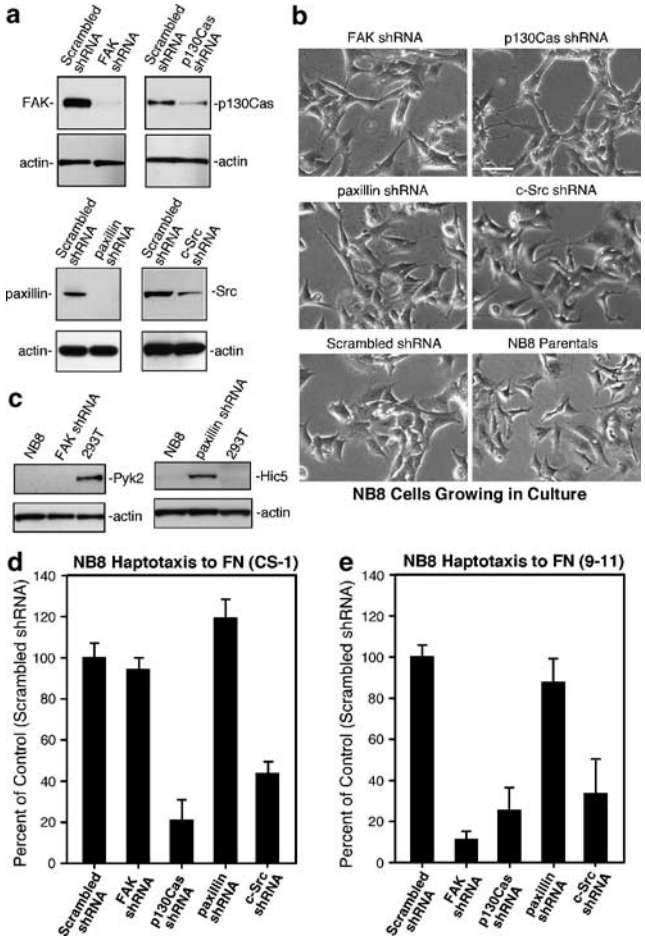


Figure 4 Src or p130Cas short-hairpin RNA (shRNA) knockdown inhibits α4β1 neuroblastoma (NB) cell motility whereas anti-focal adhesion kinase (FAK) shRNA selectively inhibits α5β1 NB cell migration. (**a**) Anti-FAK, p130Cas, paxillin and Src immunoblots of NB cell lysates stably expressing scrambled or the indicated shRNAs. Actin blotting verified equal loading. (**b**) Phase contrast images of NB8 parental or the indicated shRNA-expressing cells growing in culture. Scale bar is 20 μm. (**c**) Expression levels of Pyk2 (left panel) or Hic5 (right panel) in NB8 parental or FAK-paxillin shRNA expressing cells. 293T cell lysates were used for Pyk2 positive or Hic5 negative expression control. NB8 cell haptotaxis motility on FN-(CS-1) (**d**)* and FN-(9–11) (**e**). *See Figure 1.

versus 37±15%) and this was associated with 2.7-fold elevated motility on FN-(CS-1) (Supplementary Figure S2d). Together with the NB8 shRNA data, these results support the notion that Src and Cas expression are important for both α4β1 and α5β1 motility-promoting signaling whereas FAK is required for α5β1 but not α4β1-stimulated NB8 cell migration.

FAK promotes α5β1-stimulated Src activation and Src Y418 phosphorylation

To confirm that FAK is selectively required for α5β1- but not α4β1-stimulated NB8 cell migration, murine WT or kinase dead (KD) HA-tagged FAK were transiently expressed in NB8 FAK shRNA cells (Figure 5a). Surprisingly, both FAK constructs were phosphorylated at Y397 as detected by phospho-specific blotting and formed a complex with Src (Figure 5a). *In vitro* kinase (IVK) assays (Figure 5b) revealed that KD-FAK remained unphosphorylated at Y397 in the presence of ATP. However, addition of either purified recombinant FAK kinase domain (GST-FAK 411–686) or full-length His-tagged Src (Supplementary Figure S3) promoted KD-FAK phosphorylation at Y397 (Figure 5b). This result shows that KD-FAK can serve as a substrate for FAK and Src.

Expression of WT-FAK in NB8 FAK shRNA cells promoted >8-fold increased motility to FN-(9–11) compared with Lac-Z-expressing cells whereas KD-FAK-expressing cells remained non-motile (Figure 5c). Neither WT-FAK nor KD-FAK significantly affect NB8 motility on FN-(CS-1). When plated on FN (9–11), only low levels of Src Y418 phosphorylation were detected in NB8 FAK shRNA cells (Figure 5d). WT- but not KD-FAK promoted Src Y418 phosphorylation (Figure 5d) and Src IVK activation (data not shown) on FN-(9–11) (Figure 6d). Alternately, Src was equally activated upon FAK shRNA NB8 cells plating on FN-(CS-1) with WT- or KD-FAK re-expression (Figure 5d). These results show that FAK catalytic activity is required for α5β1-stimulated Src activation and motility whereas FAK is not required for α4β1 connections to Src and migration.

As both WT- and KD-FAK can form a complex with Src, but only WT-FAK functions to promote Src activation upon FN-(9–11) binding, the FAK catalytic domain was used to determine whether it could directly phosphorylate Src (Figure 5e). To avoid complications of intrinsic Src phosphorylation, kinase-inactive (K297M) Src was expressed in SYF fibroblasts. As analysed by anti-pY418 Src blotting after an IVK assay, the FAK kinase domain strongly promoted Src Y418 phosphorylation (Figure 5e). Thus, FAK-Src activation following α5β1-mediated cell binding may be initiated in part by FAK phosphorylation of and enhancement of Src catalytic activity.

Compensatory motility mechanisms for FAK shRNA cells on cFN

To evaluate the motility responses of shRNA-expressing NB8 cells on a natural ligand, random motility analyses and time-lapse imaging were performed with cells plated on cFN (Figure 6a). Cell tracking analyses confirmed that NB8 motility on cFN was inhibited by p130Cas and Src shRNA but not by FAK shRNA expression (Figure 6a). The lack of FAK shRNA effects on cFN motility contrasts with the partial inhibitory effects of α5 antibody addition for cFN NB8 motility (Figure 1d). Compared to parental NB8 cells (Figure 1a), FAK shRNA cells expressed lower levels of α5 but similar levels of α4 and β1 (Figure 6b). Notably, FAK shRNA cell motility was blocked by anti-α4 and anti-β1 but not by anti-α5 antibodies (Figure 6c), indicating that FAK shRNA-expressing NB8 cell motility on cFN was mediated by α4β1. Reduced FAK expression had no

