αV-class integrins exert dual roles on α5β1 integrins to strengthen adhesion to fibronectin

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Upon binding to the extracellular matrix protein, fibronectin, αV-class and α5β1 integrins trigger the recruitment of large protein assemblies and strengthen cell adhesion. Both integrin classes have been functionally specified, however their specific roles in immediate phases of cell attachment remain uncharacterized. Here, we quantify the adhesion of αV-class and/or α5β1 integrins expressing fibroblasts initiating attachment to fibronectin (≤120 s) by single-cell force spectroscopy. Our data reveals that αV-class integrins outcompete α5β1 integrins. Once engaged, αV-class integrins signal to α5β1 integrins to establish additional adhesion sites to fibronectin, away from those formed by αV-class integrins. This crosstalk, which strengthens cell adhesion, induces α5β1 integrin clustering by RhoA/ROCK/myosin-II and Arp2/3-mediated signalling, whereas overall cell adhesion depends on formins. The dual role of both fibronectin-binding integrin classes commencing with an initial competition followed by a cooperative crosstalk appears to be a basic cellular mechanism in assembling focal adhesions to the extracellular matrix.
Integrins are transmembrane receptors composed of α/β heterodimers that facilitate cell adhesion and regulate basic cellular processes such as migration, proliferation, survival and differentiation. Mammals harbour eighteen α and eight β genes. Through different combinations of α and β subunits, 24 integrins can be generated that bind to receptor sites such as vascular cell adhesion molecules and intracellular cell adhesion molecules, or extracellular matrix (ECM) proteins such as fibronectin (FN), vitronectin (VN), collagen and laminin. Individual adhesion mechanisms of integrin heterodimers with ECM substrates have been extensively studied over the past few years. However, the regulatory mechanisms through which different integrins crosstalk with each other to initiate cell adhesion are still poorly understood.

Early integrin-mediated cell adhesion is believed to follow a cascade of events that starts with integrin activation through talin and kindlin (also called integrin-inside-out signalling), followed by integrin clustering and the assembly of a large protein network at the clustered integrin cytoplasmic domain collectively called the adhesome. The adhesome comprises hundreds of proteins including talin and kindlin, which together with several adaptor and signalling molecules transduce signals from ligand-bound integrins to the cell inside (also called integrin-outside-in signalling). An important consequence of outside-in signalling is the activation of cytoskeleton containing Rho-like GTPases and their effectors such as Rho kinase (ROCK), cortical F-actin nucleators such as formins, the Arp2/3 complex and the non-muscle myosin-II.

FN consists of an array of type I, II and III modules and is one of the most abundant ECM proteins to which α5β1 and αV-class integrins adhere. Cell adhesion mediated by FN-binding integrins leads to the formation of nascent adhesions that eventually mature into large focal adhesions and then convert into central or fibrillar adhesions. While both integrin classes bind the tripleptide sequence Arg-Gly-Asp (RGD) in the 10th type III module of FN (FNIII10), α5β1 integrins also require the Pro-His-Ser-Arg-Asn (PHSRN) syntesis in the FNIII9 module, which is in close proximity to the RGD motif, to establish cell adhesion. It is not clear, whether α5β1 and αV-class integrins function individually and/or cooperate with each other during the first few seconds and minutes of adhesion initiation. Furthermore, it is also unclear whether and how the two FN-binding integrin classes signal to each other to induce and orchestrate their assembly and to strengthen adhesion to FN before nascent adhesions have formed. Interestingly, crosstalk between both integrin classes has been reported to occur at later stages (> 90 min) of cell adhesion. For example, it has been demonstrated that both integrins compete for the cytoplasmic talin pool leading to negative, trans-dominant effects, while they also strengthen adhesion to the ECM and trigger the formation of larger focal adhesions.

To provide quantitative insights into the mechanisms regulating early (≤ 120 s) fibroblast adhesion established by α5β1 and αV-class integrins to FN, we employed atomic force microscopy (AFM)-based single-cell force spectroscopy (SCFS). SCFS is well suited to characterize specific adhesion mechanisms of cells to the ECM. Compared with other methods allowing the qualitative or/and quantitative characterization of cell adhesion, SCFS offers the particular advantage to decipher early adhesion mechanisms occurring within the first few seconds to minutes of cell-ECM attachment. Therefore, we employed SCFS together with confocal microscopy to study the adhesion kinetics of α5β1 and αV-class integrins in mouse kidney fibroblasts to FN. Our results reveal a dual role of the two integrin classes upon contacting FN. First, they compete for FN binding, to which αV-class integrins bind faster, thereby preventing the engagement of α5β1 integrins. In the second phase, αV-class integrins, engaged with the substrate, signal to α5β1 integrins to establish binding to FN and to strengthen adhesion. By combining SCFS with total internal reflection fluorescence (TIRF) microscopy, we characterized that this crosstalk triggers the clustering of α5β1 integrins and recruitment of adhesome proteins. Specific perturbation experiments identified signalling pathways involved in the early crosstalk between both FN-binding integrin classes.

