β₁- and αᵥ-class integrins cooperate to regulate myosin II during rigidity sensing of fibronectin-based microenvironments

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How different integrins that bind to the same type of extracellular matrix protein mediate specific functions is unclear. We report the functional analysis of β₁- and αᵥ-class integrins expressed in pan-integrin-null fibroblasts seeded on fibronectin. Reconstitution with β₁-class integrins promotes myosin-II-independent formation of small peripheral adhesions and cell protrusions, whereas expression of αᵥ-class integrins induces the formation of large focal adhesions. Co-expression of both integrin classes leads to full myosin activation and traction-force development on stiff fibronectin-coated substrates, with αᵥ-class integrins accumulating in adhesion areas exposed to high traction forces. Quantitative proteomics linked the functional analysis of β₁-integrins to a GEF-H1–RhoA pathway coupled to the formin mDia1 but not myosin II, and αᵥβ₁ integrins to a RhoA–Rock–myosin II pathway. Our study assigns specific functions to distinct fibronectin-binding integrins, demonstrating that αᵥβ₁ integrins accomplish force generation, whereas αᵥ-class integrins mediate the structural adaptations to forces, which cooperatively enable cells to sense the rigidity of fibronectin-based microenvironments.

Integrins are α/β heterodimers that mediate cell adhesion to the extracellular matrix (ECM) and to receptors on other cells, thereby regulating numerous biological processes that are essential for development, postnatal homeostasis and pathology. The mammalian genome encodes 18 α and 8 β integrin genes, which form 24 heterodimers. Mammalian cells usually co-express several integrins, which recognize ECM components by binding specific amino-acid stretches such as the Arg-Gly-Asp (RGD) motif. RGD motifs are found in many matrix proteins including fibronectin, in which RGD mediates binding to α₅β₁ and all αᵥ-class integrins. In vivo and in vitro studies indicated that α₅β₁ and αᵥ-class integrins (for example, α₅β₃) exert both specific and redundant functions; however, how these distinct integrins accomplish their individual functions and whether these cooperate remains unclear. The signalling properties and functions of integrins are executed by specialized adhesive structures with distinct morphology, subcellular localization, lifespan and molecular composition. Nascent adhesions are short-lived adhesive structures in membrane protrusions that promote the activity of Rho–GTPases such as Rac1. Some nascent adhesions develop into large focal adhesions that initiate multiple signalling pathways, which activate effectors including myosin II. Myosin II exerts contractile forces resulting in adhesion reinforcement and recruitment of more proteins to focal adhesions, which induces a further increase in myosin II activity. This feedback signalling to myosin II critically depends on biophysical parameters such as ECM stiffness. The identity of mechanosensor(s) in focal adhesions, whether it is an integrin, a focal adhesion protein or a combination of both, is unknown. Quantitative mass spectrometry (MS) was previously used to determine the protein composition of adhesion structures (adhesomes) of cells seeded on fibronectin, and the dynamic changes on myosin-II-induced adhesion maturation. As cells recruit different integrin classes to fibronectin-induced adhesions, these studies did not assign specific proteins and signalling outputs to particular integrins.

Here we developed a cell system to investigate the protein composition and signalling properties of adhesion sites anchored selectively through α₅β₁ and/or αᵥ-class integrins. We found marked integrin-class-specific differences in the morphology of focal adhesions, in their requirement for mechanical tension, in the protein composition of their adhesomes and their signalling capacity. Furthermore, we...
identified a functional synergy between $\alpha_\beta_1$ and $\alpha_v$-class integrin signalling hubs leading to feedback amplification of myosin II activity required for focal-adhesion-mediated rigidity sensing.

RESULTS
Differential functions of $\alpha_\beta_1$ and $\alpha_v$-class integrins in adhesion formation and cell migration

To obtain cells expressing $\beta_1$- and/or $\alpha_v$-class integrins we intercrossed mice carrying conditional null mutations for the $\alpha_\iota$ and $\beta_\iota$ integrin genes and constitutive null mutations for the $\beta_1$ and $\beta_\iota$ integrin genes ($\beta_1^{-/-}, \alpha_\iota^{-/-}, \beta_\iota^{-/-}, \alpha_v^{-/-}$ mice)\(^{11}\), isolated kidney fibroblasts and immortalized them with the SV40 large T antigen (parental fibroblasts). Deletion of floxed $\alpha_\iota$ and $\beta_1$ integrin genes by adenoviral Cre transduction removed all integrins from the parental fibroblast clones (pan-knockouts, pKO; Supplementary Fig. S1a–c). Next we transduced parental fibroblasts with $\alpha_v$ or $\beta_1$ or both complementary DNAs and simultaneously transduced Cre to delete the floxed integrin alleles. This produced cells expressing $\alpha_\iota$ (pKO-$\alpha_\iota$), $\beta_1$ (pKO-$\beta_1$) or $\alpha_v$ and $\beta_1$ (pKO-$\alpha_v$/$\beta_1$) integrins, respectively (Fig. 1a). The pKO-$\alpha_\iota$, pKO-$\beta_1$, and pKO-$\alpha_v$/$\beta_1$ cells were sorted for comparable integrin surface levels to the parental cell clones (Supplementary Fig. S1d,e). Using western blotting, flow cytometry and MS we identified the following fibronectin-binding integrins: $\alpha_\iota$-$\beta_1$ in pKO-$\beta_1$ cells, $\alpha_\iota$-$\beta_\iota$ and $\alpha_\iota$-$\beta_3$ in pKO-$\alpha_\iota$ cells, and $\alpha_v$-$\beta_1$, $\alpha_v$-$\beta_\iota$ and $\alpha_v$-$\beta_3$ in pKO-$\alpha_v$/$\beta_1$ cells (Supplementary Fig. S1f,g). Calibration of our flow cytometry analysis estimated the presence of 170,000 $\alpha_\iota$-$\beta_1$, and 300,000 $\alpha_v$-class integrins on the surface of each cell, resulting in approximately equimolar surface levels for $\beta_1$, $\beta_\iota$, and $\beta_3$ integrins.

All three cell lines specifically adhered to fibronectin, whereas adhesion on vitronectin was similar for pKO-$\alpha_\iota$ and pKO-$\alpha_v$/$\beta_1$ cells.
and absent for pKO-β1 cells (Supplementary Fig. S1h). To compare the size distribution of focal adhesions we seeded cells for 90 min on fibronectin and immunostained for paxillin, integrin β1, and β1 (Fig. 1b and Supplementary Fig. S2a,b). The percentage of small nascent adhesions (<2 μm²) was significantly elevated in pKO-β1 and pKO-α/β1 cells, whereas large focal adhesions of 6–12 μm² dominated in pKO-α cells ( Supplementary Fig. S2a,b). The cell spreading area on fibronectin was significantly lower in pKO-α relative to pKO-β1 and pKO-α/β1 cells and reduced in pKO-α/β1 relative to pKO-β1 (Fig. 1c and Supplementary Fig. S1i). As cell shape and spreading area can affect cell contractility, focal adhesion size and distribution, we seeded cells on circular fibronectin-coated micropatterns surrounded by non-adhesive polyethylene glycol (PEG), and confirmed the different adhesion size distribution in the three cell lines (Fig. 1d,e). pKO-α/β1 cells contained both small nascent adhesions and large focal adhesions (Fig. 1d). pKO-β1 and pKO-α/β1 cells showed increased protrusive activity when compared with pKO-α cells (Supplementary Fig. S2a,c), which correlated with increased migration speed. The mean square displacement (MSD) of cells migrating on fibronectin showed that pKO-β1 cells migrated significantly faster than pKO-α cells, and that pKO-α/β1 cells exhibited an intermediate migration speed (Fig. 1f,g). As previously shown11,23,24, expression of α-class integrins increased migration persistence (Fig. 1h). pKO-β1 cells exhibited a significant defect in trailing edge detachment (Fig. 1i and Supplementary Fig. S2c and Videos S1–S3). These results identify a role for αβ in protrusive activities and nascent adhesion formation, whereas co-expression of α-class integrins also promotes the production of large, stable focal adhesions and trailing edge detachment in migrating cells.