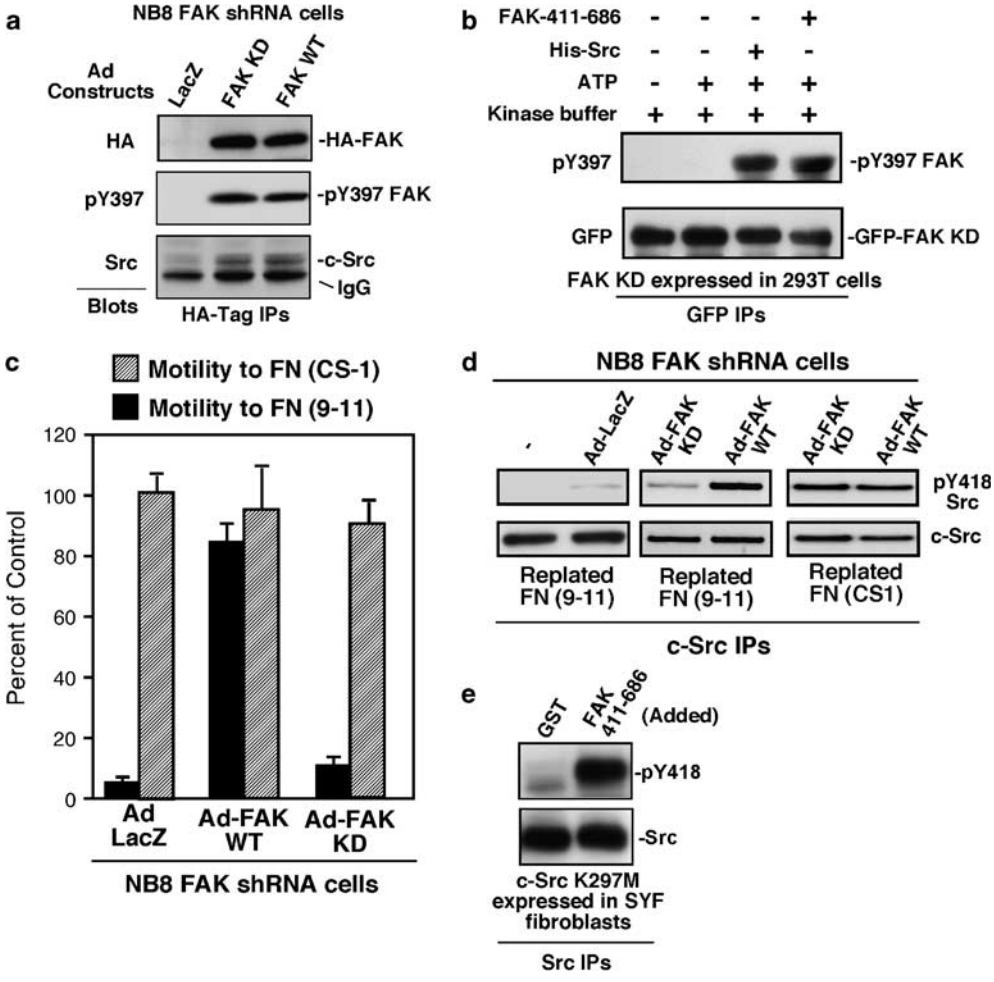


Figure 5 Intrinsic focal adhesion kinase (FAK) kinase activity is required for α5β1-stimulated NB8 cell motility and Src activation. (**a**) Anti-HA tag immunoprecipitations (IPs) from NB8 FAK short-hairpin RNA (shRNA) cells transiently expressing Ad-LacZ, Ad-WT-HA-FAK or Ad-KD-HA-FAK were analysed by anti-HA, pY397 FAK and anti-Src immunoblotting. Immunoglobulin G (IgG) indicates HA-tag IgG cross-reactivity. (**b**) From cells held in suspension to promote FAK dephosphorylation (1 h) green fluorescent protein (GFP)-kinase dead (KD)-FAK was isolated by anti-GFP IP, and incubated with or without ATP, His-Src or FAK-411–686 and analysed by anti-FAK pY397 and anti-GFP blotting. (**c**) NB8 FAK shRNA cell haptotaxis cell motility on fibronectin (FN)-(9–11) was rescued by wild type (WT)- but not KD-FAK re-expression. Cell motility on FN-(CS-1) was not affected by KD-FAK overexpression. Values presented are percent of FN-(CS-1)-stimulated cell motility and are means ± s.d. of triplicates. (**d**) NB8 FAK shRNA cells were mock treated or infected with Ad-LacZ, Ad-WT-FAK or Ad-KD-FAK, serum starved, held in suspension for 45 min and then replated onto either FN-(9–11) or FN-(CS-1) coated dishes for 30 min. Protein lysates were prepared and Src IPs were sequentially evaluated by anti-pY418 Src and total Src blotting. (**e**) Src is directly phosphorylated at Y418 by FAK. KD-(K297M)-Src was transiently expressed in SYF fibroblasts, isolated by IP and incubated in the presence of purified glutathione-*S*-transferase (GST) or GST-FAK 411–686 with ATP, washed and analysed by Src pY418 and total Src blotting.

effect on cFN-stimulated Cas tyrosine phosphorylation (Figure 6d) whereas in Src shRNA NB8 cells, Cas phosphorylation upon cFN binding was dramatically reduced (Figure 6e). Thus, α5 expression may be regulated by FAK and NB8 FAK shRNA motility on cFN is associated with α4β1-stimulated and Src-associated Cas tyrosine phosphorylation.

The α4 cytoplasmic domain promotes α4β1-stimulated NB8 motility and Src activation

To identify whether the α4 cytoplasmic domain is required for cell motility and PTK activation, NB8 cells were sorted to create a α4-null cell population and in parallel, α4 expression was reduced by lentiviral shRNA (Supplementary Figure S4a). Loss of α4 expression in NB8 cells impaired motility to cFN, prevented motility to FN-(CS-1), but did not affect motility on FN-(9–11) (Supplementary Figure S4b). Decreased cFN-stimulated motility was not associated with altered binding and NB8 cells lacking α4 did not bind to FN-(CS-1) (Supplementary Figure S4c).

To determine whether the motility-defective phenotype of α4-null and α4 shRNA cells on FN-(CS-1) was specifically due to α4 loss, wild-type human α4 (Ad-α4 WT) was transiently re-expressed (Figure 7a). Re-expression of α4 WT rescued cell motility on FN-(CS-1) (Figure 7b), whereas equivalent expression of cytoplasmic domain-truncated α4 (Ad-α4 ΔCyto) promoted

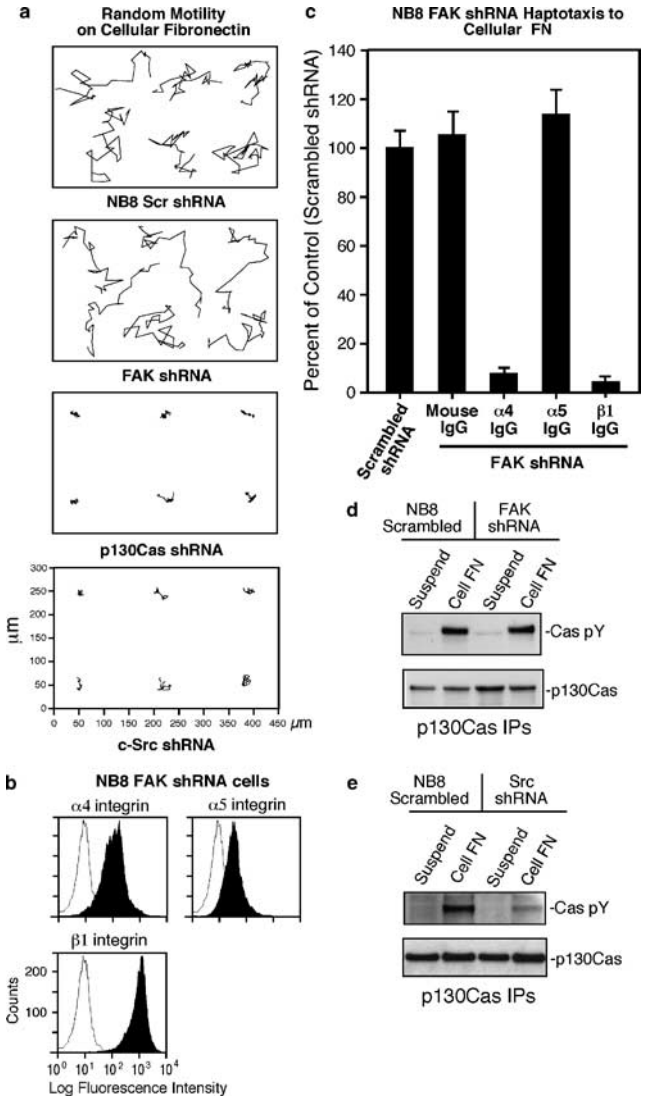


Figure 6 NB8 focal adhesion kinase (FAK) short-hairpin RNA (shRNA) cell motility on cellular fibronectin (cFN) is mediated by α4 and β1 but not by α5. (**a**) Random motility tracks of NB8 cells expressing scrambled, FAK, p130Cas, or Src shRNA. Time-lapse images were collected every 4 min for 14 h and the scale is in μm. (**b**) Flow cytometry analyses of α4, α5 and β1 integrin expression (shaded peaks) in NB8 FAK shRNA cells. Open peaks show staining with control mAb. (**c**)* Haptotaxis motility of NB8 FAK shRNA expressing cells on cFN is in the presence of anti-α4 mAb (HP2/1, 10 μg ml^{-1}), anti-β1 mAb (P4C10, 10 μg ml^{-1}) and anti-α5 mAb (P1D6, 10 μg ml^{-1}). (**d** and **e**) Anti-p130Cas IPs from NB8 scrambled or FAK shRNA cells (**d**) and Src shRNA cells (**e**) held in suspension (30 min) or plated on cFN (45 min) were analysed by anti-phosphotyrosine (pY) followed by anti-p130Cas blotting. *See Figure 1.

adhesion to FN-(CS-1) (data not shown) but did not promote cell motility to FN-(CS-1) (Figure 7b). When NB8 cells were plated onto FN-(CS-1), re-expression of α4 WT promoted Src Y418 phosphorylation equal to control NB8 cells whereas α4 ΔCyto only weakly activated Src (Figure 7c). Thus, the α4 cytoplasmic domain is required to activate Src and plays a critical role in promoting α4β1-mediated NB motility.

PTPα facilitates α4β1-stimulated Src activation, cell spreading and motility

As Src activation is accompanied by dephosphorylation of the C-terminal regulatory Tyr-529 site, and Src Tyr-529 is dephosphorylated by PTPα during FN-stimulated fibroblast migration (Zeng *et al*., 2003), we tested whether overexpression of WT or a catalytically defective (C433S/C723S) double mutant (DM) of PTPα could influence α4β1-stimulated NB8 cell motility (Figure 7d). DM-PTPα inhibited FN-(CS-1)-stimulated NB8 migration >50% with decreased cell spreading (52±11% of LacZ cell area) (Figure 7e). DM-PTPα expression resulted in elevated Src pY529 phosphorylation and decreased Src IVK activity compared to control and WT-PTPα-overexpressing cells (Figure 7f).

In contrast, WT-PTPα overexpression decreased Src pY529 phosphorylation but did not significantly affect Src catalytic activation (Figure 7f) or FN-(CS-1) cell motility (Figure 7d). As NB8 cells express moderate levels of endogenous PTPα, PTPα−/− MEFs and Ad-mediated expression of WT- or DM-PTPα was used to test the importance of PTPα in promoting α4β1-stimulated Src activation and motility (Supplementary Figures S5a and b). When PTPα−/− cells expressing α4 were plated onto FN-(CS-1), WT- but not DM-PTPα increased Src Y418 phosphorylation by 38% and decreased Src Y529 phosphorylation by 32% (Supplementary Figure S5c). Further, WT- but not DM-PTPα promoted enhanced cell spreading on FN-(CS-1) (Supplementary Figures S5d and e) and only WT-PTPα functioned to promote efficient α4β1-stimulated fibroblast motility (Supplementary Figure S5f). These results suggest that PTPα is involved in promoting α4-mediated Src activation through enhanced Src Y529 dephosphorylation and contributes to α4β1-stimulated motility.

