

Molecular mechanism of vinculin activation and nanoscale spatial organization in focal adhesions

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Focal adhesions (FAs) link the extracellular matrix to the actin cytoskeleton to mediate cell adhesion, migration, mechanosensing and signalling. FAs have conserved nanoscale protein organization, suggesting that the position of proteins within FAs regulates their activity and function. Vinculin binds different FA proteins to mediate distinct cellular functions, but how vinculin's interactions are spatiotemporally organized within FAs is unknown. Using interferometric photoactivation localization super-resolution microscopy to assay vinculin nanoscale localization and a FRET biosensor to assay vinculin conformation, we found that upward repositioning within the FA during FA maturation facilitates vinculin activation and mechanical reinforcement of FAs. Inactive vinculin localizes to the lower integrin signalling layer in FAs by binding to phospho-paxillin. Talin binding activates vinculin and targets active vinculin higher in FAs where vinculin can engage retrograde actin flow. Thus, specific protein interactions are spatially segregated within FAs at the nanoscale to regulate vinculin activation and function.

Integrin-mediated FAs are plasma-membrane-associated organelles that physically connect the actin cytoskeleton to the extracellular matrix (ECM), providing specific adhesion between cells and their surroundings to mediate tissue formation and immune responses. FAs also serve as signalling hubs where cells sense biochemical and physical cues in their environment that inform cell decision-making in the cell cycle, differentiation and death. Additionally, they serve as sites of force transmission between the cytoskeleton and the surroundings to drive tissue morphogenesis, cell movement, and ECM remodelling. These diverse functions of FAs are reflected in their biochemical complexity. FAs contain hundreds of different proteins and their composition changes in response to physical stimuli, making them important sites of mechanotransduction^{1–3}. Thus, FAs are multifaceted organelles that mediate an array of functions involving biochemical and physical interactions between the cell and its environment.

Although FAs are functionally and biochemically complex, they have conserved dynamics and structure⁴. FAs form during protrusion of the cell edge as small (<250 nm) nascent FAs containing clustered integrins, FAK and paxillin⁵. Nascent FAs undergo a process of actomyosin-dependent maturation in which they grow to several micrometres in length and change molecular composition⁶. Mature FAs exhibit variations in protein composition along their length, with

phosphorylated paxillin concentrating at their distal tips facing the cell periphery⁷, and actin-binding proteins such as vinculin, VASP and α -actinin concentrating at their proximal tips where they attach to actin stress fibres^{4,8,9}. In addition, super-resolution microscopy recently revealed that FA proteins exhibit differential nanoscale localization along the axial dimension of their 200 nm thickness⁴. This showed that proteins localize to three general FA nanodomains: a membrane-proximal integrin signalling layer (ISL) containing FAK and paxillin located within ~10–20 nm of the plasma membrane; an actin regulatory layer (ARL) containing α -actinin, VASP and zyxin that initiates ~50–60 nm from the membrane and extends upwards into the stress fibre; and a force transduction layer (FTL) containing the rod domain of talin that spans between the ISL and the ARL (ref. 4). However, the functional consequence of this organized structure has yet to be explored. The nanoscale segregation of proteins into different axial FA domains could sterically limit the possible protein–protein interactions, which in turn could dictate specific downstream functional effects. Furthermore, whether this architecture is altered to mediate distinct FA functions, or if it evolves dynamically during FA maturation is not known.

Vinculin is an essential protein required for multiple FA functions, including stabilizing and strengthening FAs and promoting their

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maturation^{10–14}, ECM mechanosensing¹⁵, regulating actin cytoskeletal dynamics¹⁶, and signalling to control cell death¹⁷. Vinculin has over 14 putative binding partners at FAs including talin¹⁸, actin¹⁹, paxillin²⁰, PIP2 (ref. 21), Arp2/3 (ref. 22) and vinexin²³, and specific vinculin–protein interactions have been ascribed to distinct FA functions. For example, vinculin interaction with paxillin mediates FA mechanosensing¹⁵, its interaction with actin is required for regulation of lamellipodial actin dynamics¹⁶, and talin binding by vinculin mediates FA strengthening¹¹. Thus, the spatiotemporal regulation of different vinculin interactions probably regulates cellular function. Additionally, vinculin's interaction with its binding partners is regulated by an auto-inhibitory, high-affinity intramolecular interaction between its head and tail domains^{24,25}, and release of auto-inhibition is believed to require simultaneous binding of multiple ligands²⁶. However, how vinculin activation and protein interactions are spatiotemporally regulated during FA formation and maturation is not known.

In this study, we sought to understand how distinct molecular interactions regulate vinculin activation and function within the context of the three-dimensional FA nano-architecture. Using super-resolution microscopy to assay vinculin nanoscale organization and a fluorescence resonance energy transfer (FRET) biosensor to assay vinculin activation, we found that inactive vinculin associates with the lower ISL by binding to phospho-paxillin, and talin binding is required to activate vinculin and target active vinculin to higher FA layers where vinculin binds actin. Furthermore, we show that vinculin distribution shifts from the ISL to the FTL and ARL as myosin II contractility promotes FA maturation. These results suggest that nanometre-scale changes in vinculin position during FA maturation facilitate vinculin activation and mechanical reinforcement of FA.

RESULTS

Vinculin is distributed throughout the three FA nanodomains

We hypothesize that the organization of vinculin into axial FA nanodomains is regulated by specific protein–protein interactions. To serve as a conceptual framework for our analysis of vinculin axial distribution within FAs, we quantitatively defined the ISL and ARL layers in human foreskin fibroblasts (HFFs) by interferometric photoactivation localization microscopy (iPALM) imaging of a plasma membrane marker (CAAX fused to tandem Eos (CAAX–tdEos)) and actin (actin fused to monomeric Eos2 (actin–mEos)), respectively, and defined the FTL as the intervening region. Fluorescent protein fusions were transiently expressed in cells plated on fibronectin-coated coverslips and fixed for iPALM imaging. For display, representative FAs were rendered with colours indicating the axial (*Z*) position of each localized molecule relative to the position of the coverslip, and the *Z*-distribution of all the molecules in the rendered FAs plotted in the accompanying histogram (Fig. 1a,b). Determination of the medians of the *Z*-distributions (Z_{med}) showed that CAAX–tdEos and actin–mEos localized to ~ 40 nm and ~ 95 nm, respectively, similar to previous reports⁴ (Fig. 1d and Supplementary Tables 1 and 2). Then, on the basis of visual comparison of averaged 1-nm-binned histograms of *Z*-distributions of each probe (Fig. 1e), we arbitrarily defined the ISL as 25–54 nm above the coverslip, where most (75%) of the CAAX–tdEos and little (13%) of the actin–mEos localized. Similarly, we defined the ARL as 85–150 nm above the coverslip where most (64%) actin–mEos

and little CAAX–tdEos resided (16%), and the intervening FTL as 55–84 nm above the coverslip (Fig. 1f). Thus, for the purpose of our analysis, the ISL consists of the 30 nm region adjacent to the plasma membrane, the ARL extends from 60 nm above the membrane into the stress fibre, and the FTL consists of the intervening 30 nm.

We next characterized the distribution of vinculin within these defined axial FA nanodomains. Transient expression of vinculin fused to tdEos at its amino terminus (WT–vinculin–N–tdEos) followed by iPALM analysis showed that WT–vinculin–N–tdEos consistently exhibited a Z_{med} of ~ 62 nm (Fig. 1c,d and Supplementary Fig. 1). Using the above definitions, we found that 35% of the vinculin molecules localized in the ISL, 41% localized in the FTL, and 24% localized in the ARL (Fig. 1e,f). Thus, vinculin is distributed throughout the three FA nanodomains, with most molecules residing in the ISL and FTL.

Vinculin is required to maintain talin in a vertically extended conformation in FA

We next sought to determine the requirement for vinculin in the nanoscale organization of its binding partners and the molecular architecture of FA. We performed iPALM analyses of cells in the presence (wild type, WT) and absence (knockdown, KD) of vinculin, examining the distribution of the ISL protein paxillin, the ARL protein actin, and talin, an extended protein that spans the FTL by binding integrin tails²⁷ in the ISL and actin²⁸ in the ARL (refs 4,29). Small interfering RNAs (siRNA) targeting the 3'UTR knocked down >95% of vinculin in HFFs (Supplementary Fig. 2a). We transiently expressed paxillin–tdEos (paxillin with N-terminally fused tdEos), actin–mEos, talin–N–tdEos (talin with N-terminally fused tdEos) and talin–C–tdEos (talin with carboxy-terminally fused tdEos) in either WT or vinculin KD background. Comparison of WT and vinculin KD cells by two-sampled Kolmogorov–Smirnov test and ANOVA analyses showed that vinculin had an insignificant effect on the localization of paxillin (Fig. 2a,b), with $\sim 50\%$ of paxillin–tdEos localized in the ISL under both conditions (Fig. 2e–g). Similarly, the loss of vinculin had an insignificant effect on the localization of actin or the N terminus of talin in FAs, with $\sim 75\%$ of actin–mEos2 localized in the ARL and $\sim 50\%$ of talin–N–tdEos localized in the ISL (Fig. 2c,d,h–l,o,p,r). In contrast, compared with WT cells, in vinculin KD, the Z_{med} of talin–C–tdEos was significantly decreased by ~ 10 nm, the fraction of molecules in the ARL was reduced, and there was a concomitant increase in the fraction of molecules in the ISL (Fig. 2m–o,q,s). Furthermore, talin–C–tdEos nanoscale position was rescued by the overexpression of vinculin–mTurquoise (Supplementary Fig. 2). Thus, in the presence of vinculin, the talin tail is localized ~ 23 nm above the head, whereas in the absence of vinculin, the tail extends only ~ 14 nm above the head. We conclude that although vinculin is not required for paxillin or actin nanoscale positioning within FA, vinculin is required for talin to be maintained in a maximal vertically extended conformation.

Vinculin is oriented in FAs with the tail above the head

We next sought to determine whether vinculin exhibits a preferred orientation in FAs by comparing the localization of the N and C termini of WT vinculin. We performed iPALM on WT–vinculin–N–tdEos and WT–vinculin–C–tdEos (Fig. 3a,b). Consistent with a previous study³⁰, we found that WT–vinculin–C–tdEos

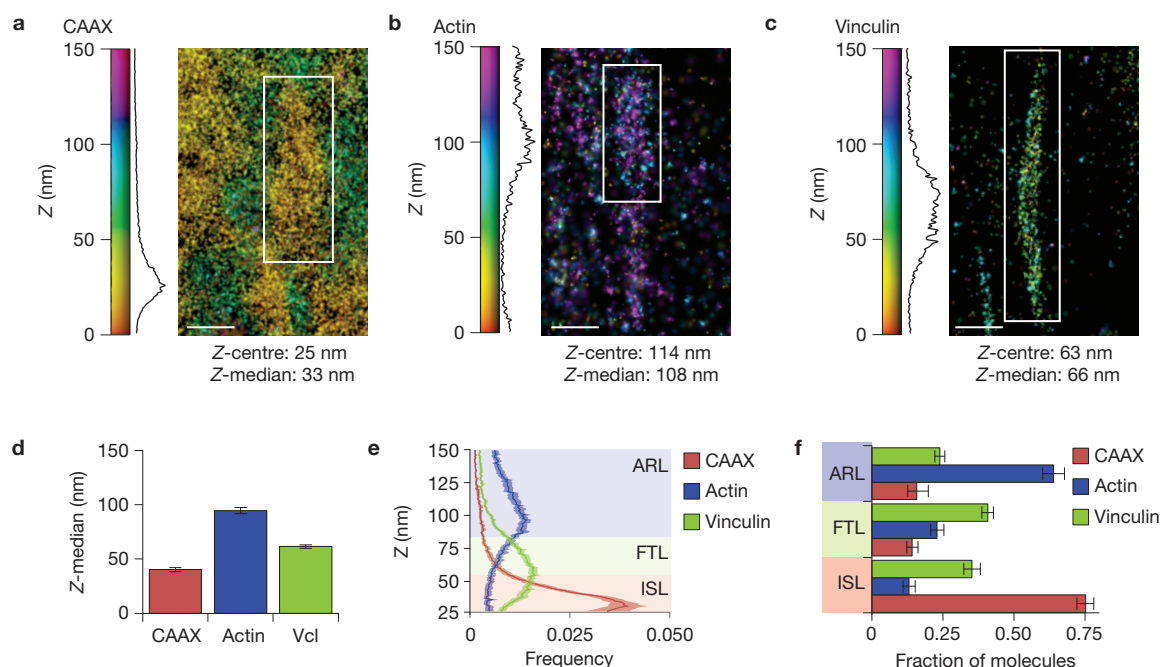


Figure 1 Vinculin is distributed throughout the three FA nanodomains. (a–c) Representative iPALM renderings from HFFs expressing CAAX-tdEos to label the plasma membrane (a), actin-mEos2 to label actin (b), or vinculin-N-tdEos (c). In a–c the colour scale represents Z-position (nm), FAs oriented with the distal tip facing up, scale bars, 1 μ m. Histograms of the Z-position of molecules within individual FAs (white rectangles in a–c) shown next to the colour scale. The measured Gaussian Z-centre and Z-median of the distribution is shown. (d) Mean of Z-median measurements from individual FAs. (e) Averaged Z-position frequency histograms of molecules within FAs. Solid line, mean frequency; shaded region, bootstrapped 95% confidence intervals. (f) Mean fraction of molecules localized to each of the three FA layers. Background colouring in e,f is used to highlight the three FA layers. ISL: integrin signalling layer (red, 25–54 nm above the coverslip), FTL: force transduction layer (green, 55–84 nm above the coverslip), ARL: actin regulatory layer (purple, 85–150 nm above the coverslip). Graphs in d–f represent measurements of $n = 27$ FAs from 5 CAAX-tdEos-expressing cells, $n = 56$ FAs from 8 actin-mEos2-expressing cells and $n = 115$ FAs from 8 vinculin-N-tdEos-expressing cells. Data in all bar graphs are represented as mean $\pm$ bootstrapped 95% confidence intervals.

localized significantly higher than WT-vinculin-N-tdEos (Fig. 3e) and had a significantly different Z-distribution (Fig. 3f), with more WT-vinculin-N-tdEos localized in the ISL and more WT-vinculin-C-tdEos localized in the ARL (Fig. 3g). As the N terminus is adjacent to the vinculin head and the C terminus is adjacent to the vinculin tail³¹, this suggests that most vinculin molecules are oriented in FAs with the tail above the head.

Activation promotes association of vinculin with the FTL and ARL

As vinculin activation affects its ability to interact with multiple binding partners including talin and actin²⁶, we next sought to determine whether vinculin activation influences its nanoscale organization within FAs. We used iPALM analysis to measure the effect of activating point mutations (vinculin N773/E775A ('CA vinculin') and vinculin D974/K975/R976/R978A ('T12 vinculin')) on the Z-position of N- and C-terminally tagged vinculin-tdEos fusion constructs in FAs (refs 16,32,33; Fig. 3c,d). We found that both CA-vinculin-N-tdEos and T12-vinculin-N-tdEos localized significantly higher in FAs and had a higher percentage of molecules localized in the ARL compared with WT-vinculin-N-tdEos (Fig. 3e,h–k). Additionally, CA-vinculin-C-tdEos localized significantly higher in FA than CA-vinculin-N-tdEos, suggesting that activated vinculin is oriented in FAs with the tail above the head

(Supplementary Fig. 3). We conclude that activation promotes an upwards shift in the distribution of vinculin within FAs.

