Talin-bound NPLY motif recruits integrin-signaling adapters to regulate cell spreading and mechanosensing

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Abstract
Integrin-dependent cell adhesion and spreading are critical for morphogenesis, tissue regeneration, and immune defense but also tumor growth. However, the mechanisms that induce integrin-mediated cell spreading and provide mechanosensing on different extracellular matrix conditions are not fully understood. By expressing β3-GFP-integrins with enhanced talin-binding affinity, we experimentally uncoupled integrin activation, clustering, and substrate binding from its function in cell spreading. Mutational analysis revealed Tyr747, located in the first cytoplasmic NPLY(747) motif, to induce spreading and paxillin adapter recruitment to substrate- and talin-bound integrins. In addition, integrin-mediated spreading, but not focal adhesion localization, was affected by mutating adjacent sequence motifs known to be involved in kindlin binding. On soft, spreading-repellent fibronectin substrates, high-affinity talin-binding integrins formed adhesions, but normal spreading was only possible with integrins competent to recruit the signaling adapter protein paxillin. This proposes that integrin-dependent cell-matrix adhesion and [...]
Talin-bound NPLY motif recruits integrin-signaling adapters to regulate cell spreading and mechanosensing

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Integrin-dependent cell adhesion and spreading are critical for morphogenesis, tissue regeneration, and immune defense but also tumor growth. However, the mechanisms that induce integrin-mediated cell spreading and provide mechanosensing on different extracellular matrix conditions are not fully understood. By expressing β3-GFP-integrins with enhanced talin-binding affinity, we experimentally uncoupled integrin activation, clustering, and substrate binding from its function in cell spreading. Mutational analysis revealed Tyr747, located in the first cytoplasmic NPLY747 motif, to induce spreading and paxillin adapter recruitment to substrate- and talin-bound integrins. In addition, integrin-mediated spreading, but not focal adhesion localization, was affected by mutating adjacent sequence motifs known to be involved in kindlin binding. On soft, spreading-repellent fibronectin substrates, high-affinity talin-binding integrins formed adhesions, but normal spreading was only possible with integrins competent to recruit the signaling adapter protein paxillin. This proposes that integrin-dependent cell–matrix adhesion and cell spreading are independently controlled, offering new therapeutic strategies to modify cell behavior in normal and pathological conditions.

Introduction

Heterodimeric receptors of the integrin family are critical to maintain the mechanical link between the ECM and the cytoskeleton. ECM-bound integrins induce also intracellular signaling, mediating cell spreading, migration, proliferation, and survival (Akiyama et al., 1994; Hynes, 2002; Green et al., 2009). Importantly, both the anchoring and signaling function of integrins are required for controlling tissue morphogenesis, causing for example tumor formation and metastasis when misregulated (Paszek et al., 2005). To treat these pathologies, it is critical to understand the adhesion-mediating cytoskeleton–integrin–matrix connection but also to reveal the mechanisms leading to integrin-mediated signaling, which is also termed mechanosensing.

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Abbreviations used in this paper: CMA, cell–matrix adhesion; FN, fibronectin; HSA, human serum albumin; NHS, N-hydroxysuccinimide; NMR, nuclear magnetic resonance; NTA, nitrilotriacetic acid; PAA, polyacrylamide; Ph(4,5)P, phosphatidylino-ositol 4,5-bisphosphate; PTB, phosphotyrosine binding; SIM, structured illumination microscopy; TIRF, total internal reflection fluorescence; VN, vitronectin.

Integrin signaling is for example manifested by the local activation of the Rac1 GTPase, which causes the formation of lamellipodia and cell spreading, at sites where exploring filopodia contact immobilized integrin ligands (Guillou et al., 2008). At the filopodia/ECM interface, the clustering of integrins, which reflects an increase in integrin concentration and nascent adhesion formation, correlates with the accumulation of the cytoplasmic adapter protein talin and subsequent recruitment of paxillin and FAK (Partridge and Marcantonio, 2006). Although talin assures the mechanical link between the integrin and the actin cytoskeleton (Wehrle-Haller, 2012), the recruitment of paxillin and FAK to nascent adhesions (Choi et al., 2011) regulates cell spreading and mechanosensing (Hagel et al., 2002; Wade et al., 2002; Friedland et al., 2009; Choi et al., 2013). However, talin appears to play a dual role because its knockout or knockdown
affected cell spreading and mechanosensing (Petrich et al., 2007b; Zhang et al., 2008; Monkley et al., 2011), which correlated with a failure to recruit paxillin and phospho-FAK, proposing a role of talin in the recruitment of these signaling adapters (Zhang et al., 2008). Indeed, talin is a key player in controlling integrin activation and the mechanical coupling of integrins to ECM ligands. To keep the integrin in an activated state, the talin head interacts with the membrane-proximal and the W/NPLY motif in the β integrin cytoplasmic tail (Tadokoro et al., 2003; Wegener et al., 2007) as well as phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) membrane lipids that open up the closed talin conformation and stabilize talin head–β integrin tail association (Goksoy et al., 2008; Saltel et al., 2009; Song et al., 2012). This results in αβ integrin tail unclasping, which leads to increased ligand binding in the integrin ectodomain, in a process called inside-out activation (Anthis et al., 2009). In turn, ECM ligands stabilize the conformational rearrangements in the integrin ectodomain in a process called outside-in activation (Zhu et al., 2013), which reinforces the ligand- and talin-bound integrin conformation (Wehrle-Haller, 2012). In addition, talin plays an important role in enhancing integrin binding to multivalent ligands by inducing integrin clustering (Bunch, 2010). Integrin clustering requires the activated integrin conformation and the PI(4,5)P₂-dependent interaction of the talin head with the membrane-proximal integrin tail (Chuzel et al., 2005; Saltel et al., 2009). Despite the critical role of talin in integrin activation and clustering, it is still not known whether talin is just keeping the integrin in a ligand-bound and signaling-competent state or whether it forms an essential part of the cytoplasmic scaffold required for recruitment of signaling adapters.

To answer this critical question, the integrin–talin association needs to be analyzed in the context of integrin signaling. As a convenient readout of integrin signaling, ligand-induced cell spreading has revealed a critical role of the W/NPLY⁷⁴⁷ motif in β3 integrin signaling (LaFlamme et al., 1994; Yläne et al., 1995; Schaffner-Reckinger et al., 1998), which further requires Rac1 GTPase activity (Berrier et al., 2000, 2002; Guillou et al., 2008). In addition, the kindlin adapter protein appears required for recruitment of signaling adapters, such as paxillin to the integrin–talin–kindlin complex.