**Differential functions of αβ and α-class integrins synergize to regulate cell contractility**

Adhesion maturation and trailing edge retraction in migrating fibroblasts requires coordinated control of myosin-II-mediated cell contractility25. We measured myosin II activity using fibronectin-coated X- or crossbow-shaped micropatterns, which report subtle changes in myosin II activity and traction forces along non-adhesive edges26–28. Parental fibroblasts cultured on X-shaped fibronectin-coated micropatterns showed a dose-dependent decrease of phosphoT18/S19-myosin light chain (pMLC), paxillin fluorescence intensities and cell area following treatment with the myosin II inhibitor blebbistatin (Supplementary Fig. S2d–g). Crossbow patterns polarize cells into a low contractile front and a highly contractile rear29. Immunofluorescence analysis revealed that pMLC and paxillin intensities were the highest in pKO-β1, lower in pKO-β1, and the lowest in pKO-α cells (Fig. 2a). Myosin II activity was low in the cell front (Fig. 2b) and high in the cell rear (Fig. 2c) and the cooperative effect of the two integrin classes on pMLC and paxillin intensities in pKO-α/β1 was most prominent in the cell rear (Fig. 2a–c). Treatment with the α-class-specific small-molecule inhibitor cilengitide reduced contractility of pKO-α/β1 cells to intermediate levels (Fig. 2h,e), confirming that the adhesive function of α-class integrins is required for the synergy with αβ. We corroborated these results with fibronectin-coated X-shapes, revealing phenotypes that resembled the rear of crossbow shapes (Supplementary Fig. S2h–j).

The ability to form large focal adhesions and stress fibres indicative of high contractile forces together with low pMLC levels in pKO-α cells was surprising. Traction-force microscopy experiments on polyacrylamide gels of 35 kPa stiffness revealed good correlation of traction forces and pMLC levels, confirming that traction forces on fibronectin-coated crossbow micropatterns are the lowest in pKO-α, the highest in pKO-α/β1 and intermediate in pKO-β1 cells (Fig. 2d). Along the cell front, traction forces were significantly higher in pKO-β1 cells when compared with pKO-α cells and the highest in pKO-α/β1 (Fig. 2e). Similar differences were observed by calculating the total contractile energy of individual cells (Fig. 2f).

**α-class integrins accumulate in areas of high traction force and mediate rigidity sensing**

The αβ integrins are known to become immobilized in large and static focal adhesions, whereas αβ integrins are mobile, separate from the αβ integrins and translocate rearward to fibrillar adhesions30,29. To investigate whether αβ and α-class integrins segregate owing to differential dependence on myosin-II-mediated tension at focal adhesions we seeded pKO-α/β1 and parental floxed cells on fibronectin-coated crossbow shapes and immunostained β1 and β3 integrins. Indeed, β3 heavily accumulated in areas that were shown to be exposed to the highest traction forces, whereas β3 levels remained very low at these sites (Fig. 3a,b). The β1 integrins in contractile focal adhesions at the cell rear were lost following blebbistatin treatment, whereas small β3-containing focal adhesions in the cell periphery were still forming ( Fig. 3a). To confirm these findings we plated pKO-α/β1 cells on 1-μm-thin fibronectin-coated lines separated by 3-μm-wide non-adhesive PEG lines. This set-up allows distinguishing ligand-bound from unbound integrins, which is impossible on uniformly coated fibronectin surfaces. Whereas the β1 integrin staining co-localized with fibronectin lines almost throughout the entire cell length, small β3 clusters overlaid with lines in the cell periphery associated with F-actin bundles. Blebbistatin treatment or inhibition of ROCK with Y-27632 disassembled the β3 integrin clusters on fibronectin lines, whereas β3 remained unchanged (Fig. 3c). The differential dependence of αβ integrins and α-class integrins on myosin-II-mediated tension at focal adhesions suggested that tension-dependent stabilization of α-class integrins contributes to rigidity sensing. In line with this hypothesis, traction-force measurements of pKO-β1 and pKO-α/β1 cells plated on micropatterned polyacrylamide gels of 3 different rigidities (1.4, 10 and 35 kPa) revealed that only pKO-α/β1, but not pKO-β1, cells were able to increase contractile energies concomitantly with the substrate rigidity. Most notably, the traction forces and contractile energies generated by pKO-β1 and pKO-α/β1 cells were similar on soft, 1.4 kPa substrates, whereas they differed significantly on stiffer substrates (Fig. 3d,e). We therefore conclude that stabilization of αβ- and fibronectin bonds through actomyosin-mediated tension is required to adjust cell contractility to defined substrate stiffnesses.

**Adhesive composition and stoichiometry is controlled by the integrin class and myosin II activity**

Cells sense their environment through integrins and numerous plaque proteins in focal adhesions17,30. The composition and stoichiometry of the adhesome in fibronectin-bound fibroblasts is controlled by myosin II (refs 19,20). We therefore reasoned that specific binding activities of the integrin cytoplasmic tails and also the differential myosin II activities in pKO-α, pKO-β1 and pKO-α/β1...
cells may contribute to their specific adhesome composition. To test this hypothesis we determined the integrin-class-specific protein composition of focal adhesions. The three cell lines were plated for 45 or 90 min on fibronectin or poly-L-lysine (PLL; permits integrin-independent adhesion) followed by chemical crosslinking and purification of focal adhesions, sample elution and quantitative MS as described previously\(^\text{19}\) (Supplementary Fig. S4a and Table S1). Isolated adhesome proteins were quantified using the label-free quantification algorithm of the MaxQuant software\(^\text{41}\). We calculated median MS intensities of 3–4 replicates and performed hierarchical clustering to compare the three cell lines at different time points with and without blebbistatin. This approach allowed identifying protein groups with high correlation of their intensity changes across different substrates, time points and cell lines. We identified a cluster containing 168 proteins significantly enriched for known (previously annotated) focal adhesion proteins. In addition to the 168 proteins, we also considered all previously annotated focal adhesion proteins\(^\text{12}\) assigned to other clusters in our analysis. This led to 245 proteins used for further analysis (Supplementary Fig. S4b). Analysis of variance (ANOVA) tests revealed that MS intensities of 62% (152/245) of them were significantly changed that MS intensities of 62% (152/245) of them were significantly changed.