Talin binding is required for vinculin activation in FAs and promotes localization of active vinculin to the FTL and ARL

The talin α -helical rod domain contains several mechanosensitive vinculin binding sites spanning the FTL that mediate vinculin activation *in vitro*³⁴ and strengthening of the integrin–actin link in cells³⁵. We thus sought to determine the role of talin in vinculin activation and nanoscale localization in FAs. To assay activation in cells, we used a vinculin activation FRET biosensor³⁶ in which donor (mTurquoise) and acceptor (NeonGreen³⁷) fluorophores were inserted into the vinculin protein such that FRET occurs when the vinculin tail interacts with the head in the auto-inhibited, closed conformation, and FRET decreases when auto-inhibition is relieved and vinculin is activated. Unlike positive and negative FRET controls (Supplementary Fig. 4a–c), WT-vinculin-FRET exhibited a significantly lower FRET ratio at FAs compared with the cytosol (Supplementary Fig. 4d,e), consistent with previous reports³⁶. In contrast, when we introduced the N773/E775A activating mutation, CA-vinculin-FRET exhibited a FRET ratio at the same low level as FA-localized WT-vinculin-FRET, independent of localization (Supplementary Fig. 4d,e). Together, these results confirm previous reports³⁶ indicating that vinculin activation is selectively increased at FAs.

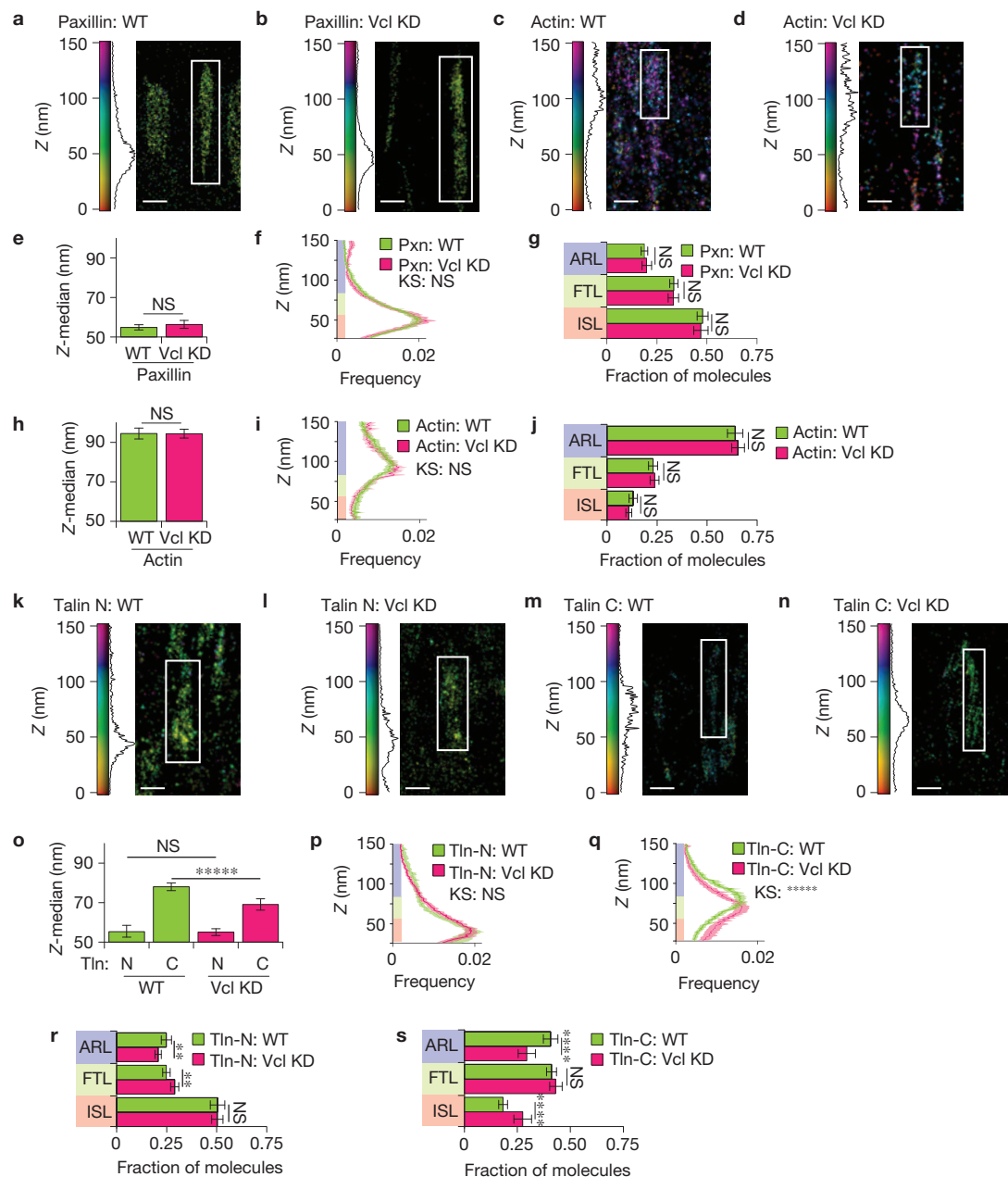


Figure 2 Vinculin is required to maintain talin in a vertically extended conformation. (a–s) iPALM images and analysis of paxillin, actin and talin from wild-type (WT) HFFs or HFFs treated with siRNAs targeting vinculin (Vcl KD). (a,c,k,m) Representative iPALM renderings from WT HFFs expressing paxillin–tdEos (Pxn, a), actin–mEos2 (c), N-terminally tagged talin–N–tdEos (k) or C-terminally tagged talin–C–tdEos (m). (b,d,l,n) Representative iPALM renderings from Vcl KD HFFs expressing paxillin–tdEos (b), actin–mEos2 (d), talin–N–tdEos (l) or talin–C–tdEos (n). In a–d and k–n, the colour scale represents Z-position (nm), FAs oriented with the distal tip facing up, scale bars, 1 μ m. Histograms of the Z-position of molecules within individual FAs (white rectangles in a–d,k–n) shown next to the colour scale. Actin–mEos2 WT data are duplicated from Fig. 1 for comparison purposes. (e,h,o) Mean of Z-median measurements from individual FAs. (f,i,p,q) Averaged Z-position

frequency histograms of molecules within FAs. Solid line, mean frequency; shaded region, bootstrapped 95% confidence intervals. Significance tested with two-sample Kolmogorov–Smirnov (KS) test. (g,j,r,s) Mean fraction of molecules localized to the three FA layers. Colouring in f,g,i,j,p–s is used to highlight the FA layers as in Fig. 1. Graphs in e–g represent measurements of $n=120$ FAs from 7 WT cells and $n=117$ FAs from 6 Vcl KD cells. Graphs in h–j represent measurements of $n=56$ FAs from 8 WT cells and $n=50$ FAs from 6 Vcl KD cells. Graphs in o–s represents data from $n=75$ FAs from 7 Tln–N WT cells, $n=107$ FAs from 5 Tln–N KD cells, $n=95$ FAs from 5 Tln–C WT cells, and $n=66$ FAs from 5 Tln–C KD cells. Data in all bar graphs are represented as mean $\pm$ 95% bootstrapped confidence intervals and significance is tested with one-way ANOVA. For statistical tests: ** $P < 0.01$, *** $P < 0.0001$, **** $P < 0.00001$, NS: not significant.

To determine the contribution of talin binding to the activation of vinculin in cells, we introduced the A50I mutation into the WT–vinculin–FRET biosensor (Fig. 4a–c). This mutation inhibits talin

binding to the vinculin head *in vitro*³³, and in cells targets to FAs, but results in reduced FA number and decreased adhesion strength and traction force¹⁰. Expression of A50I–vinculin–FRET in cells showed

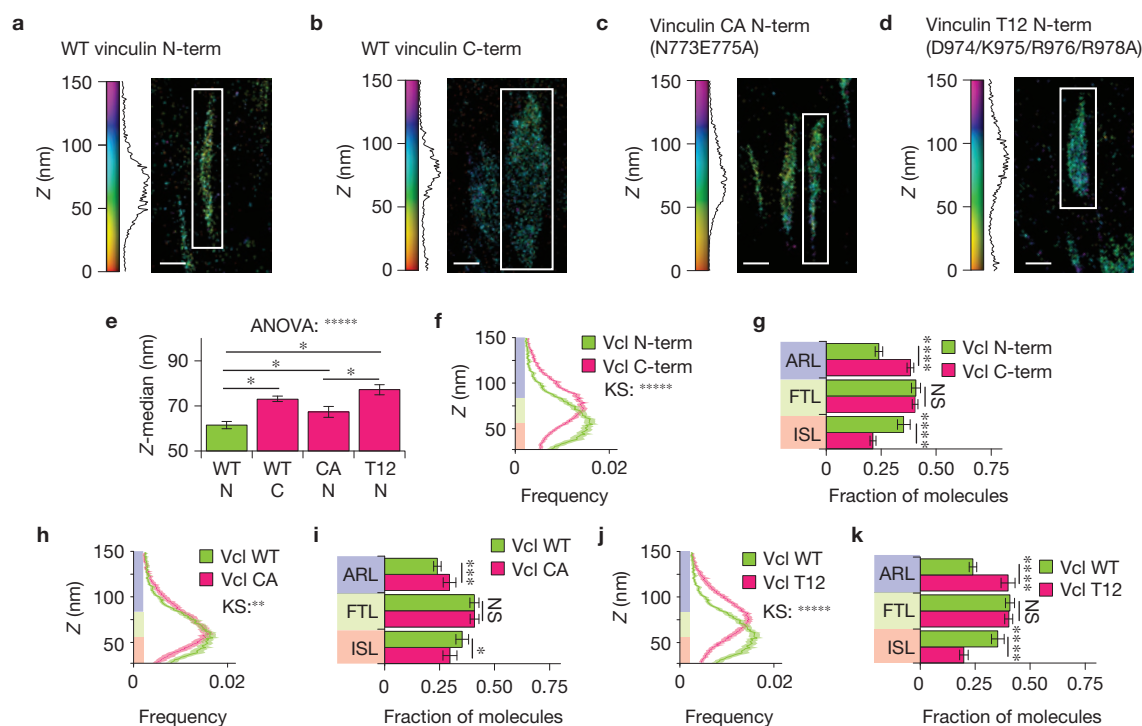


Figure 3 Vinculin is oriented in FAs with the tail above the head, and activation promotes association of vinculin with the force transduction and actin regulatory layers. (a–d) Representative iPALM rendering from HFFs expressing N-terminally tagged wild-type (WT) vinculin-N-tEos (a, data duplicated from Fig. 1 for comparison purposes), WT C-terminally tagged vinculin-C-tEos (b), constitutively active (CA, N773/E775A) vinculin-N-tEos (c), or constitutively active (T12, D974/K975/R976/R978A) vinculin-N-tEos (d). In a–d the colour scale represents Z-position (nm), FAs oriented with the distal tip facing up, and scale bars, 1 μ m. Histograms of the Z-position of molecules within individual FAs (white rectangles in a–d) shown next to the colour scale. (e–k) Quantitative analysis of data in a–d. (e) Mean of Z-median measurements of the position of molecules from individual FAs. Significance tested with one-way ANOVA.

(f,h,j) Averaged Z-position frequency histograms of molecules within FAs. Solid line, mean frequency; shaded region, bootstrapped 95% confidence intervals. Significance tested with two-sample Kolmogorov–Smirnov (KS) test. (g,i,k) Mean fraction of molecules localized to the three FA layers. Significance is tested with one-way ANOVA. Colouring in f–k used to highlight the FA layers as in Fig. 1. Graphs in e–k represent measurements of $n = 115$ FAs from 8 WT-vinculin-N-tEos-expressing cells, $n = 137$ FAs from 5 WT-vinculin-C-tEos-expressing cells, $n = 82$ FAs from 8 CA-vinculin-N-tEos cells and $n = 103$ FAs from 6 T12-vinculin-N-tEos-expressing cells. Data in all bar graphs are represented as mean $\pm$ 95% bootstrapped confidence intervals. For statistical tests: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ***** $P < 0.00001$, NS: not significant.

that in contrast to WT-vinculin-FRET that exhibited reduced FRET at FAs, the FRET ratio of A50I-vinculin-FRET was at the same high level in both the cytoplasm and FAs (Fig. 4a–c). This suggests that talin binding is required for vinculin activation at FAs.

Next, we examined the role of talin binding in regulating vinculin nanoscale localization. We knocked down vinculin and rescued with WT-vinculin-N-tEos or A50I-vinculin-N-tEos (Fig. 4d,e). iPALM analysis showed that compared with WT-vinculin-N-tEos, A50I-vinculin-N-tEos localized significantly lower in FA than WT-vinculin-N-tEos and had a significantly different Z-distribution, with reduced localization to the FTL and increased localization to the ISL (Fig. 4f–h). We conclude that talin binding promotes a shift in vinculin localization from the ISL to the FTL.

As talin binding is required for vinculin activation *in vitro*²⁶ and at FAs (Fig. 4a–c), we next examined whether talin binding was required for vinculin localization to the FTL, or whether activation was sufficient to localize vinculin to the FTL independently of talin binding. We introduced the A50I mutation into CA-vinculin-N-tEos (A50I-CA-vinculin-N-tEos) and expressed this construct in WT cells (Fig. 4j). iPALM analysis showed that compared with

CA-vinculin-N-tEos (Fig. 4i), A50I-CA-vinculin-N-tEos localized significantly lower in FAs, with significantly decreased localization to the FTL and ARL and increased localization to the ISL (Fig. 4k–m). Thus, reducing the head–tail affinity is not sufficient to rescue the localization defect of A50I-vinculin-N-tEos. Together, these FRET and iPALM results show that talin binding is required for activation of vinculin in FAs and promotes an upward shift in localization of active vinculin from the ISL to the FTL and ARL.