As a result of the close functional link between talin- and kindlin-mediated integrin activation and subsequent cell spreading and/or paxillin and FAK recruitment, the mechanism of integrin-mediated spreading has not yet been uncoupled from that of integrin activation. A major obstacle to resolving this issue is linked to the use of loss-of-function integrin mutations, which caused simultaneous failure of talin binding and loss of cell spreading. Thus, we decided to experimentally increase the talin–integrin affinity, to maintain robust cell–matrix adhesion (CMA), while screening for integrin mutations that failed to induce cell spreading. By creating β3 integrin chimeras with talin-binding peptides from layilin or PIPK1–γ, integrin–talin affinity increased by 20-fold, causing robust integrin activation but spreading only in the case of the layilin-based chimera. In this chimera, spreading, but not talin binding, was lost by a Tyr to Ala mutation in the conserved W/NPLY⁷⁴⁷ motif. Loss of spreading correlated with the failure to recruit the signaling adapter protein paxillin to focal adhesions formed by these talin-bound chimeric integrins. Kindlin-binding integrin mutations also failed to induce cell spreading, despite the recruitment of integrins into focal adhesions. In addition, enhancing the integrin–talin affinity enabled focal adhesion formation on very soft fibronectin (FN) polyacrylamide gels but spreading only in the case of the layilin chimera. These data suggest that integrin activation and focal adhesion formation is controlled by the physical state of the ECM as well as talin and kindlin binding to integrins but that cell spreading and potentially other integrin-signaling events require the recruitment of signaling adapter proteins, such as paxillin to the integrin–talin–kindlin complex.

Results

Development of a β3-GFP-integrin-dependent cell-spreading assay

To quantify integrin signaling, we developed an integrin-dependent spreading assay (Fig. 1 A), by transiently expressing wild-type and mutant forms of a validated GFP-tagged mouse β3 integrin (Ballestrem et al., 2001). Specificity was obtained by transfecting a clone of NIH-3T3 fibroblasts that exhibits very low levels of endogenous β3 integrin, when compared with other mouse fibroblasts (Fig. 1 B and Fig. S1). However, these cells express endogenous α5β1 integrins, recognized by mAbs 9EG7 and HMβ1-1, binding only activated or all β1 integrins, respectively (Fig. 1 B). After transfection and FACS sorting for β3-GFP-integrin, cells were plated in serum-free medium on glass coverslips coated with 1 µg/ml vitronectin (VN), an αβ3 integrin ligand that does not bind α5β1 integrins. The spreading kinetics was followed by phase-contrast microscopy (Fig. 1, C and D). To confirm nontoxicity of mutants, cells were plated on coverslips coated with 10 µg/ml FN, which induced spreading irrespectively of the transfected β3 integrin (Fig. 1 E). When compared with mock-transfected cells, which showed a delay in spreading of ~60 min on VN, wild-type β3-GFP-integrin–transfected cells started to spread immediately, reaching half-maximal and complete spreading at 60 and 240 min, respectively (Fig. 1 D). This spreading was identical to anti-β3 sortet cells, transfected with nontagged mouse β3 integrin (Fig. S2), suggesting that the C-terminal GFP tag was not perturbing the integrin-spreading response (Ballestrem et al., 2001).

In contrast to wild-type β3-GFP-integrin, the talin-binding-deficient Y⁷⁴⁷A β3-GFP-integrin mutant prevented spreading for ≤2 h, resulting in 30% spreading at 6 h. However, after 15 h, cells were spread similarly to wild-type and mock-transfected cells, proposing that FN secretion and endogenous α5β1 integrins (Fig. 1 B) overcame the block in spreading on VN (Fig. 1, F and G).
Figure 1. Analysis of β3-GFP-integrin–dependent cell spreading. (A) Illustration of the spreading assay. NIH-3T3 fibroblasts were transiently transfected with wild-type or mutant mouse β3-GFP-integrin constructs and FACS sorted before plating onto 1 µg/ml VN- or 10 µg/ml FN-coated glass coverslips. (B) FACS analysis for cell surface expression of β3 and β1 integrin (activated [9EG7] and total [HMβ1-1] population) in mock, wild-type, and Y747A mutant β3-GFP-integrin–transfected NIH-3T3 cells. Panels show representative results from a single experiment out of three repeats. (C) Phase-contrast images of NIH-3T3 cells expressing wild-type or Y747A mutant β3-GFP-integrin after plating for 1 h on VN. Bright and round cells were counted as nonattached, whereas dark-appearing cells (asterisks in C) were counted to obtain curves as depicted in D and E. (D and E) Spreading curves of transfected NIH-3T3 cells on VN (D) and FN (E), expressing the indicated construct. (F) Epifluorescence images of mock or β3-GFP-integrin–transfected NIH-3T3 cells plated for 1 h on VN or FN and stained for substrate-bound β1 integrin revealed by mAb 9EG7 staining. Note the recruitment of β1 integrins into CMAs, in mock-transfected cells on both VN and FN substrates and absence of CMA-recruited β1 integrins β3-GFP-integrin–expressing cells on VN. On FN, wild-type, but not Y747A, β3-GFP-integrin prevented the recruitment of β1 integrin into CMAs. (G) TIRF images of wild-type or Y747A β3-GFP-integrin cotransfected with talin1-mRFP or stained for endogenous ligand-bound β1 integrins after 6 h of spreading on VN. Insets correspond to magnified views of the boxed area as well as the respective GFP signal in this location, when indicated. wt, wild type. Bars: (C) 100 µm; (F and G, main images) 10 µm; (F and G, insets) 5 µm.
Spreading defect of Y\textsuperscript{747}A mutant β\textsuperscript{3} integrins on VN but not FN

To analyze integrin recruitment to CMAs during the initial spreading phase, ligand-bound α5β\textsuperscript{1} integrin was detected with mAb 9EG7 staining, and clustered αβ3-GFP-integrin was detected by epifluorescence and total internal reflection fluorescence (TIRF) imaging (Fig. 1, F and G). Although mAb 9EG7 staining was detected in CMA in the few mock-transfected cells spread on VN after 1 h, 9EG7 staining appeared diffuse in wild-type β3-GFP-integrin–transfected cells spread on VN and FN (Fig. 1 F). Thus, at early time points, high levels of β3-GFP-integrin prevented recruitment of endogenous α5β\textsuperscript{1} integrin into CMAs on both ligands. In contrast, the Y\textsuperscript{747}A mutant β3-GFP-integrin failed to cluster, blocked the formation of α5β\textsuperscript{1}-containing CMAs, and prevented initiation of spreading when plated for 1 h on VN (Fig. 1 F, bottom left image). However, the same cells recruited α5β\textsuperscript{1} into CMAs when spread on FN (Fig. 1 F). These data suggested ligand-specific initiation of spreading via αβ3-GFP-integrin on VN, which was not compensated by endogenous integrins (such as α5β\textsuperscript{1}) at early time points. After 6 h on VN, wild-type β3-GFP-integrin colocalized with talin1-mRFP (Fig. 1 G), whereas Y\textsuperscript{747}A mutant β3-GFP-integrin remained diffuse in the membrane without colocalization to talin1-mRFP or mAb 9EG7-reactive (α5β\textsuperscript{1} containing) CMAs (Fig. 1 G). This suggests that cells secreted their own FN, which rescued spreading in cells expressing talin-binding-defective integrins by endogenous α5β\textsuperscript{1} integrins.

