β1D-integrin splice variant stabilizes integrin dynamics and reduces integrin signaling by limiting paxillin recruitment

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Abstract

Heterodimeric integrin receptors control cell adhesion, migration and extracellular matrix assembly. While the α-subunit determines extracellular ligand specificity, the β-integrin chain binds to an acidic residue of the ligand, and cytoplasmic adapter proteins like talin, kindlin and paxillin, to form mechanosensing cell-matrix adhesions. Alternative splicing of the β1-integrin cytoplasmic tail creates ubiquitously expressed β1A and the heart and skeletal muscle specific β1D-form. To study the physiological difference between these forms, we developed fluorescent β1-integrins and analyzed their dynamics, localization, and cytoplasmic adapter recruitment and effects on cell proliferation. On fibronectin, GFP-β1A-integrin showed dynamic exchange in peripheral focal adhesions, and long, central fibrillar adhesions. In contrast, GFP-β1D-integrins exchanged slowly, forming immobile and short central adhesions. While adhesion-recruitment of GFP-β1A-integrin was sensitive to C-terminal tail mutagenesis, GFP-β1D-integrin was recruited independently of the distal NPXY-motif. In addition, the P786A mutation in the proximal, talin-binding NPXY\textsuperscript{783}-motif, switched β1D to a highly dynamic integrin. In contrast, the inverse A786P mutation in β1A-integrin interfered with paxillin recruitment and proliferation. Thus, differential β1-integrin splicing controls integrin-dependent adhesion-signaling, to adapt to specific physiological needs of differentiated muscle cells.
Introduction

Heterodimeric receptors of the integrin family play crucial roles in cell adhesion and signaling, during development and tissue homeostasis in the adult. In addition to varying subunit compositions that create different ligand specificity and affinities (Humphries et al., 2006; Hynes, 2002), certain integrins show also cell-type specific and developmentally regulated splicing (Fornaro and Languino, 1997). One of the best-known examples of alternative splicing of the ubiquitously expressed β1A-integrin, is the specific expression of the β1D-integrin isoform in cardio-myocytes and differentiated myotubes of skeletal muscle (Belkin et al., 1996; van der Flier et al., 1997; van der Flier et al., 1995; Zhidkova et al., 1995). Analysis in mice revealed that the knock-in of the β1D-variant affected primary myogenesis and led to embryonic lethality, while the deletion of β1D and thus the exclusive expression of the β1A-variant was well tolerated (Baudoin et al., 1998; Cachaco et al., 2003). Ectopic expression of the β1D-integrin isoform in myoblasts or fibroblasts revealed enhanced adhesion, integrin activation, fibronectin binding, but also reduced cell cycle progression (Belkin and Retta, 1998; Belkin et al., 1997). Some of these phenotypes, such as the enhanced cell-matrix adhesion, correlated with the increased affinity of the β1D-integrin peptide for talin in vitro (Anthis et al., 2010; Belkin et al., 1997). The particularly high affinity of the β1D cytoplasmic tail to talin2 enabled the crystallization of the complex, and NMR perturbation experiments showed that the membrane-proximal acidic and NPXY-motif of β1A/D and β3-integrins bound differentially to the talin head-domain (Anthis et al., 2010; Anthis et al., 2009). With the exception of a different rod-domain specific mechano-sensitivity, talin1 and talin2 induce similar integrin activation, cell spreading and focal adhesion formation (Austen et al., 2015). Therefore, and especially given the enhanced binding affinity for talin1 and talin2 (Anthis et al., 2010), it is not apparent why the β1D-integrin isoform cannot functionally replace β1A-integrins, resulting in embryonic mortality and developmental defects in migrating cell populations (Baudoin et al., 1998). In order to better understand how altered talin-binding affinities affected the function of different β1-integrin splice variants, we exploited extracellularly tagged fluorescent β1A and β1D-integrins (Egervari et al., 2016; Leduc et al., 2013). Extracellular
tagging of the β1A/D-integrin allowed studying their dynamic behavior in living cells, the unrestricted binding of cytoplasmic adapter proteins and quantitative analysis of their recruitment into different cell-matrix adhesion structures.

We show that extracellularly tagged β1A or β1D-integrin splice variants formed functional β1-integrins with differences in their dynamic incorporation into focal and fibrillar adhesions. C-terminal mutations revealed that the distal NPXY motif as well as the c-terminal carboxylic acid, were critical for recruitment of the β1A-variant into cell-matrix adhesions, while β1D did not require these c-terminal motifs. FRAP analysis showed dynamic remodeling of the β1A-integrin in focal and fibrillar adhesions, while β1D remained immobile. By employing an IL2-receptor-integrin chimera (Tac) (LaFlamme et al., 1994), we confirmed specific recruitment of Tac-β1D integrin to talin2-containing, central fibrillar adhesions while Tac-β1A showed less association with talin2. Despite these differences in integrin dynamics and adapter recruitment, the cell-matrix adhesion force was not significantly altered between β1A- and β1D-transfected cell populations. However, adhesion force diminished, integrin-dynamics increased and central adhesion localization was lost due to an isoform switching mutation in β1D-integrin (P786A). In contrast, the reverse mutation in β1A-integrin (A786P) affected paxillin recruitment, which was also absent from β1D-integrin containing adhesions, correlating with a delay in cell spreading and reduced cell proliferation. Our data demonstrate that integrin splicing can affect integrin dynamics and enable mechanically stronger linkage while reducing integrin signaling-capacity in terminally differentiated striated muscle cells.
Results

Extracellular GFP-tagging reveals different adhesion patterns of intracellular splice variants of β1-integrin

The use of c-terminally GFP-tagged β3-integrin receptors (β3-GFP-integrin) has allowed studying the dynamic remodeling of cell-matrix adhesion sites in migrating cells, and helped to understand how the cytoplasmic adapters such as talin and kindlin associate, in order to regulate integrin affinity and mechanosensing (Ballestrem et al., 2001; Cluzel et al., 2005; Kukkurainen, 2019; Pinon et al., 2014). While this approach has been mainly used to study the recruitment of β3-integrin into focal complexes and focal adhesions, the functional role of β1-integrin containing integrin receptors, such as the fibronectin (FN)-binding α5β1-integrin, have been less studied with live-cell imaging probes (Caswell et al., 2007; Laukaitis et al., 2001; Parsons et al., 2008). In particular, the long-term tracking of cell-surface expressed β1 or β3-integrins in focal or fibrillar adhesions, respectively, has only been possible with the insertion of the fluorescent tag into the extracellular domain (Egervari et al., 2016; Leduc et al., 2013) (see Fig. 1G for a model). In addition to providing a tool for the long-term tracking of cell surface expressed β-integrins (Tsunoyama et al., 2018), or integrin internalization (Huet-Calderwood et al., 2017), such β1-integrins, allow studying in great detail, the consequences of subtle sequence differences in the cytoplasmic tails of integrins. Thus, we used this strategy to study the functional role and dynamic differences of the alternatively spliced cytoplasmic tails of the ubiquitous β1A and muscle specific β1D-integrin, and that in the context of the FN-binding α5β1-integrin receptor. These GFP-tagged β1-integrin splice variants were transiently expressed in C2C12 myoblasts or 3T3 fibroblasts, as well as in β1-integrin-deficient GE11 and GD25 cells and compared with c-terminally tagged β1-integrin to understand their functional association with different cytoplasmic adapter proteins, such as talin and paxillin.

Expression of c-terminally tagged β1A-GFP-integrin-fusion constructs in 3T3 fibroblasts (Fig. 1B,D) and C2C12 myoblasts (Fig. 3G) showed cell surface expression. However and despite plating these cells on FN-coated coverslips, these integrins showed poor recruitment into peripheral focal adhesions and central fibrillar adhesions as compared to endogenous β1-integrin labeled with the 9EG7 monoclonal antibody, detecting the extended forms of β1-integrins (Bazzoni et al., 1995; Lenter et
al., 1993; Su et al., 2016) (Fig. 1A). The reduced recruitment of the c-terminally tagged β1A-integrins into adhesion structures was not due to the length of the linker domain, consisting of 5 or 30 amino acids in Figs 1B and 1D respectively, but potentially because the free carboxylic acid is required for kindlin interaction (Fitzpatrick et al., 2014). To avoid a non-specific interference by the cytoplasmic GFP, we decided to incorporate GFP into the extracellular domain of the β1A-integrin, similar to a strategy successfully used for Drosophila β-integrin. In these animals, the insertion of an epitope tag or fluorescent protein into a 41 amino acid long, serine-rich extracellular loop located in the hybrid-domain, was well tolerated (Bunch et al., 2006; Kendall et al., 2011; Syed et al., 2016). Although shorter in human β1-integrin, the same loop, which is localized at Pro88 (P88), offered an extracellular tagging site (Fig. 1G), which would (i) not interfere with the folding of the different structural domains of the β1-integrin, and (ii) would permit α-integrin association and fibronectin ligand binding (Egervari et al., 2016). Thus, transient expression of P88-loop tagged β1A and β1D-constructs, allowed detection of α5β1A in focal and fibrillar adhesions (Fig. 1C) and of α5β1D in many small centrally located adhesion sites for the latter construct (Fig. 1F). Interestingly, the c-terminal tagging of the β1D-splice variant (Fig. 1E) produced an identical integrin pattern as compared to P88-loop tagged GFP-β1D (Fig. 1E), with many short centrally located adhesions. Thus, while the β1A-splice variant showed poor functionality due to a c-terminal GFP-tag, the β1D-splice variant behaved similar, irrespective of the location of its tag.