Paxillin is not required for vinculin activation but promotes vinculin localization to the ISL

As paxillin binding is critical to vinculin's function in force transmission and mechanosensation¹⁵, we investigated the role of paxillin in vinculin activation and nanoscale localization in FAs. We used siRNA to knock down >95% of endogenous paxillin and ~50% of the paxillin homologue Hic5 (paxillin KD, Supplementary Fig. 5a), expressed WT-vinculin-FRET, and performed FRET analysis in control versus paxillin KD cells (Fig. 5a–c). This showed that loss of paxillin had no effect on either vinculin targeting to FAs (ref. 38) or FRET ratios, with WT-vinculin-FRET exhibiting significantly

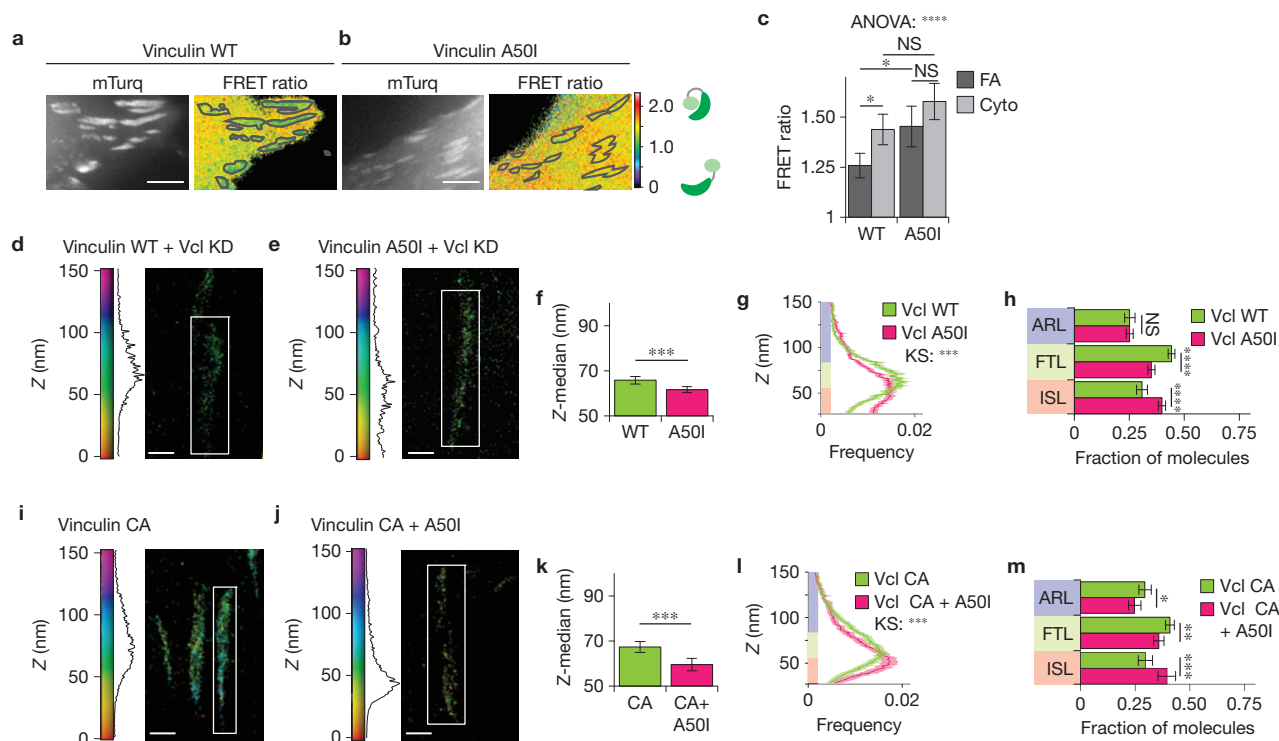


Figure 4 Talin binding is required for vinculin activation in FAs and promotes localization of active vinculin to the force transduction and actin regulatory layers. (a,b) mTurquoise (left) and processed FRET ratio image (right) of HFFs expressing either wild-type (WT) or A50I-vinculin FRET biosensor. In a,b the mask (grey lines) was created from the mTurq image and superimposed onto the FRET ratio image; scale bars, 5 μm. (c) Quantification of the mean FRET ratio value inside FAs (FA) and outside FAs (Cyto) from $n=18$ WT and $n=15$ A50I cells. (* difference is significant at $P<0.05$ cutoff, determined by Tukey test *post hoc* analysis). (d–m) iPALM images and analysis of vinculin-WT, vinculin-A50I, vinculin-CA and vinculin-CA-A50I in HFFs. (d,e,i,j) Representative iPALM renderings from HFFs treated with siRNAs targeting vinculin (KD) and additionally expressing vinculin-N-tdEos (d); or A50I-vinculin-tdEos (e); and WT HFFs expressing constitutively active (CA, N773/E775A) CA-vinculin-tdEos (i, data duplicated from Fig. 3 for comparison purpose); or CA-A50I-vinculin-tdEos (j). In d,e,i,j the colour

scale represents Z-position (nm), FAs oriented with the distal tip facing up, scale bars, 1 μm. Histograms of the Z-position of molecules within individual FAs (white rectangles in d,e,i,j) shown next to colour scale. (f,k) Mean of Z-median measurements from individual FAs. (g,l) Averaged Z-position frequency histograms of molecules within FAs. Solid line, mean frequency; shaded region, bootstrapped 95% confidence intervals. Significance tested with two-sample Kolmogorov–Smirnov (KS) test. (h,m) Mean fraction of molecules localized to the three FA layers. Colouring in g,h,i,m used to highlight the FA layers as in Fig. 1. Graphs in f–h represent measurements of $n=133$ FAs from 7 WT-vinculin-N-tdEos and $n=102$ FAs from 7 A50I-Vinculin-tdEos cells. Graphs in k–m represent measurements of $n=82$ FAs from 8 CA-vinculin-N-tdEos and $n=129$ FAs from 6 CA-A50I-vinculin-tdEos cells. Data in all bar graphs are represented as mean $\pm$ 95% bootstrapped confidence intervals with significance tested by one-way ANOVA. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$, NS: not significant.

lower FRET in FA than the cytosol in both controls and paxillin KD (Fig. 5c). We conclude that vinculin activation at FA does not require paxillin.

The above results show that paxillin has no effect on vinculin activation in FAs, and that a talin-binding mutant of vinculin localizes to FAs in an inactive conformation, begging the question of whether paxillin may recruit inactive vinculin to FAs. To address this, we assayed the ability of talin and paxillin to co-immunoprecipitate with GFP-tagged vinculin mutants (Fig. 5d and Supplementary Fig. 5b,c). We knocked down endogenous vinculin and rescued with WT-vinculin–GFP, CA-vinculin–GFP, A50I-vinculin–GFP or the double mutant A50I-CA-vinculin–GFP. Probing the immunoprecipitates for talin or paxillin showed that both proteins co-immunoprecipitated with WT and CA-vinculin, suggesting that the N773/E775A mutation does not strongly enhance binding. In contrast, whereas A50I-vinculin–GFP or A50I-CA-vinculin–GFP did not co-immunoprecipitate talin as expected¹³, paxillin was able to co-immunoprecipitate with both of these constructs (Fig. 5d). Consistent

with our observation that paxillin is not required for vinculin activation at FAs, these results show that paxillin is able to bind vinculin independently of talin binding, and support the notion that paxillin binding could target inactive vinculin to FAs.

To determine the role of paxillin in vinculin nanoscale organization, we performed iPALM analysis of WT-vinculin-N-tdEos in the presence and absence of paxillin (Fig. 5e,f). We found that compared with control, paxillin KD resulted in WT-vinculin-N-tdEos localizing significantly higher in the FAs with a significantly altered Z-distribution and decreased localization of molecules in the ISL (Fig. 5g–i), suggesting that paxillin is required to efficiently target vinculin to the ISL.

Binding to phospho-paxillin promotes vinculin localization to the ISL

We next sought to examine the role of paxillin binding in vinculin nanoscale localization in FAs. The vinculin–paxillin interaction requires paxillin phosphorylation on Tyr 31 and

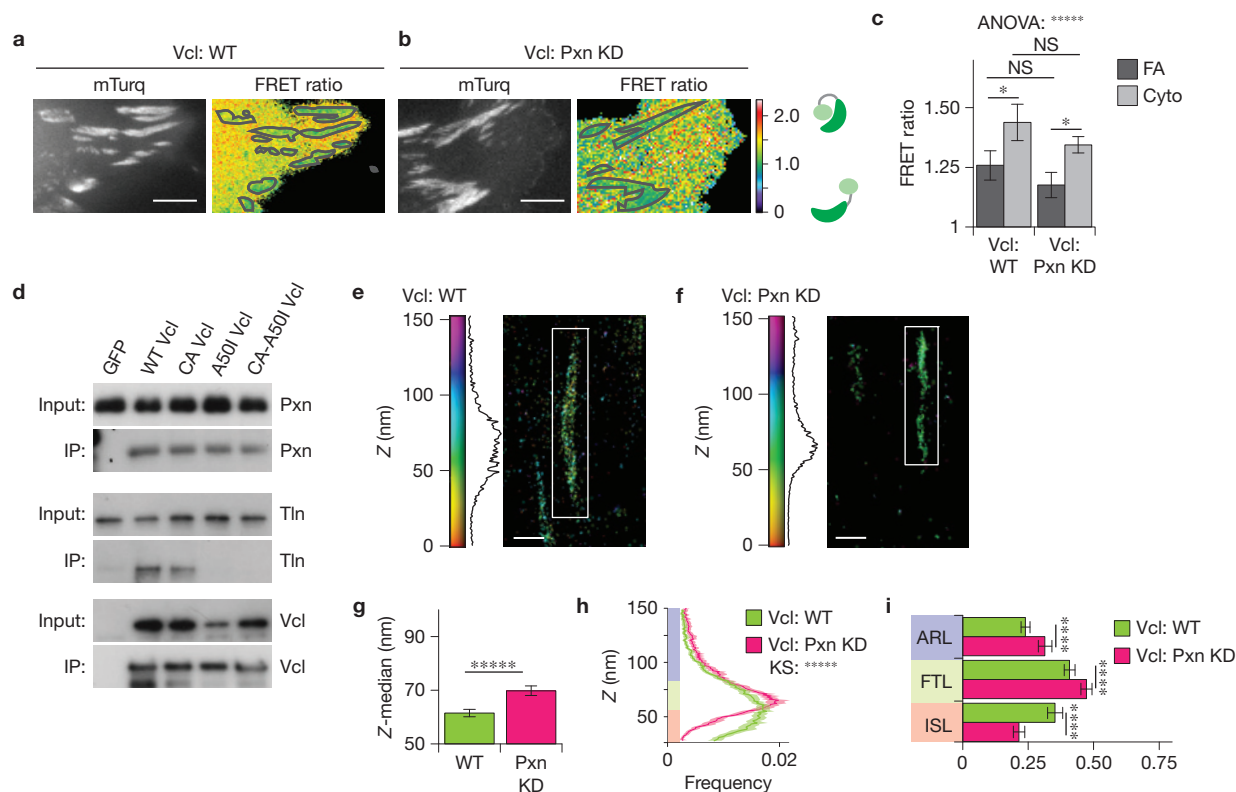


Figure 5 Paxillin is not required for vinculin activation but promotes vinculin localization to the ISL. (a,b) mTurquoise (left) and processed FRET ratio image (right) of WT or paxillin KD HFFs additionally expressing a vinculin (Vcl) FRET biosensor. In a,b the mask (grey lines) was created from the mTurq image and superimposed onto the FRET ratio image; scale bars, 5 μ m. WT-vinculin FRET data duplicated from Fig. 4 for comparison purposes. (c) Quantification of mean FRET ratio value inside FAs (FA) and outside FAs (Cyto) from $n=18$ WT and $n=16$ Pxn KD cells. (* difference is significant at $P<0.05$ cutoff, determined by Tukey test *post hoc* analysis). (d) Western blots of immunoprecipitations (IP) with anti-GFP antibodies of GFP-tagged vinculins. Input lysates and IP fractions blotted for paxillin (Pxn), talin (Tln) and vinculin (Vcl). See also Supplementary Fig. 5. (e,f) Representative iPALM rendering from HFFs expressing vinculin-N-tEos in WT (e, data duplicated from Fig. 1 for comparison purposes) or paxillin KD backgrounds (f). In e,f the colour

scale represents Z-position (nm), FAs oriented with the distal tip facing up, scale bars, 1 μ m. Histograms of the Z-position of molecules within individual FAs (white rectangles in e,f) shown next to the colour scale. (g) Mean of Z-median measurements from individual FAs. (h) Averaged Z-position frequency histograms of molecules within FAs. Solid line, mean frequency; shaded region, bootstrapped 95% confidence intervals. Significance tested with two-sample Kolmogorov–Smirnov (KS) test. (i) Mean fraction of molecules localized to the three FA layers. Colouring in h,i used to highlight the FA layers as in Fig. 1. Graphs in g–i represent measurements of $n=115$ FAs from 8 WT-vinculin-N-tEos and $n=109$ FAs from 6 Paxillin-Hic5 siRNA cells. Data in all bar graphs are represented as mean $\pm$ 95% bootstrapped confidence intervals with significance tested by one-way ANOVA. * $P<0.05$, **** $P<0.0001$, ***** $P<0.00001$, NS: not significant. Unprocessed original scans of blots are shown in Supplementary Fig. 5.

Tyr 118 by FAK in response to myosin contractility³⁸. Therefore, phosphomimetic paxillin (Y31/118E-paxillin) binds to vinculin, but non-phosphorylatable paxillin (Y31/118F-paxillin) does not³⁸. Additionally, E151Q-paxillin is a point mutant specifically defective in vinculin binding^{15,39}. All three paxillin–GFP mutants co-localize with WT paxillin at FAs and have no effect on FA size¹⁵. To confirm that neither the vinculin–paxillin interaction nor these mutations affected paxillin nanoscale localization within FAs, we introduced the mutations into paxillin–tdEos constructs and performed iPALM analysis (Fig. 6a–d). This showed that all three paxillin–tdEos constructs exhibited Z-positions and Z-distributions that were not significantly different from WT-paxillin–tdEos (Fig. 6e–k). Therefore, vinculin binding and paxillin phospho-regulation are not required to target paxillin to the ISL within FAs.

To determine whether vinculin targeting to the ISL required the vinculin–paxillin interaction, we introduced Y31/118E, Y31/118F and

E151Q mutations into paxillin–mCerulean (WT-paxillin–mCer) for co-expression with WT-vinculin-N-tEos (Fig. 6l–o). Co-expression of WT-paxillin–mCer with WT-vinculin-N-tEos in either WT or paxillin KD HFFs resulted in similar vinculin Z-position measurements (Supplementary Fig. 5d–h). When Y31/118E-paxillin–mCer was co-expressed with vinculin-N-tEos in paxillin KD cells to promote the vinculin–paxillin interaction, vinculin localized significantly lower in the FAs with significantly more molecules in the ISL compared with vinculin-N-tEos when it was co-expressed with WT-paxillin–mCer (Fig. 6p–r). In contrast, vinculin-N-tEos localized significantly higher in FAs and had less molecules in the ISL when paxillin KD was rescued with either Y31/118F-paxillin–mCer or E151Q-paxillin–mCer to disrupt the paxillin–vinculin interaction (Fig. 6p,s–v). We conclude that paxillin binding mediated by Tyr 31 and Tyr 118 phosphorylation promotes efficient targeting of vinculin to the ISL. Together, these results show that