Talin binding and integrin activation are critical for spreading initiation

To characterize additional β\textsuperscript{3} integrin tail residues required for cell spreading on VN, we expressed several talin-binding variants with mutations affecting both the W/NPLY\textsuperscript{747} and membrane-proximal motifs. Initiation of spreading was suppressed with all mutations known to affect talin binding to β3 integrin, including Y\textsuperscript{747}A, L\textsuperscript{746}A, W\textsuperscript{739}A, W\textsuperscript{739}/A/Y\textsuperscript{747}A, E\textsuperscript{748}K, and F\textsuperscript{750}A (Fig. S2). The W\textsuperscript{739}/A/Y\textsuperscript{747}A double mutant had the strongest negative effect on cell spreading, confirming the link between talin binding and cell spreading (Fig. S2). In contrast, the mutational unclamping of the inhibitory salt bridge (D\textsuperscript{723}A), which enhances integrin activation and clustering (Tadokoro et al., 2003; Cluzel et al., 2005), slightly enhanced the spreading response on VN (Fig. S2). This confirmed the link between talin-mediated integrin activation, clustering, and spreading.

High-affinity talin-integrin association does not guarantee initiation of cell spreading

Because integrin–talin interaction appeared to be critical for integrin-mediated spreading, we searched for ways to experimentally dissociate talin-mediated integrin activation from the mechanisms that induce integrin-mediated spreading (Fig. S2). To maintain integrin activation and talin linkage even in the absence of spreading, we decided to create integrin mutants with enhanced talin-binding affinity. We designed chimeric β3-GFP-integrins, in which the W/NPLY\textsuperscript{747} motif (K\textsubscript{d} = 0.3 mM; Anthis et al., 2010) was replaced with known high-affinity talin-binding motifs. Based on the interactions of layilin and PIPK-Iγ with the talin F3/phosphotyrosine-binding (PTB) domain (Barsukov et al., 2003; de Pereda et al., 2005; Kong et al., 2006; Wegener et al., 2008), two chimeras, β3-VE (layilin chimeric; similar to a β1D-derived chimera with K\textsubscript{d} = 17 nM for talin2; Anthis et al., 2010) and β3-SPLH (PIP1γ-chimeric; K\textsubscript{d} = 0.27 μM for a nonphosphorylated peptide; de Pereda et al., 2005), were designed (Fig. 2, A and B). Although these chimeras led to integrin activation (Fig. 2 E) and colocalization with talin1-mRFP in CMAs (Fig. 2, F and G), only the β3-VE chimera induced spreading on VN comparable to β3 wild type (Fig. 2 C). In stark contrast, the β3-SPLH chimera, despite enhanced binding to talin, prevented spreading similar to the Y\textsuperscript{747}A mutant (Fig. 2 C).

Y\textsuperscript{747} is required for spreading but not for high-affinity talin binding of the β3-VE chimera

To further characterize the spreading behavior and high-affinity talin interactions of the β3-VE chimera, we asked whether Y\textsuperscript{745} in the WVENPLY\textsuperscript{745} sequence plays a similar role in spreading initiation and talin binding as Y\textsuperscript{747} in the original β3 integrin sequence. To analyze this, we created the VE/Y\textsuperscript{745}A mutant (WVENPLA\textsuperscript{745}K), which showed reduced activation levels in respect to VE, which were however comparable to wild-type β3-GFP-integrin (Fig. 3, A and B). Nevertheless, the Y\textsuperscript{745}A mutation in the β3-VE chimera blocked spreading similar to the Y\textsuperscript{747}A mutation in β3 (Fig. 3 C). In contrast to the Y\textsuperscript{747}A mutant, GST–talin head pulled down the β3-GFP–VE/Y\textsuperscript{745}A mutant comparable to β3-wt, β3-SPLH, and β3-VE (Fig. 3 D and Fig. S3), despite variations of integrin expression levels observed in this experiment. To provide a more quantitative measure of binding affinities of these mutants, we performed biosensor experiments with immobilized, His-tagged talin head (1–406 aa) and purified wild-type or mutant GST–β3-tail fusion proteins. These experiments revealed a ~20-fold higher affinity of the β3-VE chimera and a ~10-fold higher affinity of the β3-VE/Y\textsuperscript{745}A mutant over wild-type β3-tail (Fig. 3 E), demonstrating that Y\textsuperscript{745} is no longer critical for talin binding in the context of the β3-VE chimera. Consistent with strong talin binding, the β3-VE/Y\textsuperscript{745}A mutant colocalized with talin1-mRFP (Fig. 3 F). This links talin binding to integrin activation and recruitment to focal adhesions but fails to reveal a direct role of talin during integrin signaling and spreading on VN.

Mutations of kindlin-binding motifs affect cell spreading

Kindlin-3 is required for platelet spreading (Moser et al., 2008). Although kindlins do not interact with the NPLY\textsuperscript{747} talin-binding motif, kindlins stimulate talin-mediated integrin activation (Ma et al., 2008), which could be essential to subsequent initiation of cell spreading. To study the role of kindlin in the regulation of cell spreading, both the distal NITY\textsuperscript{759} (Y\textsuperscript{759}A) and inter-NxxY (S\textsuperscript{756}P) kindlin-binding motifs (Ma et al., 2008; Moser et al., 2008) were mutated. Expression of both the β3-Y\textsuperscript{759}A- and β3-S\textsuperscript{756}P-GFP-integrin was slightly lower than wild type (Fig. 4 A) but caused strong β3 integrin activation defects...
Spreading-defective, high-affinity talin-binding integrins fail to recruit paxillin

Because integrin mechanosensing is associated with FAK phosphorylation (Friedland et al., 2009), which colocalizes with phosphopaxillin in nascent adhesions (Choi et al., 2011), we asked how paxillin, which is a master regulator of adhesion-mediated signaling and actin remodeling (Deakin and Turner, 2008, 2011), is recruited to spreading-defective integrins. Thus, endogenous paxillin localization was studied by TIRF immunofluorescence in β3-GFP-integrin–transfected cells spreading on VN (Fig. 5).