**Figure 2** α\(_1\)-class integrins cooperate with α\(_5\)β\(_1\) for myosin II reinforcement on stiff fibronectin-coated substrates. (a) Averaged confocal images of immunostainings (Merge: F-actin, red; pMLC, green; paxillin, blue; DAPI, blue) of the indicated cell lines plated for 3 h on fibronectin-coated micropatterns (pKO-α\(_5\); n = 55, pKO-β\(_1\); n = 36, pKO-α\(_5\)/β\(_1\); n = 71; data aggregated over 3 independent experiments). Areas with strong pMLC and paxillin fluorescent signals are marked with arrows. Scale bar, 10 μm. (b,c) Intensities of pMLC and paxillin (Pxn) fluorescence in the front (b) and rear (c) regions of individual cells (pKO-α\(_5\); n = 25, pKO-β\(_1\); n = 32, pKO-α\(_5\)/β\(_1\); n = 26; 1 representative of 3 independent experiments is shown). Optionally, cells were treated with the α\(_1\)-class integrin inhibitor cilengitide (1 μM). (d) Average traction-force fields of indicated cell types (pKO-α\(_5\); n = 54, pKO-β\(_1\); n = 86, pKO-α\(_5\)/β\(_1\); n = 68; data aggregated over 3 independent experiments). Arrows indicate force orientation; colour and length represent local force magnitude in nanonewtons. Scale bar, 10 μm. (e) Average integrated traction forces along the cell border (pKO-α\(_5\); n = 54, pKO-β\(_1\); n = 86, pKO-α\(_5\)/β\(_1\); n = 58; data aggregated over 3 independent experiments; thin lines represent s.e.m.). (f) Contractile energy of individual cells (pKO-α\(_5\); n = 54, pKO-β\(_1\); n = 86, pKO-α\(_5\)/β\(_1\); n = 68; data aggregated over 3 independent experiments). Each data point corresponds to the total contractile energy of an individual cell measured by traction-force microscopy. All statistical comparisons were t-tests (error bars represent s.e.m.), pKO-α\(_5\) (green); pKO-α\(_5\)/β\(_1\) (blue); pKO-β\(_1\) (orange); pKO-α\(_5\)/β\(_1\) + 1 μM cilengitide (black).
Figure 3 αv-class integrins accumulate in adhesion areas exposed to high traction force and cooperate with α5β1 for rigidity sensing on fibronectin. 
(a) pKO-αv/β1 cells were plated on fibronectin-coated crossbow shapes for 3 h with and without blebbistatin (BLEB) and immunostained for β1 (blue), β3 (green) integrins and F-actin (red). Scale bars, 10 μm. DAPI, white (left panel, merge). (b) Fluorescence intensity profile of the indicated stainings along the depicted linescan (3.75 μm). (c) pKO-αv/β1 cells were plated on 1 μm thin fibronectin-coated lines for 90 min with and without blebbistatin and stained for β1 (blue), β3 (green) integrin and F-actin (red). Scale bars, 10 μm. DAPI, white (merge). (d) Each data point represents the total contractile energy of individual cells measured by traction-force microscopy on gels of indicated rigidities (pKO-β1: soft n = 54, medium n = 50, stiff n = 86; pKO-αv/β1: soft n = 31, medium n = 71, stiff n = 68; data aggregated over 3 independent experiments; all pairwise statistical comparisons from t-tests are shown in Supplementary Table S5; NS, not significant). (e) Each data point represents the total integrated traction force in kilo Pascal (kPa) of individual cells measured by traction-force microscopy on gels of indicated rigidities (pKO-β1: soft n = 54, medium n = 50, stiff n = 86; pKO-αv/β1: soft n = 31, medium n = 71, stiff n = 68; data aggregated over 3 independent experiments; P values of pairwise comparisons were calculated with a t-test). pKO-αv/β1 (blue); pKO-β1 (orange).

In at least one of the three cell lines or one of the two time points (Supplementary Table S1).

In line with our previous report19, blebbistatin induced different intensity reductions in floxed fibroblasts for different classes of adhesome proteins. Following blebbistatin treatment pKO-αv/β1 and pKO-β1 cells were still able to recruit integrin-proximal proteins such as Talin-1, Kindlin-2 and ILK, whereas LIM-domain-containing proteins were reduced to background levels defined by MS intensities from cells seeded on PLL (Fig. 4a). Strikingly, blebbistatin reduced almost all focal adhesion proteins to background levels in pKO-αv cells, indicating that...
Figure 4 Composition and stoichiometry of the adhesome is determined by the individual integrin and myosin II activity. (a) Focal-adhesion-enriched fractions analysed by MS before and after blebbistatin (BLEB) treatment. The Z-scores of median MS intensities (n = 3–4) are colour coded to show relative protein abundance. A blebbistatin-insensitive cluster is marked with a red bar and blebbistatin-sensitive clusters are marked with blue bars. The arrow highlights the pronounced effect of blebbistatin on pKO-α5β1 cells. FN, fibronectin. (b) Boxplots showing MS intensity differences of 58 known focal adhesion proteins of the indicated cells relative to pKO-α5 cells cultured for 45 min without blebbistatin. A t-test revealed significant MS intensity changes after blebbistatin treatment. Boxplot whisker ends are at 1.5 interquartile range and outliers are shown as dots. (c) Focal adhesion proteins with similar Z-score profiles (colour coded) as α5β1 or αv-class integrins (selection based on Supplementary Fig. S6) were subjected to hierarchical cluster analysis. Focal-adhesion-enriched fractions were collected 45 and 90 min after plating on fibronectin. (d) SILAC ratio plot from label-inverted replicates comparing β1 with β3 tail pulldowns. Specific interactors have high SILAC ratios in the forward experiment (fwd) and low SILAC ratios in the label swapped reverse experiment (rev). The colour code shows the percentage of sequence coverage of the proteins identified by MS analysis (n = 4; 2 independent experiments): pKO-α5 (green); pKO-αv/β1 (blue); pKO-β1 (orange).
We performed stable isotope labelling with amino acids in cell culture (Fig. 4b). Furthermore, comparing the 45 and 90 min time points revealed that protein recruitment to focal adhesions was delayed in pKO-αvβ3 cells (Fig. 4a,b). Importantly, blebbistatin did not change the MS intensities of αv-class integrins, indicating that short-lived/weak αv-class integrin–fibronectin interactions are not affected by the absence of cell contractility and can be crosslinked. These findings together with those depicted in Fig. 3 indicate that αvβ3 can cluster and induce adhesome assemblies in the absence of myosin-II-mediated tension, whereas the ability of αv-class integrins to cluster and recruit adhesome proteins depends on myosin II activation and/or the stress fibre architecture at focal adhesions.

**ILK and GEF-H1 are required for myosin II reinforcement on stiff substrates**

Consulting published protein–protein interactions within the adhesome, we established a putative core interactome of fibronectin-bound αvβ3 or αv-class integrins (Supplementary Fig. S5). Hierarchical cluster analysis of MS intensities of the 125 core proteins of the integrin interactome from all conditions tested (Supplementary Fig. S6) revealed 29 proteins correlating with MS intensities of αvβ3 at both time points and 2 proteins correlating with MS intensities of αv-class integrins (Fig. 4c). In addition to this integrin interactome, we analysed the MS intensities of all actin-binding proteins in the focal-adhesion-enriched fraction and found that WAVE and Arp2/3 complexes, which drive lamellipodia formation, correlated with αvβ3, whereas the RhoA effector Dia1 (Dia1), which drives stress-fibre formation, correlated with αv-class integrins (Supplementary Fig. S7). We performed stable isotope labelling with amino acids in cell culture (SILAC)-based peptide pulldown assays with β1 and β3 integrin tail peptides and scrambled control peptides followed by MS (ref. 33) to identify which of the 29 αvβ3-enriched and 2 αv-class integrin-enriched adhesome proteins were enriched through differential associations with integrin cytoplasmic tails. Comparison of integrin-tail interactors with scrambled peptide interactors identified common and specific β3 tail- and β1 tail-binding proteins (Supplementary Fig. S8). Talin-1 showed equal binding to β1 and β3 tails and was therefore used to control the experiments. In line with the adhesome analysis (Fig. 4c) we observed very high β1-tail-specific enrichment for Kindlin-2 and a lower enrichment for the ILK/PINCH/Parvin (IPP) complex, and a high β3-tail-specific enrichment of the RhoA guanine nucleotide exchange factor GEF-H1 (Fig. 4d). Thus, the recruitment of Kindlin-2, the IPP complex and GEF-H1 to focal adhesions is controlled by the integrin tail sequence rather than the different focal adhesion architecture in pKO-β1 and pKO-αv-β3 cells. Ratiometric analysis of fluorescence intensities in focal adhesions confirmed higher Kindlin-2 and ILK levels in pKO-β1 cells and pKO-αv-β3 cells (Fig. 5a–d). To analyse GEF-H1 levels in focal adhesions we first chemically crosslinked and unroofed the cells to remove the large cytoplasmic and microtubule-associated GEF-H1 pool, and then performed immunostainings, which revealed that crosslinked GEF-H1 levels were significantly higher in pKO-αv cells and pKO-αv-β3 cells than in pKO-β1 cells (Fig. 5e–g).