The different patterns and organization of integrin adhesion sites seen with the extracellularly tagged β1-integrins could reflect functional differences in the alternatively spliced cytoplasmic tails. To corroborate this notion, we expressed the P88-loop tagged GFP-β1A and GFP-β1D-integrins also in C2C12 myoblasts, and plated them on FN (Fig. 1H,I), as well as on laminin-111 (Fig. 1J,K). While GFP-β1A-integrin formed long and thin central adhesions, resembling fibrillar adhesions, GFP-β1D-integrin formed short peripheral and centrally located cell-matrix adhesions (Fig. 1H-K). Quantification of adhesion length of C2C12 myoblasts plated on FN using a fully automated procedure (see material and methods) revealed a 3-fold shorter average adhesion length with the β1D-integrin compared to β1A-integrin (Fig. 1H, 1I, 1J, 1K).
1L), but with exaggerated adhesion length due to difficulties to separate individual adhesions. To confirm the adhesion length differences obtained by the automatic procedure, we also performed the adhesion length measurements by tracing the adhesions manually. Subsequently we plotted the length distribution profile of all the traced adhesions for cells transfected with GFP-β1A-integrin, GFP-β1D-integrin as well as β3-GFP-integrin (Fig. 1M). This analysis revealed a mean adhesion length of 6 μm for the GFP-β1A-integrin, while both the GFP-β1D-integrin and β3-GFP-integrin formed shorter adhesions of 2-3 μm (Fig. 1N). This confirmed the notion that the composition of the β1-integrin cytoplasmic tail strongly affects the shape and potential function of cell-matrix adhesions.

**β1A-integrin induces fibronectin remodeling while β1D-integrin mediates adhesion**

The increased adhesion length and central location of β1A-integrin adhesions, suggested the formation of fibrillar adhesions, which have been shown to be involved in the remodeling of FN and deposition of new FN fibers (Pankov et al., 2000; Zamir et al., 2000). To confirm this notion, we performed co-staining of β1A-integrin transfected C2C12 myoblasts plated on laminin-111 coated surfaces and evaluated the deposition of FN. In fact, anti-FN staining revealed co-localization of GFP-β1A-integrin with FN fibrils (Fig. 2A-D), suggesting FN-binding and the capacity to remodel extracellular FN by β1A-integrin. In contrast, GFP-β1D-integrin transfected C2C12 cells recruited FN into short central adhesions, localizing to the ends of stress fibers. During cell displacement, these FN-patches were deposited onto the laminin surface, without forming a network of FN-fibrils (Fig. 2E-H). This proposes that β1D-integrins are used for cell-anchorage, but fail to remodel the extracellular matrix.

In order to visualize such FN remodeling, or cell anchorage by β1A-, or β1D-integrin, respectively, we performed time-lapse and FRAP analysis of cell surface exposed GFP-β1A-, and GFP-β1D-integrin in transiently transfected C2C12 cells plated on FN-coated surfaces. GFP-β1A-integrin was found in elongated fluorescent structures or patches localized underneath the main cell body, likely representing fibrillar adhesions, as such structures were associated, but not co-localized with focal adhesions, identified as dark zones by interference reflection microscopy (Fig. 3A, asterisks). Consistent with FN-polymerization in fibrillar adhesions, GFP-β1A-
integrin patches moved and appeared to be elongated into linear structures, giving the impression that the cell translocated FN-bound α5β1A-integrins on the cell surface (Fig. 3A, arrows, insert). We also analyzed integrin dynamics within such fibrillar adhesions with fluorescence recovery after photobleaching (FRAP) and noted a recovery of the fibrillar integrin fluorescence (Fig. 3B, arrow), suggesting a dynamic linkage of GFP-β1A-integrin to FN-fibrils (Fig. 3B,E). In contrast, the analysis of GFP-β1D-integrin containing cell-matrix adhesions, revealed adhesion structures that were either directly bound to the FN-coated glass coverslip (interference reflection dark contacts)(Fig. 3C, asterisks) or organized in a patchy, or elongated manner underneath the main cell body, proposing association with short fibronectin fibrils (Fig. 3D, S2). In both cases, FRAP analysis revealed almost no fluorescence recovery over a period of 20 minutes, suggesting that the adhesive interaction of the GFP-β1D-integrin with the extracellular ligand was stable and the attachment sites were not remodeled (Fig. 3C-E). These data reveal a dynamic interaction of β1A-integrin with the cytoskeleton and are in agreement with its role in the remodeling of the extracellular matrix (Pankov et al., 2000). In contrast, the β1D-integrin fails to show such dynamic remodeling, creating highly stable and matrix-immobilized adhesion sites, which may reflect its role as a muscle-specific integrin.

To confirm that both β1A and β1D integrins were properly anchored to the actin cytoskeleton, we stained these cells with anti-vinculin (Fig. 3F-I), revealing co-localization for both of them (Fig. 3F,G). In contrast, c-terminally GFP-tagged β1A-integrin, which was present in the cell membrane, failed to be recruited into vinculin-positive adhesions (Fig. 3I). This suggests a critical role of the c-terminal tail for β1-integrin recruitment to cell-matrix adhesions, which was less important for β1D and β3-integrins (Fig. 3H).

C-terminal truncation revealed isoform-specific requirement of the distal NPXY motif

The robust recruitment of extracellularly tagged β1A-integrin into focal and fibrillar adhesions, which was not seen with c-terminally tagged β1A-integrin, suggested that the distal NPXY-motif or part of the c-terminal integrin sequence was critical for the recruitment of β1-integrins into cell-matrix adhesions sites. To test this hypothesis,
we analyzed extracellularly-tagged, c-terminally truncated β1A- and β1D-integrins for their ability to localize into cell-matrix adhesions. The deletion of the c-terminal Lys (K798) did not affect β1A-integrin recruitment into cell-matrix adhesions (Fig. 4B). In contrast, the deletion of the 3 or 4 c-terminal residues, which in the latter case included the deletion of Y795, resulted in the progressive loss in the ability of the GFP-β1A-integrin variants to be recruited into cell-matrix adhesions (Fig. 4B).

However, when performing a similar c-terminal deletion for β1D-integrin, for example removing the last 7 c-terminal residues including Y795 (β1D-delY795), the resulting mutant was still recruited to cell-matrix adhesions with a similar pattern as observed for the full-length GFP-β1D-integrin (Fig. 4C). This revealed independence of the distal NPXY-motif for β1D-integrin recruitment and explained focal adhesion recruitment of c-terminally tagged β1D-integrin (Fig. 1). The c-terminus of β1A-integrin displayed a tolerance for deletion of the last amino acid, but a requirement for Y795 and a free c-terminus for full activity was observed as previously suggested (Fitzpatrick et al., 2014).

Previous work has proposed a critical role for integrin adapter proteins such as kindlins and sorting nexins for the efficient cell surface expression of integrins (Margadant et al., 2012; Tseng et al., 2014). Since these adapters bind to the c-terminal parts of the integrin β-tails, a lack of recruitment of truncated β1A-integrin into adhesions could be easily explained by a failure of integrin maturation or cell surface expression. However due to low transfection efficiency of our constructs in 3T3 and C2C12 cells, we did not conduct further biochemical and cell biological tests at this point, but confirmed the relevance of these c-terminal sequences for β1A-integrin for recruitment into focal or fibrillar adhesions.

Proline 786 is critical for creating stable β1D-integrin adhesions

Structural analysis of the β1D/talin2 complex proposed an interaction of P786, as well as some minor interactions of N788 and N789 with the talin2 F3 domain (Anthis et al., 2010; Anthis et al., 2009). In addition, these authors measured a 2-3 fold reduction in β1D/talin-2 affinity due to P786A and Q778G mutations (Anthis et al., 2010). To test if these residues, or possibly the 3-amino acid extension in β1D, were involved in the differential integrin clustering phenotype observed between the β1A and β1D-
integrins, we created GFP-β1D-Q778G, GFP-β1A-G778Q, GFP-β1A-addAGL, GFP-β1D-P786A, and GFP-β1D-NN788/9TT mutants and expressed them in 3T3 cells (Fig. 4D). The GFP-β1D-Q778G and GFP-β1A-G778Q mutants (data not shown), as well as GFP-β1A-addAGL (Fig. 4D), showed very similar adhesion patterns comparable to their control constructs and we did not further pursue their analysis. In addition, the GFP-β1D-NN788/9TT mutant integrin showed extensive central adhesion formation, resembling the original GFP-β1D-integrin transfected cells (Fig. 4D). However, the P786A mutation in the GFP-β1D-integrin caused the loss of all centrally located adhesion sites, an increase in the cell-membrane staining and an enlargement of peripheral focal adhesions (Fig. 4D). In addition, focal adhesions had a tendency to slide and cell edges retracted easily in the β1D-P786A-integrin mutant (supplementary movie file, S5). Interestingly, localization into peripheral inward sliding adhesions resembled the phenotype observed in β3-GFP-integrin transfected 3T3 cells (Ballestrem et al., 2001; Cluzel et al., 2005) (Fig. 5C,D). Since the P786A mutation changed the behavior of the GFP-β1D-integrin, we wondered whether the reciprocal A786P conversion in the GFP-β1A-integrin had a similar strong effect. In fact, cells transiently transfected with this mutant exhibit, long thin central adhesion, reminiscent of fibrillar adhesions, but the cells appeared less anchored in their peripheries (Fig. 4D).

To further evaluate the similarities between the dynamics of the β3-GFP-integrin and the GFP-β1D-integrin-P786A, FRAP analysis of transiently transfected 3T3 cells was performed. Similarly to β3-GFP-integrin transfected 3T3 cells, this analysis revealed a complete fluorescence recovery of focal adhesion fluorescence within 10 to 15 minutes for the GFP-β1D-P786A-integrin (Fig. 5C,E), the GFP-β1A-integrin (Fig. 5A,E) and β3-GFP-integrin (Fig. 5D,E), contrasting with the immobile phenotype of the β1D-integrin isoform (Fig. 5B,E).