Paxillin efficiently localized to CMAs formed by wild-type and β3-VE chimera at 1 and 4 h (Fig. 5, A, B, and E). In contrast, paxillin colocalized only partially with β3-GFP-SPLH chimera and β3-GFP-Y745A mutant integrins in lamellipodial protrusions of spreading cells (Fig. 5, C–E). Determination of the Mander’s colocalization coefficient revealed consistently fewer β3-GFP-integrin clusters at sites of paxillin staining (Fig. 5 E), suggesting that paxillin fails to be recruited to spreading-incompetent, but talin-bound, integrins. Accordingly, such reduced recruitment could prevent or slow down the reinforcement of

**Figure 2.** Differential cell spreading by high-affinity talin-binding β3-GFP-integrins. (A) Sequence alignment of β3 integrin and the high-affinity talin-binding sequences from layilin and PIPKι-γ. Gray shading defines identical or conserved residues. (B) Sequences of the chimeric β3-GFP-integrins [β3-VE with the VE motif from layilin and β3-SPLH derived from PIPKι-γ]. Bold indicates mutated residues, and boxed letters correspond to the name of the integrin chimeras. (C) Spreading curves of NIH-3T3 cells transiently transfected with β3-GFP-VE and β3-GFP-SPLH chimera on VN and comparison with wild-type and spreading-deficient Y747A mutant β3-GFP-integrin. Double arrow indicates differential spreading of high-affinity chimeras. (D and E) β3 integrin cell surface expression levels of β3-GFP-chimera in NIH-3T3 cells [D] and increases in the β3 integrin activation index in the chimeras [E]. Numbers indicate fold increase in the activation index. (F and G) Representative TIRF images of NIH-3T3 cells spread for 6 h on 1 µg/ml VN, transiently transfected with β3-GFP-SPLH (F), β3-GFP-VE chimera (G), and talin1-mRFP. Note the talin1 colocalization with both chimeras, despite a delay in cell spreading for β3-GFP-SPLH. Error bars show standard error. wt, wild type. Bars: (main images) 25 µm; (insets) 12.5 µm.
whereas β3-GFP-integrin remained restricted to VN surfaces (Fig. 6 A), A similar situation was seen with the β3-GFP-VE chimera, with the difference that more GFP staining (integrin fusion partner) was detected on FN-coated surfaces (Fig. 6 B). However, a different image was seen in cells transfected with the β3-GFP-SPLH chimera (Fig. 6 C) or β3-GFP-VE/Y745A mutant (Fig. 6 D). GFP-positive CMAs localized on VN surfaces failed to recruit paxillin, whereas paxillin-enriched CMAs located on FN patches were devoid of GFP fluorescence (Fig. 6, C and D). This shows that β3-SPLH chimera and the β3-VE/Y745A mutant interact with VN, talin, and F-actin but fail to recruit paxillin, potentially causing the observed defect in cell spreading on VN.

Separation of integrin-dependent adhesion and mechanosensing on soft substrates

In addition to spreading, integrin signaling operates during mechanosensing, during which a tension-controlled α5β1 integrin nascent adhesions, leading to the observed delay in cell spreading (Fig. 5 F). Although the absence of paxillin is a valid explanation for the delay in spreading, the analysis is flawed by endogenous FN secretion and α5β1 integrin recruitment to lamellipodia, which could support substrate binding and integrin signaling during spreading. Thus, to circumnavigate this obstacle, and to physically separate α5β1 from αvβ3 integrin–mediated signaling during spreading, cells were plated on patterned substrates consisting of FN patches surrounded by a VN-coated surface (Fig. 6 E). Irrespective of the expressed β3-GFP-integrin construct, cells spread on this pattern, using endogenous α5β1 integrins for adhesion formation (paxillin recruitment) on FN patches. In the presence of wild-type β3-GFP-integrin, cells preferably spread on VN-coated surfaces, efficiently recruiting paxillin to CMAs (Fig. 6 A). When CMAs extended over both VN- and FN-coated surfaces, paxillin was recruited irrespective of the substrate borders, whereas β3-GFP-integrin remained restricted to VN surfaces (Fig. 6 A, inset). A similar situation was seen with the β3-GFP-VE chimera, with the difference that more GFP staining (integrin fusion partner) was detected on FN-coated surfaces (Fig. 6 B). However, a different image was seen in cells transfected with the β3-GFP-SPLH chimera (Fig. 6 C) or β3-GFP-VE/Y745A mutant (Fig. 6 D). GFP-positive CMAs localized on VN surfaces failed to recruit paxillin, whereas paxillin-enriched CMAs located on FN patches were devoid of GFP fluorescence (Fig. 6, C and D). This shows that β3-SPLH chimera and the β3-VE/Y745A mutant interact with VN, talin, and F-actin but fail to recruit paxillin, potentially causing the observed defect in cell spreading on VN.

Separation of integrin-dependent adhesion and mechanosensing on soft substrates

In addition to spreading, integrin signaling operates during mechanosensing, during which a tension-controlled α5β1 integrin
CMAs on FN-coated glass and easily detected on PAA gel surfaces (Fig. S4), suggesting that both the affinity between talin and integrins as well as the physical state of the extracellular ligand controls the conformation of the integrin and thereby its recruitment into CMAs.

To test whether high-affinity talin-binding integrins induce spreading on very soft substrates that would not allow β1-dependent spreading (<3 kPa; Yeung et al., 2005), spreading of β3-V5– and β3-GFP-SPLH–transfected fibroblasts was performed on soft (1.5 kPa) FN-coated PAA gels (Fig. 7). When plated for 2.5 h, efficient cell spreading, integrin clustering, and stress fiber formation was observed on both soft (1.5 kPa) and stiff (30 kPa) PAA substrates in the case of the β3-V5 chimera (Fig. 7, A and C). However, cells transfected with the β3-GFP-SPLH chimera failed to completely spread and flatten on soft substrates, despite the recruitment of this integrin into CMAs (Fig. 7 B and Fig. S4). In addition, SPLH-transfected cells exhibited characteristic filopodial adhesions, containing clustered integrins linked to F-actin bundles (Fig. 7 B) but failed to spread and form actin stress fibers. In contrast, on stiffer (30 kPa)
The causal link between the mechanical, integrin-mediated connection from the cytoskeleton to the ECM and reciprocal intracellular signaling is critical for morphogenesis and tissue homeostasis and is mirrored in concepts such as anoikis, metastasis (Zouq et al., 2009; Taddei et al., 2012), or FAK activation in cells exposed to rigid ECM (Paszek et al., 2005; Friedland et al., 2009). Here, we provide a model of how integrin-mediated adhesions recruit signaling adapter proteins such as paxillin to regulate cell spreading. We identified integrin mutants that, despite FN-coated PAA gels, on which endogenous α5β1 integrin signaling is active, cell spreading occurred and the β3-GFP-SPLH chimera localized to peripheral and central CMAs (Fig. 7 D and Fig. S4), associated with the formation of stress fibers. This demonstrates that the talin–integrin affinity controls the mechanical stability and tension range in which a given integrin–ligand combination is operational. On the other hand, integrin mechanosignaling requires recruitment of adapter proteins, such as paxillin, to appropriately presented NPLY747 motifs, to induce spreading, mechanosensing, and the subsequent remodeling of the actin cytoskeleton.