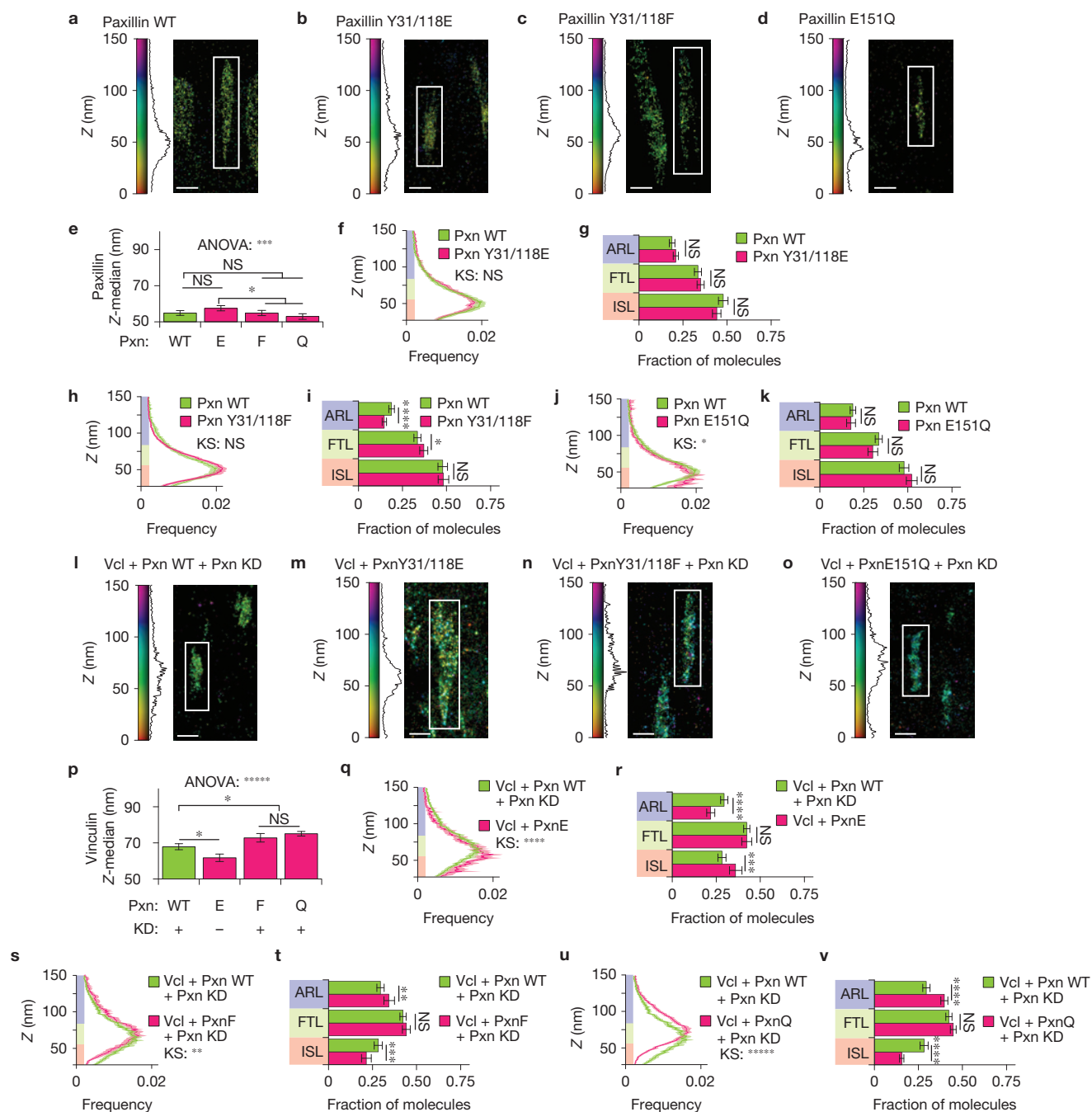


Figure 6 Binding to phospho-paxillin promotes vinculin localization to the ISL. (**a–v**) iPALM images and analysis of HFFs expressing paxillin–tdEos constructs. (**l–v**) iPALM images and analysis of HFFs expressing WT–vinculin–tdEos and paxillin–mCerulean constructs. (**a–d, l–o**) Representative iPALM renderings from HFFs expressing either wild-type (WT) paxillin–tdEos (**a**, Pxn, duplicated from Fig. 2 for comparison purposes), paxillin Y31/118E–tdEos (**b**), paxillin Y31/118F–tdEos (**c**), paxillin E151Q–tdEos (**d**), WT–vinculin–tdEos (Vcl) and WT–paxillin–mCerulean in a paxillin–Hic5 siRNA (paxillin KD) background (**l**); WT–vinculin–tdEos and paxillin Y31/118E–mCerulean (**m**), WT–vinculin–tdEos and paxillin Y31/118F–mCerulean in a paxillin KD background (**n**) or WT–vinculin–tdEos and paxillin E151Q–mCerulean in a paxillin KD background (**o**). In **a–d, l–o** the colour scale represents Z-position (nm), FAs oriented with the distal tip facing up, scale bars, 1 μ m. Histograms of the Z-position of molecules within individual FAs (white rectangles in **a–d**) shown next to the colour scale. (**e, p**) Mean of Z-median measurements from individual FAs (* difference is significant at $P < 0.05$, determined by

post hoc Tukey test). (**f, h, j, q, s, u**) Averaged Z-position frequency histograms of molecules within FAs. Solid line, mean frequency; shaded region, bootstrapped 95% confidence intervals. Significance tested with two-sample Kolmogorov–Smirnov (KS) test. (**g, i, k, r, t, v**) Mean fraction of molecules localized to the three FA layers. Colouring in **f–k** and **q–v** used to highlight the FA layers as in Fig. 1. Graphs in **e–k** represent measurements of $n = 120$ FAs from 7 WT–paxillin–tdEos-expressing cells, $n = 150$ FAs from 6 paxillin Y31/118E-expressing cells, $n = 152$ FAs from 6 paxillin Y31/118F-expressing cells, and $n = 79$ FAs from 8 paxillin E151Q–tdEos-expressing cells. Graphs in **p–v** represent measurements of $n = 110$ FAs from 5 WT–paxillin–mCerulean-expressing cells, $n = 92$ FAs from 8 paxillin Y31/118E–mCerulean-expressing cells, $n = 117$ FAs from 6 paxillin Y31/118F–mCerulean-expressing cells, and $n = 175$ FAs from 6 paxillin E151Q–mCerulean-expressing cells. Data in all bar graphs are represented as mean $\pm$ 95% bootstrapped confidence intervals with significance tested by one-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ***** $P < 0.00001$, NS: not significant.

the vinculin–phospho-paxillin interaction promotes association of vinculin with the ISL independent of vinculin activation.

Actin binding does not regulate vinculin activation or nanoscale localization in FA

As actin binding is critical for vinculin's function in regulating actin and adhesion dynamics¹⁶, we examined the role of actin in regulating vinculin activation and nanoscale localization at FAs. Two independent amino-acid point mutations (I997A and V1001A) have been shown to reduce actin binding to the vinculin tail without perturbing vinculin auto-inhibition, and result in decreased mechanical response to force in cells^{16,40}. To determine the role of actin binding in regulating vinculin activation, we introduced these mutations into the vinculin-FRET biosensor (I997A-vinculin-FRET and V1001A-vinculin-FRET, Fig. 7a,b). Analysis of FRET images showed that neither I997A-vinculin-FRET nor V1001A-vinculin-FRET had significantly different FRET values in FAs or the cytoplasm compared to WT-vinculin-FRET (Fig. 7b). Thus, actin binding is not required for vinculin activation at FAs.

To determine the effect of actin binding on vinculin nanoscale localization within FAs, we rescued vinculin-KD with WT-vinculin-N–tdEos, I997A-vinculin-N–tdEos or V1001A-vinculin-N–tdEos (Fig. 7c–e) and performed iPALM analysis. Surprisingly, we found no significant difference in Z-position or Z-distribution between WT-vinculin and the actin-binding mutants (Fig. 7f–j). Thus, although actin binding is important for regulating actin dynamics and force transduction^{16,40}, it is not required for vinculin activation or nanoscale organization at FAs.

Vinculin exhibits a gradient in activation and axial position across single FAs

Individual FAs vary in biochemical composition and distribution of traction forces across their length. Phospho-paxillin⁷ and high ECM traction forces^{15,41} are concentrated at the distal tip of FAs near the leading edge, whereas actin-associated proteins^{4,8,9} and lower forces^{15,41} are located at the proximal FA tip facing the cell centre. Therefore, we sought to determine whether vinculin activation or axial position varied along the length of individual FAs. To determine whether vinculin activation spatially varied within FAs, we measured the mean FRET ratio in the distal and proximal thirds of FAs (Fig. 8a–c). Neither the FA-targeted control probe nor CA-vinculin-FRET exhibited significant differences in FRET ratio between the distal and proximal thirds of FAs (Supplementary Fig. 6a–f). In contrast, WT-vinculin-FRET exhibited significantly higher FRET ratio values in the distal third of FAs compared with the proximal third, although both FA regions had lower FRET ratio values than the cytoplasm (compare Figs 4c and 8c). This suggests that active vinculin is concentrated in the proximal FA tip.

To determine whether vinculin nanoscale localization varied along the length of FAs, we quantified the axial position of WT-vinculin-N–tdEos in the distal and proximal thirds of FAs. iPALM analysis showed that vinculin localized significantly higher and had a significantly increased ARL localization in the proximal FA third compared with the distal FA third (Fig. 8d–g). Together these results show that vinculin exhibits gradients of low-to-high activation and axial position from the distal tip to proximal tip of single FAs.

As our results indicate that vinculin activation and nanoscale localization are regulated by interactions with talin, we hypothesized that the talin binding could mediate the observed gradient in vinculin activation and axial position across single FAs. Previous studies showed that talin and vinculin preferentially interact in the proximal tip of FAs (ref. 42). To determine whether talin vertical extension could mediate the localization of vinculin to the FTL and ARL in the proximal FA tip, we compared the axial position of talin-C–tdEos in the distal and proximal thirds of FAs (Fig. 8h–k). This showed that the talin C terminus was significantly higher in the proximal than in the distal FA tip (Fig. 8i), indicating that maximum talin vertical extension spatially corresponds with higher vinculin axial position within individual FAs.

Vinculin is recruited initially to the ISL and later to the FTL and ARL during myosin II-mediated FA maturation

Previous reports showed that vinculin activation increases during FA dynamics and growth³⁶, which together with our current results suggests that vinculin nanoscale localization and binding partners may evolve during FA maturation. To obtain a synchronized population of FAs at different stages of maturation, we performed washouts of the myosin II ATPase inhibitor, blebbistatin, and performed iPALM analysis of vinculin axial position during contractility-induced FA growth (Fig. 8l–q). We first characterized the kinetics of contractility-induced FA maturation in cells co-expressing paxillin–mCherry and vinculin–GFP. Blebbistatin treatment (50 μ M, 2 h) inhibited FA maturation and reduced vinculin localization at FAs (refs 38,43). Replacement of blebbistatin-containing with drug-free media during live-cell total internal reflection fluorescence (TIRF) microscopy revealed that immature, diffraction-limited FAs at the cell edge underwent coordinated growth, with vinculin recruited to maturing FAs as early as 1 min after washout (Fig. 8l). By 5 min post-washout, FAs were elongated and the cell edge had advanced, and by 7 min, a new row of nascent FAs formed. Thus, we analysed vinculin at 1 and 5 min after blebbistatin washout to ensure that all FAs had begun maturation simultaneously.

To determine the nanoscale localization of vinculin during FA maturation, we expressed WT-vinculin-N–tdEos in cells, treated them with blebbistatin, and fixed at 1 min or 5 min after blebbistatin washout (Fig. 8m,n). iPALM analysis showed that at 1 min after washout, vinculin localized $\sim$ 57 nm above the coverslip, with half of the molecules localized to the ISL (Fig. 8o–q), but by 5 min after induction of FA maturation, vinculin was localized $\sim$ 20 nm higher with most molecules in the FTL and ARL (Fig. 8p,q). Thus, vinculin localizes to the ISL at the onset of myosin II activity, and vinculin distribution shifts upward as FAs undergo contractility-induced maturation.

To determine whether paxillin phosphorylation could be responsible for recruitment of vinculin to the ISL in nascent FAs, we characterized the dynamics of paxillin phosphorylation on Tyr 118 (pY118-paxillin) during myosin II-induced FA maturation (Fig. 8r). Western blot analysis showed that, as reported previously³⁸, blebbistatin treatment significantly reduced pY118-paxillin. One minute after blebbistatin washout, pY118-paxillin increased more than twofold with no additional increase at 5 min post-washout. Therefore, consistent with earlier findings⁴⁴, contractility-dependent phosphorylation of paxillin

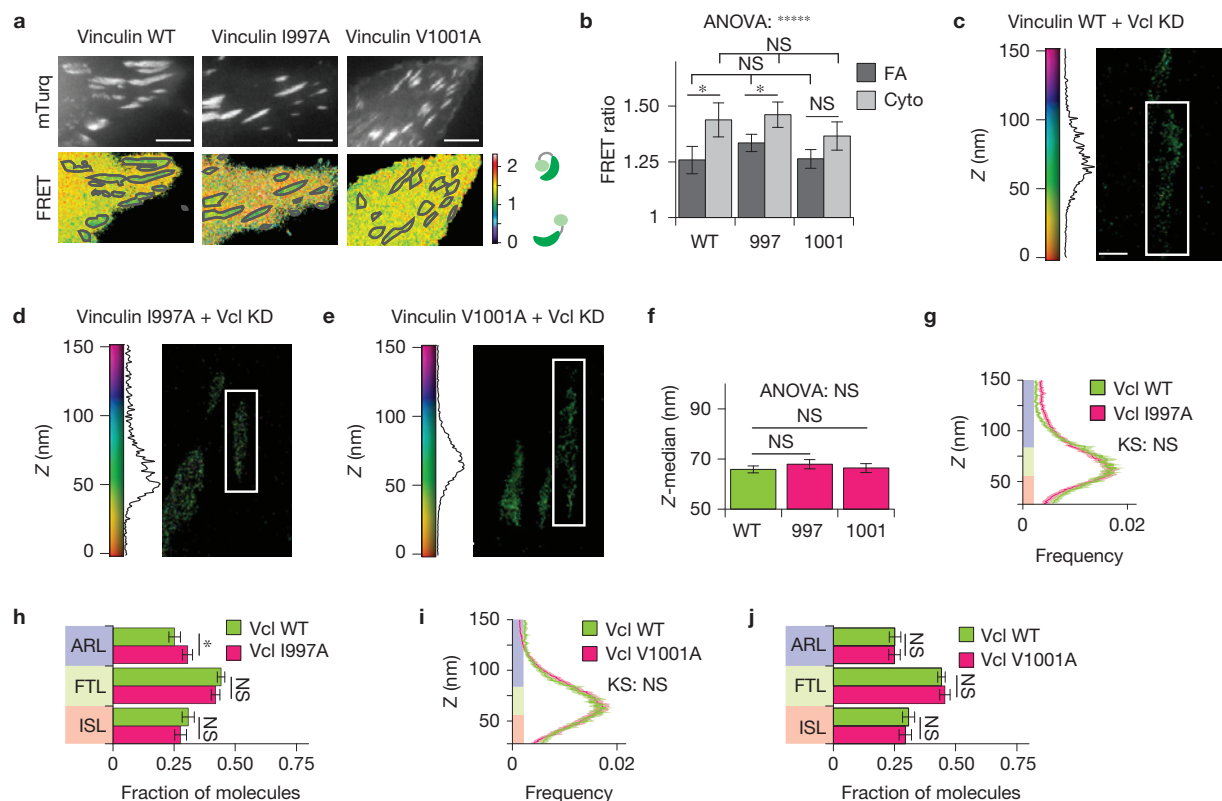


Figure 7 Actin binding does not regulate vinculin activation or nanoscale localization. (a) mTurquoise (top) and processed FRET ratio (bottom) images of wild-type (WT) HFFs expressing WT or actin-binding mutant (I997A and V1001A) vinculin FRET biosensor. Scale bar, 5 μ m. The mask (grey lines) was created from the mTurquoise image and superimposed onto the FRET ratio image. WT data are duplicated from Fig. 4 for comparison purposes. (b) Quantification of the mean FRET ratio value inside FAs (FA) and outside FAs (Cyto) from $n = 18$ WT, $n = 21$ I997A, and $n = 16$ V1001A cells. (* difference is significant at $P < 0.05$, determined by *post hoc* Tukey test). (c–e) Representative iPALM renderings from HFFs expressing vinculin-N-tdEos (c), I997A-vinculin-tdEos (d), or V1001A-vinculin-tdEos (e) in a vinculin siRNA (vinculin KD) background. In c–e the colour scale represents Z-position (nm), FAs oriented with the distal tip facing up, scale bars, 1 μ m. Histograms of the Z-position of molecules within individual FAs

(white rectangles in c–e) shown next to the colour scale. WT vinculin data duplicated from Fig. 4 for comparison purposes. (f) Mean of Z-median measurements from individual FAs. (g–j) Quantitative iPALM analysis of Vcl KD HFFs expressing WT-vinculin-tdEos, I997A-vinculin-tdEos or V1001A-vinculin-tdEos. (g,i) Averaged Z-position frequency histograms of molecules within FAs. Solid line, mean frequency; shaded region, bootstrapped 95% confidence intervals. Significance tested with two-sample Kolmogorov–Smirnov (KS) test. (h,j) Mean fraction of molecules localized to the three FA layers. Colouring in f–k and q–v used to highlight the FA layers as in Fig. 1. Graphs in f–j represent measurements of $n = 133$ FAs from 7 WT-vinculin-N-tdEos, $n = 155$ FAs from 6 I997A-vinculin-tdEos cells, and $n = 182$ FAs from 8 V1001A-vinculin-tdEos cells. Data in all bar graphs are represented as mean $\pm$ 95% bootstrapped confidence intervals with significance tested by one-way ANOVA. * $P < 0.05$, **** $P < 0.00001$, NS: not significant.