Results

Differential contributions of α5β1 and αV-class integrins. To determine how α5β1 and αV-class integrins contribute to the initiation of cell adhesion, we quantified the adhesion forces of α5β1 and/or αV-class integrin-expressing mouse kidney fibroblasts to FN by SCFS (Fig. 1a). The cell lines were derived from pan-integrin deficient fibroblasts (pKO) reconstituted with either αV-class (pKO-αV), or β1 (pKO-β1), or both classes of integrins (pKO-αV/β1). To visualize the binding of FN by receptors other than α5β1 and αV-class integrins, that is, syndecans, we used the FN fragment FNIII10-10, which contains the RGD and PHSRN motifs. For SCFS, a single fibroblast was attached to concanavalin A (ConA)-functionalized AFM cantilever and incubated for 7–10 min to ensure firm adhesion of the fibroblast to the cantilever. The rounded fibroblast bound to the cantilever was then brought into contact with the FNIII10-10 substrate for contact times ranging from 5 to 120 s. Subsequently, the fibroblast was separated from the substrate to measure the fibroblast-substrate adhesion force at maximum cantilever deflection (Supplementary Fig. 1a). To obtain statistically firm results, the single-cell experiments were repeated multiple times using different cantilevers, fibroblasts and FNIII10-10-coated substrates. Our measurements revealed that the three reconstituted pKO fibroblast lines showed characteristic integrin-specific adhesion profiles to FNIII10-10, whereas non-reconstituted pKO fibroblasts displayed negligible adhesion (Fig. 1a). pKO-αV/β1, pKO-αV and pKO-β1 fibroblasts significantly strengthened adhesion with increasing contact times to FNIII10-10. Interestingly, while the adhesion strength of pKO-αV/β1 and pKO-αV fibroblasts was similar, pKO-β1 fibroblasts established much stronger adhesion to FNIII10-10, which doubled at 120 s contact time compared with pKO-αV and pKO-αV/β1 fibroblasts. Importantly, the stronger adhesion of pKO-β1 fibroblasts was also observed with full-length FN (Supplementary Fig. 1b). Furthermore, pKO-αV/β1, pKO-αV and pKO-β1 fibroblasts reduced adhesion to RGD-deleted FN fragments (FNIII10-10ARGD) to integrin-unspecific levels confirming that the adhesion strengthening was integrin-dependent (Supplementary Fig. 1c). In summary, our results showed that pKO-β1 fibroblasts established much stronger adhesion and strengthened adhesion much faster to FN compared with pKO-αV/β1 fibroblasts, indicating that the presence of αV-class integrins prevented adhesion strengthening to FN via α5β1 integrins.

Next, we tested whether blocking α5β1 integrins with an α5β1 integrin-blocking antibody (4B18) or αV-class integrins with cyclic RGD (clenitigide, CIL) alters adhesion of pKO-αV/β1 fibroblasts to FNIII10 (Fig. 1b). We found that blocking α5β1 integrins did not alter the adhesion of fibroblasts to FNIII10-10, while blocking αV-class integrins, increased the adhesion of pKO-αV/β1 fibroblasts to levels observed for pKO-β1 fibroblasts (Fig. 1b). One hypothesis for αV-class integrins hindering adhesion strengthening of α5β1 integrins could be the preferential binding of talin and kindlin to the β-tail of αV-class integrins. Hence, upon blocking αV-class integrins, talin...
fibroblasts are shown. single
s.d. Statistical significances were calculated with two-tailed Mann–Whitney analysed to detect single binding event. Bars show mean and error bars the
a (green). Adhesion forces of pKO-
1 fibroblasts to FNIII7-10 in presence of a

applying two-tailed Wilcoxon tests. (**

significant differences between the slopes (**

0.0001; ***P<0.001; **P<0.01; *P<0.05; NS, non-
significant, P≥0.05.

and kindlin became available to bind and activate ζ5β1 integrins.21,22 Thus, we performed cytoplasmic β-tail pull-down assays (Supplementary Fig. 1d), which in line with a recent study23, confirmed that talin equivalently bound to both β3 and β1 subunits, while kindlin-2 preferentially associated with the cytoplasmic domain of the β1 subunit. A second hypothesis could be that ζV-class integrins have higher binding rates and therefore, compete with ζ5β1 integrins for substrate binding. Thero, we performed SCFS with single molecule sensitivity24 (Supplementary Fig. 1e) to determine the binding probability of both ζV-class and ζ5β1 integrins with FN. This binding probability allowed estimating if one or both FN-binding

integrins bind RGD in pKO-αV/β1 fibroblasts, upon initial contact. Therefore, the contact time of the fibroblasts to FNI17-10 was reduced to ≈100 ms and the probability of single-integrin binding events, in the presence of either β1AB or CIL (Fig. 1c), was determined. The experiments revealed an integrin binding probability of 0.25 ± 0.07 (mean ± s.d., n = 3,529) per unperturbed pKO-αV/β1 fibroblast as compared with an unspecific binding probability of 0.10 ± 0.05 (n = 1,636) per pKO fibroblast. In the presence of β1AB, the binding probability increased to 0.40 ± 0.21 (n = 2,244), while in the presence of CIL, the binding probability decreased to 0.12 ± 0.06 (n = 3,218), comparable to that of pKO fibroblasts lacking FN-binding integrins.