**Discussion**

The causal link between the mechanical, integrin-mediated connection from the cytoskeleton to the ECM and reciprocal intracellular signaling is critical for morphogenesis and tissue homeostasis and is mirrored in concepts such as anoikis, metastasis (Zouq et al., 2009; Taddei et al., 2012), or FAK activation in cells exposed to rigid ECM (Paszek et al., 2005; Friedland et al., 2009). Here, we provide a model of how integrin-mediated adhesions recruit signaling adapter proteins such as paxillin to regulate cell spreading. We identified integrin mutants that, despite
Figure 6. Analysis of paxillin recruitment on FN/VN patterned substrates. (A–D) Merged RGD planes of SIM images of NIH-3T3 cells spread for 4 h on FN/VN patterned substrates, representing the FN-coated surface (squares) and VN-coated surfaces without stain (grid). Cells express wild-type β3-GFP-integrin (A), β3-GFP-VE (B), β3-GFP-SPLH (C), and β3-GFP-VE/Y745A (D), and antipaxillin reactivity. Magnified views of the boxed areas in A–D showing β3-GFP (in green) and antipaxillin staining (magenta) localized over FN-coated areas (dotted squares). Note the colocalization of antipaxillin staining with β3-GFP-integrin clusters on VN and endogenous integrins on FN surfaces in A and B but the absence of antipaxillin staining in β3-GFP-integrin clusters on VN in C and D (asterisks). pax, paxillin. (E) Alexa Fluor 647–labeled FN- and anti-VN–stained patterns reveal the specificity of the coating strategy. (F) Schematic view of integrin signaling complexes in respect to integrin ligand and mutation. Integrins 1 and 5 correspond to α5β1, and 3 and V correspond to αvβ3. T, talin; P, paxillin; K, kindlin. Images in A–D were taken from one out of three similar experiments. Bars: (A–D) 20 µm; (E) 10 µm; (insets) 4 µm.
Figure 7. **Cell spreading on soft and stiff FN-coated PAA gels.** (A–D) ApoTome images of NIH-3T3 cells plated for 2.5 h on FN-functionalized (1 mg/ml) soft (1.5 kPa; A and B) and stiff (30 kPa; C and D) PAA gels, transiently transfected with β3-GFP-VE (A and C) and β3-GFP-SPLH chimeras (B and D). Cells were fixed and stained for F-actin (middle column) or imaged for GFP expression (left column). A magnified view of the boxed areas in A–D is shown on the right. Note the spreading of β3-GFP-VE–transfected cells on soft, FN-coated gels, whereas β3-GFP-SPLH accumulates in filopodial CMAs in round, nonspread cells. Please note that the UV-induced FN-coating method was used in this figure, which was repeated three times with similar phenotypes. Act, actin; mer, merge. Bars: (main images) 20 µm; (insets) 5 µm.
enhanced talin-binding and focal adhesion formation, failed to induce cell spreading and to recruit paxillin, a critical player in cell spreading, FAK activation, and regulation of Rho family GTPases (Wade et al., 2002; Deakin and Turner, 2008, 2011; Choi et al., 2011). We found that paxillin recruitment can be blocked by tyrosine mutation in the highly conserved talin-binding W/NPLY motif, even when bound to talin as in the V/E/Y745A chimera, suggesting that paxillin is recruited to β integrin tails when presented in a talin-bound state. In addition, adjacent β integrin tail sequences, known to bind kindlin are also required for cell spreading, thus proposing an explanation for the critical role of kindlins in immune defense, morphogenesis, and tumor growth (Moser et al., 2009; Pluskota et al., 2011; Sin et al., 2011). Furthermore, high-affinity talin-binding integrins induced cell adhesion and spreading, on soft, spreading-repellent ECM, proposing that mechanosensing of the ECM is directly linked to the stability of the integrin–talin complex and its ability to recruit signaling adapter proteins. These findings are key for evaluating and predicting the behavior of normal and tumor cells in response to pathological changes in matrix stiffness (Paszek et al., 2005; Engler et al., 2006), while offering new therapeutic strategies to differentially controlling integrin-mediated adhesion versus adhesion signaling.

Linking the integrin-talin-kindlin complex to cell spreading

Talin has emerged as the critical regulator of integrin activation and clustering (Tadokoro et al., 2003; Saltel et al., 2009). In addition, knockdown of talin or cell type–specific knockouts demonstrated a critical role in adhesion signaling, synergies with growth factors, and cell spreading (Miyamoto et al., 1995, 1996; Monkley et al., 2000, 2011; Petrich et al., 2007b). Moreover, the correlation between talin- and kindlin-mediated αIβ3 integrin activation, platelet spreading, and blood clotting proposes that the integrin–talin–kindlin complex induces intracellular signaling (Montanez et al., 2008; Moser et al., 2008, 2009). Interestingly, distinct platelet-spreadig and bleeding phenotypes are observed between two β3 integrin mutants similarly defective in talin binding (Y747A, strong bleeding defect, and L746A, weak effect; Petrich et al., 2007a). In light of our data, these phenotypes might be caused by defects in integrin-mediated spreading.

Kindlins contribute to integrin activation (Ma et al., 2008), which involves binding to the inter NxxY region and distal NxxY motif (Bledzka et al., 2012; Yates et al., 2012). However, kindlins also mediate integrin signaling because the deletion of kindlin-2 in embryonic stem cells blocks spreading even in the presence of Mn2+-activated integrins (Montanez et al., 2008). Thus, we propose that the distinct roles of kindlin in integrin activation and signaling are reflected by the Y759A and S752P mutations, respectively, showing differing phenotypes when combined with the activating D753A mutation (Fig. 4 C). However, whether loss of kindlin interaction is indeed responsible for the phenotypes of these mutants needs to be determined.

Recruitment of signaling adapters and mechanosensing

Although a paxillin domain deletion study identified LIM (Lin-11, Isl-1, and Mec-3) domains 2 and 3 as the focal adhesion–targeting site, it is still not understood how paxillin is recruited to focal adhesions (Brown et al., 1996; Deakin and Turner, 2008). In fact, the absence of paxillin recruitment to lamellipodia in talin knockdown cells suggested that talin could be involved in paxillin recruitment (Zhang et al., 2008). Our data suggest that paxillin is recruited in a kindlin-regulated manner, either directly or indirectly to an interface formed by the talin-bound NPLY peptide. Although paxillin is recruited through its C-terminal LIM domains, the N-terminal LD domains can interact with multiple structural and signaling focal adhesion proteins, such as vinculin, parvin, FAK, and the GIT–PIX–PAK–NCK complex, creating an integrin-bound signaling nexus (Deakin and Turner, 2008). Moreover, phosphorylation of paxillin is required for FAK recruitment to nascent adhesions (Choi et al., 2011), suggesting that paxillin targets FAK to focal adhesions.

In contrast to this model, it was recently proposed that FAK recruits talin to nascent adhesions (Lawson et al., 2012). However, this is not consistent with FAK recruitment to integrin–talin complexes in filopodia (Partridge and Marcantonio, 2006) and the localization of FAK and vinculin to CMA in response to the C-terminal talin rod domain (Wang et al., 2011). The talin rod domain exhibits numerous vinculin binding sites (Gingras et al., 2005), which are activated by mechanical force (Hyttönen and Vogel, 2008; del Rio et al., 2009), thereby linking mechanosensing and FAK phosphorylation at Y397 to tensional stress along the ECM–integrin–talin–F-actin axis (Shi and Boettiger, 2003). Notably, our data also explain the phenotype of the myosporoid180A mutant in Drosophila melanogaster, lacking both NPXY motifs but retaining the membrane-proximal talin–integrin binding site, which enables recruitment of talin but not that of paxillin to muscle attachment sites (Tanentzapf and Brown, 2006).