To verify that the differential dynamics of GFP-β1A and GFP-β1D-integrin were not due to endogenously expressed β1A-integrin, we stably expressed these GFP-β1-integrins in β1-integrin deficient GD25 cells. Similarly to the behavior in C2C12 and 3T3 cells, the fast and slow dynamic exchange of GFP-β1A and GFP-β1D-integrin,
respectively, was also found in GD25 cells (Fig. 5F), proposing that integrin dynamics is controlled by its c-terminal tail sequence. The difference in adhesion dynamics in these GD25 transfected cells was also illustrated by measuring the displacement of the edges of cell-matrix adhesion, analyzed from 5 hour time lapse recordings. The mean adhesion speeds of all measured adhesions was about 1.5x faster for GFP-β1A-integrin adhesions compared to GFP-β1D-integrin adhesions (n=6 cells; >2000 measurements per cell, 18.0 μm/h (+/- 5.1 SEM) for GFP-β1A-, and 11.5 μm/h (+/- 0.9 SEM) for GFP-β1D-integrin). This trend was even more pronounced for the fraction of the 50% fastest moving adhesions (25.3 μm/h (+/- 7.8 SEM), for GFP-β1A-, and 14.8 μm/h (+/-1.2 SEM) for GFP-β1D-integrin).

Reduction of cell attachment forces by the β1D-P786A mutant

In order to analyze whether the enhanced integrin dynamics of the β1D-P786A-integrin would affect the β1-integrin-dependent cell-matrix attachment forces, we tested the resistance to fluid shear forces by employing a spinning disc assay (Friedland et al., 2009; Tabone-Eglinger et al., 2014). Transiently transfected β1-integrin deficient GE-11 cells were plated on FN-coated coverslips and exposed to fluid shear stress in a spinning disk device. A soluble DsRed-construct was co-expressed with extracellular tagged GFP-β1A- or GFP-β1D-integrin in GE-11 cells for 72 hours. Transiently transfected cells were then detached and plated onto circular coverslips for 30 and 60 minutes, prior to subjection to shear forces in the spinning disc device for 5 minutes. In order to prevent β3-integrin-dependent cell attachment to FN, we incubated cells in the presence of the αvβ3 and αvβ5 specific inhibitor S-36578-2 (Maubant et al., 2006), efficiently reducing FN-mediated cell attachment forces of non-transfected GE-11 cells at concentrations of 400 nM (data not shown). Under these conditions, which blocked the adhesive response by endogenous β3-integrin, attachment forces for GFP-β1A- or GFP-β1D-integrin transfected GE-11 cells were remarkably similar. On the other hand, the expression of the GFP-β1D-P786A-integrin receptor in GE11 cells led to a twofold reduction in shear resistance (Figure 5G). Thus, despite the reported differences in talin-binding affinities between the β1A and β1D-integrin, and the different integrin turnover rates in adhesions, the
two β1-integrin splice variants induced the same cellular resistance to mechanical shear forces. Nevertheless, a reduction in shear resistance was seen in the β1D-P786A-integrin isoform. These findings suggest that the mechanical adhesion to matrix is an integrated response in which reduced adapter binding affinities can be compensated by changes in avidity, or integrin clustering, and may also include integrin-dependent signaling to maintain the focal adhesion, in order to resist an increase in shear forces.

*Lack of correlation between talin/integrin affinity and cell-matrix adhesion behavior*

In order to understand whether integrin/talin affinity differences were responsible for the above-mentioned changes in cell-matrix adhesion phenotypes, we decided to directly measure the binding affinity of GST-fused β1-integrin tails to immobilized talin1-head domain (1-406). The talin head was immobilized on a sensor surface and the binding of GST-β1A integrin tail fusion proteins were analyzed by biolayer interferometry as previously described for β3-integrin tails (Pinon et al., 2014). We observed enhanced binding of β1D- over β1A- and β3-integrin tails, to the immobilized talin head domain (Fig. 5H). The β1D-P786A, as well as the β1D-NN788/9TT integrin tail showed about half the talin-binding capacity, when compared to β1D-integrin, but still twice as much as the β1A- and β3-integrin tails. Interestingly, the binding affinity for the triple mutant (β1D-P786A/N788/9TT) was again identical to the β1D-integrin, demonstrating that the talin/integrin affinity is not easy to predict based on individual mutations (Fig. 5H).

*Talin2 induces recruitment of β1D-integrins to central adhesions*

In order to further evaluate the recruitment mechanisms of β1D or β1A-integrins into central and peripheral adhesions we tested the localization of the two talin isoforms in C2C12 cells. As previously reported (Austen et al., 2015; Rahikainen et al., 2017), the expression of talin1-mCherry was mainly restricted to peripheral adhesions (Fig. 6A), where β3- and β1A-integrin can be detected in fibroblast cells. Interestingly, the expression of a talin2-YPet construct (Austen et al., 2015) revealed centrally located fibrillar adhesions, as well as staining in peripheral focal adhesions (Fig. 6B), similar
to the data reported by Praekelt et al. (Praekelt et al., 2012). Quantification of the adhesion length and diameter showed significant differences in the aspect ratio (major axis/minor axis) of the talin1 and talin2 adhesions (Fig. 6C). With the help of previously described fusion proteins of the extracellular domain of the IL2-receptor alpha (Tac) (LaFlamme et al., 1994) and the transmembrane and cytoplasmic domain of either β1A or β1D-integrins (Tac-β1A and Tac-β1D), we probed the intracellular recruitment of these construct to the membrane-proximal cytoskeleton of C2C12 cells. While Tac-β1A was recruited to peripheral and centrally located large and wide focal adhesions (Fig. 6D), Tac-β1D localized to thin elongated fibrillar adhesion-like structures (Fig. 6E), very similar to the adhesions labeled with talin2. We confirmed this differential pattern by establishing the aspect ratio of the measured Tac-localization, showing similar patterns for talin2 and Tac-β1D (Fig. 6F). Furthermore, talin2 transfection in stably Tac-β1A or Tac-β1D transfected C2C12 cells revealed extensive co-localization in both central and peripheral adhesions for the latter, but only an overlap in staining of peripheral adhesions for the former situation (Fig. 6G,H). Thus, using Tac-integrin chimeras as affinity probes, our data suggested that β1D-integrin is recruited to central and peripheral adhesions, in a talin2-dependent manner, while β1A-integrin shows a preferred interaction for talin1, localized in peripheral focal adhesions.

As shown above, when bound to their extracellular ligands, β1D-integrin forms short and substrate anchored adhesions in the cell center, while β1A-integrin forms centrally located, long and thin FN-associated fibrillar adhesions (see Fig. 2). This suggests that retention of β1A-integrin in fibrillar adhesions requires talin2 and additional cytoskeletal elements. On the other hand, mutations targeting the residue P786 in the β1D-integrin (Fig. 4D) may cause the loss of recruitment to such talin2-enriched central adhesions. Therefore, we further evaluated the connection of the GFP-β1D-P786A-integrin mutant with other elements of the cytoskeleton. Transiently transfected 3T3 cells were stained for endogenous vinculin, in order to potentially identify changes in adapter interactions. Indeed, the P786A mutation led to the loss of GFP-β1D-integrin co-localization with vinculin-expressing central fibrillar adhesions (Fig. 6I), proposing that a specific centrally located F-actin interaction capacity has been lost due to the P786A mutation in GFP-β1D-integrin. However,
when the P786A loss of function mutation was combined with the NN788/9TT mutation (GFP-β1D-P786A/NN788/9TT), long and thin, centrally located cell-matrix adhesions were again detected (Fig. 6J), resembling the phenotype of GFP-β1A-integrin. In addition, these central adhesions were also positive for anti-vinculin staining (Fig. 6J), proposing that a loss in β1D-integrin affinity for talin2 due to the P786A mutation, could be at the origin of the β3-GFP-integrin-like adhesion behavior. In contrast, the capacity of GFP-β1A-integrin to assemble FN-fibers might be critically linked to residues T788 and T789 that are expressed in the β1A-integrin in the context of alanine at position 786. Interestingly the latter two threonines T788 and T789 have been identified to be required for binding to the integrin adapter protein kindlin (Moser et al., 2008), suggesting that kindlin-recruitment to the integrin tail is also determining the differential organization and dynamics of focal and fibrillar adhesions.

**Pro786 prevents paxillin recruitment and cell spreading in β1D-integrin**

Since the integrin adhesion dynamics are also regulated by the cellular signaling activity (Tabone-Eglinger et al., 2012) and not only integrin/adapter protein affinity, we analyzed the signaling capacity of cell-matrix adhesion sites by staining with anti-paxillin and anti-phospho-tyrosine antibodies, showing the qualitatively same result. In contrast to anti-vinculin, anti-phospho-tyrosine staining did not reveal centrally located fibrillar adhesions in stably GFP-β1A-integrin transfected GD25 cells, but stained mainly the peripheral focal adhesions (Fig. 7A). In contrast to anti-vinculin staining in GFP-β1D-integrin expressing 3T3 cells, phospho-tyrosine proteins were not efficiently recruited to GFP-β1D-integrin containing adhesions in GD25 cells (Fig. 7B). Interestingly, expressing the β1D-integrins in GD25 cells, had also a negative effect on their spreading capacity. Moreover, after 6 hours of spreading on fibronectin, a reduction in the cell area was observed for GFP-β1D-integrin expressing cells (Fig. 7I).