Tyr 118 occurs within the first minute of FA maturation, mirroring the kinetics of vinculin localization to the ISL during contractility-induced FA maturation. Together, our findings demonstrate that vinculin nanoscale localization evolves during myosin II-dependent FA maturation and suggest that vinculin localizes first to the ISL by interaction with phospho-paxillin in nascent FAs. As FAs grow, vinculin molecules exhibit increased localization to the FTL and ARL, probably by talin-mediated activation.

DISCUSSION

This study provides the first demonstration of protein activity and function regulated by the nanoscale molecular architecture of FAs. Although vinculin has many characterized molecular interactions that regulate distinct FA functions, it was previously unknown how vinculin's protein interactions are spatiotemporally regulated within FAs during maturation. Using super-resolution microscopy and a FRET biosensor in combination with point mutants with well-characterized

in vitro protein binding defects and cellular phenotypes, we dissected the roles of paxillin, talin and actin in vinculin's activation, orientation and nanoscale position within FAs. Our work highlights the mind-blowing power of light microscopy for determining protein activity and interactions with nanometre precision within organelles in intact cells. Our results suggest that inactive vinculin is recruited to the membrane-proximal ISL by binding to phospho-paxillin. Talin binding promotes vinculin activation and targeting of active vinculin to the FTL where vinculin can engage actin in the ARL to modulate retrograde flow and strengthen adhesion. Thus, the spatial segregation of paxillin, talin and actin along the Z axis of the FA regulates vinculin recruitment, activation and function during FA maturation.

On the basis of our study and the work of others, we propose a model in which upwards redistribution of vinculin within the FA structure during maturation facilitates vinculin activation and mechanical reinforcement of FAs (Supplementary Fig. 7). At the leading edge, integrins are activated in the protruding lamellipodium

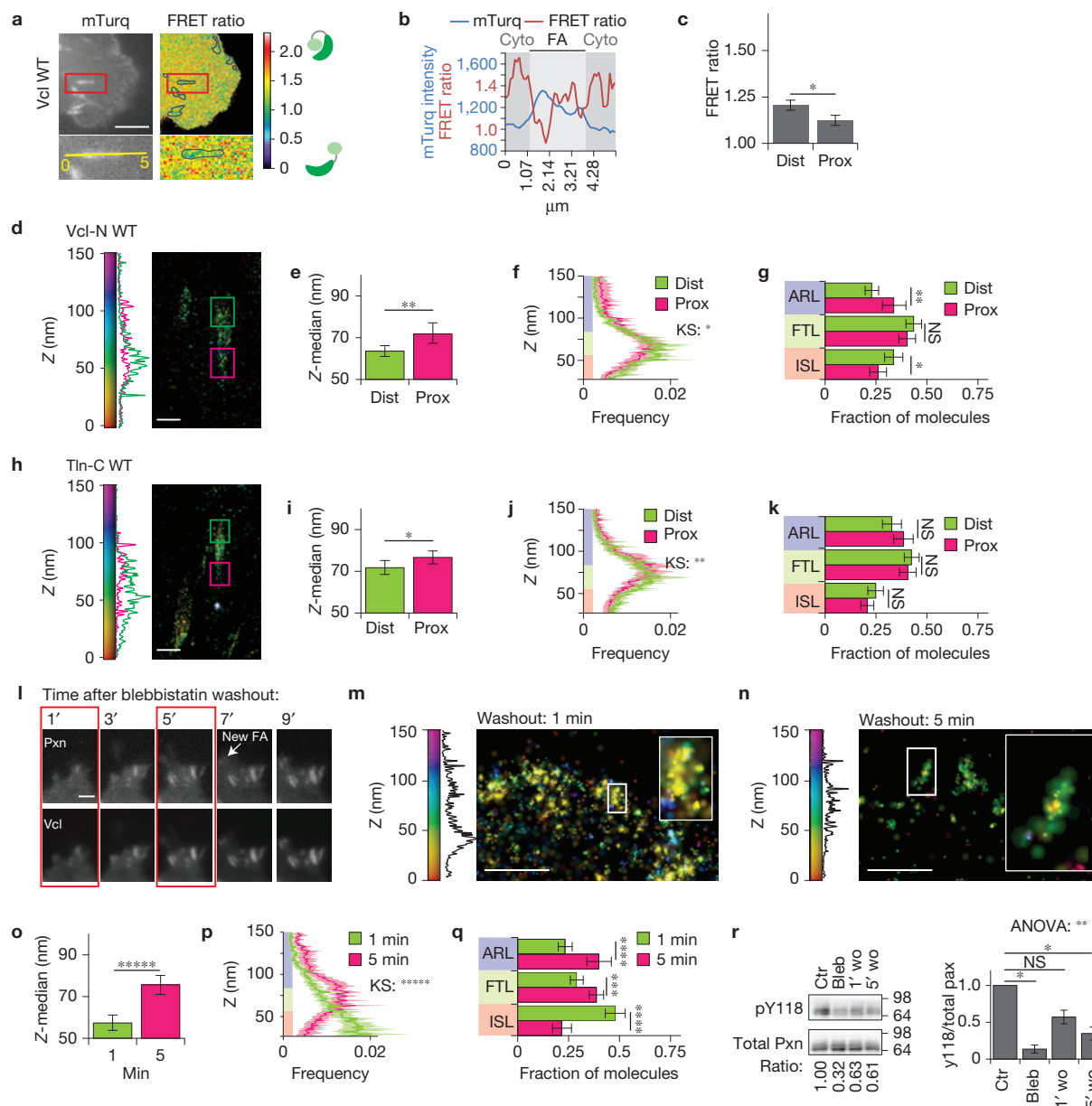


Figure 8 Vinculin activation and nanoscale localization are spatiotemporally regulated. **(a)** Top: mTurquoise (mTurq) and processed mTurquoise/NeonGreen FRET ratio images of an HFF cell expressing the vinculin FRET biosensor. Bottom: Zoom of outlined area. FA mask (grey lines) created from the mTurq image and superimposed onto FRET ratio image. **(b)** mTurquoise intensity and FRET ratio along the 5 μ m long line in the zoom in **a**. Dark shaded areas are cytosolic (Cyto) regions adjacent to the FA (light shaded area). **(c)** Mean FRET ratio quantification of the distal (Dist, facing cell edge) 1/3 and proximal (Prox, facing cell centre) 1/3 of FA line scans. **(d-k)** iPALM images and analysis of HFFs expressing WT-vinculin-N-tdEos or talin-C-tdEos. **(d,h)** Representative iPALM renderings of HFFs expressing vinculin-N-tdEos (**d**) or talin-C-tdEos (**h**). Histograms of the Z-position of molecules within Dist and Prox thirds shown next to the colour scale. **(e,i)** Mean of Z-median measurements from Dist or Prox thirds. **(f,j)** Averaged Z-position frequency histograms of molecules. Solid line, mean frequency; shaded region, bootstrapped 95% confidence intervals. **(g,k)** Mean fraction of molecules localized to the three FA layers. **(l)** Time-lapse TIRF micrographs of FAs during blebbistatin washout. HFFs expressing paxillin-mCherry (top) and vinculin-EGFP (bottom) treated with 50 μ M blebbistatin for 2 h. Blebbistatin washout at $t=0$ min, arrow: newly formed FA. Outlined areas: time points for iPALM analysis. Scale bars, 2 μ m. **(m,n)** Representative iPALM renderings

of vinculin-N-tdEos at 1 min (**m**) and 5 min (**n**) after blebbistatin washout. Histograms of Z-positions of molecules within individual FAs (rectangles in **m-n**) shown next to the colour scale. **(o)** Mean Z-median measurements from individual FAs. **(p)** Averaged Z-position histograms of molecules within FAs. Solid line, mean frequency; shaded region, bootstrapped 95% confidence intervals. **(q)** Mean fraction of molecules localized to the three FA layers. **(r)** Left: Representative immunoblots of HFFs after 2 h of DMSO (Ctr) or 50 μ M blebbistatin treatment (Bleb), or 1 min or 5 min after blebbistatin washout (1' wo, 5' wo) probed with antibodies against total or pY118-paxillin. Ratio: ratio of pY118/total paxillin, normalized to control. Right: Quantification of ratios. Graphs in **c** represent measurements of $n=17$ FAs from 6 cells; **e-g**, $n=40$ FAs from 10 cells; **i-k**, $n=33$ FAs from 5 cells; **o-q**, $n=61$ FAs from 6 cells and $n=52$ FAs from 7 cells fixed for 1 and 5 min after blebbistatin washout respectively; **r**, $n=3$ experiments. Colouring in **f,g,j,k,p,q** highlights FA layers as in Fig. 1. Data in **e,g,i,k,o,q** are mean $\pm$ 95% bootstrapped confidence interval, in **c,r** mean $\pm$ s.e.m., significance tested by one-way ANOVA. Significance tested with two-sample Kolmogorov-Smirnov (KS) test in **f,j,p** and with Tukey's *post hoc* test in **r**. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ***** $P < 0.00001$, NS: not significant). Unprocessed original scans of blots are shown in Supplementary Fig. 6.

by talin-mediated⁴⁵ engagement of actin retrograde flow^{46,47} to form nascent FAs and bind the ECM. FAK and paxillin are quickly recruited⁵, and tension on engaged integrins⁴⁸ promotes FAK activation³⁸ and rapid tyrosine phosphorylation of paxillin^{7,44}. Paxillin phosphorylation in nascent FA mediates the recruitment of inactive vinculin to the ISL (refs 7,38), although other pathways for recruitment are possible. Interaction with phospho-paxillin puts vinculin into proximity with its activating ligands, including PIP2 in the membrane²¹, talin³², and actin²⁶, where it acts as a coincidence detector^{26,31}. As tension builds, talin stretches to unmask vinculin binding sites³⁵, allowing talin and a second ligand to mediate vinculin activation and promote vinculin association with the upper FA layers where it binds actin to engage retrograde flow¹⁶ and bolster the force-transmitting link between talin, integrin and the ECM (refs 11,16). Vinculin binding stabilizes talin in a vertically extended conformation, allowing for further extension of the talin rod, exposing additional cryptic vinculin binding sites⁴⁹ and further recruitment of active vinculin, allowing for positive feedback to mechanically reinforce FAs. In mature FAs, inactive vinculin remains associated with phospho-paxillin in the ISL in the distal FA tip, and active vinculin is distributed in the FTL and ARL in the proximal FA tip.

Our results suggest that talin is a master regulator of vinculin nanoscale localization and function. We show that talin binding is required for activation and localization to the FTL and ARL *in vivo*, but surprisingly, actin binding is not. Functionally, talin binding is critical for vinculin's ability to strengthen FAs and promote FA growth^{10,11,13}, suggesting that vinculin performs these jobs in the upper layers of the FAs. Talin's capacity to regulate vinculin's association with the ARL also suggests that it controls vinculin's ability to engage retrograde actin flow and mechanically reinforce FAs (ref. 16). Furthermore, although *in vitro* experiments have shown that vinculin binding prevents refolding of stretched talin^{34,49}, our results provide the first *in vivo* evidence that vinculin binding is required to maintain talin in a vertically extended conformation.

Our data suggest that vinculin dynamics are spatiotemporally regulated by distinct protein interactions to mediate ECM mechanosensing. As the vinculin–phospho-paxillin interaction promotes traction force oscillations in FAs that facilitate mechanosensing and durotaxis¹⁵ the dynamics of vinculin recruitment and nanoscale localization mediated by this interaction are also probably critical for mechanosensing. Fluorescence recovery after photobleaching studies suggest that the vinculin phospho-paxillin interaction is very labile, whereas activated vinculin is more stably associated with FAs (refs 33,38). Paxillin and vinculin have also been observed to interact in both the FA and the cytoplasm⁵⁰, which could allow rapid loading of preassembled complexes into the FA from the cytoplasm. Thus, dynamic traction oscillations in FAs could be mediated by rapid activation and redistribution of the labile, paxillin-bound pool of inactive vinculin to the upper FA layers where it reinforces FA traction.

In conclusion, the distinct vertical distributions of paxillin, talin and actin allow for the segregation of vinculin–protein interactions into nanodomains that spatiotemporally regulate vinculin activation and function during FA maturation. We suggest that spatial segregation of different protein–protein interactions into molecularly distinct nanodomains within organelles could be a broader mechanism for regulating protein activity in cells. □

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

L.B.C. and C.M.W. conceived the study and wrote the manuscript with input from all authors. L.B.C., C.M.W. and S.L.C. designed experiments. L.B.C. performed and analysed most experiments. L.B.C. and G.S. performed iPALM imaging. M.A.B. and M.W.D. designed new cDNA constructs and performed cloning. G.S. and H.F.H. conceived of, built and maintained iPALM instrumentation and developed iPALM processing tools. L.B.C., M.A.B., M.W.D. and C.M.W. designed the summary cartoon.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Kuo, J.-C. C., Han, X., Hsiao, C.-T. T., Yates III, J. R. & Waterman, C. M. Analysis of the myosin-II-responsive focal adhesion proteome reveals a role for β -Pix in negative regulation of focal adhesion maturation. *Nat. Cell Biol.* **13**, 383–393 (2011).
2. Byron, A., Humphries, J. D., Bass, M. D., Knight, D. & Humphries, M. J. Proteomic analysis of integrin adhesion complexes. *Sci. Signal.* **4**, pt2 (2011).
3. Schiller, H. B., Friedel, C. C., Boulegue, C. & Fässler, R. Quantitative proteomics of the integrin adhesome show a myosin II-dependent recruitment of LIM domain proteins. *EMBO Rep.* **12**, 259–266 (2011).
4. Kanchanawong, P. *et al.* Nanoscale architecture of integrin-based cell adhesions. *Nature* **468**, 580–584 (2010).
5. Choi, C. K. *et al.* Actin and α -actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. *Nat. Cell Biol.* **10**, 1039–1050 (2008).
6. Zaidel-Bar, R., Ballestrem, C., Kam, Z. & Geiger, B. Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. *J. Cell Sci.* **116**, 4605–4613 (2003).
7. Zaidel-Bar, R., Milo, R., Kam, Z. & Geiger, B. A paxillin tyrosine phosphorylation switch regulates the assembly and form of cell-matrix adhesions. *J. Cell Sci.* **120**, 137–148 (2007).
8. Zamir, E. *et al.* Molecular diversity of cell-matrix adhesions. *J. Cell Sci.* **112**, 1655–1669 (1999).
9. Wolfenson, H. *et al.* A role for the juxtamembrane cytoplasm in the molecular dynamics of focal adhesions. *PLoS ONE* **4**, e4304 (2009).
10. Diez, G., Auernheimer, V., Fabry, B. & Goldmann, W. H. Head/tail interaction of vinculin influences cell mechanical behavior. *Biochem. Biophys. Res. Commun.* **406**, 85–88 (2011).
11. Dumbauld, D. W. *et al.* How vinculin regulates force transmission. *Proc. Natl Acad. Sci. USA* **110**, 9788–9793 (2013).
12. Saunders, R. M. *et al.* Role of vinculin in regulating focal adhesion turnover. *Eur. J. Cell Biol.* **85**, 487–500 (2006).
13. Humphries, J. D. *et al.* Vinculin controls focal adhesion formation by direct interactions with talin and actin. *J. Cell Biol.* **179**, 1043–1057 (2007).
14. Carisey, A. *et al.* Vinculin regulates the recruitment and release of core focal adhesion proteins in a force-dependent manner. *Curr. Biol.* **23**, 271–281 (2013).
15. Plotnikov, S. V., Pasapera, A. M., Sabass, B. & Waterman, C. M. Force fluctuations within focal adhesions mediate ECM-rigidity sensing to guide directed cell migration. *Cell* **151**, 1513–1527 (2012).
16. Thievsen, I. *et al.* Vinculin-actin interaction couples actin retrograde flow to focal adhesions, but is dispensable for focal adhesion growth. *J. Cell Biol.* **202**, 163–177 (2013).
17. Subauste, M. C. *et al.* Vinculin modulation of paxillin-FAK interactions regulates ERK to control survival and motility. *J. Cell Biol.* **165**, 371–381 (2004).
18. Burridge, K. & Mangeat, P. An interaction between vinculin and talin. *Nature* **308**, 744–746 (1984).