Discussion

Here, we analysed the signaling properties of endogenous α4β1 and α5β1 integrins on NB cells using recombinant ligands to selectively activate α4β1 or α5β1 and find that α5β1-mediated NB8 cell migration required both Src and FAK PTK activities whereas α4β1-stimulated NB8 cell motility required Src but not FAK. Moreover, we identified a novel and overlooked mechanism of direct FAK-mediated Src activation through FAK phosphorylation of Src within the catalytic region at Y418.

Current models of α5β1-stimulated FAK-Src signaling place FAK activation and autophosphorylation at Y397 as a receptor-proximal event (Mitra and Schlaepfer, 2006). FAK Y397 phosphorylation promotes Src SH2 domain binding to FAK, presumably leading to conformational Src activation with a FAK-Src complex. Alternatively, integrin-regulated dephosphorylation of Src at Y529 can enhance Src activation (Zeng *et al*., 2003). In both models, it remains undetermined how maximal Src activation occurs via kinase domain Y418 phosphorylation. Although this may occur via Src autophosphorylation (Roskoski, 2005), we found that Src

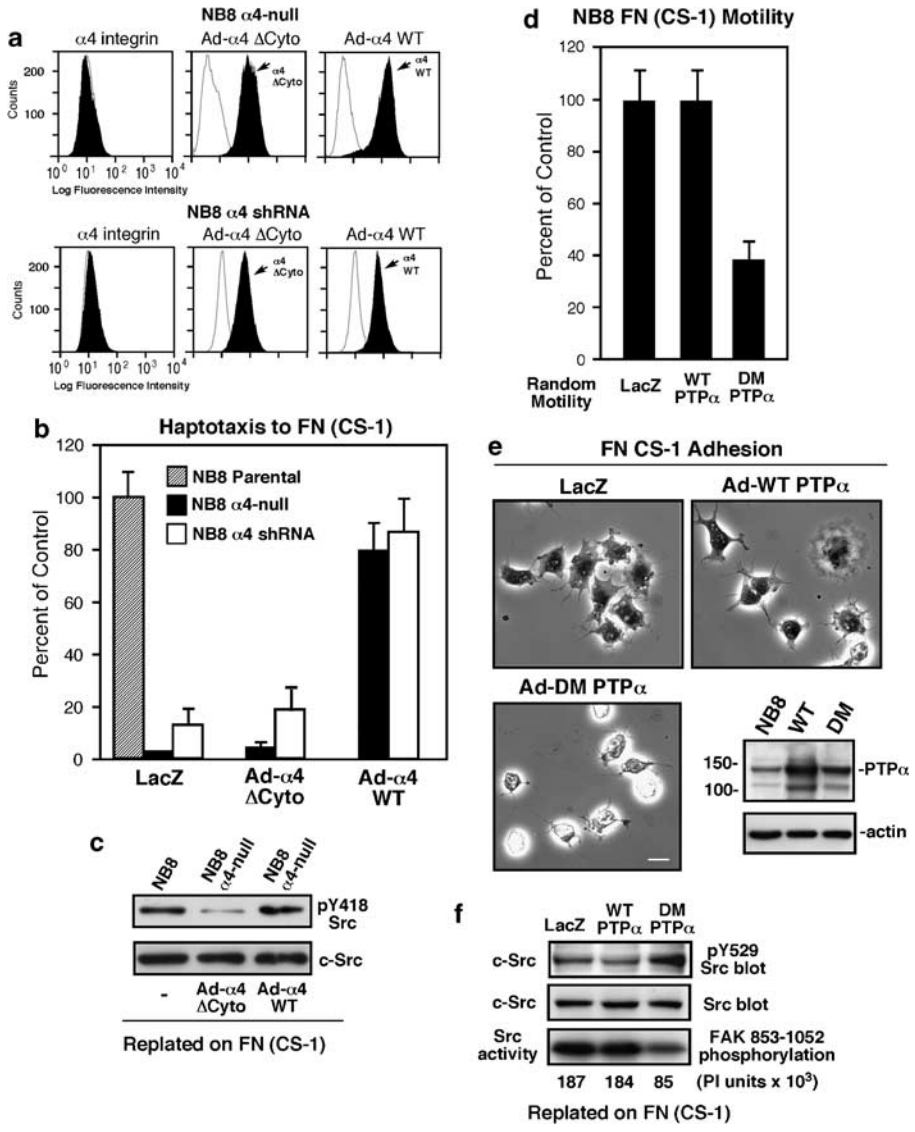


Figure 7 α4 cytoplasmic domain and receptor protein-tyrosine phosphatase-α (PTPα) are important for α4β1-stimulated Src activation and NB8 cell motility on fibronectin (FN)-(connecting segment-1 (CS-1)). (**a**) α4 flow cytometry analyses of NB8 α4-null or NB8 α4 short-hairpin RNA (shRNA)-expressing cells transiently reconstituted Ad-α4 WT or Ad-α4 ΔCyto (shaded peaks). Open peaks show control mAb staining. (**b**)* Haptotaxis motility assays on FN-(CS-1). Values are percent of NB8 parental motility (Control). (**c**) Src IPs from protein lysates from NB8 parental or NB8 α4-null cells transiently re-expressing α4 ΔCyto or α4 wild type (WT) and plated on FN-(CS-1) for 45 min. Src IPs were sequentially evaluated by pY418 Src and total Src blotting. (**d**)* NB8 cells were transduced with Ad-LacZ, Ad-WT-PTPα or a catalytically inactive mutant PTPα double mutant (DM) and assessed for random motility on FN-(CS-1). Values are percent of Ad-LacZ-transduced NB8 cells (control). (**e**) Phase contrast images showing adhesion and spreading on FN-(CS-1) of NB8 cells transduced with Ad-LacZ, Ad-WT-PTPα or Ad-DM-PTPα at 15 min after plating. Scale bar is 10 μm. Inset, protein lysates of Ad-transduced NB8 cells reveals moderate WT-PTPα and DM-PTPα overexpression compared with endogenous PTPα expression. Actin blotting verified equal loading. (**f**) NB8 cells were transduced with the indicated Ad constructs, plated onto FN-(CS-1) for 15 min and Src IPs analysed for pY529 Src phosphorylation and expression. In parallel, Src IPs were evaluated for IVK activity using GST-FAK 853–1052 as a substrate. Phosphoimager (PI) units are an average from two experiments. *See Figure 1.

Y418 phosphorylation only weakly occurred in FAK shRNA NB8 cells after α5β1 stimulation. Re-expression of WT- and KD-FAK formed a complex with Src, but only WT-FAK functioned to promote α5β1-stimulated Src Y418 phosphorylation and cell motility. As recombinant FAK kinase domain could directly phosphorylate Src Y418 *in vitro*, we conclude that α5β1-stimulated Src activation occurs via direct FAK phosphorylation of Src. As Src has been shown to phosphorylate FAK kinase domain residues Y576/Y577 leading to maximal FAK activation (Calalb *et al*., 1995), our results suggest that the formation of a FAK-Src complex after α5β1-stimulation leads to mutual PTK activation through corresponding trans-phosphorylation events.

Although previous studies have implicated integrin β1 in mediating FAK-Src activation after α5β1-stimulation of cells (Mitra and Schlaepfer, 2006), we find that it is the α4 cytoplasmic domain in combination with β1

which facilitates Src PTK activation in a FAK-independent manner to promote NB8 cell motility. Accordingly, abolishing α4 integrin expression in NB8 cells inhibited motility on cFN, and prevented binding to FN-(CS-1), but did not significantly impact migration on an α5β1-selective ligand FN-(9–11). This suggests that minimal crosstalk exists between α4β1 and α5β1 receptors on NB8 cells. Additionally, knockdown of FAK blocked only α5β1-stimulated NB8 migration whereas knockdown of Src or p130Cas prevented both α4β1- and α5β1-initiated cell motility. Fibroblast reconstitution experiments showed that p130Cas, which binds to both FAK and Src, is needed for both α5β1 and α4β1 cell motility. These NB studies extend results obtained using FAK−/− fibroblasts where exogenous human α4 expression was sufficient to form a functional α4β1 receptor that rescued FAK−/− cell motility defects (Hsia *et al*., 2005), and support the existence of a conserved α4-specific signaling linkage promoting Src activation and cell motility.

It is known that integrins can generate intracellular signals through the lateral association with other receptors or the clustering of signaling proteins with integrin cytoplasmic domains (Ruoslahti, 1999). It is interesting that α4-associated signaling is dependent on the integrity of the α4 cytoplasmic domain. This differs from α5β1-stimulated signaling that is generated through protein binding interactions with the β1 cytoplasmic domain. In α4-null or α4-shRNA NB8 cells, α4β1-stimulated motility and Src activation were rescued by re-expression of wild type but not a cytoplasmic domain-truncated mutant of α4 (α4 ΔCyto). Our finding that α4 ΔCyto re-expression in NB8 cells promoted adhesion but not Src activation or motility do not support a dominant role for the β1 subunit within an α4β1 signaling complex.