Despite of equivalent binding of talin with β3 and β1 subunits, ζ5β1 integrins exhibited lower on-rates compared with ζV-class integrins. This suggested for a role of integrin-inhibitory adapter proteins, such as the integrin cytoplasmic associated protein 1 (ICAP-1)25,26, which delays the activation of ζ5β1 integrins and confers them lower on-rates. Hence, we performed SCFS experiments with ICAP-1-deficient mouse embryonic fibroblasts (ICAP-1 KO MEFs, Supplementary Fig. 1f). Indeed, while control WT MEFs showed similar adhesion to FN as that of pKO-αV/β1 fibroblasts, ICAP-1-deficient MEFs adhered stronger to FN at all contact times, with adhesion forces comparable to those observed for pKO-β1 fibroblasts. These findings suggest that ICAP-1 curbs FN binding of ζ5β1 integrins and hence available talin/kindlin readily binds ζV-class integrins instead, during adhesion initiation.

Thus, the higher binding rates of ζV-class versus ζ5β1 integrins, the negligible expression and undetectable functional role of ζVβ1 integrins for early adhesion to FN (Supplementary Figs 2 and 3), together with the similar surface expression of ζ5β1 integrins on pKO-β1 and pKO-αV/β1 fibroblasts6 demonstrate that ζV-class integrins outcompete ζ5β1 integrins likely due to inactivity of ζ5β1 integrins and thereby prevent pKO-αV/β1 fibroblasts to fully strengthen adhesion to FN.

ζV-class integrins stimulate fibroblast adhesion to FN. Although we report an outcompeting of ζ5β1 integrins by ζV-class integrins during early FN adhesion, cooperation of both integrin classes during late FN adhesion (>45 min) was reported.21 To test the possibility whether engaged ζV-class integrins crosstalk with non-outcompeted FN-binding ζ5β1 integrins via signalling to regulate early fibroblast adhesion, we coated the cantilever with VN, which enabled adhesion, integrin clustering and phospho-tyrosine induction of ζV-class integrins in pKO-αV and pKO-αV/β1 fibroblasts (Fig. 2a, Supplementary Fig. 4a,b). To pertain the high adhesion strength of fibroblast to FNI17-10, we functionalized the cantilever with 5 μg ml⁻¹ VN diluted in ConA. Control experiments excluded ConA as co-signalling receptor to VN (Fig. 2a). After 20 s of contact time, pKO-αV/β1 fibroblasts attached to VN-coated cantilevers established faster and stronger adhesion to FNI17-10 compared with pKO-αV/β1 fibroblasts attached to ConA only. After a contact time of 120 s to FNI17-10, VN-stimulated fibroblasts further increased the adhesion compared with non-stimulated pKO-αV/β1 (Fig. 2b) and non-
stimulated pKO-β1 (Supplementary Fig. 5) fibroblasts, which indicates that VN-engaged ζV-class integrins promotes fibroblast adhesion to FN. Furthermore, the concomitant decrease in sequestering of ζV-class integrins, by reducing the concentration of VN on the cantilever, increased adhesion to the VN substrate (Supplementary Fig. 4c). These results suggest that unoccupied ζV-class integrins on VN-stimulated pKO-αV/β1 fibroblasts also bind to FN to strengthen adhesion (Fig. 2c). Moreover, the reduced adhesion of VN-stimulated pKO-αV/β1 fibroblasts to
**Figure 2** | Engagement of αV-class integrins reinforces adhesion of α5β1 integrins to FN. (a) Immunofluorescence of pKO-αV/β1 fibroblasts seeded on VN-, ConA-, or FNII7-10-functionalized substrates. Fibroblasts adhering to 50 μg·mL⁻¹ VN (VNHIGH), 5 μg·mL⁻¹ VN diluted in ConA (VN), ConA, CIL and FNII7-10 for 10 min were stained for αV-class integrin (green), actin (red) and phospho-tyrosine (ptyr, blue) using β3 integrin-specific antibodies for the detection of αVβ3 integrins, phallolidin and ptyr antibody, respectively ('Methods' section). Immunostaining of αV-class integrins and phospho-tyrosine in pKO-αV/β1 fibroblasts adhering to FNII7-10-coated substrates is used as a positive control. Scale bars, 10 μm. (b) αV-class integrins engaged to VN augment fibroblasts adhesion to FNII7-10. pKO-αV/β1 fibroblasts were either attached to ConA (yellow)- or to VN (blue)-coated cantilevers for 7–10 min, then approached to the FNII7-10-coated substrate for defined contact time and finally retracted to measure the adhesion force. (c) αV-class integrins engaged to FNII7-10 could also strengthen fibroblast adhesion to FNII7-10. pKO-αV/β1 fibroblasts were either attached to ConA (yellow)- or to VN (blue)-coated cantilevers for 7–10 min, then approached to the FNII7-10-coated substrate for defined contact time and finally retracted to measure the adhesion force. (d) VN-stimulated fibroblasts enhance adhesion to FNII7-10 via α5β1 integrins. pKO-αV/β1 fibroblasts were incubated with a α5β1 integrin-blocking antibody (β1AB) for 30 min, then attached to VN-coated cantilevers and finally approached to FNII7-10 for defined contact time (5–120 s). Dots show adhesion forces of single fibroblasts (n=10 for each condition) and red bars their median. Statistical significances were calculated with two-tailed Mann–Whitney U-tests (**P<0.001; ***P<0.001; **P<0.01; *P<0.05; NS, non-significant, P≥0.05).
FN, in the presence of α5β1 integrin-blocking antibody (Fig. 2d), indicates that the increased adhesion of pKO-α2V/β1 fibroblasts was primarily mediated by α5β1 integrins and to a lesser extent by αV-class integrins.