To investigate whether the IPP complex and GEF-H1 contribute to myosin II regulation by αvβ3 and αv-class integrins we seeded ILK0/0 (control) and ILK−/− fibroblasts34 on fibronectin-coated X-shapes and stained for pMLC. ILK−/− fibroblasts had similarly low pMLC signals as pKO-αv cells (Fig. 5h,i). Furthermore, inhibition of αvβ3 with blocking antibodies or αv-class integrins with cinglutoide in ILK0/0 cells significantly reduced pMLC levels (Fig. 5h,i), confirming that both fibronectin-binding integrin classes are required to activate myosin II. To examine whether GEF-H1 regulates integrin-mediated activation of myosin II on fibronectin-coated X-shapes we depleted GEF-H1 messenger RNA using short interfering RNA (siRNA; Fig. 5j) and found significantly reduced pMLC levels in GEF-H1-silenced pKO-αv-β3 cells, slightly reduced levels in pKO-β1 cells, and unaffected levels in pKO-αv cells (Fig. 5k,l) indicating that GEF-H1 reinforces myosin II activity in a αvβ3-dependent manner.

The IPP complex and GEF-H1 have been implicated in cell contractility regulation by tuning RhoA GTPase35–37. Therefore, we investigated whether the activity of RhoA and Rac1 are affected in our cell lines. Seeding the three cell lines for 45 min on fibronectin induced a significantly higher RhoA activity in pKO-αv, cells when compared with pKO-β1 and pKO-αv-β3 cells (Fig. 5m). Rac1 activity was the lowest in pKO-αv cells, higher in pKO-β1 and the highest in pKO-αv-β3 cells (Fig. 5n). As the high GEF-H1 and RhoA levels in focal adhesions of pKO-αv cells are not able to promote high pMLC, we conclude that only αvβ3 can elicit signals for mediating RhoA-driven myosin II activation.

**Integrin-specific signalling pathways cooperate for feedback regulation of myosin II**

The coupling of active RhoA to its effector Rock requires unknown signalling events that depend on cell adhesion, cell shape and cytoskeletal tension38–40. To uncover integrin-specific regulators of myosin II upstream and downstream of active RhoA we performed SILAC-based quantitative phosphoproteomics of adhesion signalling on fibronectin. We quantified a total of 3,180 proteins (Supplementary Table S2) and 7,529 phosphorylation sites (Supplementary Table S3) in the three cell lines seeded for 45 min on fibronectin. ANOVA tests of triplicate experiments identified 150 proteins and 1,010 phosphorylation events as significantly regulated in at least one of the three cell lines (Fig. 6a and Supplementary Fig. S9, Tables S2 and S3). Hierarchical cluster analysis of the SILAC ratios of the 1,010 phosphorylation events revealed clusters dominated by αvβ3 complexes and clusters dominated by αv-class integrins. We also observed clusters regulated oppositely by αvβ3 and αv-class integrins, indicating antagonistic regulation, and clusters regulated by both integrin classes, indicating synergistic regulation. Using ratio thresholds for the different pairwise comparisons allowed assignment of 646 of the 1,010 determined phospho-sites into either the antagonistic, dominant or synergistic category (Fig. 6b and Supplementary Table S4).

We searched for phospho-sites that influence myosin II activity in an integrin-dependent manner and found that pKO-β1 and pKO-αv-β3 cells showed increased phosphorylation of the RhoA/Rock targets S693-myosin phosphatase-1 (Mypt1; Fig. 6c–e) and S3-collin (Fig. 6c–e). Myosin II activity is also induced by Mlck, whose activity is controlled by Ca2+ or Erk2 in focal adhesions38,39. We observed synergistic downregulation of S364-Mlck and synergistic...
Figure 5 αv- and β1-mediated activation of myosin II requires ILK and GEF-H1. (a–f) Cells were plated on fibronectin-coated glass coverslips for 90 min and immunostained for: Talin-1 (Tln; red) and Kindlin-2 (Kind2; green) (a); ILK (red), paxillin (Pxn; green) and F-actin (white) (b); or GEF-H1 (red) and paxillin (green) (c); scale bars, 10 µm. DAPI, blue (a,b). Ratios of thresholded fluorescence intensities (FI) were calculated for Kindlin-2 and Talin-1 (pKO-αv, n = 12, pKO-β1, n = 22, pKO-αv/β1, n = 22; results are aggregated over 3 independent experiments) (c), and ILK and paxillin (pKO-αv, n = 33, pKO-β1, n = 40, pKO-αv/β1, n = 40; aggregated over 3 independent experiments) (d). The correlation coefficient for GEF-H1 and paxillin staining (pKO-αv, n = 11, pKO-β1, n = 10, pKO-αv/β1, n = 15; aggregated over 3 independent experiments) was determined (f). (g) Total fluorescence intensity of focal-adhesion-retained GEF-H1 after crosslinking and unroofing of cells (pKO-αv, n = 11, pKO-β1, n = 10, pKO-αv/β1, n = 15; aggregated over 3 independent experiments). (h) ILK−/− and ILK-flox fibroblasts plated for 3 h on fibronectin-coated X-shapes stained for pMLC, F-actin and paxillin. Scale bar, 10 µm. (i) Quantification of the relative fluorescence intensities for pMLC to untreated ILK-floxed cells (ILK-flox n = 26; ILK-null n = 12; ILK-flox + Cil n = 23; ILK-null + Cil n = 12; ILK-flox + anti-αβ1; n = 16, ILK-null anti-αβ1; n = 10; data aggregated over 2 independent experiments). (j) siRNA-mediated depletion of GEF-H1 confirmed by western blotting. (k) Cells were plated on fibronectin-coated X-shapes and stained for pMLC, F-actin and paxillin. Scale bar, 10 µm. (l) Quantification of the relative fluorescence intensities for pMLC in siRNA-treated cells (pKO-αv, + control siRNA n = 24, pKO-β1, + control siRNA n = 48, pKO-αv/β1, + control siRNA n = 56, pKO-αv, + GefH1 siRNA n = 22, pKO-β1, + GefH1 siRNA n = 34, pKO-αv/β1, + GefH1 siRNA n = 59; data aggregated over 2 independent experiments). (m) Relative RhoA-GTP loading in cells plated for 45 min on fibronectin (n = 9; 1 representative of 3 independent experiments is shown). (n) Relative Rac1-GTP loading in cells plated for 45 min on fibronectin (n = 9; 1 representative of 3 independent experiments is shown). Error bars represent s.e.m. and P values were calculated using a t-test. pKO-αv (green); pKO-αv/β1 (blue); pKO-β1 (orange).
Figure 6 Integrin-specific phosphorylation landscapes on adhesion to fibronectin. (a) Hierarchical cluster analysis of SILAC ratios of 1,010 significantly regulated (ANOVA test and Benjamini/Hochberg false discovery rate) phosphorylation events in the indicated cells plated for 45 min on fibronectin from 3 independent replicates. The colour code depicts the normalized log₂ SILAC ratio between cell lines. (b) The bar graph shows the number of phosphorylation events grouped into different modes of regulation based on the indicated SILAC ratio threshold criteria. AG, antagonistic; DO, dominant; SY, synergistic. (c) SILAC ratios for selected phosphorylation events. The bar graph depicts the median of 3 independent experiments with error bars showing the s.d. (d) A selection of differentially regulated phosphorylation events confirmed by western blotting using phospho-site-specific antibodies. (e) Signalling network with differentially regulated phosphorylation events shown to be functionally relevant in cell protrusion or contraction. Sites dominated by αvβ1 or synergistically upregulated in pKO-αv/β1 cells are shown. (f,g) Mean pMLC fluorescence intensity (f) and mean cell area (g) on fibronectin-coated X-shapes before and after treatment with ML-7 (25 µM) to inhibit Mlck, UO126 (50 µM) to inhibit ROCK, or Y-27632 (10 µM) to inhibit ROCK. (pKO-αv: untreated n = 12, +ML-7 n = 10, +U0126 n = 15, +Y-27632 n = 16; pKO-β1: untreated n = 16, +ML-7 n = 17, +U0126 n = 19, +Y-27632 n = 21; pKO-αv/β1: untreated n = 11, +ML-7 n = 18, +U0126 n = 19, +Y-27632 n = 30; 1 representative of 3 independent experiments is shown; all pairwise statistical comparisons using t-tests are shown in Supplementary Table S5; error bars represent s.e.m.). pKO-αv, green; pKO-αv/β1, blue; pKO-β1, orange.