One of the central signaling adapter proteins in focal adhesions is paxillin, whose recruitment to β3-integrin-containing adhesions correlates with cell spreading (Pinon et al., 2014). While anti-paxillin staining was detected in peripheral GFP-β1A-
integrin containing focal adhesions (Fig. 7C), anti-paxillin staining was barely detected in GFP-β1D-integrin, GFP-β1A-A786P, as well as in GFP-β1D-P786A-integrin containing adhesion sites (Fig. 7D-F). Interestingly, the GFP-β1A-integrin with a proline mutation at position A786 (A786P) was still able to form centrally located fibrillar adhesions (Fig. 7D). When the localization of paxillin to GFP-β1-integrin containing adhesion sites was evaluated by the pearson coefficient (Fig. 7J), a significant reduction in paxillin recruitment was observed for both the β1D- and the β1A-A786P mutant integrins as compared to β1A. Additionally, and in order to exclude the “contamination” of GFP-β1-integrin with endogenous β3-integrin, and thus with β3-integrin-dependent recruitment of paxillin, we used FN/VN patterned substrates (Pinon et al., 2014), to spatially dissociate the β1A- and β1D-integrins from endogenous β3-integrins (Fig. 7G, H). Quantification of the GFP-integrins in respect to paxillin staining revealed a reduction of paxillin recruitment onto GFP-β1D-integrin containing adhesions on FN (Fig. 7K), confirming the results obtained by the evaluation of the localization correlation analysis on non-patterned surfaces (Fig. 7J).

Thus, in addition to the increased talin binding affinity for the β1D-integrin tail in vitro and slowed down integrin turnover, β1D-integrin caused reduced paxillin recruitment and decreased cell spreading.

**Paxillin recruitment to β1-integrin correlates with cell proliferation**

The reduced recruitment of paxillin to β1D-integrin in GD25 cells was further analyzed to understand its potential impact on other integrin-dependent signaling events, such as cell proliferation. In order to reveal a β1-integrin-dependent proliferation activity in the SV-40 transformed GD25 cells, we used the xCELLigence System and analyzed cell proliferation by measuring the increase in the electric impedance over 75 hrs as an indication for increasing substrate coverage and therefore cell number. GD25 cells stably transfected with GFP-β1A-integrin or with GFP-β1D-integrin (Fig. 7M) showed no dramatic changes in impedance during the first 20 hrs (Fig. 7N). Afterwards, GFP-β1A-integrin expressing cells started proliferating quicker than GFP-β1D-integrin expressing cells, leading to a continuous delay in proliferation of the latter cell line of 7 hrs (Fig. 7N,O). Additionally, we tested cell
proliferation in this setting by BRDU incorporation. We reduced the serum-mediated stimulation of proliferation by starving the cells for 20 hrs in 1% serum-containing medium, prior to the BRDU incubation for 4 hrs (Fig. 7L). Again, GD25 cells stably transfected with GFP-β1A-integrin showed enhanced proliferation as indicated by increased BRDU incorporation compared to non-transfected control cells, or cells that expressed β1D-GFP-integrin. Moreover, the expression of GFP-β1A-integrin carrying the A786P suppressed BRDU incorporation, even below that seen with non-transfected control cells. This proposes that P786 has an important role in β1D-integrin to mechanically stabilize the talin/β1D-integrin interaction, while preventing the recruitment of the paxillin signaling adapter protein, to prevent adhesion-signaling and adhesion-turnover of β1D-integrins in differentiated myotubes.

**Discussion**

The development and regeneration of muscle tissues is intimately linked to changes in the expression of integrin family of receptors and their association with different ECM structures. During development, myoblasts of somitic origin (Chevallier, 1979) invade the limb-mesenchyme along migration pathways that provide an ectoderm or fibroblast secreted FN-containing matrix (Chiquet et al., 1981; de Almeida et al., 2016). While individual myoblasts can also secrete and organize their own FN-network, the FN network is lost from the surfaces of fused myotubes (Chen, 1977; Chiquet et al., 1981). Moreover, the interaction with FN is stimulating the proliferation of myoblasts, while also delaying their fusion into myotubes (Podleski et al., 1979). In addition, the regeneration of myotubes from satellite cells require the contact with FN, which is also stimulating the rejuvenation of aging satellite cells (Lukjanenko et al., 2016). Importantly, the ability of FN to trigger myoblast regeneration, proliferation and FN matrix remodeling is linked to the expression of the α5- and β1-integrins (Qiao et al., 2014; Rozo et al., 2016; Taverna et al., 1998). Moreover, the FN binding α5β1A-integrin stimulates proliferation and paxillin phosphorylation, which is counteracted by the cytoplasmic tail of α6 integrin, leading to α6β1-mediated adhesion to laminin and myoblast differentiation (Sastry et al., 1999). When myoblasts fuse and differentiate into myotubes, a switch to the laminin-
binding $\alpha 7$ subunit is observed to create $\alpha 7\beta 1$-integrins that associate with the dystroglycan complex to anchor myotubes to the surrounding basement membrane (Guo et al., 2006). Similarly, upon fusion of myoblasts into myotubes, the $\beta 1D$-integrin isoform is expressed creating $\alpha 5\beta 1D$ and $\alpha 7\beta 1D$ subpopulations (Nawrotzki et al., 2003; van der Flier et al., 1997). One of the hallmarks of the $\beta 1D$-integrin is its higher affinity to talin and hence previous studies have proposed that enhanced mechanical linkage of myotubes to the ECM could be the major functional role of the $\beta 1D$-integrin isoform (Anthis et al., 2010; Belkin et al., 1997).

Although, we mainly propose in this manuscript that the differential cytoplasmic $\beta 1$-tail sequences are responsible for the observed differential biological effects, assuming an equal pairing of the integrin splice forms with a given $\alpha$-subunit (Lenter and Vestweber, 1994), we cannot exclude that some of the reported observations are due to a differential $\alpha$-integrin subunit association, which could be induced by integrin adapter proteins, recognizing specific pairs of cytoplasmic clasped (inactive) tails of the integrin heterodimers (Tseng et al., 2018). While this is an interesting hypothesis, we would like to propose that the specific cytoplasmic features of the $\beta 1D$-integrin are causing its biological effects.

Thus, in addition to enhanced talin binding (Anthis et al., 2010; Belkin et al., 1997), $\beta 1D$-integrin is also less prone to be modulated by phosphorylation at the inter-NPXY-motif. In $\beta 1A$, the phosphorylation of the $T^{788}T^{789}$ motif, or its PP2A-mediated dephosphorylation, induce focal adhesion disassembly or formation, respectively (Kim et al., 2004). Switching the $T^{788}T^{789}$ motif in $\beta 1A$ to the $N^{788}N^{789}$ motif found in $\beta 1D$, created integrin-dependent adhesions insensitive to conditions that destabilized $\beta 1A$-integrin by Ser/Thr phosphorylation. Notably activation of CAMKII has been directly associated with the phosphorylation of the TT-motif and the destabilization of the $\beta 1A$-integrin (Bouvard et al., 1998; Suzuki and Takahashi, 2003). In addition, CAMKII phosphorylation of Icap-1 led to its activation, which then caused competition with kindlin for binding to $\beta 1A$-integrins and subsequent integrin-dissociation from focal adhesions (Brunner et al., 2011; Millon-Fremillon et al., 2013). The potential competition between Icap-1 and kindlin2 for $\beta 1A$-integrin
binding is relevant as kindlin2, is critical for integrin activation (Montanez et al., 2008; Theodosiou et al., 2016). Interestingly, biochemical binding assays also revealed that mutation of the T\(^{788}\)-T\(^{789}\)-motif (TT-AA) blocked binding of the closely related kindlin3 to GST-β1A-tail fusion proteins (Moser et al., 2008). Although the T\(^{788}\)/N-T\(^{789}\)/N mutations were not specifically tested in the kindlin binding assay, kindlin3 binding to β1D cytoplasmic tail has been found reduced compared to β1A (Yates et al., 2012). The reduced binding of kindlin to the β1D tail in biochemical assays, as well as the failure of c-terminal tail truncations of the β1D tail to affect integrin recruitment to focal adhesions, propose that kindlins are not required for the activation and function of the β1D-integrin. This strongly suggests that β1D-integrin is not only independent of the regulation by Ser/Thr kinases and PP2A, but also insensitive to c-terminal β1A-integrin regulators such as Icap-1 and kindlins and is mainly regulated by talin1, as well as talin2 binding that are critical for the function of muscles (Conti et al., 2008).

In addition to the non-responsiveness to Ser/Thr phosphorylation at the inter-NPXY region (Kim et al., 2004), we also show here that paxillin is not efficiently recruited to β1D-integrin containing adhesions. Some residual recruitment of paxillin to FN-localized β1D-integrin containing adhesions in GD25 cells, could be linked to residual β3-integrin binding on FN (Bachmann et al submitted; 2019), and previously observed for high-affinity talin-binding β3-integrins (Pinon et al., 2014), or linked to a different mechanosensitive threshold. This might be explained by reduced kindlin binding to the c-terminal integrin tail (Yates et al., 2012), and that could also affect the assembly of FN-fibrils (Fig. 2). Nevertheless, the delayed spreading of β1D-integrin expressing GD25 cells on fibronectin, or their reduced proliferation, suggests that the altered recruitment of paxillin to this integrin affects intracellular signaling, while maintaining at the same time strong talin-mediated cell-matrix adhesions of the myotubes to the tendons and basement membranes that are not prone to be affected by raises in CAMKII activity due to elevated intracellular Ca\(^{2+}\) levels.