To evaluate, whether FN-engaged αV-class integrins also signal and enforce adhesion of α5β1 integrins to FN, we attached pKO-α2V/β1 fibroblasts to a cantilever coated with FNIII7-10 carrying a mutation in the synergy site (FNIII7-10-mSyn), to which α5β1 integrins poorly bind20. FNIII7-10-mSyn-stimulated pKO-α2V/β1 fibroblasts strengthened adhesion to FNIII7-10 substrates similar to VN-stimulated pKO-α2V/β1 fibroblasts (Fig. 2c, Supplementary Fig. 6a) or pKO-α2V/β1 fibroblasts attached to FN-coated cantilevers (Supplementary Fig. 6b) indicating that αV-class integrins, stimulated either by VN or FN, induced α5β1 integrin-mediated cell adhesion to FN.

Next, we tested whether signalling by and/or the fast binding rates of αV-class integrins on pKO-α2V/β1 fibroblasts induced the strong adhesion of α5β1 integrins to FN-coated substrates. To this end, we attached pKO-α2V/β1 fibroblasts to cantilevers coated with either VN, to which αV-class integrins bind but elicit relatively less signalling response, if any, compared with VN-bound αV-class integrins20 (Fig. 2a). The experiments revealed that GIL-attached pKO-α2V/β1 fibroblasts failed to adhere to VN-coated substrates (Fig. 2e), suggesting that efficient sequestering of αV-class integrins prevented adhesion to VN at the opposite side of the fibroblast. However, GIL-attached pKO-α2V/β1 fibroblasts adhered to FN-coated substrates at similar strengths (Fig. 2e) as GIL-treated ConA-attached pKO-α2V/β1 fibroblasts (Fig. 1b) but at lower strength compared with VN-attached pKO-α2V/β1 fibroblasts (Fig. 2b). These results suggest that the functional state of αV-class integrins, upon sequestration to the cantilever, governs cell adhesion to FN and that αV-class integrin-mediated outcompeting of α5β1 integrins and αV-class integrin-mediated signalling act together to orchestrate α5β1 integrin-mediated adhesion strengthening.

Engagement of αV-class integrins clusters α5β1 integrins. We have observed that αV-class integrin signalling contributed to α5β1 integrin-mediated fibroblast adhesion to FN. Next, we tested whether αV-class integrin engagement induces clustering of α5β1 integrins, by combining SCFS with TIRF microscopy to visualize GFP-tagged paxillin clusters in fibroblasts adhering to FNIII7-10 substrates, for contact times ranging from 5 to 500 s (Fig. 4)29. Irrespective, whether pKO-α2V, pKO-β1 and pKO-α2V/β1 were attached on VN- or ConA-coated cantilevers, the size and occurrence of the paxillin-positive clusters increased for the first ≈40 s of contact time and remained constant thereafter. Strikingly, the intensity/size of paxillin-positive clusters in VN-stimulated pKO-α2V/β1 fibroblasts adhering to FNIII7-10 was higher compared with any other condition, indicating that VN-binding of αV-class integrins at the cantilever triggered robust α5β1 integrin clustering at the opposing FNIII7-10 substrate (Fig. 4, Supplementary Table 1). Interestingly, ConA-attached pKO-α2V and ConA-attached pKO-α2V/β1 fibroblasts showed comparable intensities of paxillin clusters, further supporting that αV-class integrins dominate early fibroblast adhesion to FN. Surprisingly, although ConA-attached pKO-β1 fibroblasts exhibited higher adhesion to FNIII7-10, compared with pKO-α2V and pKO-α2V/β1 fibroblasts (Fig. 1a), they assembled paxillin-positive adhesion clusters with lowest intensities (Fig. 4), suggesting that the affinity of α5β1 integrin for FN is influenced by the absence of αV-class integrins.