upregulation of pT183/pY185-Erk2 activities in pKO-αv/β1 cells (Fig. 6c–e). Western blotting using phospho-site-specific antibodies corroborated these results (Fig. 6d). We uncovered three pathways (Erk2, Rock, Mlck) that were differentially regulated by the two integrin classes following adhesion to fibronectin, and reasoned that inhibition of either one or any combination of these pathways would abrogate synergistic myosin II reinforcement. Indeed, the cooperative activation of myosin II in pKO-αv/β1 cells was blocked by inhibiting Erk (UO126), Rock (Y-27632) or Mlck (ML-7; Fig. 6f,g). To confirm the relevance of this finding, we overexpressed constitutively active (ca-) kinase...
Figure 7 Activation of Rock is αvβ3-dependent. (a–c) Total cell lysates of cells plated for 90 min on fibronectin in the indicated conditions and analysed by western blotting with phospho-specific antibodies. The levels of pErk2 (b) and pMLC (c) were quantified using densitometry (n = 3). (d) A representative western blot analysis of cells transfected with myc-tagged ca-RhoA or myc-tagged ca-ROCK constructs and probed with the indicated antibodies. (e) Densitometric quantification of western blots (n = 3). The bar graphs show ratios of pMLC signals from cells expressing ca-RhoA or ca-Rock over the empty vector control. NS, not significant. (f) Confocal image of indicated cells transfected with a myc-tagged ca-ROCK construct, seeded on fibronectin-coated crossbow shapes and immunostained with Myc (red), pMLC (green), F-actin (blue) and DAPI (white). Scale bar, 25 µm.

DISCUSSION

We reconstituted pan-integrin-deficient fibroblasts with β3- and/or αv-class integrins and correlated integrin-class-specific cellular phenotypes with integrin-class-specific adhesion composition and signalling events. Fibroblasts exploring fibronectin-based microenvironments engage αvβ3 and αv-class integrins to orchestrate membrane protrusions, cell contractility and cell migration. Our cell line analyses revealed a series of signalling events accomplished by αvβ3 integrins, which activate Rac1, induce membrane protrusions, assemble nascent adhesions and generate RhoA/Rock-mediated myosin II activity. In conjunction with these events, mechanosensitive αv-class integrins accumulate in areas subjected to high tension and reinforce adhesive sites to induce further activation of myosin II and development of large focal adhesions and actomyosin bundles (Fig. 8). Our study uncovers a sequence of tightly integrated biophysical and biochemical events induced by αvβ3 and αvβ5-integrins.
αβ₁ and α₁-class integrin cooperation during rigidity sensing. αβ₁ integrins adhere to fibronectin, and assemble Kindlin-2- and ILK-rich small peripheral adhesions in a myosin-II-independent manner. The protein assembly in αβ₁-containing adhesions activates Rac1, Wave and Arp2/3-driven actin polymerization to induce membrane protrusions, and RhoA/Rock-mediated myosin II activation to induce tension. This tension increases the adhesion lifetime of αβ₁-class integrins bound to ligand on stiff substrates, which reinforces and stabilizes focal adhesions. αβ₁-class integrins recruit GEF-H1 to focal adhesions, which reinforces RhoA/myosin II in a αβ₁-dependent manner, and increases RhoA activity to promote mDia-mediated stress fibre formation. The combination of αβ₁-class integrin-mediated structure (focal-adhesion anchoring and stress-fibre formation) with the αβ₁-mediated force generation (myosin II activity) constitutes a synergistic system, which is important for adapting cellular contractility and architecture to the rigidity of fibronectin-based microenvironments.

Adaption to mechanical and topographic constraints—ECM-stiffness-dependent modulation of both force production (myosin) and coupling (focal adhesion size and stress fibres)

Figure 8 Model of αβ₁ and α₁-class integrin cooperation during rigidity sensing. αβ₁ integrins adhere to fibronectin, and assemble Kindlin-2- and ILK-rich small peripheral adhesions in a myosin-II-independent manner. The protein assembly in αβ₁-containing adhesions activates Rac1, Wave and Arp2/3-driven actin polymerization to induce membrane protrusions, and RhoA/Rock-mediated myosin II activation to induce tension. This tension increases the adhesion lifetime of αβ₁-class integrins bound to ligand on stiff substrates, which reinforces and stabilizes focal adhesions. αβ₁-class integrins recruit GEF-H1 to focal adhesions, which reinforces RhoA/myosin II in a αβ₁-dependent manner, and increases RhoA activity to promote mDia-mediated stress fibre formation. The combination of αβ₁-class integrin-mediated structure (focal-adhesion anchoring and stress-fibre formation) with the αβ₁-mediated force generation (myosin II activity) constitutes a synergistic system, which is important for adapting cellular contractility and architecture to the rigidity of fibronectin-based microenvironments.

α₁-class integrins that adjust fibroblast contractility to the rigidity of fibronectin-coated substrates. The cooperation of αβ₁ and α₁-class integrins to sense the rigidity of fibronectin-based microenvironments predicts that cell migration towards a rigidity gradient, called durotaxis, may also depend on the cooperation of both integrins. These findings have potential ramifications for certain pathologies, such as fibrosis and tumour metastasis where rigidity sensing of fibronectin matrices is crucial in disease progression.

To better understand how distinct integrin classes individually and cooperatively probe the biophysical properties of a fibronectin-based microenvironment, we established a cell model system and used proteomics methods to characterize their focal adhesion composition, phospho-signalling and proteome changes. Our comprehensive proteomic data set of adhesion signalling revealed that integrin-class-specific adhesomes and phospho-proteomes are enriched with integrin-specific adapter proteins and signalling intermediates. Several well-known integrin outside-in signalling pathways, including the Rac1/Wave/Arp2,3 and RhoA/Rock pathways, were dominated by αβ₁ integrins. Interestingly, the pKO-β₁ cells developed very few stress fibres, indicating that αβ₁-induced RhoA activity was preferably used for production of myosin-II-mediated force but not formin-mediated stress-fibre formation. In contrast, the pKO-α₁ cells exhibited high RhoA activity, which in turn induced the formation of thick stress fibres, most likely through the activation of mDia, but did not activate Rock/pMLC/myosin II. The coupling of active RhoA to different downstream effectors by distinct integrin classes was unanticipated. The underlying mechanism(s) are unclear, but probably involve specific mark(s) either attached to active RhoA or to the effectors enabling differential interactions with GTP-bound RhoA.