19. Jockusch, B. M. & Isenberg, G. Interaction of α -actinin and vinculin with actin: opposite effects on filament network formation. *Proc. Natl Acad. Sci. USA* **78**, 3005–3009 (1981).
20. Turner, C. E., Glenney, J. R. & Burridge, K. Paxillin: a new vinculin-binding protein present in focal adhesions. *J. Cell Biol.* **111**, 1059–1068 (1990).
21. Gilmore, A. P. & Burridge, K. Regulation of vinculin binding to talin and actin by phosphatidylinositol-4-5-bisphosphate. *Nature* **381**, 531–535 (1996).
22. DeMali, K. A., Barlow, C. A. & Burridge, K. Recruitment of the Arp2/3 complex to vinculin: coupling membrane protrusion to matrix adhesion. *J. Cell Biol.* **159**, 881–891 (2002).
23. Kioka, N. *et al.* Vinexin: a novel vinculin-binding protein with multiple SH3 domains enhances actin cytoskeletal organization. *J. Cell Biol.* **144**, 59–69 (1999).
24. Johnson, R. P. & Craig, S. W. An intramolecular association between the head and tail domains of vinculin modulates talin binding. *J. Biol. Chem.* **269**, 12611–12619 (1994).
25. Johnson, R. P. & Craig, S. W. F-actin binding site masked by the intramolecular association of vinculin head and tail domains. *Nature* **373**, 261–264 (1995).
26. Chen, H., Choudhury, D. M. & Craig, S. W. Coincidence of actin filaments and talin is required to activate vinculin. *J. Biol. Chem.* **281**, 40389–40398 (2006).
27. Calderwood, D. A. *et al.* The talin head domain binds to integrin subunit cytoplasmic tails and regulates integrin activation. *J. Biol. Chem.* **274**, 28071–28074 (1999).
28. Goldmann, W. H. *et al.* Examining F-actin interaction with intact talin and talin head and tail fragment using static and dynamic light scattering. *Eur. J. Biochem.* **250**, 447–450 (1997).
29. Critchley, D. R. Biochemical and structural properties of the integrin-associated cytoskeletal protein talin. *Annu. Rev. Biophys.* **38**, 235–254 (2009).
30. Rubashkin, M. G. *et al.* Force engages vinculin and promotes tumor progression by enhancing PI3K activation of phosphatidylinositol (3,4,5)-triphosphate. *Cancer Res.* **74**, 4597–4611 (2014).
31. Bakoliitsa, C. *et al.* Structural basis for vinculin activation at sites of cell adhesion. *Nature* **430**, 583–586 (2004).
32. Cohen, D. M., Chen, H., Johnson, R. P., Choudhury, B. & Craig, S. W. Two distinct head-tail interfaces cooperate to suppress activation of vinculin by talin. *J. Biol. Chem.* **280**, 17109–17117 (2005).
33. Cohen, D. M., Kutscher, B., Chen, H., Murphy, D. B. & Craig, S. W. A conformational switch in vinculin drives formation and dynamics of a talin-vinculin complex at focal adhesions. *J. Biol. Chem.* **281**, 16006–16015 (2006).
34. Ciobanasu, C., Faivre, B. & Le Clairche, C. Actomyosin-dependent formation of the mechanosensitive talin-vinculin complex reinforces actin anchoring. *Nat. Commun.* **5**, 3095 (2014).
35. Del Rio, A. *et al.* Stretching single talin rod molecules activates vinculin binding. *Science* **323**, 638–641 (2009).
36. Chen, H., Cohen, D. M., Choudhury, D. M., Kioka, N. & Craig, S. W. Spatial distribution and functional significance of activated vinculin in living cells. *J. Cell Biol.* **169**, 459–470 (2005).
37. Shaner, N. C. *et al.* A bright monomeric green fluorescent protein derived from *Branchiostoma lanceolatum*. *Nat. Methods* **10**, 407–409 (2013).
38. Pasapera, A. M., Schneider, I. C., Rericha, E., Schlaepfer, D. D. & Waterman, C. M. Myosin II activity regulates vinculin recruitment to focal adhesions through FAK-mediated paxillin phosphorylation. *J. Cell Biol.* **188**, 877–890 (2010).
39. Brown, M. C., Perrotta, J. A. & Turner, C. E. Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. *J. Cell Biol.* **135**, 1109–1023 (1996).
40. Thompson, P. M. *et al.* Identification of an actin binding surface on vinculin that mediates mechanical cell and focal adhesion properties. *Structure* **22**, 697–706 (2014).
41. Gardel, M. L. *et al.* Traction stress in focal adhesions correlates biphasically with actin retrograde flow speed. *J. Cell Biol.* **183**, 999–1005 (2008).
42. Bachir, A. I. *et al.* Integrin-associated complexes form hierarchically with variable stoichiometry in nascent adhesions. *Curr. Biol.* **24**, 1845–1853 (2014).
43. Wolfenson, H., Bershadsky, A., Henis, Y. I. & Geiger, B. Actomyosin-generated tension controls the molecular kinetics of focal adhesions. *J. Cell Sci.* **124**, 1425–1432 (2011).
44. Schneider, I. C., Hays, C. K. & Waterman, C. M. Epidermal growth factor-induced contraction regulates paxillin phosphorylation to temporally separate traction generation from de-adhesion. *Mol. Biol. Cell* **20**, 3155–3167 (2009).
45. Tadokoro, S. *et al.* Talin binding to integrin β tails: a final common step in integrin activation. *Science* **302**, 103–106 (2003).
46. Astrof, N. S., Salas, A., Shimaoka, M., Chen, J. & Springer, T. A. Importance of force linkage in mechanochemistry of adhesion receptors. *Biochemistry* **45**, 15020–15028 (2006).
47. Jiang, G., Giannone, G., Critchley, D. R., Fukumoto, E. & Sheetz, M. P. Two-piconewton slip bond between fibronectin and the cytoskeleton depends on talin. *Nature* **424**, 334–337 (2003).
48. Friedland, J. C., Lee, M. H. & Boettiger, D. Mechanically activated integrin switch controls $\alpha 5 \beta 1$ function. *Science* **323**, 642–644 (2009).
49. Yao, M. *et al.* Mechanical activation of vinculin binding to talin locks talin in an unfolded conformation. *Sci. Rep.* **4**, 4610 (2014).
50. Hoffmann, J.-E., Fermin, Y., Stricker, R. L., Ickstadt, K. & Zamir, E. Symmetric exchange of multi-protein building blocks between stationary focal adhesions and the cytosol. *eLife* **3**, e02257–e02257 (2014).

METHODS

Cell culture and transfection. HFF cells were obtained from American Type Culture Collection (ATCC) and maintained at 37 °C at 5% CO₂ in DMEM supplemented with 15% FBS (Gibco). Transfections of 2 µg of plasmid DNA were performed using a Nucleofector (NHDF solution, program U-020; Lonza). For FRET experiments, cells were plated for 6–8 h before imaging on 22 × 22 mm no. 1.5 coverslips pre-coated with 15 µg ml⁻¹ fibronectin (2 h at 37 °C, Millipore). Imaging was performed in growth media without phenol red and supplemented with 20 mM HEPES and 30 units ml⁻¹ Oxyrase. For iPALM experiments, fluorescent fiducials are affixed to the coverslip to provide a constant internal reference for calibration, alignment, and spatial drift correction. Gold (Au) nanoparticles (80 to 100 nm) were sparsely adsorbed (~2,000 per mm²) to the coverglass surface and immobilized by 30–50 nm of sputtered SiO₂, as previously described⁴. Before experiments, fiducial coverslips were ultraviolet-sterilized for 15 min, rinsed with Dulbecco's phosphate-buffered saline (DPBS, Invitrogen), coated with 15 µg ml⁻¹ fibronectin (2 h at 37 °C, Millipore), and incubated with 1% heat-inactivated bovine serum albumin (1 h, 37 °C, Sigma-Aldrich) before a final rinse with DPBS. For iPALM experiments, cells were transfected for ~36 h and then replated onto fibronectin-coated coverslips for 6–8 h, allowing time for cells to spread and migrate with minimal ECM fibrillogenesis. Cells were fixed with 4% PFA in PHEM buffer (PIPES 60 mM, HEPES 25 mM, EGTA 10 mM, MgCl₂ 2 mM, pH 6.9) for 15 min. Imaging was performed in PHEM buffer supplemented with 2 mM trolox (Calbiochem) and 0.04 mg ml⁻¹ catalase (Sigma-Aldrich) to scavenge oxygen and inhibit photodamage and bleaching. We imaged focal-adhesion-containing lamella areas, typically no greater than 20 µm from the cell edge, containing at least three fiducials for calibration and drift correction.

Pharmacological treatments. Blebbistatin (50 µM) was used to inhibit myosin II ATPase activity (Toronto Research Chemicals).

Plasmids. Plasmids encoding actin-mEos2, talin-N-tEos, talin-C-tEos, paxillin-N-tEos, WT-vinculin-N-tEos, CAAX-tEos, vinculin-EGFP and paxillin-mCherry were previously described^{4,16,38}. All fluorescent protein expression vectors were generated using a C1 or N1 (Clontech-style) cloning vector backbone. To construct the fluorescent protein cloning vectors, the cDNA encoding mCerulean, tEos, mNeonGreen and mTurquoise was amplified with a 5' primer encoding an AgeI site and a 3' primer encoding a BspEI site (C1) or a NotI site (N1) for insertion into the corresponding vector backbone. The resulting PCR products were purified, digested, and ligated into similarly treated EGFP-C1 and EGFP-N1 vector backbones, yielding C1 and N1 cloning vectors for mCerulean, tEos, mNeonGreen and mTurquoise.

To generate the chicken paxillin (NM_204984.1) vector, an advanced EGFP variant mEmerald (wtGFP + F64L, S65T, S72A, N149K, M153T, I167T, A206K) was initially used to characterize the fusion. To produce a paxillin fusion with a 22-amino-acid linker separating the C terminus of paxillin from the fluorescent protein, paxillin cDNA was PCR amplified with a 5' primer encoding an EcoRI site and a 3' primer encoding a NotI site. The resulting PCR product and mEmerald-N1 were both digested by the appropriate restriction enzymes, gel purified and ligated to yield mEmerald-paxillin-22. On sequence verification of the vector, mEmerald-paxillin-22 and mCerulean-N1 were digested by EcoRI and NotI, gel purified, and ligated to yield mCerulean-paxillin-22.

To prepare the vinculin fusion, human vinculin (NM_003373.3) was PCR amplified with a 5' primer encoding a NheI site and a 3' primer encoding an EcoRI site. The resulting PCR product and mEmerald-N1 were digested by the appropriate restriction enzymes, gel purified and ligated to yield mEmerald-vinculin-21. On sequence verification of the vector, mEmerald-vinculin-21, tEos-N1, mNeonGreen-N1 and mTurquoise-N1 were digested with NheI and EcoRI, gel purified, and ligated to yield tEos-vinculin-21 (WT-vinculin-C-tEos), mNeonGreen-vinculin-21 and mTurquoise-vinculin-21.

S. W. Craig generously provided cDNAs of ECFP-EYFP-vinculin FRET probes³⁶. To generate the mTurquoise-mNeonGreen-vinculin tail probe, the ECFP-EYFP-vinculin tail probe was PCR amplified with AgeI forward and BspEI reverse primers (Supplementary Table 3). The resulting PCR product was digested, gel purified, and ligated into a similarly treated C1 cloning vector. On sequence verification, mTurquoise was PCR amplified with KpnI forward and XhoI reverse primers (Supplementary Table 3). The PCR fragment was digested, purified, and ligated to the similarly treated ECFP-EYFP-vinculin tail probe described above. This yielded the mTurquoise-EYFP-vinculin tail probe intermediate. Following sequence verification, mNeonGreen was PCR amplified with NotI forward and NotI reverse primers (Supplementary Table 3). The PCR fragment was digested, purified, and ligated to a similarly treated mTurquoise-EYFP-vinculin tail probe intermediate. This resulted in the mTurquoise-mNeonGreen-vinculin tail probe.

To generate the vinculin control probe, HindIII forward and KpnI reverse primers (Supplementary Table 3) were used to PCR amplify ECFP and amino acids

(1–419) of vinculin. The resulting PCR product was purified, digested, and ligated to a similarly treated mNeonGreen C1 cloning vector. This resulted in an ECFP-mNeonGreen-vinculin control intermediate. Following sequence verification, mTurquoise was PCR amplified using HindIII forward and EcoRI reverse primers (Supplementary Table 3). The PCR fragment was digested, purified, and ligated to a similarly treated ECFP-mNeonGreen-vinculin control probe intermediate. This resulted in the mTurquoise-mNeonGreen-vinculin control probe.

To construct the mTurquoise-mNeonGreen-FRET-10 probe, NheI forward and AgeI reverse primers were used to PCR amplify mNeonGreen. The resulting PCR product was digested, gel purified, and ligated to a similarly treated mTurquoise-N1 cloning vector, generating a 10-amino-acid linker (SGLRSPPVAT) separating the fluorescent proteins. This yielded mTurquoise-mNeonGreen-FRET-10.

All fusion DNA was purified using the Plasmid Maxi kit (QIAGEN) and sequence verified (The Florida State University Bioanalytical and Molecular Cloning DNA Sequencing Laboratory). To ensure proper localization, all fusion proteins were characterized by transfection in HeLa cells (CCL2 line; ATCC) using Effectene (QIAGEN) and ~1 µg plasmid DNA. Transfected cells were grown on coverglass in DMEM/F12, fixed after 48 h, and mounted with Gelvatol.

Mutagenesis to generate vinculin and paxillin mutants. The vinculin and paxillin point mutants were generated from the WT constructs using the Quick-change II site-directed mutagenesis kit (Agilent Technologies) and the forward primers listed in Supplementary Table 3 and their reverse complements.

siRNA knockdown of endogenous protein. To inhibit paxillin expression in HFF cells, we used the ON_TARGETplus SMART-pool for Human PXN (cat. no. L-005163-00-0005), and to inhibit Hic5 expression we used the ON_TARGETplus SMART-pool for Human TGFBI/1 (cat. no. L-006565-00-0005, Dharmacon). To inhibit vinculin expression we pooled two custom siRNAs targeted to the 3'UTR of vinculin (sequences in Supplementary Table 3). (Dharmacon). siRNA was transfected into cells using the Nucleofector and knockdown was achieved after 48 h (paxillin and Hic5) or 72 h (vinculin).