Previously, we were able to dissociate the talin-mediated mechanical link of β3-integrins to F-actin from the capacity of ECM and talin-bound integrins to induce cell spreading (Pinon et al., 2014). Lack of cell spreading correlated with an absence of
paxillin recruitment to mutant forms of β3-integrins, which included the Y747A mutant in the proximal NPXY motif, the talin binding motif from PIPKιγ (WDTANNPLY747KEA to WVYSPLH745YSA) (Pinon et al., 2014; Wegener et al., 2007), as well as the S752P mutant identified in a patient with Glanzman thrombastenia (Ma et al., 2008; Pinon et al., 2014). Interestingly, in the latter two cases, the Y747 sidechain critical for β3-integrin signaling (Petrich et al., 2007a), is either absent (Pinon et al., 2014), or the corresponding H745 inaccessible due to a tight turn of the PIPKιγ sequence (Wegener et al., 2007). This situation is quite similar to the Y783-sidechain in β1D-integrin, which is projected against the talin F3 domain surface, stabilized by additional β1D/talin contacts at residue P786 (Anthis et al., 2010; Anthis et al., 2009). We observed P786A and A786P mutations causing a switch in the adhesive and signaling behavior of β1D-, and β1A-integrins, respectively, which could well reflect the 5-fold differences in affinity for talin2, therefore also changing the preferred intracellular localization of these integrins (Anthis et al., 2010), as well as the potential accessibility of the Y783-sidechain. Therefore, the localization of the β1A-integrin mutant A786P outside of peripheral focal adhesions, could reflect a “talin2-binding mode”. In this mode, the sidechain of Y783 and thus paxillin recruitment and cell proliferation is affected by talin-2 association, potentially modifying the context and folding of the integrin peptide on the surface of the talin-adapter protein, further confirming the critical role of talin in integrin-mediated signaling (Conti et al., 2008; Petrich et al., 2007b).

Recently a different mechanism for paxillin recruitment to integrin-containing nascent adhesions has been proposed and shown to require the binding to kindlin2 (Bottcher et al., 2017; Theodosiou et al., 2016). Moreover kindlin and paxillin interactions were able to stabilize the activated state of integrin αIIbβ3 (Gao et al., 2017). This mechanism of paxillin/kindlin interaction would correlate (i) with the failure of kindlin recruitment to the S752P mutant in β3 (Ma et al., 2008) and the respective loss in cell spreading despite its forced activation and talin colocalization in the D723A/S752P mutant (Pinon et al., 2014), and (ii) with the weak binding of kindlin3 to β1D-integrin (Yates et al., 2012), thereby affecting paxillin recruitment and integrin signaling. However, the paxillin-binding defect of the SPLH or Y747A mutant in β3-integrin will not fit this model, since the kindlin-binding motif is not affected in these
mutants (Bledzka et al., 2012; Moser et al., 2008). Similarly, the A786P mutation in β1A-integrin may only slightly affect the binding affinity to kindlin2, as inferred from the recent crystal structure of kindlin2 (Li et al., 2017). In a cellular context however, kindlin2 binding could become strongly affected by adjacent high-affinity talin2 binding at the P786 residue. In fact, the crystal structure of the β1D peptide bound to talin2 (Anthis et al., 2010; Anthis et al., 2009), proposes a steric effect on the inter NPXY-residues (T788T), thereby affecting kindlin binding and subsequent paxillin recruitment.

Nevertheless, in all the reported paxillin-binding deficient integrin sequences, either the modification, or accessibility of the Tyr-residue in the talin-binding NPXY-motif (Y747A, or SPLH), or the inter-NPXY sequence is altered by proline residues. Since proline residues are affecting the secondary structure of the integrin peptide, it is likely that their presence is either perturbing kindlin-recruitment or affects sterically the recruitment of paxillin to the integrin/talin/kindlin complex. In this regard, the phenotype of the β1D P786A mutation is interesting. On the one hand, the P786A mutation in β1D-integrin is affecting the stability of the integrin within adhesions (FRAP), while also correlating with a 2-fold reduction in biochemical interaction with talin1 (Fig. 5H), and a 5-fold reduced affinity for talin2 as reported by Anthis et al. (Anthis et al., 2010). Although the reduction in talin1 binding is significant, it does not reach the low levels of β1A and β3-integrin tails, which however recruit paxillin normally when present in focal adhesions (Fig. 5H). On the other hand, the rescue of the P786A mutant in β1D, which has lost its affinity for talin2 (Anthis et al., 2010), by the expression of the kindlin-binding T788T789 motif (PNN/ATT mutant) (Moser et al., 2008) suggests that a high-affinity interaction of kindlin with the inter-NPXY motif is critical for β1A-integrin recruitment into fibrillar adhesions, as well as paxillin recruitment, and integrin-dependent signaling in peripheral focal adhesions. How paxillin recruitment is regulated by integrin sequences such as Y747, and/or kindlin-binding will require further studies (Gao et al., 2017). Nevertheless our study provides evidence for the regulation of integrin-dependent intracellular signaling by alternative splicing. Specifically, the β1D-integrin splice variant provides a higher affinity for the talin2-adapter protein, but with the expense of failing to support de novo fibronectin fiber assembly, as well as failing to induce integrin-dependent
signaling. This is highly relevant for the proper mechanical function of differentiated myotubes, in which the control of myosin-contractility should be uncoupled from mechanosensing mechanisms observed in other cells of the body.

Material and Methods

cDNAs and site-directed mutagenesis
The cDNA encoding human β1A-integrin was obtained from RZPD (Deutsches Ressourcenzentrum für Genomforschung GmbH) Clone ID: IRATp970E0719D6. It was cloned into pCDNA3 (Invitrogen) using XbaI and EcoRI sites. Mutations were introduced by primer overlap extension and subsequently verified by automated sequencing. C-terminally tagged β1A was constructed identical to the previously published β3-EGFP-integrin (Ballestrem et al., 2001), by replacing the stop codon with a PinAI and XbaI encoding linker that was used to insert the EGFP-sequence (5-CAGTCTAGAGACCGGTTTTCCCTCATACTTCGG-3). The resulting -SPVAT-linker (-aa- in Fig. 1) was much shorter than a previously published one (-bb- in Fig. 1), which we received as a kind gift from M. Humphries, (Manchester) (Parsons et al., 2008). The extracellularly tagged β1A integrin was constructed by introducing PinAI and XhoI sites in a loop in the hybrid-domain by duplicating proline at position 88 (AEGKP88EDIT), which resulted in the AEGKPVSRGPGEDIT sequence that was used to insert the EGFP sequence (Fig. 1G) (forward: 5-AACCGGTCTCTCGAGGACCAGAGGATATTACTCA-3; reverse: 5-TCCCTCGAGACCGGTTTGGAGCTCTTCTGCTGTT-3). The β1D sequence was introduced into the different β1A-integrin constructs in two steps; first by exchanging Gly with Gln at position 778, and then by the modification of the sequence 3’ of S785, using the following overlapping primers (forward: 5-GTCCTATTAATAATTTCAAGAATCCAAACTACGGACGT-3; reverse: 5-TGTTCTAGATTAGACCAGACCGTTTACGCTCCTGTT-3). The pcDNA3-based vectors were used for transient transfections of 3T3, C2C12 and GE11 cells. For stable transfections of GD25 cells, and in order to avoid an excessive, but maintaining a moderate, long-term transcription in stably transfected cells, the CMV promoter of the
pcDNA3 vector was replaced by a 1kb fragment of the matrix attachment region of chicken lysozyme (MAR, kind gift of N. Mermod, Lausanne, Switzerland) (Girod et al., 2005; Loc and Stratling, 1988), and a 1.4 kb fragment (NheI-EcoRI) of the human β-actin promoter. Since GD25 cells are resistant to G418, the neomycin resistance gene in the modified β1A(D)-integrin containing pcDNA3 (sm-pcDNA3) was swapped for that of the puromycin gene (smp-pcDNA3). This same vector was also used to express the IL2-receptor alpha β1-fusion constructs, as well as the generation of puromycin resistant clones in C2C12. The extracellular domain of Tac (Paulhe et al., 2009) was fused to the transmembrane and cytoplasmic domains of β1A and β1D-integrins using unique BglII and XbaI sites. Talin1-mcherry and talin2-YPet constructs were previously published and the latter a kind gift of Dr. Carsten Grashoff, Muenster, Germany) (Austen et al., 2015; Rahikainen et al., 2017). DNA sequence analysis was performed for all constructs and mutants to ensure error-free amplification and correct base replacement.

**Cell culture, transient and stable transfections**

Mouse C2C12 myoblasts cells, 3T3 NIH fibroblast, as well as β1-integrin deficient GD25 and GE11 cells in (Fassler et al., 1995) (a kind gift of Dr. R Faessler, Munich) were grown in DME containing 10% FCS, Gln, and antibiotics, as previously described (Ballestrem et al., 2001). For the 3T3-NIH and GD25 cells, transfections were performed with Jet PeI (Polyplus Transfection) according to the manufacturer’s recommendation. For the C2C12 cells and GE11 cells, electroporation was done with the Microporator from Axonlab following the guidelines from the manufacturer. For selection of stably transfected GD25 cells, medium was supplemented with puromycin, and subsequently sorted by FACS for the surface expression of the human β1A, or β1D-integrin by reactivity with mAb Ts2/16. Jet Prime was used for the transient transfection of C2C12 cells with talin1/2, Tac-β1A and Tac-β1D-constructs, according to the manufacturer’s recommendation (Polyplus Transfection).