Although forces play an important role in the assembly of focal adhesions, pKO-α₁ cells induced the largest focal adhesions among the three cell lines and also exhibited the lowest myosin II activities and traction forces. Focal adhesion size is not the sole predictor of traction forces and the final focal adhesion size can also be determined by an mDia-dependent mechanism. Therefore, we propose that the large size of focal adhesions in pKO-α₁ cells depends on RhoA/mDia-induced stress fibres rather than on myosin II. However, although the final focal adhesion size in pKO-α₁ cells was myosin-II-independent, their formation and/or stability were strictly myosin-II-dependent, evidenced by the pronounced destabilization of α₁-class integrin adhesions with blebbistatin. A role for α₁-class integrins for focal adhesion stabilization has also been obtained from single-protein tracking experiments of β₁ and β₁ integrins, which showed that β₁ integrins are more mobile than α₁ integrins. The necessity of αβ₁ for cell stiffening following force application has also been postulated. Similarly, the recruitment of GEF-H1 to focal adhesions and Erk2 activity was reported as necessary for cell stiffening following force application. Our results link these observations and suggest that force-mediated stabilization of α₁-fibronectin bonds will reinforce focal adhesions, increase local concentrations of GEF-H1 and activate RhoA following αβ₁-induced Erk2 activation. Therefore, α₁-class integrins could be capable of forming stronger extracellular catch bonds with fibronectin than αβ₁ integrins do, resulting in longer bond lifetimes of α₁-class integrins with fibronectin when force is applied. However, as the influence of force on the on and off rates of αβ₁ and α₁-class integrins with fibronectin have not been systematically studied, this hypothesis awaits future testing.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.F. initiated the project; R.F. and H.B.S. designed the experiments and wrote the paper; H.B.S., M-R.H., T.Y., S.Z., J.P., Z.S. and A.R. performed experiments; H.B.S., M-R.H., T.Y., S.Z., K-E.G., C.C.F. and R.F. analysed data; J.P., M.T., K-E.G. and M.M. provided important reagents and/or analytical tools; all authors read and approved the manuscript.
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METHODS

Isolation, immortalization, viral reconstitution and transfection of cell lines. Mouse pKO fibroblasts and reconstituted pKO−α, pKO−β, and pKO−β1, cell lines were generated from fibroblasts (flexed parental) derived from the kidney of 21-day-old male mice carrying floxed α1 and β1 alleles (α1lox/lox, β1lox/lox), and constitutive β2, and β2 null alleles (β2+/−, β2−/−; ref. 21). Individual kidney fibroblast clones were immortalized by retroviral delivery of the SV40 large T. The immortalized flexed fibroblast clones were then retrovirally transduced with mouse α1, and/or β1 integrin cDNAs and the endogenous flexed β2 and α1 integrin loci were simultaneously deleted by adoviral transduction of the Cre recombinase. Reconstituted cell lines were FACS sorted to obtain cell populations with comparable integrin surface levels to the parental cell clones. Transduction of ca-RhoA (myc–RhoA pcDNA3.1) and ca-ROCKD4 (myc–ROCKD4 pcDNA3.1) was carried out with Lipofectamine 2000 (Invitrogen through Life Technologies) according to the manufacturer’s instructions. The transfection control was an empty pcDNA3.1 vector.

Adhesion and cell migration analysis. Adhesion assays were carried out as previously described. Briefly, cells were plated for 20 min in 96-well plates coated with varying concentrations of ECM ligands. After washing the plates the number of adhered cells that remained on the plate was quantified using attenuance at 595 nm.

To analyse random movement, cell culture dishes were coated with fibronectin (5 μg ml−1 in PBS; 2 h at room temperature) and blocked with 1% BSA in PBS. After seeding, video time-lapse microscopy was performed using phase contrast at ×20 magnification. A total of 12 migrating pKO−α, 12 migrating pKO−α/β, and 14 migrating pKO−β cells from 5 independent movies were analysed. One pixel in each cell nucleus was marked manually and served as the cell’s coordinate. Each tracked cell j with a track length Nj was recorded by its xi, j and yi, j position for every frame i. A tracking point was made every Δt = 1 min. The time difference between the tracking coordinates xi, j and x′,j−1 = t − Δt, where n is the frame number. The mean squared displacement (msd) of the cell j at time t = nΔt was calculated by

$$\text{msd}(t) = \frac{1}{N_j - N} \sum_{i=1}^{N_j} (x_{i+1,j} - x_{i,j})^2 + (y_{i+1,j} - y_{i,j})^2$$

All msd values were calculated for all cells and averaged. The used propagated uncertainty for the msd(t) is the standard deviation of the mean. For an increasing n the number of given tracks contributes to msd(t) decreases as well as the propagated uncertainty caused by the tracking uncertainty increases. Therefore, the msd(t) has been cut at n = 90. To determine the persistence time P and the diffusion constant D, Furcht’s formula

$$\text{msd}(t) = 4D \left( t - P \left(1 - \exp \left(-\frac{t}{P}\right)\right)\right)$$

has been fitted through the data. The mean velocity of a cell j was computed as the average of the distance travelled each time step divided by the time step.

Micropatterning and immunostainings. Micropatterns were generated on PEG-coated glass coverslips with deep-ultraviolet lithography. Glass coverslips were incubated in a 1 M solution of a linear PEG, CH2═(O−CH−(CH3)2)x−1−NH−CO−(NH−CH2−CH2)=(Si(OEt)3) in dry toluene for 20 h at 80 °C under a nitrogen atmosphere. The substrates were removed, rinsed intensively with ethyl acetate, methanol and water, and dried with nitrogen. A peglated glass coverslip and a chromium-coated quartz photomask (MLSC, Jena) were immobilized with vacuum and the mask holder, which was immediately exposed to deep ultraviolet light using a low-pressure mercury lamp (NIQ 60/35 XL longlife lamp, quartz tube, 60 W from Heraeus Noblelight) at 5 cm distance for 7 min. The patterned substrates were then incubated overnight with 0.1% PFA in PBS for 5 min at room temperature, washed with PBS, blocked with 1% BSA in PBS for 1 h at room temperature and then incubated with antibodies. The fluorescent images were collected with a laser scanning confocal microscope (Leica SP5).

Acrylamide micropatterning. Micropatterns were first produced on glass coverslips as previously described. Briefly, 20 mm square glass coverslips were oxidized through oxygen plasma (FEMTO, Diener Electronics) for 10 s at 30 W before incubating with 0.1 mg ml−1 poly-L-lysine (PLL)–PEG (PLL20K–PEG2K, JenKem) in 10 mM HEPES, pH 7.4, for 30 min. Acrylamide gels were exposed to 165 nm ultraviolet (UVO) cleaner, gel) and coated glass coverslips with deep-ultraviolet lithography. A tracking point was made every 5 min. Then, coverslips were incubated with 20 mg ml−1 of fibronectin (Sigma) and 2 mg ml−1 of rhodamine-labelled fibronectin (Cytoskeleton) in 100 mM sodium bicarbonate solution for 30 min. Acrylamide solution containing acrylamide and bisacylamide (Sigma) was degassed for 20 min under house vacuum and mixed with passivated fluorescent beads (Invitrogen) by sonication before addition of APS and TEMED. A 25 μl drop of this solution was put directly on the micropatterned glass coverslip. A silanized coverslip was placed over the drop and left polymerizing for 30 min (fluorescent beads passivation and glass silanization were performed as previously described). The sandwich was then put in 100 mM sodium bicarbonate solution and the gel was gently removed from the patterned glass coverslip while staying attached to the other coverslip owing to the silanization treatment. This process transferred the protein micropatterns onto the gel as previously described. Three different solutions of 3%/0.225%, 5%/0.225%, 8%/0.264% acrylamide/bisacylamide were used. The corresponding Young’s modulus of the gels was 1.4, 9.6 and 34.8 kPa respectively as measured using AFM. Coverslips were mounted in magnetic chambers (Cytoo) and washed with sterile PBS before plating cells.

AFM measurements of the Young’s modulus of acrylamide gels. We measured gel stiffness throught nanonindentation using an atomic force microscope (Bruker Nanoscope) mounted with silica-bead-tipped cantilevers (r(head) = 2.5 μm, nominal spring constant 0.06 N m−1, Novascan Technologies). Initially, we determined the sensitivity of the photodiode to cantilever deflection by measuring the slope of a force distance curve while pressing the cantilever onto a glass coverslip, and the force constant of the cantilever using the thermal noise method included in the Bruker Nanoscope software. For each acrylamide/bisacylamide ratio used in the traction-force microscopy measurements we acquired 27 force curves in 3 by 3 grids (2 μm spacing between points) at three different locations on the gels. Before and during indentation experiments gels were kept in PBS. To obtain stiffness values from force curves we used the NanoScope Analysis software. Specifically, we corrected for baseline tilt, and used the linear fitting option for the Hertz model with a Poisson ration of 0.48 on the indentation curve.