Western blot. Cells lysed in 2× SDS Protein Gel Loading Solution (Quality Biological) were separated by SDS-PAGE and electro-transferred to Immobilon-P PVDF membrane (Millipore). Membranes were blocked with 3% non-fat dry milk in TBS-T buffer (20 mM Tris pH 7.6, 137 mM NaCl, 0.1% (vol/vol) Tween-20) incubated with primary antibody overnight at 4 °C, washed with TBS-T, incubated with appropriate HRP-conjugated secondary antibodies and visualized with Immobilon chemiluminescent HRP Substrate (Millipore). Antibodies and dilutions: mouse anti-paxillin (1:1,000, BD Transduction Labs cat. no. 610051); mouse anti-talin (1:1,000, Abcam cat. no. ab11188); mouse anti-vinculin (1:1,000, Sigma-Aldrich cat. no. V4505); rabbit anti-gfp (1:5,000, Abcam cat. no. ab290); rabbit anti-Y118 paxillin (1:500, Invitrogen cat. no. 44-722G); HRP-conjugated anti-mouse (cat. no. 715-035-150) or anti-rabbit (cat. no. 711-035-152), 1:5,000 (Jackson ImmunoResearch Laboratories). To quantify paxillin phosphorylation levels, unsaturated exposures of the western blots were acquired with a myECL Imager (Thermo Fischer). The background-corrected mean intensity was measured for each band using the myECL software. The phospho-paxillin intensity was divided by the total paxillin intensity, and values were normalized relative to control treatment.

Immunoprecipitation. HFF cells were transfected with vinculin siRNA for 60 h. Cells were then transfected with GFP (nonspecific control) or vinculin-GFP constructs, plated in 10 cm tissue culture plates pre-coated with 15 µg ml⁻¹ fibronectin, and supplemented with 15% FBS for 12 h. Cells were collected in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 5% glycerol, 1% Triton-X + complete mini-EDTA free protease inhibitor cocktail and PhosSTOP phosphatase inhibitor (Roche Diagnostics)) for 20 min at 4 °C. Lysates were clarified by 20 min centrifugation at 13,000g. Supernatants were incubated with rabbit anti-GFP antibody (1:300, Abcam cat. no. ab290) and rotated overnight at 4 °C. The following day, the solution was incubated with 30 µl proteinA Dynabeads (Invitrogen) with rotation at 4 °C for 1 h. Beads were washed two times with lysis buffer, 1 time with DPBS and resuspended in 30 µl 2× SDS Protein Gel Loading Solution (Quality Biological). Samples were boiled for 10 min and analysed by western blotting using the appropriate antibodies.

TIRF microscopy. Dual-colour time-lapse TIRF microscopy of EGFP-vinculin and mCherry-paxillin in living cells was performed at 37 °C using an Apo TIRF ×100 1.49 NA oil-immersion objective lens (Nikon Instruments) on an inverted Eclipse Ti microscope system (Nikon Instruments)⁵¹. Pairs of EGFP (using 488 nm laser illumination, Coherent) and mCherry (using 561 nm laser illumination, Coherent) images were captured in rapid succession at 20 s intervals using a CCD (charge-coupled device; Coolsnap HQ2; Photometrics) operated in the 5 MHz

readout mode. Blebbistatin washout during high-resolution imaging was performed using an RC-30 perfusion chamber (Warner Instruments) as previously described⁴⁴.

FRET imaging and analysis. Widefield epifluorescence imaging of FRET biosensors in living cells was performed at 37 °C using a $\times 60$ 1.40 NA PlanApo oil-immersion objective lens (Nikon Instruments) on an inverted TE2000-E microscope system (Nikon Instruments). Illumination was provided by a solid-state white light (Lumencor SOLA SE) using the CFP/YFP/HcRed-3XA Sedat filter set with a 444/521/608 dichroic mirror (Semrock). The following filters were used: mTurquoise image: excitation 427/10, emission 472/30; FRET image: excitation 427/10, emission 542/27; NeonGreen image: excitation 504/12, emission 542/27. Cells were located and brought into focus with mTurquoise filters to prevent bleaching of the acceptor fluorophore. Images were captured in rapid succession (mTurquoise, then FRET, then NeonGreen) using a CCD (Coolsnap HQ2, Photometrics). For each channel, ten images were acquired in a cell-free area to use for shade correction. Ten dark-current images were acquired using mTurquoise filters without illumination.

The raw mTurquoise, FRET and NeonGreen images were aligned using the rigid body method of the StackReg ImageJ plugin⁵². The FRET ratio was calculated using MATLAB to run the Danuser Lab Biosensors processing software 2.1 (available for download: <http://lccb.hms.harvard.edu/software.html>). Briefly, images were manually thresholded to create a binary mask of the cell. Dark-current images were filtered with a 3×3 median filter, averaged, and subtracted from each raw image. Shade correction images were filtered with a 3×3 median filter and averaged. The dark-current-corrected images were divided by the averaged shade correction image. Using the mask of the cell to define the background, the average background intensity was subtracted from the image. Bleed-through of mTurquoise and NeonGreen into the FRET channel was corrected with experimentally determined bleed-through coefficients. The FRET ratio image was created by dividing the corrected FRET image by the corrected mTurquoise image. The mask of the cell was applied so that the ratio value outside the cell was zero.

For each cell, FAs were segmented using a combination of Otsu and Rosin thresholding of the raw mTurquoise image. The resulting mask was applied to the processed FRET ratio image to determine the mean FRET ratio value within FAs (inside the masked regions) and in the cytoplasm (outside the masked regions). To compare the distal and proximal regions of FAs, a line scan was manually drawn along the long axis of individual FAs. The FA was defined as the pixels along the line scan where the intensity was $>2 \times$ s.d. of the adjacent cytoplasm intensity. The FA line scan was divided into thirds, and the mean FRET values in the distal 1/3 and proximal 1/3 were computed.

iPALM imaging. Instrumentation associated with the iPALM method has been described in detail previously⁵³. The sample was mounted between two dual-opposed infinity-corrected objectives (Nikon CFI Apochromat TIRF $\times 60$, NA = 1.49, Nikon Instruments) with index matching oil (Cargille type DF, Cargille Laboratories). We used light from a 50 mW 405 nm diode laser (Coherent), attenuated by neutral density filters, to achieve single fluorophore-level Eos activation and a 150 mW 561 nm diode pumped solid-state laser (Crystalaser) for excitation of activated fluorophores. Narrow bandwidth laser line filters (MaxLine LL01-561-12.5 and MaxDiode LD01-405/10-12.5, Semrock) were placed before the objectives to reject emission noise from the laser and autofluorescence generated in the optical path. The activation and excitation beams entered the back focal planes of the objectives through slots in custom turning mirrors, and the radial positions of the activation and excitation beams were adjusted to produce a near total internal reflection condition. The emission fluorescence signal was collected by the two objective lenses, and mirrors were used to direct the emitted light from the objectives into the custom-designed beam splitter (Rocky Mountain Instruments). The output beams from the 3-way beam splitter were then focused on three separate EMCCD cameras (Andor iXon DU-897, Andor Technology) by achromatic lenses (01LA0799, CVI Melles Griot). Long-pass and band-pass optical filters (RazorEdge LP02-568RU-25 and BrightLine FF01-588/21-25, Semrock) were placed in front of the cameras to reject the excitation light. Typical iPALM data acquisition consisted of 20,000–40,000 images acquired with three Andor iXon EMCCD cameras operated in EM gain, frame-transfer mode with a 50 ms exposure per image. Data acquisition was carried out using software written in LABVIEW (National Instruments). Data analysis, image processing and rendering was performed using software written in IDL (ITT Visual Information Solutions) and run on a Linux computational cluster at HHMI Janelia Farm Research Campus.

Gold (Au) nanoparticles (25–100 nm) were used for drift correction, image registration, and interferometry calibration. For each iPALM image set, the optics were aligned using localization of a single fiducial. The focal plane was found with the bottom objective, which was then held constant, while the top objective was adjusted to the same focal plane and lateral position. To properly align the optics for

interference, the sample holder was continuously oscillated along the Z axis over a range of 400 nm. The beam splitter was then translated up or down until the intensity of the fiducial oscillated owing to the interferometric effect. A calibration curve was acquired by translating the sample in discrete Z-steps of 1 nm over a range of 250 nm. The intensity of the fiducial would then oscillate with a similar period but with phase differences between the cameras. Fine alignment of the position and tilt of the bottom mirror in the beam splitter was performed to obtain a $\sim 120^\circ$ phase difference between each camera. Once alignment was achieved and a final calibration curve was taken, the acquisition of raw image sets could proceed.

After image acquisition was complete, the raw data sets were processed to localize individual molecules and extract their X-, Y- and Z-coordinates, as described previously^{4,53}. First the individual camera images were aligned and added together to form a sum image. Individual fluorescent particles in the sum image were fitted to a two-dimensional Gaussian by nonlinear least-squares fitting to obtain X- and Y-coordinates. The peak amplitude of fluorescent particles in each individual camera image was determined with two-dimensional Gaussian fit. These amplitudes were used to extract the Z-coordinate of each fluorescent molecule from the calibration curve. Sample drift was corrected by tracking the position of fiducials. Lateral sample drift was typically less than 5 nm and vertical (Z-coordinate) sample drift varied between 10 and 60 nm. iPALM images were rendered from the processed list of three-dimensional molecular coordinates. The position of the XY localization was represented by a normalized two-dimensional Gaussian with a width proportional to lateral localization uncertainty, and the position of the Z-coordinate was represented by colour.

Analysis of processed iPALM data. The measured molecular X-, Y- and Z-coordinates were exported into a .txt file for analysis in MATLAB. Processed iPALM data sets included the coordinates of fluorescent molecules localized in FAs and the surrounding cytoplasm as well as autofluorescent molecules inside and outside the cells. To quantify the spatial distribution of the proteins specifically residing within individual FA regions, we manually segmented FAs from the top-view XY iPALM images such that each rectangular ROI contained a single FA and the immediately adjacent space. For each individual FA, the vertical Z-coordinates contained in the ROI were plotted in a 1-nm-binned histogram. As described previously⁴, in addition to the main peak representing molecules in the FA, each histogram contained a smaller peak of autofluorescent molecules from the substrate surface. We determined the centre vertical positions (Z-centre) and width parameter (σ) of each peak using a Gaussian fit. We subtracted the coverslip Z-centre from the FA Z-centre, so that FA Z-centre measurements represent the distance from the local coverslip. We previously found that integrin cytoplasmic tails are ~ 25 – 30 nm above the coverslip and the FAs extend ~ 150 nm above the coverslip⁴. Therefore, molecules with X–Y coordinates within the ROI and Z-coordinates between 25 and 150 nm above the local coverslip Z-centre were considered 'FA-associated'. For each ROI, we calculated the median Z-position of FA-associated molecules and subtracted the coverslip Z-centre from this value to obtain the Z-median relative to the coverslip. Finally, for each segmented FA we calculated the percentage of FA-associated molecules in each of the defined FA layers (ISL: 25–54 nm, FTL: 55–84 nm, ARL: 85–150 nm). Thus, we are able to calculate the Z-centre, Z-median and Z-distribution for each individual FA in the cell.

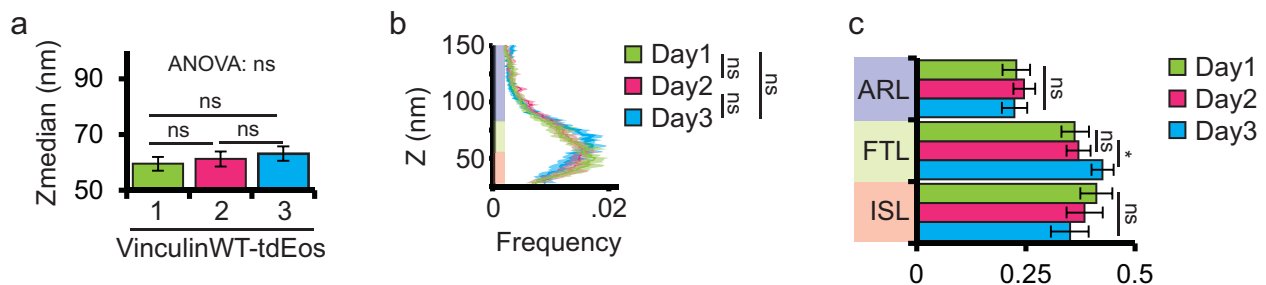
To compare different conditions, we calculated the average Z-median, average Z-distribution and average percentage of molecules in each FA layer, and we computed 95% confidence intervals by resampling our data sets 10,000 times using the bootstrapping method. Confidence intervals are shown as error bars on bar graphs or as lightly shaded regions on the average Z-distribution plots.

Statistical analysis. Differences between iPALM Z-median, percentage of localization to each layer, and mean FRET ratios were determined by an analysis of variance (ANOVA) followed by Tukey *post hoc* analysis and differences were considered significant at $P < 0.05$. Average iPALM Z-distributions were statistically compared using a two-sample Kolmogorov–Smirnov test. Unless otherwise stated, all data are presented as mean $\pm$ 95% confidence interval of the mean. All representative microscopy images are presented with quantification of the entire data set. In Figs 2–8 the number of FAs was assessed from 5–10 cells from 2 independent sample preparations per condition; in Supplementary Figs 1–3, 5 and 6 the number of FAs was assessed from 2–8 cells. Detailed information on replication of experiments can be found in the figure legends and Supplementary Table 2.

51. Shin, W. *et al.* in *Live Cell Imaging: A Laboratory Manual* (eds Goldman, R., Swedlow, J. & Spector, D.) 2nd edn (Cold Spring Harbor Press, 2010).

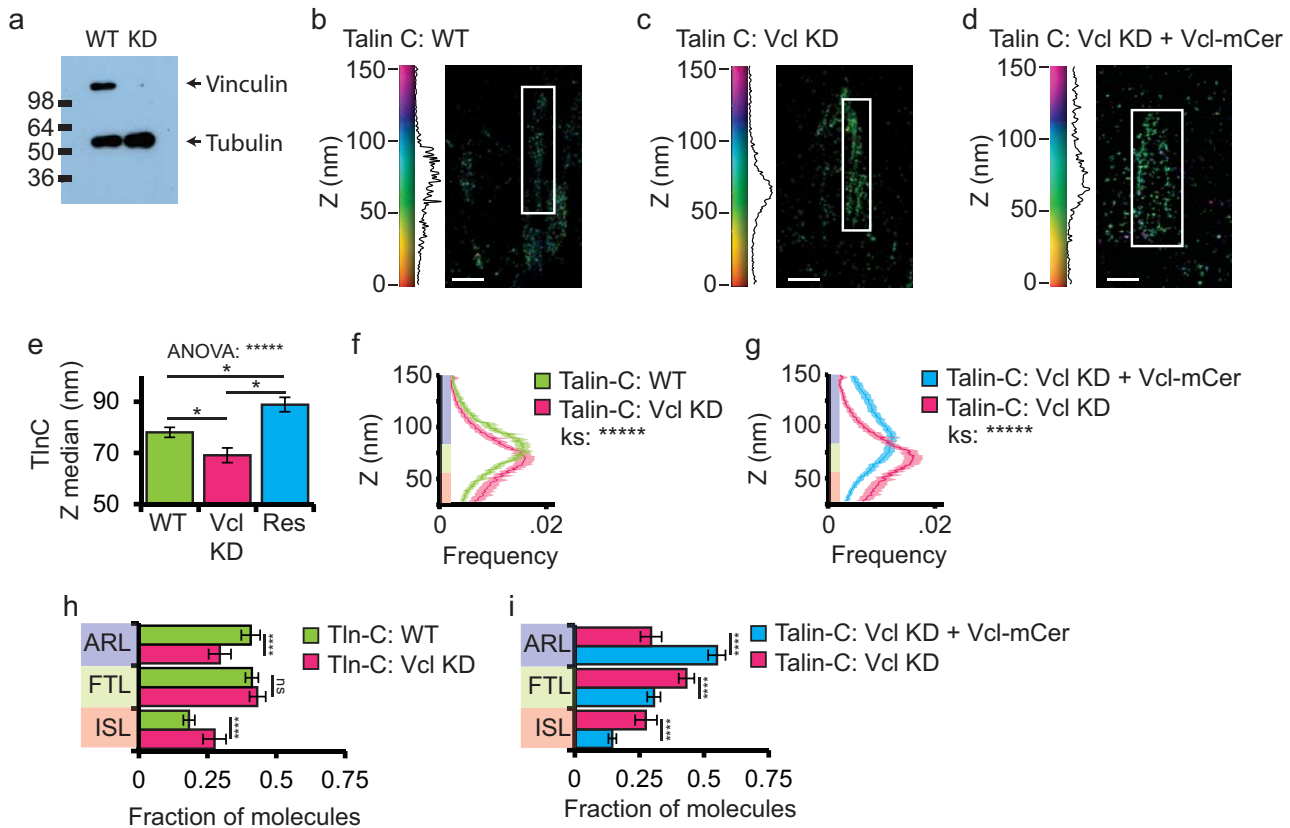
52. Thévenaz, P., Ruttimann, U. E. & Unser, M. A pyramid approach to subpixel registration based on intensity. *IEEE Trans. Image Process.* **7**, 27–41 (1998).