In addition to the cytoplasmic tail of integrins, the synergy site in FN, as well as the catch bond of the α5β1 integrin, is involved in mechanosensing, during which α5β1 integrin is a specific force transducer that can induce FAK signaling (Friedland et al., 2009; Kong et al., 2009; Boettiger, 2012). Modifying the cell type–specific force regimen by either softening or stiffening the ECM affects spreading and adhesion signaling (Yeung et al., 2005) as well as the differentiation of stem cells (Engler et al., 2006). Different adhesion strength between α5β1 and αvβ3 led to the proposal that α5β1 is mainly adhesive, whereas αvβ3 mediates mechanosensing (Roca-Cusachs et al., 2009). However, this idea contrasts with a study showing mechanosensing activity for both integrins (Shi and Boettiger, 2003). Thus, it is possible that these two integrins work under different force regimes. Although αvβ3 integrin enables spreading on stiff substrates, such as bone matrices and cross-linked tumor stroma (Paszek et al., 2005), α5β1 integrin functions within soft ECM conditions. That the β3-VE-integrin induces robust cell spreading on soft FN proposes that high talin–integrin affinity can compensate for weakly tethered integrin ligands, thus tuning mechanosensing to a different tensional state of the ECM.
high-affinity structures, the W-V-Yp-S/N-P/E-L/I peptide forms a hydrophobic clamp (underlined) stabilized by electrostatic interactions between E/Yp and K357/R358 of talin (Fig. 8A). On the one hand, this high-affinity binding motif enables talin binding in the absence of Y747, while on the other hand, precisely positioning Y747 into a shallow hydrophobic pocket, a conformation that is also adopted for binding of α1D to talin2 (Fig. 8A; Anthis et al., 2009). Interestingly, this shallow binding of Y747 creates a hydrophobic surface, potentially enabling interactions with integrin-, PIPKI-γ-, and layilin-derived peptides (Fig. 8A), the proposition of a spreading-competent integrin peptide configuration should be possible. Structures of the high-affinity talin-binding peptides of PIPKI-γ (WVYpSLHYSA; Barsukov et al., 2003; de Pereda et al., 2005; Kong et al., 2006; Wegener et al., 2007) as well as of layilin (WVENEIYY; Wegener et al., 2008) have confirmed the role of W739 (W775 in α1 integrin) to bind to a hydrophobic pocket created by talin R358 (R361 in talin2; García-Alvarez et al., 2003; Anthis et al., 2009). Moreover, in these high-affinity structures, the W-V-E/Yp-S/N-P/E-L/I peptide forms a hydrophobic clamp (underlined) stabilized by electrostatic interactions between E/Yp and K357/R358 of talin (Fig. 8A). On the one hand, this high-affinity binding motif enables talin binding in the absence of Y747, while on the other hand, precisely positioning Y747 into a shallow hydrophobic pocket, a conformation that is also adopted for binding of β1D to talin2 (Fig. 8A; Anthis et al., 2009). Interestingly, this shallow binding of Y747 creates a hydrophobic surface, potentially enabling interactions with...
signaling adapters, such as paxillin (Fig. 8 B). For this site to be accessible, however, the integrin peptide needs to be in a flattened conformation. This is prevented in the PIPK1-γ-derived peptide (β3-3SplH chimera), as a result of looping of the integrin-bound peptide (Wegener et al., 2007), and thereby potenti- 
al obstructing access for signaling adapters. Such a scenario would explain the lack of spreading by the β3-3SplH chimera as well as the critical role of kindlin in binding the C-terminal integrin peptide to allow unrestricted access of a signaling adapter to the talin-bound NPLY peptide (Moser et al., 2008; Harburger et al., 2009; Bledzka et al., 2012; Yates et al., 2012). Consistent with this model, the D740TAN sequence of PIPK1-K738WVENPLY745KEAT (lined; Saltel et al., 2009) was obtained by replacing the D740TAN sequence of PIPK1- 
duced by primer overlap extension and verified by automated sequencing. Integrin point mutations were intro-
A cytomegalovirus promoter–driven pcDNA3/EGFP vector has been previ-
ously described (Ballestrem et al., 2001). Integrin point mutations were intro-
duced conformation no longer compatible with kindlin binding (Ma et al., 2008) and adapter recruitment, such as paxillin. Thus, we prop-
estral OBRA (University of Wisconsin School of Medicine, Madison, WI). A red fluorescent version was generated by exchanging EGFP with a 
cytokine-driven pcDNA3/mRFP vector has been previ-
ously described (Ballestrem et al., 2001). Integrin point mutations were intro-
duced by primer overlap extension and verified by automated sequencing. The β3-3SplH chimera with the high-affinity, talin-binding sequence from PIPK1-γ was constructed by replacing the β3-NPLY3 motif with residues from the C-terminus of PIPK1-γ (Wegener et al., 2007): K738WDTANNPLY747KEAT (β3 integrin) to K738WVYSPN740SFAT (β3 chimeric; modified residues underlined; Saltel et al., 2009). The β3-VE talin-binding, high-affinity chimera was obtained by replacing the D740TAN sequence of β3 integrin with the VE sequence from the fynkin protein: K738WDTANNPLY747KEAT (β3 integrin) to K738WVENPLY745KEAT (β3-VE chimera). Full-length mouse N-terminal EGFP-tagged talin 1 was obtained from A. Huttenlocher (University of Wisconsin School of Medicine, Madison, WI). A red fluorescent version was generated by exchanging EGFP with mRFP (talin 1-mRFP) and expressed in a pcDNA3 vector under the cytomeg-
avirus promoter control. GST–talin head (1–435 aa) and GST–β3-3peil (716–762 aa) fusion constructs used for pull-down and Octet biosensor ex-
periments were obtained after cloning of PCR-amplified fragments of the C terminus of PIPK1-K738WVENPLY745KEAT (lined; Saltel et al., 2009). Empty pGex-2T vector was used to produce GST for control experiments. His-tagged human talin head was generated by inserting residues 1–406 of human talin 1 into the pTrcHisC vector at the BamHI site. DNA sequence analysis was performed for all 
sequence analysis was performed for all 
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with noncoupled glutathione-Sepharose 4B beads (GE Healthcare) at 4°C for 1 h. Pre cleared lysates where then incubated at 4°C for 1 h with GST or GST–talin head-loaded glutathione beads, obtained by incubation with bacterial lysates according to standard protocols. After incubation, beads were washed three times with lysis buffer and boiled in standard reducing SDS-PAGE sample buffer. SDS-PAGE and Western blotting were performed according to standard protocols. GFP-tagged proteins were detected with mouse anti-EGFP mAb (Covance) and revealed by anti–mouse HRP-coupled antibody (Jackson ImmunoResearch Laboratories, Inc.).