Traction-force microscopy and image analysis. Confocal acquisition was performed on an Eclipse Ti-E Nikon inverted microscope equipped with a CSUX1-A1 Yokogawa confocal head and an Evolve EMCCD camera (Roper Scientific, Princeton Instrument). A CPI Plan APO VC oil 100×/1.4 objective (Nikon) was used. The system was driven by the Metamorph software (Universal Imaging).

Traction-force microscopy was performed as previously described. Displacement fields describing the deformation of the polyacrylamide substrate are determined from the analysis of fluorescent bead images before and after removal of the adhering cell with trypsin treatment. Images of fluorescent beads were first aligned to correct experimental drift using the Align slices in stack ImageJ plugin. The displacement field was subsequently calculated by a custom-written particle image velocimetry (PIV) program implemented as an ImageJ [http://rsb.info.nih.gov/ij] plugin. The PIV was performed through an iterative scheme. In all iterations the displacement was calculated by the normalized correlation coefficient algorithm, so that an individual interrogation window was compared with a larger searching window. The next iteration takes into account the displacement field measured previously, so that a false correlation peak due to insufficient image features is avoided. The normalized cross-correlation also allowed us to define an arbitrary threshold to filter out low correlation values due to insufficient beads present in the window. The resulting final grid size for the displacement field was 2.67 × 2.67 μm. The erroneous displacement vectors due to insufficient beads present in the window were filtered out by their low correlation value and replaced by the median value from the neighbouring vectors. With the displacement field obtained from the PIV analysis, the traction-force field was reconstructed by the Fourier transform PIESCO (cytometry (FTTC) method, with regularized scheme on the same grid (2.67 × 2.67 μm) without further interpolation or remapping. The regularization parameter was set at 1 × 10−11 for all traction-force reconstructions. The Fourier transform traction cytometry code was also written in Java as an Imagej plugin, so that the whole traction-force microscopy procedure from PIV to force calculation could be performed with Imagej. The entire package of traction-force microscopy software is available at https://sites.google.com/site/qingzongtseng/tfm.Contractile

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energy was then computed as the integral under the cell of the scalar product of force and displacement vectors using a custom-written code in MatLab. Force profiles along the cell front were generated by integration of the traction maps over the width of the circular part of the pattern. Average pictures were generated after alignment using the Align plugin (in stack ImagJ). PicoAdhesion intensity profiles were generated by integration of the paxillin intensity along the border of the circular part of the micropattern.

Rho–GTPase assays. Cells were serum-starved overnight, detached with trypsin–EDTA and kept in suspension in serum-free medium for 1 h. Cells were then plated on fibronectin-coated dishes (blocked with 1% BSA) in serum-free medium for 45 min. Cell lysis and active Rho–GTPase pulldown was performed using the active Rac1 Pull-Down and Detection Kit or the active Rho Pull-Down and Detection Kit (Cat#16118, 16116, Pierce) according to the manufacturer’s instruction. The active GTpase signal was normalized to total protein level of the GTpase. Western blots were quantified with Totallab.

RNA interference. Cells were transiently transfected with a final concentration of 300 nM siRNA (stealth RNAi; Invitrogen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol, using the targeting sequence sense-5' -CCCGGAACGUGUGCUACCAUGGUA-3' for GFI-1. As a control we used the scrambled sequence sense-5' -CCUCAAUGUUGCAUCACGGUUU-3'.

MS. For proteome and phosphoproteome analysis fibroblasts were cultured in lysis/arginine-free DMEM with 10% FBS, oxidized methionine, N-carbamidomethylated DSP protein and carboxymethylated lysine were searched as variable modifications. Finally, the label-free quantification algorithm implemented in the MaxQuant software was used as described previously. SILAC-based peptide pulldowns were carried out with the cytoplasmic tails of β1 integrin (5’-HDREFRAFEERERKRWDXTAWNPLYKEATS-TTNITRYGRT-3’) and the tails of β3 integrin (5’-HDREFRAFEERERARWDXTAWNPLYKEATS-TTNITRYGRT-3’). The tail peptides were de novo synthesized with a deshithiobiote on the amino terminus, coupled to magnetic streptavidin beads (MyOne Streptavidin C1—Invitrogen) and pulldowns from SILAC-labelled cell lysates were performed as described previously. After a mild wash the bound proteins were eluted from the magnet using 16 mM biotin (Sigma-Alrich). After protein precipitation and in-solution digestion, LC-MS/MS and data analysis was performed as described above. The peptide pulldown experiments were done as reverse SILAC labelling experiments in duplicate (4 biological replicates). We generally considered outliers with high SILAC ratios and high sequence coverage/identity as more significant than proteins that had only a high SILAC ratio.

Bioinformatics and statistics. ANOVA analysis of the cellular proteome and phosphoproteome was performed using the Perseus bioinformatics toolbox of MaxQuant (J. Cox et al.; manuscript in preparation). Multiple testing corrections were performed using the inbuilt permutation method and significant hits were identified at a significance level of 0.01 and 0.05, respectively. ANOVA analysis of the 245 core adhesome proteins was performed using the statistical programming language R (http://www.R-project.org) with the adaptive Benjamini and Hochberg step-up false discovery rate-controlling procedure for multiple testing and a significance level of 0.05. Hierarchical clustering was performed using an average linkage approach and Euclidean distances. Enrichment analysis of clusters for Gene Ontology (GO) terms, KEGG pathways and PFAM and InterPro protein domains was performed with the DAVID webserver using the multiple testing correction method by Benjamini and Hochberg and a significance level of 0.05. Protein–protein interactions (PPIs) were compiled from different sources including: PPI databases (DIP (ref. 56; version of December 2009), IntAct (ref. 57) and MINT (ref. 58) (both downloaded on 19 May 2010), BIOGRID (ref. 59; version 3.0.64) and HPRD (ref. 60; Release 9)); the adhesome network database and the KEGG pathway database. For the adhesome network database, we distinguished between undirected PPIs and directed activating and inhibiting interactions as annotated in the adhesome database and in KEGG. Human and mouse interactions were combined using the orthologue tables of the Mouse Genome Database (MGD) to the human genome. For the adhesome database, we used the adhesome network database, we distinguished between undirected PPIs and directed activating and inhibiting interactions as annotated in the adhesome database and in KEGG. Human and mouse interactions were combined using the orthologue tables of the Mouse Genome Database (MGD) to the human genome. The confidence network of PPIs from public databases contained only interactions reported in at least two separate publications. Networks were visualized using the Cytoscape software. Bar graphs throughout the study were generated in Microsoft Office and depict, unless otherwise indicated, the means and standard errors of the means. Box plots and dot plots were generated using the SigmaPlot software or the MatLab software.
Data deposition. Raw data for the phosphoproteome and proteome analyses of the three cell lines are deposited in the Tranche database (https://proteomecommons.org/tranche/) with the following accession numbers: Schiller_Integrins_Phosphoproteome, on33gw4tEXu5YErn5zrp; Schiller_Integrins_Proteome, EvAbqut9c7fC90OQTyawI.