**Antibodies and immunofluorescence**

Cells grown in complete medium were fixed with 4% PFA in PBS for 10 min, permeabilized, and blocked in 0.1% Triton X-100 and 1% BSA in PBS for 30 min. Mouse mAbs to vinculin (V9131; Sigma-Aldrich), paxillin (P13520; BD
Transduction Laboratories), mouse mAb 7G7 against the Tac epitope, mouse mAb anti-phospho-tyrosine (4G10 Platinum, Millipore, Billerica, MA, USA), rabbit-polyclonal antibody to fibronectin (1801, a gift from Matthias Chiquet, Bern, Switzerland) and the Rat mAb 9EG7 (553715, BD transduction Laboratories) that detects the activated β1-integrin were applied in 1% BSA-PBS for 1 h. After being washed in blocking solution, Texas–red conjugated goat anti–mouse antibodies (Jackson Immuno Research Laboratories, West-Grove, PA, USA), or Cy5 conjugated goat anti-rabbit (anti-FN) or Texas-red conjugated goat anti-rat were applied for 1 h and subsequently washed as above. F-actin was detected with Texas red–phalloidin (Invitrogen). Preparations were stored in PBS and images were collected at RT using a 63X NA 1.40 objective on an LSM510 inverted microscope. In addition, background and contrast were adjusted using the adjust Level command in Photoshop (Adobe).

**TIRF microscopy**

Intra-objective TIRF microscopy was performed on an inverted microscope (Axiovert 100M; Carl Zeiss, Inc.) equipped with a combined epifluorescence/TIRF adapter (TILL Photonics) and a 100x NA 1.45 objective (Carl Zeiss, Inc.). EGFP fusion proteins were excited with the 488-nm line of a 150-mW argon-ion laser (Reliant 150m; Laser Physics), and red dyes were excited with the 535-nm line of a 20-mW diode laser (Compass 215M-20; Coherent, Inc.). Laser output at the end of the optical fiber was set to 5mW each, to assure equal illumination of biological samples with the blue or green laser light. Openlab software (PerkinElmer) controlled image capture by a 12-bit charge-coupled device camera (Orca 9742-95; Hamamatsu Photonics) as well as the operation of the laser shutters and microscope. The background and contrast were adjusted using the “Level” command in Photoshop (Adobe).

**Live cell imaging and FRAP**

Cells were cultured in FN-coated glass bottom dishes for 24 h in complete culture medium prior to analysis. Medium was replaced by F12 medium containing glutamine, antibiotics and 10% FCS prior to live cell imaging and FRAP analysis. FRAP experiments with NIH-3T3 and C2C12 cells were performed at 37°C on an LSM510 inverted microscope equipped with a heated stage and CO₂ control essentially as described (Ballestrem et al., 2001; Wehrle-Haller, 2007). To reduce loss of fluorescence due to bleaching during the recovery period, we reduced the laser power of the 488-nm
line to 0.5% at a maximal laser output of 50%. To improve light collection, the pin hole was partially opened (to 200 µm). To ensure maintenance of the focus during the recovery period, an IRM image was recorded simultaneously. Time-lapse analysis was performed using the same settings and images scans were performed every 60 seconds.

For stable transfected GD25 cells, culture and live-cell imaging conditions were identical as above, but measurements were performed on a Nikon A1r inverted confocal microscope equipped with an autofocus module and a 60x oil objective NA 1.4, pinhole of 2.3 (CFI Plan Apochromat VC WD:0.13mm), at zoom 4, and with a 488nm 50mW laser at 37°C, 10% CO₂. For FRAP experiments in GD25 cells, 12-bit confocal images were acquired every 20 seconds over 17 min. Grey level intensities of bleaching zones, as well as of non-bleached adhesion sites to compensate for bleaching during recovery period, and empty control background areas were analyzed by ImageJ and values were treated essentially as described (Wehrle-Haller, 2007).

**FACS**

Stably transfected GD25 cells were detached with trypsin/EDTA, blocked in culture medium and incubated with the first antibody, mRat 9EG7 or the anti-human β1-integrin (Ts2/16; Biolegend), for 45 minutes on ice. Cells were washed and re-suspended in DMEM and incubated with the secondary antibody, goat anti mouse PE (Phycoerythrin) conjugated or goat anti rat PE for 30min on ice. Cells were washed twice and re-suspended in PBS for the FACS analysis. FACS was performed with a BD FACSIAria II instrument (selecting between 10,000 and 100,000 cells for reseeding).

**Substrate coating and microcontact printing with extracellular matrix proteins**

For spreading experiments on defined protein substrates, glass coverslips were coated for 1 h at room temperature with purified horse-serum-derived FN (a gift from M. Chiquet, Bern), or EHS-tumor derived laminin-I (a gift from J. Engel, Basel) diluted in PBS at the indicated concentrations, followed by washing and blocking of the coated surfaces with 1 mg/ml human serum albumin (Sigma-Aldrich).

Silicone stamps for microcontact printing of differential substrates were produced as previously described (Lehnert et al., 2004). Binary choice substrates were produced with human plasma FN (Sigma-Aldrich, #F2006 or Millipore, #FC010) and human plasma VN (Sigma-Aldrich, #V8379). Silicone stamps were incubated for 10 min with a solution of 5 µg/ml FN and 45 µg/ml heat-inactivated FN in PBS. Alexa Fluor
647 labeled FN was added to visualize the FN pattern. After nitrogen drying, the stamp was pressed onto a glass cover slip for 10 min before it was released. Next, the pattern was covered with VN at a concentration of 5 µg/ml in PBS for 1 hour at room temperature. Heat-inactivated FN was produced by heating FN for 30 min to 90°C and was added to block VN adsorption in the stamped area. Cells do not spread on heat-inactivated FN. After the final incubation step, patterns were washed with PBS and used directly for cell seeding. Detachment from culture flasks was stopped with trypsin inhibitor (Sigma-Aldrich) and cells were seeded in DMEM without FCS if not stated otherwise.

**Automated and manual integrin adhesion length measurements**

Integrin transfected cells were incubated for 8hrs on FN coated glass coverslips and fixed for 10 min in 4% PFA/PBS. TIRF images were taken in PBS. Adhesion length measurements were obtained from 12-bit images after selection of the cell area and background subtraction, using MetaMorph software (MDS Analytical Technologies). Integrin adhesions were identified with the “angiogenesis tool” and subsequently skeletonized to one pixel width to obtain the adhesion length measurements of individual adhesions. The average adhesion length per cell was calculated from all the adhesions identified in one cell and the measurement performed on at least 20 cells per experiments, which were repeated 4 times.

The adhesion length distribution analysis was performed manually on TIRF images of GFP-β1-integrin fluorescence, with the help of the line tool in the image analysis software ImageJ. Each adhesion length distribution profile was grouped into decile borders and these decile values were averaged over one experiment (n=8 cells, 3 experiments). To test how much influence the choice of images for quantification and personal interpretation of adhesion lengths would have, two different investigators quantified independently from each other the cell adhesion length distributions of different pictures from the same experiment. Both investigators obtained the same trends for adhesion length distributions and mean values. These results let us conclude that adhesion length distributions measurements can be reproduced and image choice does not alter the result.
Recombinant protein purification and Octet biosensor analysis

Purification of GST-β1-tail chimeras and His₆-tagged talin head domain (residues 1-406) were performed as described (Pinon et al., 2014). In brief, proteins were produced in *E. coli* BL-21-Star cells (Invitrogen) and induced with 1mM IPTG for 5 hrs at 37 °C, and lysed by homogenization (Emulsiflex C3, Avestin Inc. Ottawa, Canada) in PBS containing protease inhibitor cocktail. GST-tagged proteins were purified on Glutathione Sepharose™ (GE Healthcare 4 Fast Flow), detached in 50 mM Tris-HCl, containing 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 UV/Vis absorption analysis. The His₆-tagged talin head domain lysates were generated in 20 mM sodium phosphate buffer (1 M NaCl, 20 mM imidazole, pH 7.4) and purified on HisTrap FF affinity columns (GE, Healthcare) prior to elution with a linear imidazole gradient 0-700 mM. Eluted fractions were further purified by cation exchange chromatography using HiTrap SP FF columns (GE, Healthcare), and eluted with a linear NaCl gradient prior to analysis of purity and concentration as mentioned for the GST-chimeras.

Biosensor analysis was performed on a Fortebio Octet RED384 instrument (Pall, Menlow Park, CA) at 25 °C and stirring speed of 1000 rpm, using Ni-NTA sensors. Sensors were pre-wetted with buffer (50 mM NaH₂PO₄, 150 mM NaCl pH 7.2) in order to get baseline prior to immobilization. Samples or buffers were pipetted either into 96- or 384-well plates at a volume of 200 µl or 80 µl per well, respectively. Sensors were chemically activated by immersing them in 0.05 M EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide) and 0.1 M NHS (N-Hydroxysuccinimide) in H₂O for 100 s. Talin head (50 µg/ml) was immobilized on the sensor’s surface for 300 s. The excess NHS esters were quenched by 1 M ethanolamine pH 8.5 for 100 s. Serially diluted GST-β1-tail chimeras were then applied on the talin-coated sensors in concentrations of 20, 80, 320, 1250 and 5000 nM to obtain the relative affinity of integrin-GST to talin. The binding of each concentration of integrin-GST to the sensor was measured for 300 s before moving the sensor into well containing higher integrin-GST concentration. GST alone was used as negative control with the same conditions. In data analysis, the GST control response signal was subtracted from the measured binding response.
**SIM-Microscopy and image analysis**

SR-SIM imaging was performed on a non-serial Zeiss Elyra PS.1 microscope with a 63x/1.4NA oil immersion objective and an Andor iXon EMCCD camera. The grid for SR-SIM was rotated three times and shifted five times leading to 15 frames raw data out of which a final SR-SIM image was calculated with the structured illumination package of ZEN software (Zeiss). Values for calculation were selected for best resolution without causing image artifacts. Channels were aligned by using a correction file that was generated by measuring channel misalignment of fluorescent tetraspecs (ThermoFischer, #T7280). Colocalization was analyzed with the Fiji software package (Schindelin et al., 2015). Colocalization to FN was quantified by measuring Mander’s coefficient of thresholded images by using the Fiji plugin JACoP (Bolte and Cordelières, 2006).