Discussion

The establishment of cell adhesion is a tightly regulated process, which is governed by the binding of integrins to the ECM. Here, we report that different FN-binding integrin classes establish distinct adhesion profiles during the initiation of cell adhesion to FN. In the early phase of adhesion formation (<2 min), fibroblasts expressing only α5β1 integrins establish stronger adhesion to FN compared with αV-class integrins, which is in line with previous reports reporting stronger adhesion promoting function of α5β1 integrins compared with αV-class integrins30. However, we also observe that fibroblasts expressing both FN-binding integrin classes establish considerably lower adhesion strengths compared with fibroblasts expressing only α5β1 integrins. This finding of a ‘differential integrin-dependent adhesion’ was surprising since both fibroblast lines express comparable numbers of αV-class and α5β1 integrins on their cell surface and expression of both integrins was shown to establish strongest adhesion after a contact time of more than an hour8.

Integrin cross-talk among each other and other cell adhesion molecules, such as ephrins and cadherins30–32, to perfectly adjust cell adhesion to the ECM. Our experiments identify a novel cross-talk between αV-class and α5β1 integrins to establish and strengthen early cell adhesion to FN. When initiating cell
**Figure 3 | Role of signalling molecules for the development of α5β1 and αV-class integrin-mediated adhesion forces.** Adhesion forces of knockout (KO) or pKO-αV/β1 fibroblasts to FNIII7-10 were determined both in the absence and presence of specific chemical inhibitors. pKO-αV/β1, talin KO, kindlin KO, ILK KO fibroblasts and pKO-αV/β1 fibroblasts treated with chemical inhibitors were attached to either ConA (yellow)- or VN (blue)-coated cantilevers. If not stated pKO-αV/β1 fibroblasts were used for experiments. Fibroblasts were adhered to FNIII7-10-coated substrates for 120 s. Chemical inhibitors were added at indicated concentrations to pKO-αV/β1 fibroblasts starting 30 min before experiments, with the exception of C3 toxin, which was added 3 h before. S-trityl-L-cysteine (STC), glycerol (Gly) and DMSO were used as negative controls (CTR) to measure the adhesion of pKO-αV/β1 fibroblasts. For VN-stimulating fibroblasts, cantilevers were coated by 5 μg ml⁻¹ VN diluted in ConA. Dots show adhesion forces of single fibroblasts (n≥10 for each condition) and red bars their median. Statistical significance was determined to compare unperturbed and perturbed adhesion for each (non-stimulated and VN-stimulated) condition by two-tailed Mann–Whitney U-tests (** P<0.001; *** P<0.0001; **** P<0.00001; NS, non-significant, P≥0.05).

**Figure 4 | Engagement of αV-class integrins induces α5β1 integrin clustering.** (a) Time series of TIRF images of GFP-labeled paxillin expressed in pKO-αV/β1 (blue), pKO-αV (yellow) and pKO-β1 (green) fibroblasts adhering to FNIII7-10-coated substrates. To record the images, single fibroblasts were attached to ConA (non-stimulated) or VN-coated (stimulated) cantilevers, incubated for 7-10 min and then approached to the FNIII7-10 coated substrate. Paxillin-GFP-intensity was detected by TIRF microscopy after 5 s and then after every 20 s for up to 500 s contact time with the substrate. To stimulate fibroblasts by VN, cantilevers were coated using 5 μg ml⁻¹ VN diluted in ConA. Scale bars, 10 μm. (b) Paxillin-GFP-intensity over contact time. The data were taken from TIRF images such as shown here and the statistical analysis of the data is given in Supplementary Table 1. Dots show mean fluorescence intensities of fibroblasts and error bars show s.e.m. (n≥10 for each condition).
adhesion to FN, αV-class integrins compete with zβ1 integrins for substrate binding. We observe that αV-class integrins show a higher binding rate (on-rate) to FN, which initially prevents zβ1 integrins from binding. In line with our results, earlier studies reported that αVβ3 integrins prevent the recruitment of zβ1 integrins to adhesion sites at early cell spreading. This competition for FN binding could be due to differences in extracellular ligand binding and/or interactions of integrin β-tails with cytoplasmic proteins such as talin, kindlin, and ILK and/or inhibitory adapter proteins. It has been demonstrated that despite the presence of αVβ3 integrins, mutation in the talin binding site in β3-integrin leads to the predominant engagement of zβ1 integrins to FN. In our results β1- and β3-tails showed equivalent binding to talin and the adhesion of ICAP-1-deficient fibroblasts to FNIIII7-10 was higher than that of wild-type fibroblasts. This finding suggests that ICAP-1 hinders the binding of talin or kindlin to β1-tail during adhesion initiation and thereby increases the available pool of talin/kindlin for αV-class integrins to bind and to initiate adhesion. After initiating adhesion and engaging the substrate, αV-class integrins signal to zβ1 integrins to induce their clustering and to establish adhesion to FN, which is much stronger and faster than the adhesion established by both integrin classes in the absence of the crosstalk (Supplementary Fig. 5). Eventually, the adhesion strengthens with time and develops into adhesion sites, in which zβ1 and αV-class integrins separate into different compartments. Although, the distinct roles of and the cooperativity among β1- and αV-class integrins have been extensively studied during adhesion maturation and in response to force, here, we show that both FN-binding integrins interplay from the early onset of adhesion.