The most simplistic mechanism for α4 to activate Src is via a direct binding interaction. Nonetheless, pull down assays using a recombinant α4 cytoplasmic domain revealed only a weak binding between α4 and Src (data not shown). However, Src can become partially activated by integrins through the dephosphorylation of the C-terminal regulatory Y529 site mediated via the protein tyrosine phosphatases such as PTPα (Zeng *et al*., 2003). The cytoplasmic tails of α integrins, α1 and αL, can associate with T-cell PTP in HeLa cells and with the CD45 PTP in leukocytes, respectively (Geng *et al*., 2005; Mattila *et al*., 2005). We found that overexpression of a catalytically inactive mutant of PTPα inhibited α4-associated Src activation and NB8 cell motility. In complimentary studies, WT PTPα re-expression in PTPα−/− MEFs (but not catalytically inactive PTPα) enhanced α4β1-stimulated Src activation, spreading and motility. However, as α4-expressing PTPα−/− MEFs partially spread and migrated on FN-(CS-1), PTPα is likely important but not essential for α4-Src-associated cell motility. It remains unclear how α4 facilitates Src Y418 phosphorylation in a FAK-independent manner.

Distinct from the widely expressed α5β1 pair, α4β1 is more selectively expressed in inflammatory, endothelial progenitor and in a subset of tumor cells (Rose *et al*., 2002). In addition to cFN, α4β1 binds to VCAM-1 that is upregulated at sites of inflammation and present within the bone marrow; a common site of NB tumor metastasis (Brodeur, 2003). α4β1 engagement with ligand stimulates cell spreading but does not readily promote cell contraction and mature focal adhesion formation (Chan *et al*., 1992; Pinco *et al*., 2002); thus potentially permitting rapid changes in cell movement. This differs from α5β1 that promotes both cell spreading and focal contact maturation into stable adhesion structures. Moreover, α5β1 but not α4β1 signaling has been linked to enhanced FN matrix assembly (Chan *et al*., 1992; Wu *et al*., 1995; Na *et al*., 2003). As FAK has been shown to be a critical signaling component associated with FN matrix assembly (Ilic *et al*., 2004), the differential activation of FAK by α5β1 may be a key discriminatory event distinguishing the biological outcomes of α4β1- and α5β1-initiated signaling events.

Materials and methods

Tumor immunostaining

NB tumors were obtained at the time of surgery with informed patient/guardian consent and according to the regulations of the Institutional Review Board at Memorial Sloan-Kettering Cancer Center. Tumors were embedded in OCT and cryopreserved. Frozen tissue sections were fixed in 3% paraformaldehyde and processed for mouse Immunoglobulin G (IgG)-specific staining to co-detect either α4 integrin (IgG1, Chemicon MAB1383, Temecula, CA, USA) or α5 integrin (IgG1, Chemicon MAB1969) with anti-GD2 NB marker (IgG3, PHB781) followed by secondary IgG-specific antibodies (flourescein goat anti-mouse IgG1 and rhodamine goat anti-mouse IgG3 from Jackson Immuno Research, West Grove, PA, USA). Draq5 (Alexis Biochemicals, Lausen, Switzerland) was used for nuclear staining. Confocal images were captured using Nikon Eclipse C1 microscope and background fluorescence was determined with tissues stained with only secondary antibodies. Three images were assessed for intensity in the RGB channels using Image J software, and the average intensity was calculated. The ratio of staining in the red and green channels was assessed relative to controls, normalized to blue channel (nuclei) and scored positive based upon a threshold at twice of the sampled background.

Adenovirus production and infection

Recombinant Ad for LacZ, human α4 integrin, cytoplasmic domain-truncated α4 integrin were used as described (Hsia *et al*., 2005). FAK WT, FAK R454 KD, FRNK and FRNK S1034 are HA-tagged, cloned into pADtet7 and protein expression was performed as described (Hsia *et al*., 2005). Ad-PTPα-WT and Ad-PTPα-DM (C4333S/C723S) were used as described (Chen *et al*., 2006). In Ad-LacZ-transduced cells, staining for β-gal activity using X-gal as a substrate revealed LacZ expression in >85% of cells.

Short-hairpin RNAs, lentivirus production and infection

The sequences (see Supplementary Information) used to inhibit human FAK, p130Cas, Src or paxillin expressions were cloned into pLentiLox3.7. For induction of lentiviruses, 293T cells were transfected with plentilox3.7 shRNA construct, CMV-VSVG envelope vector, pMD.G, RSV-Rev and

pMDL g/p RRE as described (Hsia *et al*., 2005). Integrin α4 shRNA lentivirus was from Sigma (St Louis, MO, USA). Lentivirus-containing media was used to infect NB8 cells in the presence of polybrene (5 μg ml^{-1}) for 2 days. Infected cells expressing GFP were obtained by FACS and maintained as a pooled population. α4 shRNA-infected cells were selected with 2 μg ml^{-1} puromycin for 5 days and maintained as a pooled population.

Cell migration

Haptotaxis motility and random motility were performed as described using MilliCell chambers (Millipore, Billerica, MA, USA, 8 μm pores for fibroblasts and 3 μm pores for NB cells) (Hsia *et al*., 2005).

Abbreviations

Ad, adenovirus; BSA, bovine serum albumin; cFN, cellular fibronectin; CS-1, connecting segment-1; DM, double mutant; FACS, fluorescence activated cell sorting; FAK, focal adhesion kinase; FBS, fetal bovine serum; FN, fibronectin; GFP, green fluorescent protein; GST, glutathione-*S*-transferase; IP, immunoprecipitation; KD, kinase dead; LacZ, β-galactosidase; mAb, monoclonal antibody; MEFs, mouse embryonic fibroblasts; moi, multiplicity of infection; NB, neuroblastoma; PTK, protein-tyrosine kinase; PTPα, receptor protein-tyrosine phosphatase-α; pTyr, phosphotyrosine; VCAM-1, vascular cell adhesion molecular 1; WCL, whole cell lysate; WT, wild type.

Acknowledgements

We thank the laboratory of Mark Ginsberg for various α4 fusion proteins and we greatly appreciate the administrative assistance provided by Theresa Villalpando. Y Lim was supported in part by Korea Research Foundation Grant (M01-2005-000-10071-0). This work was supported by grants from the NIH to David Schlaepfer (CA75240, CA87038, CA102310), to Dwayne Stupack (CA107263) and from the Canadian Institutes of Health Research to Catherine Pallen (MOP-49410). Nai-Kong Cheung is supported by CA106450 and the Robert Steel Foundation. D Schlaepfer is an American Heart Association Established Investigator (0540115N). This manuscript is dedicated to the memory of Jaewon Han Ph.D. whose work stimulated our interest into the novel aspects of α4 integrin signaling.

References

Brodeur GM. (2003). Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* **3**: 203–216.

Calalb M, Polte T, Hanks SK. (1995). Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for the Src family kinases. *Mol Cell Biol* **15**: 954–963.

Chan BM, Kassner PD, Schiro JA, Byers HR, Kupper TS, Hemler ME. (1992). Distinct cellular functions mediated by different VLA integrin alpha subunit cytoplasmic domains. *Cell* **68**: 1051–1060.

Chen M, Chen SC, Pallen CJ. (2006). Integrin-induced tyrosine phosphorylation of protein-tyrosine phosphatase-alpha is required for cytoskeletal reorganization and cell migration. *J Biol Chem* **281**: 11972–11980.

Geng X, Tang RH, Law SK, Tan SM. (2005). Integrin CD11a cytoplasmic tail interacts with the CD45 membrane-proximal protein tyrosine phosphatase domain 1. *Immunology* **115**: 347–357.

Guo W, Giancotti FG. (2004). Integrin signalling during tumour progression. *Nat Rev Mol Cell Biol* **5**: 816–826.

Hsia DA, Lim ST, Bernard-Trifilo JA, Mitra SK, Tanaka S, den Hertog J *et al* (2005). Integrin α4β1 promotes focal adhesion kinase-independent cell motility via α4 cytoplasmic domain-specific activation of c-Src. *Mol Cell Biol* **25**: 9700–9712.

Huttenlocher A. (2005). Cell polarization mechanisms during directed cell migration. *Nat Cell Biol* **7**: 336–337.

Ilic D, Kovacic B, Johkura K, Schlaepfer DD, Tomasevic N, Han Q *et al* (2004). FAK promotes organization of fibronectin matrix and fibrillar adhesions. *J Cell Sci* **117**: 177–187.

Kil SH, Krull CE, Cann G, Clegg D, Bronner-Fraser M. (1998). The α4 subunit of integrin is important for neural crest cell migration. *Dev Biol* **202**: 29–42.

Mattila E, Pellinen T, Nevo J, Vuoriluoto K, Arjonen A, Ivaska J. (2005). Negative regulation of EGFR signalling through integrin-alpha1beta1-mediated activation of protein tyrosine phosphatase TCPTP. *Nat Cell Biol* **7**: 78–85.

Mitra SK, Hanson DA, Schlaepfer DD. (2005). Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol* **6**: 56–68.

Mitra SK, Schlaepfer DD. (2006). Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr Opin Cell Biol* **18**: 516–523.

Mostafavi-Pour Z, Askari JA, Parkinson SJ, Parker PJ, Ng TT, Humphries MJ. (2003). Integrin-specific signaling pathways controlling focal adhesion formation and cell migration. *J Cell Biol* **161**: 155–167.

Na J, Marsden M, DeSimone DW. (2003). Differential regulation of cell adhesive functions by integrin alpha subunit cytoplasmic tails *in vivo*. *J Cell Sci* **116**: 2333–2343.

Pankov R, Yamada KM. (2002). Fibronectin at a glance. *J Cell Sci* **115**: 3861–3863.