**Cell spreading analysis**

Stable β1-integrin expressing GD25 cells were plated for 5 hrs on 10μg/ml FN coated coverslips in 10% FCS containing DMEM. Subsequent time-lapse videos were started and phase contrast pictures were taken every 5 min with a 10x long distance objective on a Zeiss-Axiovert 100M microscope at a constant temperature of 37°C and 10% CO2 levels. For image analysis and detection of cell area, the cell borders were defined by the threshold tool in ImageJ and subsequently cell area and perimeter quantified.

**Pearson correlation of integrins and paxillin**

TIRF images of GFP-tagged β1-integrin stable transfected GD25 cells were acquired after overnight culture on FN-coated glass coverslips. Cells were fixed, permeabilized and stained with anti-paxillin antibodies. Pair of images were analyzed in Image J using the co-localization module to evaluate the pearson correlation coefficient of 20 cells per data point.
**BRDU-incorporation and cell proliferation tests**

Stable transfected and FACS-sorted GD25 cells were plated for 6 hrs in medium containing 5% FBS on glass coverslips, previously coated with 10 μg/ml of FN for 1 hr at room temperature. The serum content of the medium was then reduced to 1% for 20 hrs and then incubated for 4 hrs in the same medium containing BRDU, in concentrations according to the manufacturers recommendation. Cells were fixed and stained with DAPI and antibodies to BRDU according to standard protocols. 5 random selected areas were imaged with a 10x objective and percentage of BRDU expressing nuclei manually counted from a total of 250 to 500 cells per condition. Proliferation of GD25 cell lines stably transfected with GFP-β1A-integrin or GFP-β1D-integrin was measured with a xCELLigence System (ACEA Biosciences Inc.). 15 000 cells of the respective cell lines were applied to specialized electrode-coated wells from ACEA Biosciences that allowed impedance measurements as a proxy for cell adhesion and increase in cell number. Impedance values were corrected for baseline levels of the medium and were converted to arbitrary units by the company software for comparison of different conditions. Experiments were performed with duplicated wells and in culture medium with 10% FCS. Impedance was measured every 15 min for 75 hrs and is shown as mean of three independent experiments.

**Spinning disc analysis**

Application of shear stress and subsequent data analysis was performed with a spinning disc device, as previously described (Boettiger, 2007; Tabone-Eglinger et al., 2014). β1-integrin deficient GE11 cells were transiently co-transfected with GFP-tagged β1A-integrin and red fluorescent protein, both in pcDNA3 vectors, and plated for different times on FN-coated round coverslips. Then coverslips were mounted in the spinning disc device. Coverslips were spun with the spinning disc device and shear stress was applied in PBS containing Ca²⁺ and Mg²⁺ during 5min at room temperature. Coverslips were then fixed and mounted in Fluoromount. Automated red-fluorescent protein and phase contrast images were taken with an inverted Nikon microscope integrated into an ImageXpress Micro High Content Screening System (Molecular Devices, Sunnyvale, CA, USA). Counting and determination of the radial position of cells was performed with the Metamorph software (Molecular Devices,
Sunnyvale, CA, USA). Final plotting of normalized cell density and $T_{50}$ determination was performed with Prism6 (GraphPad Software, Inc. CA, USA).

To reduce the influence of endogenous $\alpha_v\beta_3$-integrin expressed in GE11 cells, non-transfected GE11 cells were first incubated in the presence of various concentrations of the $\beta_3$-integrin inhibitor (S 36578-2), to determine conditions in which non-transfected GE11 cell adhesion to FN-coated coverslips was minimal. We then selected 400nM inhibitor for 30 and 60 minutes of cell incubation with the coated coverslips prior to shear stress application.

Statistical analysis
Experiments were performed at least in triplicates and the mean and SEM were determined. Paired student t-test (for direct sample comparison), or one-way ANOVA analysis (for comparison of multiple samples) were performed with Excel and PrismGraph. Significant differences were assumed when p-values were below 0.05. If not specifically mentioned p-values were indicated as follows (p<0.01 by **, p<0.001 by ****, or p<0.0001 by ****).
Author contribution
MSR, BK and MB contributed equally to construct cloning, mutagenesis, cell culture and life-cell analysis. LA performed the Octet analysis and MB the binary choice substrate analysis and SIM-imaging. Spinning disc experiments were performed by MSR and DBoe. MC provided technical help, and DBoe, DBou, MB and VH provided funding, discussions and conceptual input. BWH wrote the manuscript. All authors revised and accepted the final version of the manuscript.

Competing interest
None of the authors declares a competing interest.

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References


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Figures

Figure 1:

Expression of GFP tagged β1-integrin splice variants and adhesion length analysis. Representative TIRF images from 3 independent experiments, showing endogenous β1-integrin (A) or transiently transfected GFP-tagged β1-integrin splice variants in NIH-3T3 (A-F) and C2C12 cells (H-K) cultured for 8hrs on FN (A-F, H, I) and LN coated (J,K) glass coverslips. Endogenous, substrate-bound β1-integrin was revealed with 9EG7 antibody staining, showing centrally located fibrillar adhesions and peripheral focal adhesions (A). Cell expressing a c-terminally GFP-tagged β1A-integrin, with a linker previously used for β3-GFP-integrin (Ballestrem et al., 2001).
(B), or extracellularly GFP-tagged β1A-integrin according to Egervari et al., (Egervari et al., 2016) (C, G). The integrin staining patterns were also compared to cells transfected with a c-terminally tagged β1A-integrin with a 30 amino acid, highly flexible linker (D), described in (Parsons et al., 2008). Centrally located short adhesions were observed with both c-terminally (E), and extracellularly tagged β1D-integrin splice forms (F). (G) Structure based model of extracellularly GFP-tagged α5β1-integrin receptor with insertion site at Pro88, based on published structures α5β1 (3VI4), GFP (1C4F) and fibronectin type III repeats 7-10 (1FNF). An animated view of the integrin-GFP fusion model is shown as supplementary data. (H-K) Expression of extracellularly tagged β1A- (H, J) and β1D-integrin (I, K) splice variants in mouse C2C12 cells plated for 8 hours on FN (H, I) or LN (J, K) coated coverslips. (L-N) Quantification of extracellular-tagged β1A- and β1D-integrin adhesion length distributions from TIRF-images of transiently transfected C2C12 cells (n=3), plated for 8 hrs on FN-coated glass coverslips, using a non-supervised segmentation (L) (3x 20 cells analyzed, mean +/-SEM, t-test), or a blinded, manual tracking approach (M, N) (Adhesions from 3x 8 cells analyzed, mean +/-SEM, ANOVA), revealing a shift in individual (M) and mean (N) adhesion length distribution to longer β1A-adhesions, compared with β1D-adhesions, or adhesions positive for β3-GFP-integrin (Ballestrem et al., 2001).
**Figure 2:**

*FN-assembly of extracellularly GFP-tagged β1A and β1D-integrins in C2C12 cells.*

Representative confocal images (A-H) of transiently transfected GFP-β1A-, or GFP-β1D-integrin in C2C12 cells plated overnight on laminin-coated glass coverslips (A-H) and imaged for β1A-integrin (GFP; A), β1D-integrin (GFP; E), F-actin (Phalloidin-texas-red (TR); B,F), or deposited fibronectin (anti-FN, C,G), and their merge (D,H). Line profiles were drawn through the magnified area to reveal co-localization of F-actin, FN and β1-integrin.
Figure 3:

FN-assembly, turnover dynamics and vinculin adapter recruitment of extracellularly GFP-tagged β1A and β1D-integrins in C2C12 cells.

Representative confocal images, of time lapse or FRAP analysis of transiently transfected C2C12 cells plated on FN-coated (A-D, F-I) glass coverslips. (A) Time-lapse analysis of C2C12 cells transfected with GFP-β1A-integrin and cultured for 72 hrs, revealed dynamic changes in integrin distribution, illustrated by an elongating patch of GFP-β1A-integrin fluorescence (between arrows, and boxed region shown as a kymograph in the last frame). Please note the localization of integrin positive patches (asterisks) close to interference reflection-dark zones of cell-substrate adhesions. (B-E) FRAP analysis (bleached regions indicated by dashed lines) of C2C12 cells transfected with either GFP-β1A-integrin (B) or GFP-β1D-integrin (C,D) and plated on FN-coated coverslip for 30hrs (B), 5 hrs (C) and 24 hrs (D). An interference reflection image is shown at the beginning of each sequence.
showing whether the GFP-labeled structures are directly bound to glass-coated FN (interference reflection-dark areas) (C, asterisks), or partially-detached FN-fibrils (B,D). A quantification of the fluorescence recovery of bleached adhesion structures (as indicated by an arrow in (B)) is shown in (E). Data points represent the mean +/-SEM of 3 to 4 cells per condition, with each 3 to 5 analyzed adhesions. With the exception of the first data-point at 40 seconds, a t-test showed significant differences (p<0.05) for all later time points. (F-I) Anti-vinculin stained (magenta) C2C12 cells transfected with different integrins and plated overnight on FN-coated coverslips revealing extracellularly tagged GFP-β1A-integrin (F), GFP-β1D-integrin (G), β3-GFP-integrin (Ballestrem et al., 2001) (H), and c-terminally GFP-tagged β1A-aa-GFP-integrin as in Fig. 1B (I). Note the membrane staining of the c-terminally tagged β1A-aa-GFP-integrin, and apparent absence from vinculin positive central adhesions (compare F with I).
Figure 4:

Mutational analysis of GFP-β1A- and GFP-β1D-integrin adhesion formation in NIH-3T3 cells.