Our data demonstrates that already within the first two minutes of early fibroblast adhesion, FN-binding integrins critically depend on integrin-associated proteins such as talin, kindlin, and ILK. However, it was surprising to observe that formin inhibition also affected early cell adhesion. A recent report showing that the formin homology 2 domain containing formin inhibition also affected early cell adhesion. A recent study reported that formin inhibition also affected adhesion. In response to αV-class integrin engagement, zβ1 integrins enhanced binding to FN and formed adhesion clusters that considerably strengthened early fibroblast adhesion within ≤120 s. Although, we clearly observed the formation of paxillin clusters in VN-stimulated fibroblast adhering to FN, the lateral resolution limit of TIRF did not allow us to determine their sizes. Hence, SFCS combined with super resolution microscopy will be necessary to further characterize the assembly of adhesion clusters.

In summary, our study provides direct evidence that αV-class integrins adhering to VN- or FN-coated cantilevers signal to zβ1 integrins to bind FN at the opposite side of the fibroblast and to form adhesion clusters. Hence, we deduced a model to depict the two-step process by which αV-class integrins crosstalk with zβ1 integrins to establish and to strengthen cell adhesion to FN (Fig. 5). In the first step, αV-class integrins initiate cell adhesion by binding FN quicker than zβ1 integrins. The engagement of αV-class integrins to VN (or FN) clusters, recruits and activates adhesion-specific proteins including talin, kindlin, ILK, and formins to mediate the link to the actin cytoskeleton (Fig. 5a,b). In a second step, the engaged αV-class integrins activate signalling involving the RhoA/Rock and the Rac1/Wave/Arp2/3 pathways that finally promote zβ1 integrins binding to FN. Gradually, the adhesion strengthens and matures by clustering and separating zβ1 integrins via myosin-II into different FA compartments (Fig. 5c,d).

**Methods**

**Cell culture.** pKO, pKO-αV/β1, pKO-αV/β1 lifeact-mCherry paxillin-GFP, pKO-αV, pKO-αV lifeact-mCherry paxillin-GFP, pKO-β1, pKO-β1 lifeact-mCherry paxillin-GFP, talin KO, kindlin KO1 and ILK KO2 and mouse kidney fibroblasts, ICAP-1 KO, ICAP-1 WT2 mouse embryonic fibroblast cell lines (generated in house by R. Fässler) were maintained in DMEM (Gibco-Life technologies, NY, USA), supplemented with 1% (v/v) fetal calf serum (FCS, Sigma, Steinheim, Germany), 100 units/ml penicillin and 100 μg/ml streptomycin (both Gibco-Life technologies). Fibroblasts were grown on fibronectin, Calbiochem-Merck, Darmstadt, Germany) coated tissue culture flasks (Jet BioFil, Guangzhou, China) in a humidifying incubator with 5% CO2 at 37 °C. For SFCS, fibroblasts were grown on 24-well plates (Thermo Scientific, Roskilde, Denmark) and serum-starved overnight before measurements. The fibroblasts were regularly tested for mycoplasma contamination.

**Expression and purification of fibronectin fragments.** FN fragment FNIII7-10, RGD-deleted fragment FNIII7-10RGD and synergy mutated FN fragment FNIII7-10mSYN were expressed from plasmids pET15b-FNIII7-10 and pET15b-FNIII7-10mSYN (generated by R. Fässler) in Escherichia coli (DE3) pLysS. Briefly, cells growing in L broth (Invitrogen, Carlsbad, USA) supplemented with 100 μg/ml ampicillin (Sigma-Aldrich, Buchs, Switzerland) at 37 °C. Expression was induced with 1 mM isopropyl thiogalactoside (IPTG, Sigma) at optical density (OD)600 = 0.6. Cells were collected after 4 h, re-suspended in buffer (20 mM Tris–HCl, 150 mM NaCl, pH 8.0), and broken by sonication. Cell debris was removed by ultracentrifugation at 40,000g for 45 min. The solubilized protein fraction was bound to nickel-nitrilotriacetic acid resin (Protino Ni-NTA, Macherey-Nagel, Düren, Germany) for 1 h at 4 °C. The resin was then loaded onto a column and washed with buffer (20 mM Tris–HCl, 150 mM NaCl, 10 mM imidazole, pH 8.0). FN fragments were eluted with elution buffer (200 μg/ml 1 M imidazole, pH 8.0). Fractions were pooled and dialyzed against washing buffer (20 mM Tris–HCl, 150 mM NaCl, pH 8.0). The protein concentration was adjusted to 1.0 mg/ml with dialyzing buffer and aliquots were stored at −20 °C.