Parsons JT. (2003). Focal adhesion kinase: the first ten years. *J Cell Sci* **116**: 1409–1416.

Pinco KA, He W, Yang JT. (2002). 4β1 integrin regulates lamellipodia protrusion via a focal complex/focal adhesion-independent mechanism. *Mol Biol Cell* **13**: 3203–3217.

Rose DM, Han J, Ginsberg MH. (2002). Alpha4 integrins and the immune response. *Immunol Rev* **186**: 118–124.

Roskoski Jr R. (2005). Src kinase regulation by phosphorylation and dephosphorylation. *Biochem Biophys Res Commun* **331**: 1–14.

Ruoslahti E. (1999). Fibronectin and its integrin receptors in cancer. *Adv Cancer Res* **76**: 1–20.

Stupack DG, Teitz T, Potter MD, Mikolon D, Houghton PJ, Kidd VJ *et al* (2006). Potentiation of neuroblastoma metastasis by loss of caspase-8. *Nature* **439**: 95–99.

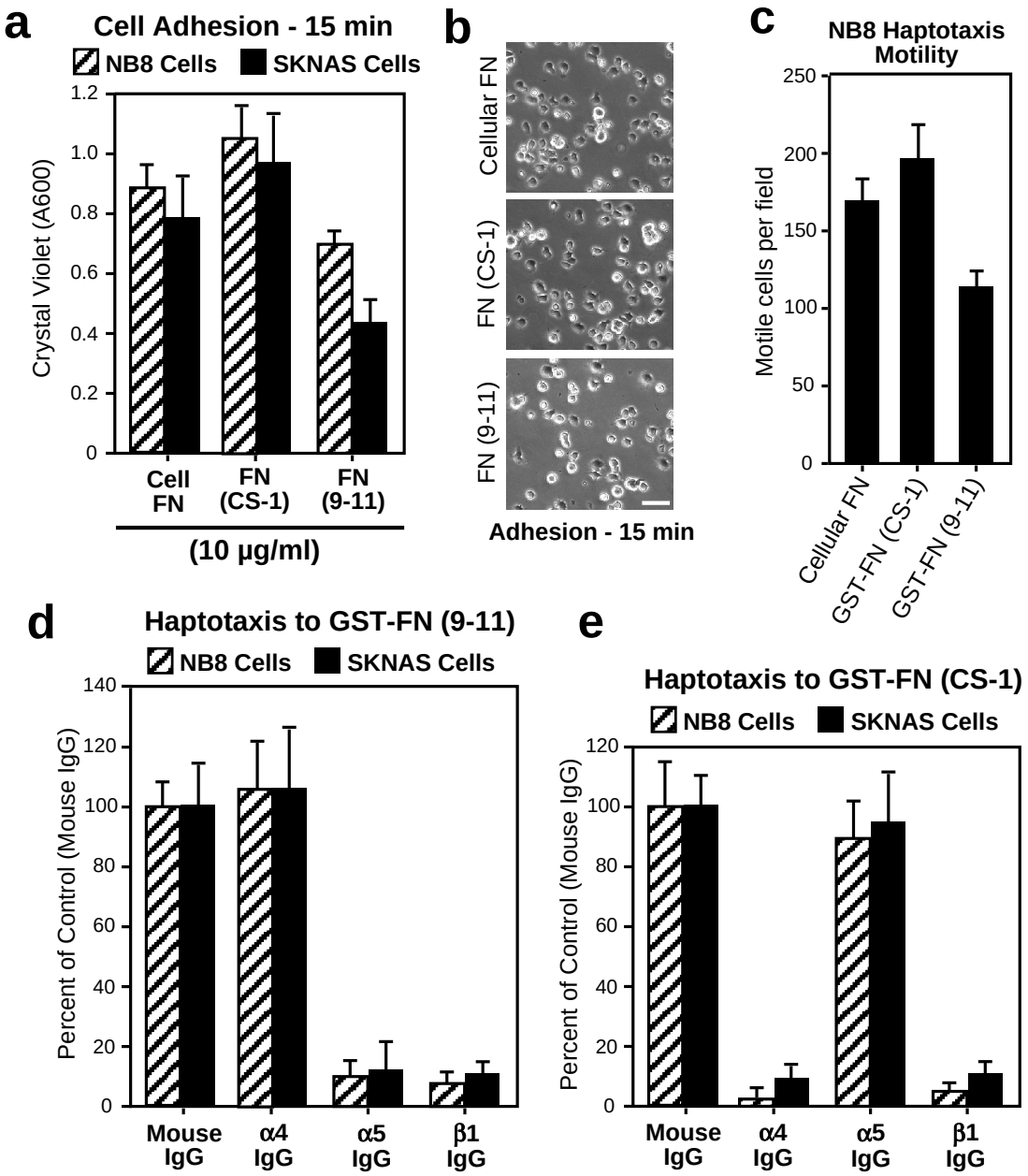
Testaz S, Duband JL. (2001). Central role of the α4β1 integrin in the coordination of avian truncal neural crest cell adhesion, migration, and survival. *Dev Dyn* **222**: 127–140.

Wu C, Fields AJ, Kapteijn BA, McDonald JA. (1995). The role of α4β1 integrin in cell motility and fibronectin matrix assembly. *J Cell Sci* **108**(Part 2): 821–829.

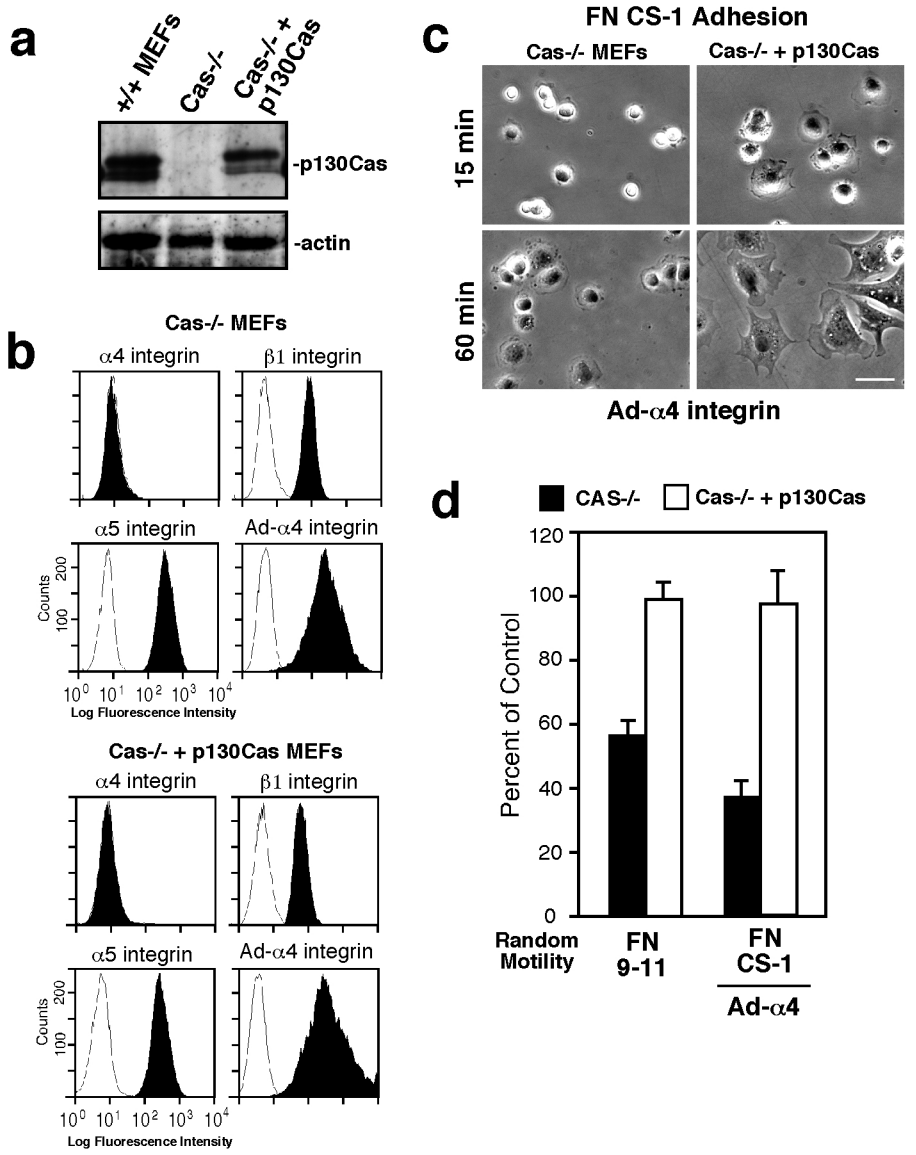
Zeng L, Si X, Yu WP, Le HT, Ng KP, Teng RM *et al* (2003). PTPα regulates integrin-stimulated FAK autophosphorylation and cytoskeletal rearrangement in cell spreading and migration. *J Cell Biol* **160**: 137–146.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).