**Recombinant protein purification**

Expression of GST-β3-tail chimeras and GST control protein in Escherichia coli BL21 Star cells (Invitrogen) was induced by 1 mM IPTG for 5 h at 37°C, after which the collected cells were lysed by homogenization (EmulsiFlex C3; Avestin, Inc.) into PBS. After clarification by centrifugation, lysates were incubated in glutathione–Sepharose [4 Fast Flow; GE Healthcare] suspension overnight. After washing with PBS, proteins were eluted using 50 mM Tris-HCl and 20 mM reduced glutathione, pH 8, and dialyzed into 50 mM sodium phosphate buffer (150 mM NaCl, pH 7.2) before analysis by SDS-PAGE, Coomassie blue staining, and concentration determination by UV/visible spectrometry (A280).

His-tagged tail head domain (residues 1–406) was produced in BL21 Star cells as described in the previous paragraph for GST-integrins. Cells were lysed in 20 mM sodium phosphate buffer (1 M NaCl and 20 mM imidazole, pH 7.4). After clarification by centrifugation, lysates were loaded into affinity columns (HiTrap FF; GE Healthcare) using a liquid chromatography system (ÄKTA Purifier; GE Healthcare), washed, and eluted with a linear imidazole gradient 0–700 mM. Eluted fractions were further purified by cation exchange chromatography using HiTrap SP FF columns (GE Healthcare), by loading pooled peak fractions diluted 1:10 in 20 mM Tris-HCl and 20 mM NaCl, pH 7.5. The bound proteins were eluted with a linear NaCl gradient in the loading buffer. Elution of tail head was observed at ~550 mM NaCl. Eluted fractions were concentrated by a 30-kD filter, analyzed by SDS-PAGE and Coomassie blue staining and estimated to be >95% pure.

**Octet biosensor analysis**

 Biosensor assays were performed on a ForteBio Octet RED384 instrument (ForteBio Life Sciences) using a Ni–nitrilotriacetic acid (NTA) sensor chip. A temperature of 25°C and a stirring speed of 1,000 rpm were used throughout the experiment. Sensors were chemically activated by immersing them in 0.1 M EDC and 0.05 M N-hydroxysuccinimide (NHS; Thermo Fisher Scientific) in H2O for 100 s. 50 µg/ml His–tail head was applied in 50 mM NaPO3 and 150 mM NaCl, pH 7.2, resulting in a binding response of ~8 nm after 300 s. The remaining activated groups were then quenched by 1 M ethanolamine, pH 8.5, for 100 s. Control experiments were performed without cross-linker, demonstrating identical results, requiring, however, more extensive baseline corrections as a result of leakage of tail head from the Ni–NTA.

To obtain relative affinities of different GST-β3-tail chimeras for tail head, specifically SPI-3 GST fusion proteins were applied on the tail-coated sensors in concentrations of 20–1,250 nM. GST binding to the sensor was measured for 300 s before applying the next higher protein concentration.

**Microcontact printing of patterned substrates**

Masters and stamps for microcontact printing were produced as previously described (Jehnert et al., 2004). In brief, masters were fabricated from silicon wafers by low-voltage electron beam lithography using a positive tone. The resulting resist pattern was inverted using a lift-off process and reactive ion etching to yield a master with rectangular, 650-nm-deep holes in the silicon surface. Silicon stampes were produced by the thin stamp technique using Syylgard 184 (Corning).

Stamps were incubated with nine parts unlabeled human FN at 5 µg/ml and one part Alexa Fluor 647–labeled bovine FN (protein-labeling kit; Life Technologies) for 10 min. After drying under nitrogen, stamps were pressed onto glass coverslip for 10 min. Protein-free regions were coated by incubation with 5 µg/ml human VN (Sigma-Aldrich) for 1 h. Coverslips were rinsed once with PBS before seeding and culturing of transiently transfected NIH-3T3 cells in DMEM containing 10% FCS. After 4 h, cells were fixed and stained with antipaxillin as described in the following paragraphs.

**Structured illumination microscopy (SIM)**

SIM was performed at room temperature with a Plan Achromat 63x, 1.40 NA oil differential interference contrast objective (Carl Zeiss) on a nonmolecular prototype (ELVRA PS1). Images were acquired in superresolution SIM mode, comparable to a commercial PS1 microscope.

**PAA gel cell culture substrates**

Thin PAA gel cell culture substrates were made according to published protocols (Buxboim et al., 2010). Cleared and dried glass coverslips were aminosilanized under vacuum for 1 h with (3-aminopropyl)triethoxysilane (Sigma-Aldrich). Coverslips were then activated with 0.5% glutaraldehyde (in PBS) for 30 min followed by extensive washing with H2O. Degasosed acrylamide/bisacrylamide mixtures were prepared in 50 mM Hepes-buffered solution to obtain soft (5/0.025% ~1.5 kPa) and stiff gels (8/0.1% ~30 kPa; Yeung et al., 2005; Tse and Engler, 2010) and mixed with ammonium persulfate and tetramethylethylenediamine (Sigma-Aldrich) to final concentrations of 0.06% wt/vol and 0.4% vol/vol, respectively. Mixtures were immediately added onto the glutaraldehyde-activated coverslips, covered with a cleaned, nontreated glass coverslip, and left to polymerize overnight at ~500 µm thick after polymerization, coverslip removal, and rinsing, gel surfaces were incubated with the cross-linker Sulfo-SANPAH (Thermo Fisher Scientific; 1 mg/ml in 50 mM Hepes; 80 µl per coverslip). The cross-linker-coated gels were photoactivated by exposure to UV for 2 x 1.5 min, quickly rinsed, and then incubated with a 1-mg/ml FN (YQ Proteins) solution for 1 h at 37°C.

To compensate for lower FN cross-linking with UV-activated activation on soft PAA gels, we also used an alternative FN cross-linking strategy (Rajagopalan et al., 2004) that provided similar results (Fig. S4). During the polymerization of the PAA gels, 59 µM NHS acrylic acid was included into acrylamide/bisacrylamide mixture. FN was cross-linked by covering the polymerization mixture with coverslips first coated with 30 mg/ml BSA and subsequently with 250 µg/ml FN. During polymerization, the acrylic acid/BSA mixture incorporated into the PAA gel and simultaneously cross-linked the FN onto the surface of the gel. For both procedures, gels were washed in PBS and conditioned for 1 h before the addition of cells in HSA-containing DMEM.

**Immunostaining of fixed cells**

Transfected transiently transfected cells were plated for the indicated time on specific substrates (VN- or FN-coated glass or FN-coated PAA gels), fixed with 4% PFA/PBS, and washed with PBS. Cells were blocked and permeabilized for 30 min with a solution of PBS containing 1% BSA and 0.2% Triton X-100. The primary antibody was diluted, applied in PBS containing 1% BSA and incubated for 3 h at room temperature. After washing, the incubation was subjected for 30 min with the secondary antibody in the same buffer. Finally, cells were washed, imaged, and stored in PBS.