Figure S1 Generation of pKO-αv, pKO-β1 and pKO-αv/β1 cell lines. (a) Workflow of the generation of pKO kidney fibroblasts (strategy 1) and integrin reconstituted pKO fibroblasts (strategy 2). (b) Phase contrast image of the floxed and pKO cells plated on FN. Scale bar 20 µm. (c) Integrin profile of floxed and pKO cells analysed by flow cytometry. (d) Cell surface levels of indicated integrins analysed by flow cytometry. (e) Relative fluorescence intensities of indicated integrins from three independent stainings analysed by flow cytometry. The means (n=3) and standard deviations are shown. (f) Western blots for αv and β1 integrins. GAPDH was used as loading control. (g) Cell lysates and immunoprecipitates of β1 integrin were immunoblotted for αv, α5 and β1 integrins. Note that αv does not associate with β1 in pKO-αv/β1 cells. (h) Adhesion assay on fibronectin (FN) or vitronectin (VN). Numbers of adherent cells 20 minutes after seeding are shown as relative values of OD=595nm. The bar graph shows the mean and s.e.m. (n=3; one representative out of 2 independent experiments is shown). (i) Cells plated on FN and time-lapse imaged using a phase contrast microscope at 20x magnification. Scale bar 100 µm. pKO-β1 (green); pKO-αv (blue); pKO-αv/β1 (orange); parental β1/αv floxed cell (red).
Figure S2  

αvβ1 and αv-class integrins induce different spreading areas, membrane protrusions and adhesion sites on FN. (a) Cells were plated on FN for 90 minutes and immunostained with the indicated antibodies. Arrowheads indicate cortactin-positive lamellipodia and arrows mark the small NAs in lamellipodia. Scale bar 10μm.  

(b) Size distribution of adhesive sites of cells stained with Paxillin calculated with the Metamorph software. Boxplots show the percentage of adhesions in the depicted size classes (pKO-αv n=15; pKO-αv/β1 n= 29; pKO-β1 n=23; one representative out of 2 independent experiments is shown). Boxplot whisker ends are at 1.5 interquartile range and outliers are shown as dots. Significance was calculated using a t test (*=p<0.05; ***=p<10 E-06).  

(c) Still pictures taken from supplementary movies S1-S3 showing trailing edge detachment defects indicated by the arrows. Scale bar 100 µm. (d) Floxed cells cultured 3 hours on FN-coated X-shapes treated for 1 hour with indicated concentrations of blebbistatin (BLEB), and then stained for Paxillin, pMLC and f-actin. Scale bar 10 µm.  

(e) Fluorescence intensities of pT18/S19-MLC, (f) Paxillin (Pxn) and (g) cell areas after blebbistatin treatment (n=20 cells; error bars represent s.e.m.).  

(h) Cells plated on FN-coated X-shapes and stained for pMLC, Paxillin and f-actin. Scale bar 10 µm. (i) Fluorescence intensities of pS18/T19-MLC and (j) cell areas (pKO-αv n=46, pKO-β1 n=46, pKO-αvβ1 n=21, pKO-αvβ1 +Cil n=10; one representative out of 3 independent experiments is shown; error bars represent s.e.m.). Cilengitide (Cil) was used to block αv-class integrins. Significance was calculated using a ttest.  

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Figure S3 Adhesome analysis of pKO-αv, pKO-β1 and pKOαvβ1 cells. (a) Workflow for isolation of FA enriched fractions and analysis of adhesome components. (b) Adhesomes derived from cells plated on indicated substrates for 45 or 90 minutes were examined by non-supervised hierarchical cluster analysis of Z-scores of median MS intensities (n=3-4). The labels on the right indicate significantly enriched gene ontology (GO) terms.
Figure S4 α5β1- and αv-class-specific PPIs and phosphosites. (a) The PPI network derived from FA-enriched samples. Integrin subunits are in the centre and their direct and indirect interactors are in the inner and outer circles, respectively. Black lines between nodes indicate high confidence PPI, red arrows indicate activating interactions and blue lines indicate inhibiting interactions. The nodes were labelled with gene symbols and colour-coded according to the MS intensity ratio of pKO-αvβ1 versus pKO-β1. Node edges were colour-coded according to the SILAC ratio of the maximally regulated phosphosite on each significantly regulated protein. (b) The PPI-network was derived as in (a). The nodes and node edges were colour-coded according to the MS intensity ratio of pKO-αv versus pKO-αv/β1. (c) The PPI-network was derived as described in (a). The nodes and node edges were colour-coded according to the MS intensity ratio of pKO-αv versus pKO-β1.
Figure S4 continued
Figure S4 continued
Figure S5: Integrin-specific differences in the "core integrin interactome". The Z-scores of median MS intensities (n=3-4) of the 125 core integrin-interactome proteins (Fig. S4) were subjected to hierarchical clustering. The black bars on the left indicate α5β1-dependent FA proteins, while the green bar indicates the αv-class integrin-dependent FA proteins selected for the clustering in Fig. 4c.
**Figure S6** Network analysis of actin binding proteins enriched in the adhesome preparations. (a) Actin binding proteins were extracted from the adhesome dataset using gene ontology annotations. Black lines between nodes indicate high confidence PPI, red arrows indicate activating and blue lines indicate inhibiting interactions. The nodes were labelled with gene symbols and colour coded according to the log2 MS intensity ratio of pKO-α\textsuperscript{v} over the pKO-β\textsuperscript{1} sample. Node edges were colour-coded according to the log2 SILAC ratio of the maximally regulated phosphosite on each significantly regulated protein. The box marks components of the WAVE and Arp2/3 complex, while the arrowhead marks the formin mDia. (b) The graph was generated as in (a), except that the nodes were colour-coded according to the log2 MS intensity ratio of pKO-α\textsuperscript{v/β} over the pKO-β\textsuperscript{1} sample.
**Figure S7** Integrin tail peptide pull-downs. (a) Sequence of synthetic desthiobiotinylated peptides used for the pull down experiments. (b) SILAC ratio plot from label inverted replicates (specific interactors have high SILAC ratio in the forward experiment and low SILAC ratios in the label swapped reverse experiment) comparing the β1-tail peptide with a scrambled control. The table shows the most intense β1-specific interactors with high sequence coverage that were reproducibly enriched versus the scrambled control peptide (scr) (n=4; 2 independent experiments). (c) SILAC ratios of proteins from inverted replicates comparing the β3-tail peptide with a scrambled control. The table shows the most intense β3-specific interactors with high sequence coverage that were reproducibly enriched versus the scrambled control peptide (scr) (n=4; 2 independent experiments).
Figure S8 Cellular proteome of pKO-αv, pKO-β1 and pKO-αv/β1 cells. (a) SILAC labelled cells cultured on FN for several passages were analysed by MS. SILAC ratios of 150 significantly regulated proteins (ANOVA, Benjamini/Hochberg FDR) were subjected to non-supervised hierarchical cluster analysis and colour coded. The bars depict differentially regulated clusters of proteins. (b) Gene names of the 3 differentially regulated groups (a) are shown. Known FA proteins are marked with an asterisk. (c) Scatter plot showing SILAC ratios. Previously annotated FA proteins are labelled in red.
Figure 5c

IB: anti GEF-H1

IB: anti GAPDH

Figure 6d

IB: anti pMLC (S19/Thr18)

IB: anti total MLC

IB: anti pMypt1 (Thr696)

IB: anti total Mypt1

IB: anti pMEK1/2 (S217/221)

IB: anti total MEK1/2

IB: anti pErk (Thr202/Tyr204)

IB: anti total Erk

IB: anti pCofilin (S3)

IB: anti total Cofilin

Figure S9 Uncropped western blots.
Supplementary video legends

**Video S1** Time-lapse movie of pKO-α cells plated on FN. Cells were plated on FN coated (5 µg/ml; blocked with 1% BSA) tissue culture dishes in presence of 10% serum and video tracked over 20 hours with a frame rate of 1 picture every 4 minutes. Pictures were acquired with a phase contrast microscope at magnification 20x.

**Video S2** Time-lapse movie of pKO-α/β1 cells plated on FN. Cells were plated on FN coated (5 µg/ml; blocked with 1% BSA) tissue culture dishes in presence of 10% serum and video tracked over 20 hours with a frame rate of 1 picture every 4 minutes. Pictures were acquired with a phase contrast microscope at magnification 20x.

**Video S3** Time-lapse movie of pKO-β1 cells plated on FN. Cells were plated on FN coated (5 µg/ml; blocked with 1% BSA) tissue culture dishes in presence of 10% serum and video tracked over 20 hours with a frame rate of 1 picture every 4 minutes. Pictures were acquired with a phase contrast microscope at magnification 20x.