Wu et. al., Supplemental Figure 1

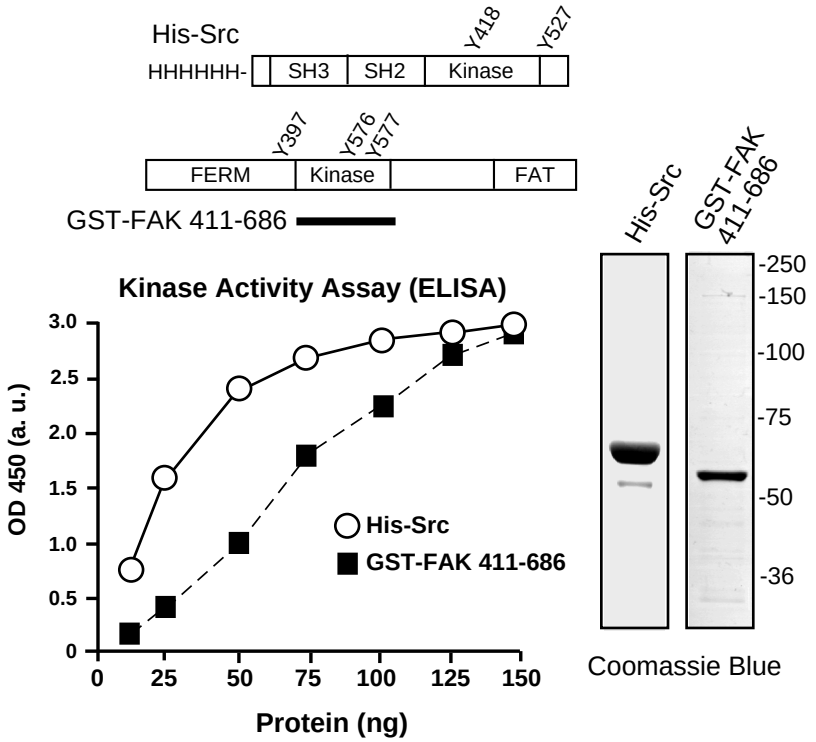


Supplemental Figure 1. Recombinant FN (CS-1) or FN (9-11) stimulate α4β1- or α5β1-mediated NB cell motility, respectively. (**a**) Quantitative analysis of NB8 and SKNAS cell adhesion after 15 min to cellular FN, FN (CS-1), and FN (9-11). NB8 equally adhere to cellular FN and GST FN (CS-1) with slightly less adhesion to GST FN (9-11). SKNAS show strong adhesion to cellular FN and to FN (CS-1) and two-fold less adhesion to FN (9-11). (**b**) Phase contrast images of NB8 cells adhesion on cellular FN, FN (CS-1), and FN (9-11). Scale bar is 25 µm. (**c**) NB8 haptotaxis motility performed on cellular FN, FN (CS-1), and FN (9-11). Both FN (CS-1) and FN (9-11) functioned to promote cell motility at 125% and 67% levels compared to cellular FN, respectively. (**d**) NB cell haptotaxis motility on FN (CS-1) is blocked by anti-α4 mAb (HP2/1, 10 µg/ml) and anti-β1 mAb (P4C10, 10 µg/ml) but not by anti-α5 mAb (P1D6, 10 µg/ml). (**e**) NB haptotaxis cell motility on FN (9-11) is blocked by anti-α5 mAb (P1D6, 10 µg/ml) and anti-β1 mAb (P4C10, 10 µg/ml), but not by anti-α4 mAb (HP2/1, 10 µg/ml). (**a**, **c**, **d**, and **e**) Values are means +/- SD of triplicates from at least two separate experiments.



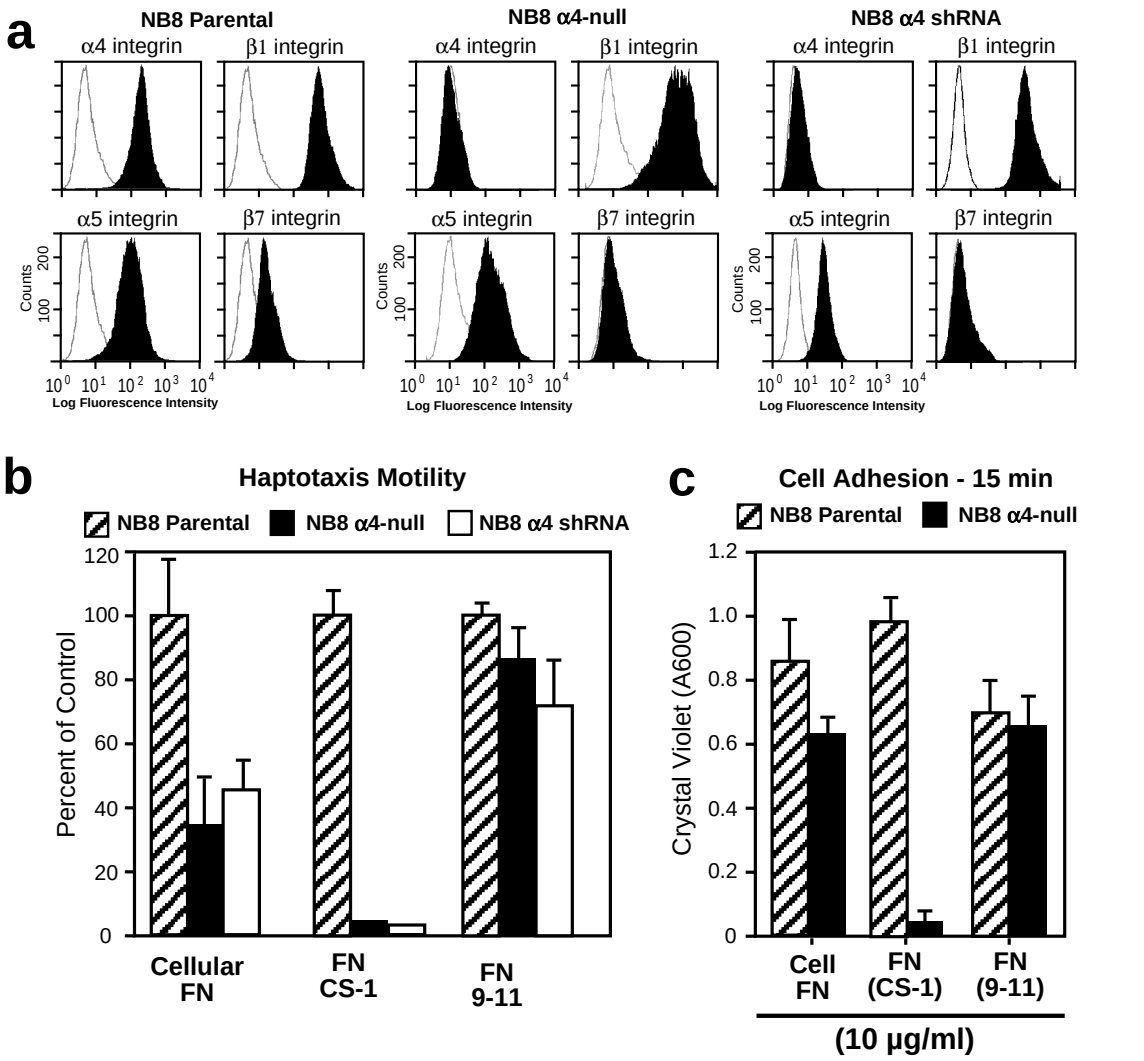
Supplemental Figure 2. Human α4 integrin expression in Cas-/- and Cas-/- +p130Cas fibroblasts. (**a**) Expression of p130Cas in normal mouse embryonic fibroblasts (MEFs), Cas-/-, and Cas-/- +p130Cas fibroblasts were analyzed anti-p130Cas immunoblotting. Actin blotting verified equal loading. (**b**) Flow cytometry analyses of endogenous murine α4, β1, α5, and adenovirus (Ad)-expressed human α4 integrin in Cas-/- and Cas-/- +p130Cas cells (shaded peaks). Staining with control mAb (open peaks). (**c**) Phase contrast images showing adhesion and spreading on FN (CS-1) of Cas-/- and Cas-/- +p130Cas cells with human α4 integrin expression at indicated time points. Scale bar is 15 μm. (**d**) Random motility assay on FN (9-11) or FN (CS-1) of Cas-/- and Cas-/- +p130Cas cells with or without human α4 integrin expression revealed that p130Cas promotes α5β1- and α4β1-mediated cell motility.