The following antibodies were used for the different experiments: mouse mAb–anti–chicken paxillin (clone 349; BD; reacting with mouse paxillin), rat mAb anti–mouse β1 integrin (clone 9EG7; BD), mouse IgM anti-VN (Sigma-Aldrich), mouse mAb antivinculin (V9131; Sigma-Aldrich), rabbit polyclonal antibody to FN (1801; gift from M. Chiquet, University of Bern, Bern, Switzerland), DyLight 549–conjugated goat–anti–mouse (Jackson ImmunoResearch Laboratories, Inc.), Cy3-conjugated goat anti–mouse (BD), Cy3-conjugated goat anti–mouse IgM (Dianova), Texas red–conjugated goat anti–rabbit (Jackson ImmunoResearch Laboratories, Inc.), and Texas red–conjugated goat anti–rat (Jackson ImmunoResearch Laboratories, Inc.). F-actin staining was performed using an Alexa Fluor 546–conjugated phalloidin (Molecular Probes).

Epifluorescence images of stained cells were taken in PBS at room temperature, using a 63x, NA 1.4 oil immersion objective on a microscope (Axiovert 100M) equipped with a 10-bit charge-coupled device camera (Orcia 9742–95; Hamamatsu Photonics) and the Openlab software (PerkinElmer). Cells grown on PAA gels were mounted in Vectashield and visualized with an microscope (AxioImager ApoTome; Carl Zeiss) equipped with a camera (AxioCam MRm; Carl Zeiss) using a 63x, NA 1.25 or 40x, NA 1.3 Plan Neofluor oil immersion objective (Carl Zeiss).

**Antipaxillin colocalization analysis**

TIRF images of β3-GFP–integrin and antipaxillin DyLight 549 fluorescence were acquired sequentially. Before colocalization analysis and when required, images were aligned using the RGD alignment plugin in Fiji (National Institutes of Health; Schindelin et al., 2012). Then, a mask was created to analyze only the peripheral areas of the cells containing lamellipodial and focal extensions. Using this mask, the Fiji colocalization threshold plugin was used to calculate the Manders’ colocalization coefficient with an automatic threshold to determine the degree of GFP fluorescence at sites of paxillin staining. A box and whisker plot was drawn using Prism (GraphPad Software) to represent the distribution of the obtained data (n = 30–50 cells per condition), which was however biased to maximal colocalization in cells expressing high levels of integrins. This was particularly relevant for the β3-GFP–SPLH chimera at 4 h, at which relatively large numbers of high-expressing cells were present (Fig. 6 E).


Kieffer, N., C. Melchior, J.M. Guinet, S.Michels, V. Gouon, and N. Bron. 1996. Serine 752 in the cytoplasmic domain of the beta 3 integrin subunit is


Figure S1. **Evaluation of β3 integrin expression on various mouse fibroblast cell lines.** (A) FACS histogram of different mouse fibroblast cell lines, mouse B16F1 melanoma, and middle T-transformed mouse thymus endothelial cells (mTend). Cells were stained with hamster anti-mouse β3 integrin mAb (clone 2C9.G2) and goat anti-hamster R-phycoerythrin-conjugated secondary antibody. For the staining control, the first antibody was omitted. (B) Histogram analysis of total endogenous cell surface β1 integrin in mock- and high-affinity talin-binding β3-GFP-integrin-transfected NIH-3T3 cells. Transfected cells were stained with hamster anti-mouse β1 integrin mAb (clone HMβ1-1) and goat anti-hamster R-phycoerythrin-conjugated secondary antibody. The data shown in A and B are each from a single representative experiment out of three repeats.
Figure S2. Spreading analysis of cells expressing integrins with mutations modifying talin interaction. (A) Schematic representation of integrin activation by ligand, talin, and kindlin binding (left side) and hypothetical models of integrin signaling that require exchange or “competition” between activating and signaling adapter proteins or a “synergistic” model in which signaling adapters are recruited to an activated integrin–adapter complex. (B) Sequences of the cytoplasmic tails of β3 and αv integrins, indicating the membrane-proximal clasp (dashed box), the binding sites for talin (membrane proximal and W/NPLY), and kindlin (key residues are enlarged and numbered). (C) Mean β3 integrin cell surface expression levels of mock or various β3-GFP-integrin-transfected NIH-3T3 cells. (D) Activation index of wild-type or mutant forms of β3-GFP-integrins transiently expressed in NIH-3T3 cells. Note the increase in integrin activation caused by the mutation of the membrane-proximal salt bridge (D723A). Fold increase over wild type is indicated for the activating mutation D723A. (E) Spreading curves of NIH-3T3 cells transfected with wild-type GFP or nontagged β3 integrin and compared with cells expressing the activated D723A β3-GFP-integrins. (F) Spreading curves of NIH-3T3 cells transfected with β3-GFP-integrins carrying different single or double mutations in the W/NPLY747 motif. (G) Spreading curves of NIH-3T3 cells transfected with β3-GFP-integrins carrying mutations in the membrane-proximal (MP) talin binding site [data points represent the mean of three experiments ± SEM]. T, talin; P, paxillin; K, kindlin; wt, wild type.
Figure S3. **Western blot of lysates and GST-talin head pull-downs of β3-GFP-integrins.** As shown in part in Fig. 3 D, wild-type and mutant β3-GFP-integrins were transiently expressed in COS-7 cells, lysed, and added to glutathione–Sepharose previously loaded with GST protein alone (control) or with the GST-talin head (1–435 aa). Beads were washed and eluted with sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes, and revealed with mouse anti-GFP mAb and HRP-conjugated secondary reagent. The lysate control was performed on 1/10 of the material used for the pull-down. Note that despite lower transfection efficiency in this experiment, the β3-VE construct is pulled down comparable to wild type. Blots were processed and revealed to be identical and in parallel. Lanes showing molecular mass marker migration on the same membrane before immunodetection have been overlaid for reference. ctr, control; wt, wild type.
Figure S4. **Differential recruitment of β3-GFP-integrin into focal adhesions on FN-coated glass versus PAA gels.** Glass coverslip or stiff (30 kPa) and soft (1.5 kPa) PAA gels were covalently functionalized with FN using the NHS-acrylic acid-based method (Rajagopalan et al., 2004), and NIH-3T3 fibroblasts expressing the indicated integrin constructs were plated for 2.5 h. Cells were fixed, and the β3-GFP fluorescence was imaged using a 40×, NA 1.3 oil immersion objective on a microscope (AxioImager). Five typical cells per condition were selected to represent the degree of cell spreading as well as the level of β3-GFP-integrin recruitment to CMAs. Magnifications are identical for all images. WT, wild type. Bar, 30 µm.

Reference