Sequence comparison between the cytoplasmic tails of β1A-, β1D-, and β3-integrins and indication of the conserved proximal and distal NXXY motifs (A). Residues differing from β1A-integrin are highlighted in red and blue for β1D-, and β3-integrins respectively.

Representative TIRF images of 3T3 cells plated for 8 hrs on FN-coated glass coverslips, transiently transfected with wildtype or mutant versions of extracellularly GFP-tagged β1A-, or β1D-integrins as indicated. (B, C) representative images of c-terminal deletion
series of β1A- (B), or β1D-integrins (C). Note the progressive loss of adhesion formation in c-terminal truncated β1A-, but not β1D-integrins. (D) Sequence swapping between β1A- and β1D-integrins, revealing a partial loss-of-function phenotype in the β1D-P786A-integrin mutant.
Confocal imaging and FRAP analysis of extracellularly tagged wildtype GFP-β1A-integrins (A), GFP-β1D-integrins (B), GFP-β1D-P786A-integrins (C), and c-terminally tagged β3-GFP-integrin (Ballestrem et al., 2001), transiently transfected into NIH-3T3 cells and plated onto FN-coated glass coverslips prior to FRAP analysis (E). FRAP curves were established from at least 3 independent cells with 3 different adhesions per cell (mean +/-SEM). In order to exclude an influence of expression levels, or of endogenous β1A-integrins, the FRAP analysis was repeated in stable transfected GD25 cells, expressing extracellularly GFP-tagged β1A- and β1D-integrins (F) (n=5 cells with 3-5 analyzed adhesions; mean +/-SEM, t-test), showing significantly different fluorescence recovery during the recovery phase (****) or at the end of the analysis period for the mobile fractions (**). (G) spinning disk analysis of the shear force resistance of GE11 cells on FN-coated coverslips, transiently transfected with different β1-integrins and inhibited with

Figure 5:

FRAP and cytoplasmic adapter recruitment analysis in wildtype and P786A-mutant β1D-integrins.
an αvβ3-inhibitor. Note the lack of significant differences in shear force resistant between GFP-β1A- and GFP-β1D-integrins, and more substantial, but still non-significant reduction for the GFP-β1D-P786A-integrin mutant (mean +/- SEM, ANOVA). (H) Biolayer interference analysis (Octet-sensor) of GST-β-integrin tail fusion protein binding to his-tagged Talin1-FERM domain (residues 1-405) (n=3, mean +/- SEM). Note the 2-fold reduction in talin affinity for the β1D-P786A-mutant, but 5-fold lower affinities of β1A- and β3-integrin tails as compared to integrin β1D.
Figure 6:
Analysis of central adhesions for Talin2 and β1D-integrin recruitment
Representative confocal and TIRF images from 3 independent experiments showing transiently or stable transfected C2C12 (A-H), or NIH-3T3 cells (I-J), expressing c-
terminally tagged talin1, talin2, as well as the Tac-β1A or Tac-β1D integrin chimera. (A,B) Transient transfections of C2C12 cells with Talin1-mcherry (A) and Talin2-YPET (B), and the evaluation of the aspect ratios of talin-positive adhesions (n=3) with 8 cells per condition (mean +/-SEM, t-test) (C). (D,E) Anti-Tac-antibody staining (mAb 7G7) of stably Tac-β1A (C) and Tac-β1D (D) expressing and bulk sorted C2C12 cells. Cells were plated on serum coated glass coverslips for 24 hrs prior fixation and imaging. Subsequent aspect ratios of Tac-positive adhesion structures were determined from three different batches of plated cells (n=3, with 8 cells per condition, mean +/-SEM, t-test) (F). (G,H) Transient transfection of Talin2-YPET into stably Tac-β1A (G), or Tac-β1D (H) expressing C2C12 cells. Representative cells were selected from 3 independent experiments and co-localization was determined by confocal microscopy and comparison of the individual signals of Talin2-YPET (G’,H’) and anti-Tac (G’’,H’’). Please note the extensive overlap of central (upper panels) and peripheral adhesions (lower panels) between the Talin2 and Tac-β1D construct (H), and reduced overlap of Talin2 and Tac-β1A construct in central adhesions (G’,G’’, upper panels). (I, J) TIRF imaging of anti-vinculin stained NIH-3T3 cells transiently transfected with extracellular GFP-tagged β1D-P786A (I) and β1D-P786A/NN788/9TT (J) mutant integrins. Note the absence of recruitment to central adhesions in the β1D-P786A-mutant, and recovery of fibrillar adhesion like central adhesions in the triple β1D-P786A/NN788/9TT-mutant, respectively.
Figure 7:

Reduction in cell spreading and proliferation of β1D-integrin expressing cells correlates with reduced paxillin recruitment.

Representative images from 3 independent experiments showing TIRF-imaging of stably transfected GD25 cells (A-F), plated for 6 hrs (A,B), or overnight (C-F) on FN-coated glass coverslips and stained with anti-P-tyr (A, B) and anti-paxillin antibodies (C-F). Note the absence of P-tyr and paxillin staining in central fibrillar adhesions in wildtype β1A-integrin transfected cells (A,C), but also lack of co-localization in peripheral adhesions in
GFP-tagged β1A-A786P-integrin (D), β1D-integrin (E), and β1D-P786A-integrin (F) transfected cells. SIM-microscopy of GD25 cells stably expressing GFP-β1A-, or GFP-β1D-integrins, plated on binary-choice VN/FN patterned substrates obtained by microcontact printing (G, H, K). (I) Quantification of the cell spreading area of GD25 cells stably transfected with GFP-β1A-, or GFP-β1D-integrin on FN-coated coverslips, 6 hrs after plating (15 cells per condition, n=3; mean +/-SEM, t-test and significance represented by the P-value). Quantification of the pearson coefficient from TIRF-images comparing anti-paxillin staining and GFP-β1-integrin fluorescence (J) from cells plated overnight on FN-coated coverslips (10 cells analyzed per condition, n=3; mean +/-SEM, ANOVA). Quantification of co-localization of GFP-β1A-, or GFP-β1D- integrins with paxillin and FN on VN/FN-binary choice patterns (10 cells for each condition, n=3, mean +/-SEM, t-test) (K). (L) BRDU-incorporation assay into GD25 cells stably expressing GFP-β1A-, GFP-β1D-, or GFP-β1A-A786P-mutant integrin (n=3, >250 cells per condition, mean +/-SEM, ANOVA). Note the significant reduction in BRDU incorporation of the A786P-mutant compared to wildtype β1A-integrin. Although similar to non-transfected cells, the reduction of BRDU-incorporation of β1D-integrin was not significantly different from wildtype β1A-integrin transfected cells. (M) Representative FACS and dot-blot analysis of stably transfected GD25 cells exhibiting GFP-β1A-integrin, or GFP-β1D-integrin on their surfaces. The intensity of GFP (x-axis) is plotted against the surface staining with mAb Ts2/16, which does not stain non-transfected GD25 cells. (N,O) 75 hrs growth curve of stably transfected GD25 cells (n=3, mean +/-SEM, t-test), revealed by impedance measurement in an xCELLigence System. The histogram in O, shows the impedance measurement of both cell lines at the time the half-maximal level of impedance was reached by the GFP-β1A-integrin transfected cells (n=3, mean +/-SEM, t-test).
**Movie 1** (.mov) to Fig. 1G (structural model of the extracellular tagged GFP-β1-integrin fusion protein). The animation gives a 3D impression of the GFP-tagged (green) ligand-binding domain of α5β1-integrin (RGD-peptide in yellow). Model based on the ligand-binding extracellular fragment of α5β1 corresponding to 3VI4, and green fluorescent protein (GFP) corresponding to 1C4F.
Movie 2 (.avi) to support Fig. 3D. C2C12 cell transiently expressing the GFP-β1D-integrin construct for 72 hrs. Although the recovery of bleached GFP-β1D-integrin structures on the surface of that cell is very slow, the individual GFP-positive fibers move on the surface of the cell (40 seconds between frames).
Movie 3 (.mov) to support Fig. 5A. Transiently transfected 3T3 cell expressing the GFP-β1A-integrin construct for 72 hrs. Fluorescent structures are bleached and recovery can be observed over time (40 seconds between frames).
**Movie 4 (.mov)** to support Fig. 5B. Transiently transfected 3T3 cell expressing the GFP-β1D-integrin construct for 72 hrs. Fluorescent structures are bleached and recovery can be observed over time (40 seconds between frames).
Movie 5 (.mov) to support Fig. 5C. Transiently transfected 3T3 cell expressing the GFP-β1D-P786A-mutant-integrin construct for 72 hrs. Fluorescent structures are bleached and recovery can be observed over time (40 seconds between frames).
**Movie 6 (.mov)** to support Fig. 5D. Transiently transfected 3T3 cell expressing the β3-GFP-integrin construct for 72 hrs. Fluorescent structures are bleached and recovery can be observed over time (40 seconds between frames).
**Movie 7 (.avi)** to support Fig. 5F. Representative example of a stably transfected GD25 cell expressing the GFP-β1A-integrin construct. Fluorescent structures are bleached and recovery was followed over time to establish the data in Fig. 5F (20 seconds between frames. Width of field 53µm).
**Movie 8 (.avi)** to support Fig. 5F. Representative example of a stably transfected GD25 cell expressing the GFP-β1D-integrin construct. Fluorescent structures are bleached and recovery was followed over time to establish the data in Fig. 5F (20 seconds between frames. Width of field 53µm).