Wu et. al., Supplemental Figure 3

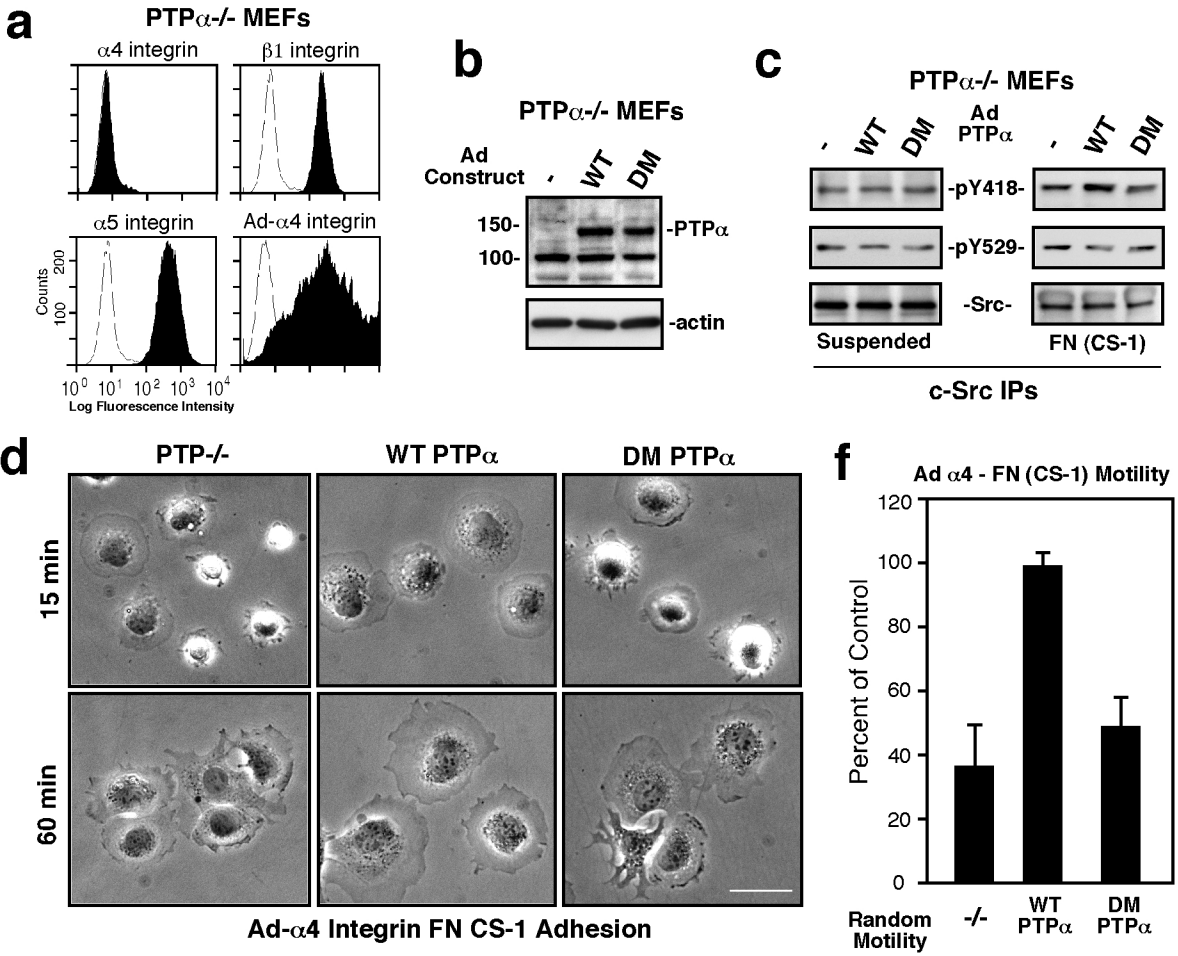


Supplemental Figure 3. Activity profile of recombinant His-Src and GST-FAK 411-686. Schematic of His-tagged Src and the FAK kinase domain region (411-686) expressed in baculovirus and initially isolated by Ni-NTA and glutathione agarose, respectively. After size fractionation chromatography, the purity of His-Src and GST-FAK 411-686 was >90% as visualized by SDS-PAGE and Coomassie Blue staining. Shown is an ELISA-based activity assay measuring the phosphorylation of poly Glu:Tyr (4:1).

Wu et. al., Supplemental Figure 4



Supplemental Figure 4. Abolishing α4 integrin expression inhibits α4β1- but not α5β1-stimulated NB motility. (**a**) Flow cytometry analyses of α4, α5, β1, and β7 integrin expression (shaded peaks) in NB8 parental, NB8 α4-null sorted, and α4 shRNA-expressing cells. Open peaks show staining with control mAb. (**b**) Haptotaxis motility assays show that loss of α4 integrin expression prevents FN (CS-1) but not FN (9-11)-stimulated NB cell motility and inhibits migration on cellular FN. Values presented are percent of NB8 parental cell motility on cellular FN (Control). (**c**) Quantitative analysis of NB8 parental and α4-null cell adhesion to cellular FN, FN (CS-1), and FN (9-11) after 15 min. (**b** and **c**) Values are means +/- SD of triplicates from two separate experiments.



Supplemental Figure 5. Human α4 expression in PTPα-/- fibroblasts reveals that PTPα activity promotes α4β1-stimulated cell spreading and motility. (**a**) Flow cytometric analyses of endogenous murine α4, β1, α5, and adenovirus (Ad)-expressed human α4 integrin in PTPα-/- cells (shaded peaks). Open peaks show staining with control mAb. PTPα-/- MEFs express high levels of endogenous α5 and β1 but not α4 integrins. (**b**) Ad-PTPα re-expression was verified by anti-PTPα blotting of whole cell lysates after 24h infection. Actin blotting verified equal loading. (**c**) PTPα-/- cells were infected with human Ad-α4 and as indicated, also transduced with either Ad-PTPα (WT) or catalytically-inactive PTPα (DM). After 24 h, cells were serum starved overnight, trypsinized, held in suspension for 45 min or plated onto FN (CS-1) for 45 min prior to cell lysis. Src IPs were sequentially analyzed by Src pY418, Src pY529, and total Src blotting. (**d**) As in panel C, PTPα-/- cells were transduced with Ad-α4 WT and the indicated Ad-PTPα constructs, and replated onto FN (CS-1). Representative phase contrast images show cell spreading differences at 15 min and 60 min. Scale bar is 15 μm. (**e**) Cell area on FN (CS-1) was measured by ImageJ software and expressed as percentage of WT PTPα cell area at 60 min. Values are means +/- SD from three to four different image fields in which >15 cells were measured per condition. The difference between WT PTPα and PTP-/- or DM PTPα cells is statistically significant (* $p < 0.001$). (**f**) Random motility on FN (CS-1) of PTPα-/- and PTPα-/- re-expressing wild type PTPα (WT) or PTPα (DM) with human α4 integrin re-expression revealed that PTPα activity promotes α4β1-mediated cell motility. Values are means +/- SD of triplicates from two separate experiments.

Materials and Methods

Antibodies and reagents

Anti-pTyr (4G10) monoclonal antibody (mAb) was obtained from UBI. p130Cas (clone 21), Pyk2 (clone 11), paxillin (clone 349), human integrin β7 (555943), Hic-5 (clone 34), and FAK (clone 77) mAbs were from BD Biosciences. β-actin mAb (clone AC-74), Flag mAb (M2), and integrin α4 shRNA plasmid targeting human α4 sequence 559-579 (gctccgtgttatcaagattat) were purchased from Sigma. Polyclonal antibodies to p130Cas (C20), Src (Src-2 and N-16), and HEF1/Cas-L were obtained from Santa Cruz Biotechnology. Phospho-specific antibodies to the FAK pY397 motif, Src pY418 motif, and to Src pY529 were from Invitrogen-BioSource. Polyclonal antibodies to FAK (5904 and 5592) and mAb specific to Src (clone 2-17) or to the HA-tag (12CA5) were used as described (Sieg et al., 1998). Mouse mAbs to human integrin α4 (clones HP2/1 and P1H4), integrin α5 (clones P1D6 and NKI-SAM-1), integrin α6 (clone 4F10), and rat anti-human mAb to integrin α6 (clone NKI-GoH3) were from Chemicon. Rat anti-mouse mAbs to integrin α5 (clone 5H10-27), integrin αv (clone RMV-7), integrin α4 (clone 9C10), and integrin β1 (clone KMI6) were from BD Biosciences. Mouse anti-human mAbs to integrin β1 (clone P4C10) and to integrin β5 (clone P1F6) were used as described (Hsia et al., 2005). Anti-Fyn mAb (clone 301) was from Wako Pure Chemicals, Japan. Purified human cellular FN (341633), compounds PP2 and PP3 were from Calbiochem. Rabbit anti-PTPα antibody has been described (Zeng et al., 2003). Rabbit polyclonal anti-GFP was raised against recombinant 6-His-tagged-GFP produced from baculovirus culture.

Cells and constructs

Cas-/- and Cas-/- +p130Cas fibroblasts were provided by the late H. Hirai (University of Tokyo, Tokyo, Japan). RPTPα-/- fibroblasts were generated as described (Zeng et al., 2003). SYF fibroblasts were from American Type Culture Collection (ATCC). Human SKNAS (ATCC), NB8 (Teitz et al., 2000) and other neuroblastoma cells were from NK Cheung (Memorial Sloan-Kettering Cancer Center, New York, NY 10021). Cell media was supplemented with 10% FBS, 1 mM non-essential amino acids, 1 mM sodium pyruvate, 1000 U/ml penicillin, and 1000 µg/ml streptomycin sulfate. Bacterial GST expression vectors for human FN CS-1 region and FN repeats 9-11 were used as described (Hsia et al., 2005). The coding region of the human α4 cytoplasmic domain region (32 residues) was cloned into pGEX4T as a 134 bp EcoRI-XhoI fragment generated by PCR using the primers 5'-aaaGAATTCaaggctggcttctttaaaag-3' 5'-aaaCTCGAGctgttttccattctctc-3'. All constructs were verified by DNA sequencing.

Flow cytometric analysis

Cells were trypsinized, enumerated, and incubated with specific primary antibodies (10^6 cells/ µg antibody) for 20 min on ice in 100 µl of PBS followed by pelleting and washing using cold PBS. Alexa 488-conjugated goat anti-rat IgG (Molecular Probes) or Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes) were used as labeled secondary antibodies for visualization. The conditions for secondary antibody incubation

and washing were the same as above. Analyses were performed using a FACScan or FACS Calibur machines (BD Biosciences). Negative controls used mouse or human IgG in the primary incubation. Integrin α4-null NB8 cells were generated by 5 sequential rounds of sorting with α4 antibody HP2/1 antibody, collecting the negative cell population, and expansion of the sorted cells. FACS analysis was used to confirm the lack of α4 surface expression.