**Cantilever and substrate functionalization.** For fibroblast attachment, cantilevers were plasma cleaned (PDC-32G, Harrick Plasma) and then incubated
inactive integrins cluster to strengthen adhesion. This crosstalk eventually leads to the formation of nascent adhesions. Recent structural investigations suggested we here illustrate inactive integrins in the bent confirmations (grey) and active/engaged integrins in the extended conformation (coloured).

proteins mediate and strengthen the attachment of integrins to the actomyosin cortex. To strengthen fibroblast adhesion, activated integrins and recruits integrin-associated proteins such as talin, kindlin, ILK and formins to the adhesion site. Upon recruitment, the integrin-associated fibroblasts initiate contact with FN, 

Figure 5 | αV-class integrins compete with α5β1 integrins for the binding of FN and crosstalk to α5β1 integrins to strengthen adhesion to FN. (a) As fibroblasts initiate contact with FN, αV-class integrins successfully compete with α5β1 integrins to bind the substrate. (b) This binding engages αV-class integrins and recruits integrin-associated proteins such as talin, kindlin, ILK and formins to the adhesion site. Upon recruitment, the integrin-associated proteins mediate and strengthen the attachment of integrins to the actomyosin cortex. To strengthen fibroblast adhesion, activated αV-class integrins via the RhoA/ROCK/myosin-II and Arp2/3 pathway signal to (c) α5β1 integrins to bind FN thereby forming new adhesion sites. (d) Consequently, α5β1 integrins cluster to strengthen adhesion. This crosstalk eventually leads to the formation of nascent adhesions. Recent structural investigations suggested that inactive α5β1 integrins can co-exist in bent and unbent conformations, while inactive αV-class integrins have shown to be bent51. Thus, for simplicity we here illustrate inactive integrins in the bent conformations (grey) and active/engaged integrins in the extended conformation (coloured).

Single-cell force spectroscopy. For SCFS, we mounted an AFM (Nanosurf II equipped with CellHesion Module, JPK Instruments, Berlin, Germany) on an inverted fluorescence microscope46 (Observer.Z1/A1, Zeiss, Germany). The temperature was kept at 37°C throughout the experiment by a Petri dish heater (JPK Instruments). Two hundred micro litre long tip-less V-shaped silicon nitride cantilevers having nominal spring constants of 0.06 N m⁻¹ (NP-0, Bruker, USA) were used. Each cantilever was calibrated prior the measurement by determining its sensitivity and spring constant using the thermal noise analysis of the AFM57. To adhere a single fibroblast to the AFM cantilever, overnight serum-starved fibroblasts with confluency up to ~80% were washed with PBS and detached from the culture flask with 0.25% (w/v) trypsin (Sigma-Aldrich), up to 2 min. Trypsinized fibroblasts were suspended in SCFS media (DMEM supplemented with 20 mM HEPES) containing 1% (v/v) FCS, pelleted and resuspended in serum free SCFS media48. Fibroblasts were allowed to recover for at least 30 min from trypsin treatment49. Functionalized Petri dishes were washed with SCFS media to remove unbound proteins. Adhesion of a single fibroblast to the free cantilever end was achieved by pipetting the fibroblast suspension onto the functionalized Petri dishes. The functionalized cantilever was lowered onto a fibroblast with a speed of 10 μm s⁻¹ until a force of 5 nN was recorded. After ~5 s contact, the cantilever was retracted with 10 μm s⁻¹ for 50 μm and cantilever bound fibroblast was incubated for 7–10 min to assure firm binding to the cantilever. Using optical microscopy (DIC and phase contrast), the morphological state of the fibroblast was monitored. Adhesion measurements were only conducted using rounded fibroblast before they spread on the cantilever. For adhesion force experiments, the rounded fibroblast bound to the cantilever was lowered onto the coated substrate with a speed of 5 μm s⁻¹ until a contact force of 2 nN was recorded. For contact times of 5, 20, 50 or 120 s, the cantilever height was maintained constant. Subsequently, the cantilever was retracted at 5 μm s⁻¹ and for >90 μm until the fibroblast and substrate were fully separated. After the experimental cycle, the fibroblast was allowed to recover for a time period equal to contact time before measuring the adhesion force for a different contact time. A single fibroblast was used to probe the adhesion force for all contact times or until morphological changes (that is, spreading) was observed. The sequence of contact time measurements and area of the substrate were varied. The adhesion of at least 10 fibroblasts was measured per condition to obtain statistically firm results. Adhesion forces were determined after baseline correction of force–distance curves with JPK software (JPK Instruments). For single molecule sensitivity, we modified SCFS with low contact force (200 pN) and zero contact time. Force-distance curves were analysed to determine binding probability using JPK software.

Statistical tests comparing the adhesion forces and slopes. Unpaired t-tests: two-tailed Mann–Whitney tests were applied to determine significant differences between the median adhesion forces at the given contact times among different conditions. Tests were done using Prism (GraphPad, La Jolla, USA). To compare adhesion strengthening among different fibroblast lines, we determined the differences in their slopes describing the adhesion force over time. We defined the (discrete) slope between contact times t₁ and t₂ with corresponding adhesion force measurements F₁ and F₂ as sₓ,y = (F₂ - F₁)/(t₂ - t₁). For each fibroblast cell line, we defined the slope of the adhesion force–time data as the average slope of all adjacent time points. We generated 100 bootstrap samples from the original data to obtain samples of equal size and tested for differences in slope between fibroblast cell lines using two-tailed Wilcoxon’s test.

Analysis of statistical interactions between integrins. For each time point, the statistical interaction strength between the αV-class and α5β1 integrins with respect to adhesion force is defined as e = F_KO−αVβ1 + F_KO−α5β1 - F_KO−β1, where F denotes the adhesion force. The quantity e is the deviation of the expected
effect of both integrins under an additive null model, namely \( F_{\text{pKO-x}} + F_{\text{pKO-y}} \), from the observed effect, namely \( F_{\text{pKO-x},y} + F_{\text{pKO}} \). If \( \mu = 0 \), then there is a positive, whereas if \( \mu < 0 \), then there is a negative correlation between S9-class and \( \xi_1 \) integrins. Statistical testing of the null hypothesis of no interaction (\( \mu = 0 \)) was performed on 100 bootstrap samples using two-tailed Wilcoxon’s test.

**Immunoprecipitation of integrins.** For immunoprecipitation of \( \beta_1 \) integrin, pK0 fibroblasts were washed twice with PBS and incubated with fresh crosslinking solution—0.5 mM diethio-bis succinimidylpropionate (DSP, Thermo scientific, USA) for 30 min at room temperature (RT). DSP was quenched with 50 mM Tris–HCl pH 7.5 for 10 min. Fibroblasts were lysed in lysis buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 1% Triton X-100, 0.05% sodium deoxycholate) with protease and phosphatase inhibitors and sonicated. The samples were pre-cleaned with A/G Plus Agarose (Santa Cruz, Germany) protein for 15 min at 4°C. After centrifugation, 30 μl of anti-\( \beta_1 \) integrin antibody (rabbit–polyclonal, homemade) for 1 h at 4°C, followed by the addition of 50 μl of A/G agarose protein for another hour, in an end-over-end rotor. After three washes with lysis buffer, the crosslink was reversed with 50 μl of 2x Laemmli sample buffer (homemade) containing 5 mM DTT (Thermo Scientific, USA) for 30 min at 37°C. After this, 1 μl of beta-mercaptoethanol (Sigma) was added and samples were incubated for 5 min at 95°C. Samples were subjected to SDS-PAGE and western blot analysis against integrins using specific \( \nu \)Nt antibody (AB1930, Millipore, Germany), \( \nu_5 \) integrin (#4705, Cell Signaling Technology, Germany) and \( \beta_1 \) integrin (homemade) antibodies with the dilution of 1:1,000.

**Integrin \( \beta \)-tail peptide pull downs.** Pull downs were performed with the following \( \beta_1 \) peptides: wild-type cytoplasmic tail peptide (HDRIIFAKE-KKMKNAVKTGDPNYIKSVATTNVNYKEGK), \( \beta_3 \) wild type cytoplasmic tail peptide (HDRIIFAKEFEEERAKWDTANNLYKEEDAEFTDHTDINAK-EOH). All peptides were desialylated. Before use, peptides were immobilized on Dynabeads MyOne Streptavidin C1 (Millipore, USA) for 30 min, before the experiments. SCFS was conducted in the presence of the respective drug/antibody in the stated concentrations. Combined TIRF and SCFS (CellHesion200) mounted on an inverted microscope (Observer.Z1, Zeiss, Germany) and loaded the supernatant on a 4–20% SDS–PAGE gel. Samples were analysed by western blot with antibodies to functionalized 24-well glass bottom plates and allowed to spread for 10 or 30 min. TIRF images were acquired at initial 5 s and thereafter at 20 s intervals for the duration of 1:50 in blocking buffer overnight at 4°C. Secondary antibodies used for integrin \( \beta_3 \) and phospho-tyrosine were anti-rat Alexa Fluor 488 (Ab91006, Life Technologies, USA) and anti-mouse Alexa Fluor 647 (Ab1500115, Life Technologies, USA), respectively, diluted 1:100 in blocking buffer for 1 h at RT. Actin was stained using rhodamine-phalloidin (Life Technologies, USA) in dilution of 1:500 in blocking buffer for 1 h at RT. Fibroblasts were washed thrice with PBS after every step. Stained fibroblasts were treated with Prolong gold anti-fade reagent (Invitrogen AG, Switzerland) for 24 h at RT and analysed with inverted confocal microscope (Nikon Ti-E, Switzerland) using a ×63/1.4 oil objective. Signals were collected sequentially and images were analysed with NIS software (Nikon).

**Code availability.** The procedures for comparing adhesion slopes and for assessing interactions were implemented in a code in the statistical programming language R, which is included in Supplementary Note 1.

**Data availability.** The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

M.B. performed and analysed most experiments. N.S. contributed to TIRF experiments. J.H. helped with experimental set-up and data analysis. H.B.S. and R.F. provided important reagents and/or analytical tools. G.P.C. performed immunoprecipitation experiments. N.B. wrote the -code for statistical analysis. All authors discussed the experiments, read and approved the manuscript.

Additional information

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