Cell lysis, immunoprecipitation, pull down assays, and blotting

Cells were solubilized in modified RIPA lysis buffer containing 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS (Sieg et al., 1998). For immunoprecipitations, antibodies were incubated with lysates for 3 hours at 4°C and collected by binding to Protein G-plus (Calbiochem) or Protein A (Repligen) agarose beads. SDS-PAGE, antibody blotting, and sequential membrane re-probing was performed as described (Sieg et al., 1998). The intensity of the bands were analyzed using ImageJ v1.37 (NIH).

Short hairpin RNAs sequence

Sense oligos used were as follows: FAK 5'-tGA ACC TCG CAG TCA TTT ATT tca aga gAA TAA ATG ACT GCG AGG TTC *ttt ttt* c-3', p130Cas 5'-tGT ATG GCC AGG AGG TGT ATT tca aga gAA TAC ACC TCC TGG CCA TAC *ttt ttt* c-3', Src 5'-tGC ACT ACA AGA TCC GCA AGT tca aga gAC TTG CGG ATC TTG TAG TGC *ttt ttt* c-3', paxillin 5'-tGA GAA GCC TAA GCG GAA TGT tca aga gAC ATT CCG CTT AGG CTT CTC *ttt ttt* c-3', and Srambled 5'-tGT CTC CGA ACG TGT CAC GTT tca aga gAA CGT GAC ACG TTC GGA GAC *ttt ttt* c-3'. The 5' t belongs to U6 promoter, and lower letters represent components of shRNA and italicized run of t's is a terminator sequence. The 3'C was added to generate an Xbal site. Antisense oligos used were as follows: FAK 5'-tcg aga aaa aag aaC CTC GCA GTC ATT TAT Tct ctt gaA ATA AAT GAC TGC GAG GTT Ca-3', p130Cas 5'-tcg aga aaa aaG TAT GGC CAG GAG GTG TAT Tct ctt gaA ATA CAC CTC CTG GCC ATA Ca-3', Src 5'-tcg aga aaa aaG CAC TAC AAG ATC CGC AAG Tct ctt gaA CTT GCG GAT CTT GTA GTG Ca-3', paxillin 5'-tcg aga aaa aaG AGA AGC CTA AGC GGA ATG Tct ctt gaA CAT TCC GCT TAG GCT TCT Ca-3', and Scrambled 5'-tcg aga aaa aag tcT CCG AAC GTG TCA CGT Tct ctt gaA ACG TGA CAC GTT CGG AGA Ca-3'. Additional 4 nucleotides at the 5' end were added to generate an Xbal site. The oligos were annealed and ligated into the Hpal/Xbal site of pLentilox 3.7 (Rubinson et al., 2003).

Cell adhesion

For cell adhesion, glass cover slips were pre-coated with 5 or 10 µg/ml GST-FN (9-11), GST-FN (CS-1), or cellular FN in PBS overnight at 4°C and then blocked with 1% heat-denatured BSA for 1 h at 37°C. Cells were used 24 hours after Ad infection and were serum-starved (0.5% FBS) between 18 and 24 h. Equal numbers of suspended cells in DMEM plus 0.5% BSA (Migration Media) were plated, incubated at 37°C, and at 15 or 60 min, unattached cells were washed away with PBS and the attached cells were fixed with 3.7% paraformaldehyde. Cell spreading values were quantified by ImageJ using

ROI (region of interest) manager tool. Data were expressed as percentage of p130Cas or WT PTPα re-expressing cells on FN at 60 minutes. To quantify cells adhesion, adherent cells on FN were fixed with paraformaldehyde, stained with 0.05% Crystal Violet, and washed with PBS. Stain was eluted with 10% acetic acid and the absorbance measured at 600 nm.

Time-lapse motility analyses

NB8 cells stably expressing FAK, p130Cas, Src, or scrambled shRNA were serum-starved overnight. 200,000 cells were plated onto glass coverslips coated with 2 μg/ml cellular FN in 5% FBS-containing growth media. After 1 h, media was replaced with 199 Media (Invitrogen) and the glass coverslip-associated cells were placed into chamber maintained at 37°C (20/20 Technology Inc.) and GFP-expressing cells were visualized using a 20X objective (Olympus IX51, NA= 0.5) with a monochrome CCD camera (Hamamatsu Orca-ER) on an inverted fluorescent microscope (IX51, Olympus). Images were collected every 4 minutes for 10 h and individual cell tracking performed using OpenLab software (Improvision)

Baculovirus Src, FAK, and in vitro kinase assays

His-tagged Src was generated by PCR using primers 5'-cgggatccatgcatcaccatcaccatcacatgggtagcaacaagagcaagcccaaggatg-3' 5'cgggatccctataggttctccccgggctggtactgtgg-3', cloned into the pVL1393 baculovirus vector (Pharmingen) at BamHI site. The FAK catalytic domain region (411-686) was generated by PCR using the primers 5'-cgatcgaattctcgaccagggattatgagattca-3' 5'-tagctgtcgacttactgcaccttctcctcctccagg-3', cloned into pGEX4T vector at EcoRI and SalI sites, and moved into the pAcG2T baculovirus GST fusion expression vector (Pharmingen). Recombinant His-Src and GST-FAK 411-686 were generated using the Baculogold System (Pharmingen). Virus clones were identified by plaque assays and amplified. For protein expression, SF9 cells were infected at a multiplicity of infection (m.o.i) of 2-5 pfu/cell and cultured at 27°C for 48 h. Ni-NTA and glutathione agarose affinity chromatography were used to purify His-Src and GST-FAK (411-686) respectively, and both proteins were further purified by size fractionation using hiload 16/60 Superdex chromatography (GE Healthcare). The proteins were concentrated and stored frozen in Kinase Buffer (20 mM Tris pH 7.4, 400 mM NaCl, 0.5 mM Na_3VO_4, 25 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM EDTA, 5 mM β-mercaptoethanol, and 5% glycerol). The purity of His-Src and GST-FAK 411-686 were estimated at >90% by SDS-PAGE and Coomassie Blue staining. His-Src and GST-FAK in vitro kinase activity were measured using the K-LISA PTK screening kit (Calbiochem) with poly Glu-Tyr (4:1) as a substrate and both proteins showed dose-dependent phosphorylation activity.

Kinase-inactive Src (K297M) was subcloned into pCDNA3.1 vector as described previously (Hauck et al., 2001). SYF cells were transfected with 2.5 µg of pCDNA3.1 Src K297M using Lipofectamine 2000 (Invitrogen) and after 48 h, cells were harvested by trypsin-EDTA treatment, lysed in modified RIPA buffer, and Src K297M isolated by IP using Src (clone 2-17) mAb. For GST-FAK phosphorylation of Src, Src IPs were

washed once in 1% Triton Lysis buffer, twice in HNTG buffer, and once in Kinase Buffer with 100 μM ATP. Kinase reactions were initiated by adding either ~100 ng GST or GST-FAK 411-686, incubation at 32°C for 15 min, and kinase reactions terminated by SDS-PAGE and analyses by anti-pY418 and total Src immunoblotting. Similar assays were performed with 100 ng GST-FAK or His-Src for phosphorylation of KD FAK followed by anti-pY397 and anti-GFP blotting. For measurements of Src-associated activity, Src IPs were washed as above and resuspended in Src kinase buffer (20 mM Pipes pH 7.0, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM DTT). Kinase assays were initiated by ATP (20 μM, 20 μCi/nmol ATP) and 1 μg GST-FAK 853-1052 (Schlaepfer et al., 1994) addition, incubated at 32°C for 15 min, and then resolved by SDS-PAGE. GST-FAK phosphorylation was visualized by autoradiography and quantified using a phosphoimager (Molecular Dynamics).

References

Hauck CR, Hunter T, Schlaepfer DD (2001). The v-Src SH3 domain facilitates a cell adhesion-independent association with focal adhesion kinase. *J. Biol. Chem.* **276:** 17653-17662.

Hsia DA, Lim ST, Bernard-Trifilo JA, Mitra SK, Tanaka S, den Hertog J *et al* (2005). Integrin {alpha}4{beta}1 Promotes Focal Adhesion Kinase-Independent Cell Motility via {alpha}4 Cytoplasmic Domain-Specific Activation of c-Src. *Mol. Cell. Biol.* **25:** 9700-12.

Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J *et al* (2003). A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat. Gene.t* **33:** 401-6.

Schlaepfer DD, Hanks SK, Hunter T, van der Geer P (1994). Integrin-mediated signal transduction linked to Ras pathway by Grb2 binding to focal adhesion kinase. *Nature* **372:** 786-791.

Sieg DJ, Hauck CR, Schlaepfer DD (1999). Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *J. Cell Sci.* **112:** 2677-2691.

Sieg DJ, Ilic D, Jones KC, Damsky CH, Hunter T, Schlaepfer DD (1998). Pyk2 and Src-family protein-tyrosine kinases compensate for the loss of FAK in fibronectin-stimulated signaling events but Pyk2 does not fully function to enhance FAK^- cell migration. *EMBO J.* **17:** 5933-5947.

Teitz T, Wei T, Valentine MB, Vanin EF, Grenet J, Valentine VA *et al* (2000). Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. *Nat. Med.* **6:** 529-35.

Zeng L, Si X, Yu WP, Le HT, Ng KP, Teng RM *et al* (2003). PTP alpha regulates integrin-stimulated FAK autophosphorylation and cytoskeletal rearrangement in cell spreading and migration. *J Cell Biol* **160:** 137-46.