53. Shtengel, G. *et al.* Imaging cellular ultrastructure by PALM, iPALM, and correlative iPALM-EM. *Methods Cell Biol.* **123**, 273–294 (2014).



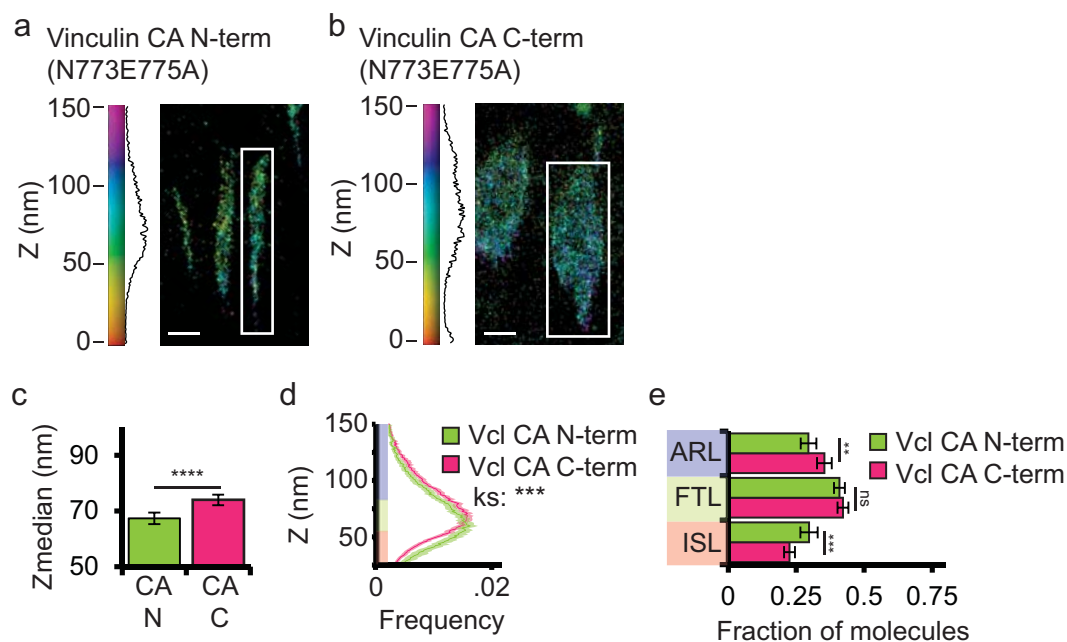
Supplementary Figure 1 Vinculin WT iPALM measurements do not significantly vary between experiments. **(a)** Mean of Z-median measurements from individual FAs in cells expressing WT vinculin-N-tdEos and imaged in three independent experiments. **(b)** Averaged Z-position frequency histograms of molecules within FAs. Solid line, mean frequency; Shaded region, bootstrapped 95% confidence about the mean. Significance tested with two-sample KS test. **(c)** Mean fraction of molecules localized to each

of the three FA layers in FAs. Colouring in (b-c) used to highlight the three FA layers as in Fig. 1. Graphs in (a-c) represent measurements of $n=21$ FAs for 2 cells (Day1), $n=60$ FAs from 3 cells (Day2), and $n=35$ FAs from 3 cells (Day3). Data in all bar graphs are represented as mean $\pm$ 95% bootstrapped confidence intervals. Significance tested with one way ANOVA followed by post-hoc Tukey test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ***** $p<0.00001$, ns: not significant.



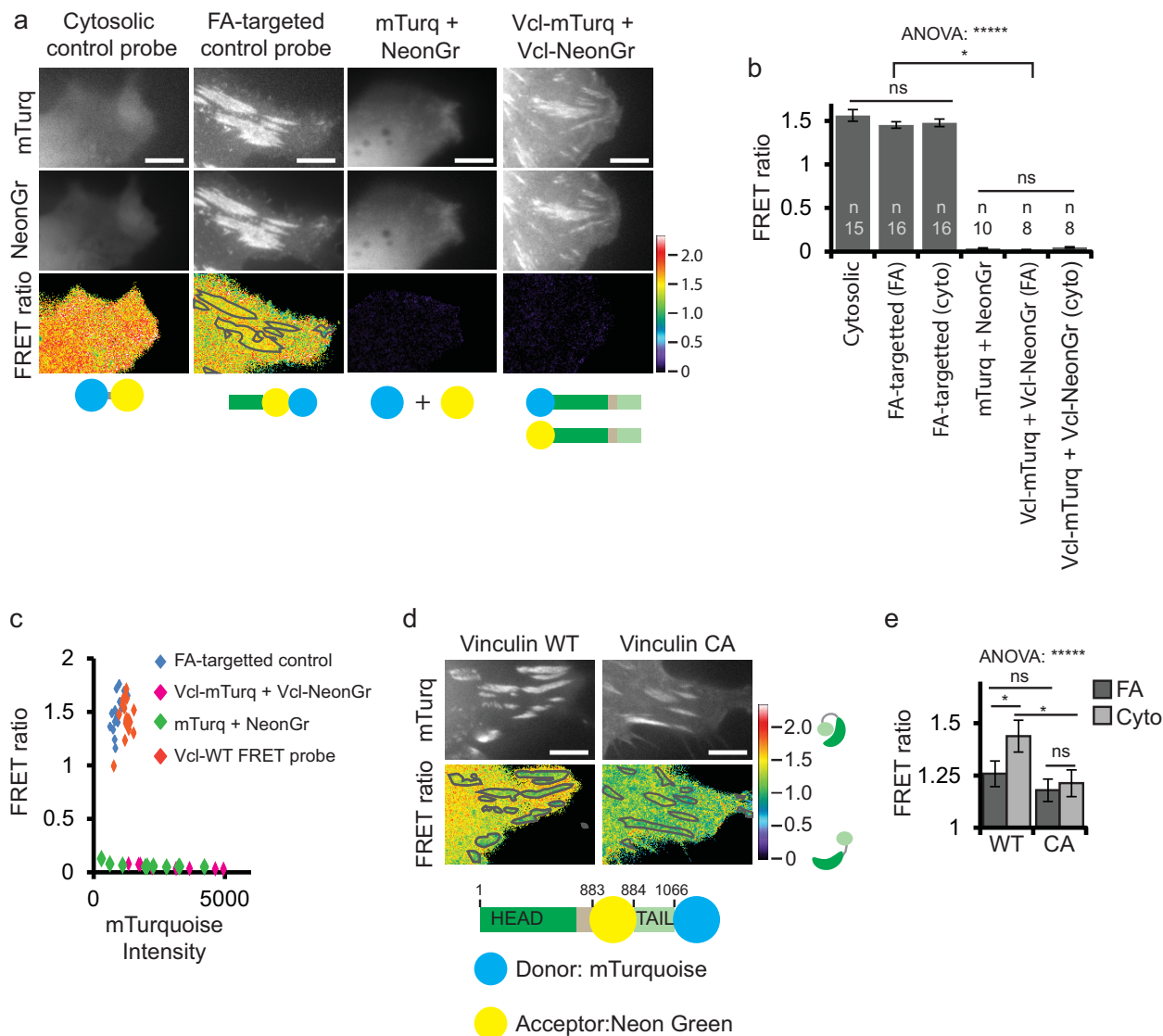
Supplementary Figure 2 Talin nano-scale position is rescued by re-expression of WT Vinculin. **(a)** Western blots of vinculin (top) and tubulin (loading control, bottom) protein in lysates of WT HFFs versus HFFs expressing vinculin siRNA for 72 hours (KD). **(b)** Representative iPALM renderings from wild-type (WT) HFF cells expressing Talin-C-tdEos **(c-d)** Representative iPALM rendering from HFF cells treated with siRNAs targeting vinculin (Vcl KD) and additionally expressing Talin-C-tdEos (c) or Talin-C-tdEos with Vinculin-mCerulean (c). (a,c data duplicated from figure 2 for comparison purposes). In (b-d) the colourscale represents Z-position (nm), FAs oriented with the distal tip facing up, and scale bar = 1 micron. Histograms of the Z-position of the molecules within individual FAs (white boxes in b-d) displayed next to the colourscale. **(e)** Mean of Z-median measurements of the position of molecules from individual FAs in cells expressing Talin-C-

tdEos (TlnC) in WT HFFs (WT), TlnC in vinculin KD HFFs (Vcl KD) or in cells expressing TlnC and vinculin-mCerulean in vinculin KD HFFs (Res). **(f, g)** Averaged Z-position frequency histograms of molecules within FAs. Solid line, mean frequency; Shaded region, bootstrapped 95% confidence about the mean. Significance tested with two-sample KS test. **(h, i)** Mean fraction of molecules localized to each of the three FA layers in FAs from cells in (e). Colouring in (f-i) used to highlight the three FA layers as in fig. 1. Graphs in (e-i) represent measurements of $n = 95$ FAs from 5 TlnC WT cells, $n = 66$ FAs from 5 TlnC KD cells, and $n = 79$ FAs from 5 TlnC rescue cells. Data in all bar graphs are represented as mean $\pm$ 95% bootstrapped confidence intervals with significance tested with one way ANOVA followed by post-hoc Tukey test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ***** $p < 0.00001$, ns: not significant.



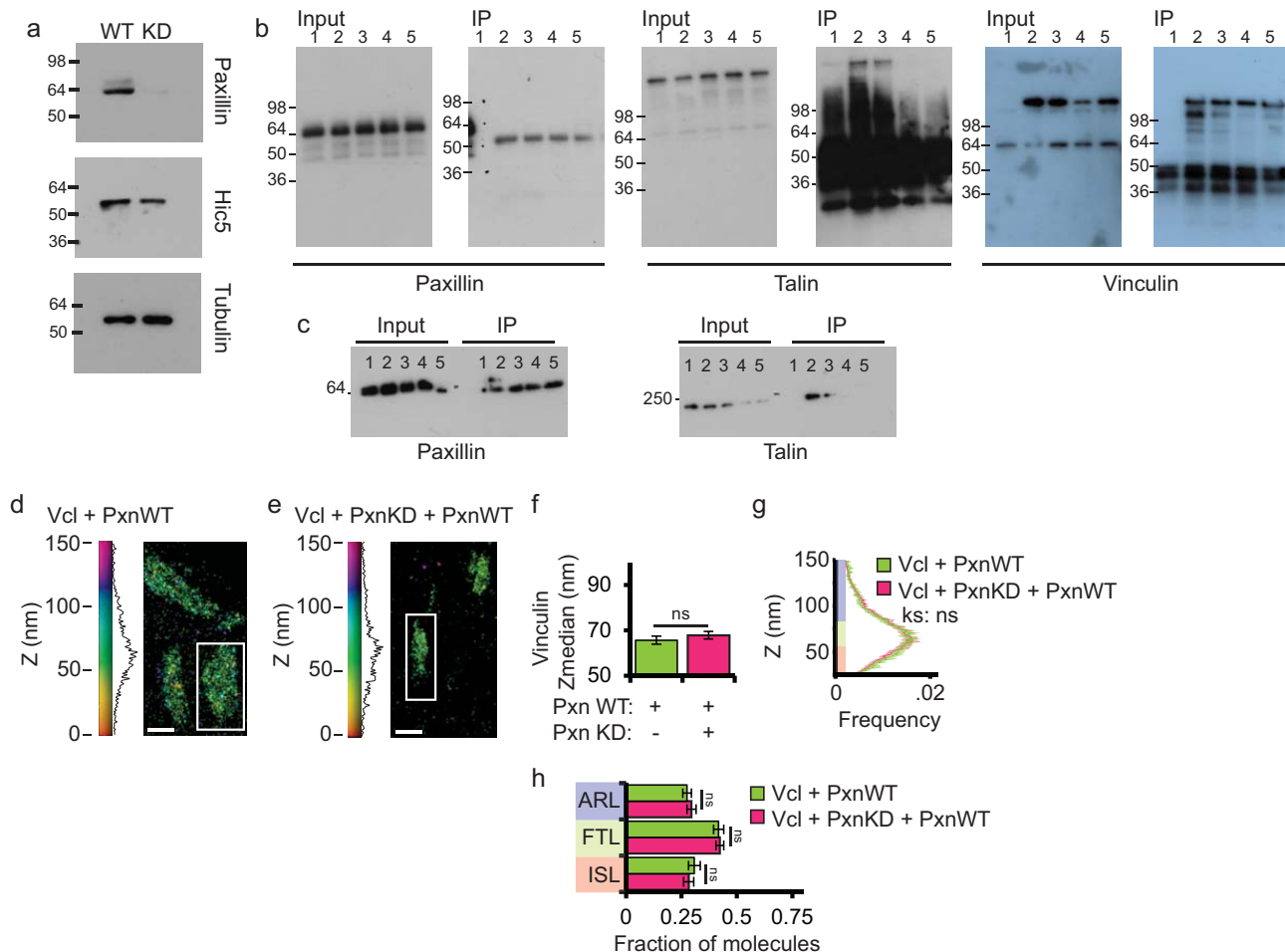
Supplementary Figure 3 Active vinculin is oriented with the tail above the head. **(a-b)** Representative iPALM rendering from an HFF cell expressing N-terminally tagged wild-type constitutively active (CA, N773/E775A) vinculin-N-tdEos (a, data duplicated from figure 3 for comparison purposes), or C-terminally tagged CA-vinculin-C-tdEos (b). In (a-b) the colourscale represents Z-position (nm), FAs oriented with the distal tip facing up, and scale bar = 1 micron. Histograms of the Z-position of the molecules within individual FAs (white boxes in a-b) displayed next to the colourscale. **(c)** Mean of Z-median measurements from individual FAs **(d)** Averaged Z-position frequency histograms of molecules within FAs.

Solid line, mean frequency; Shaded region, bootstrapped 95% confidence about the mean. Significance tested with two-sample KS test. **(e)** Mean fraction of molecules localized to each of the three FA layers. Colouring in (d-e) used to highlight the three FA layers as in Fig. 1. Graphs in (c-e) represent measurements of n=82 FAs from 8 CA-vinculin-N-tdEos cells and n=148 FAs from 6 CA-vinculin-C-tdEos expressing cells. Data in all bar graphs are represented as mean± 95% bootstrapped confidence intervals with significance tested with one way ANOVA. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001, ***** p<0.00001, ns: not significant.



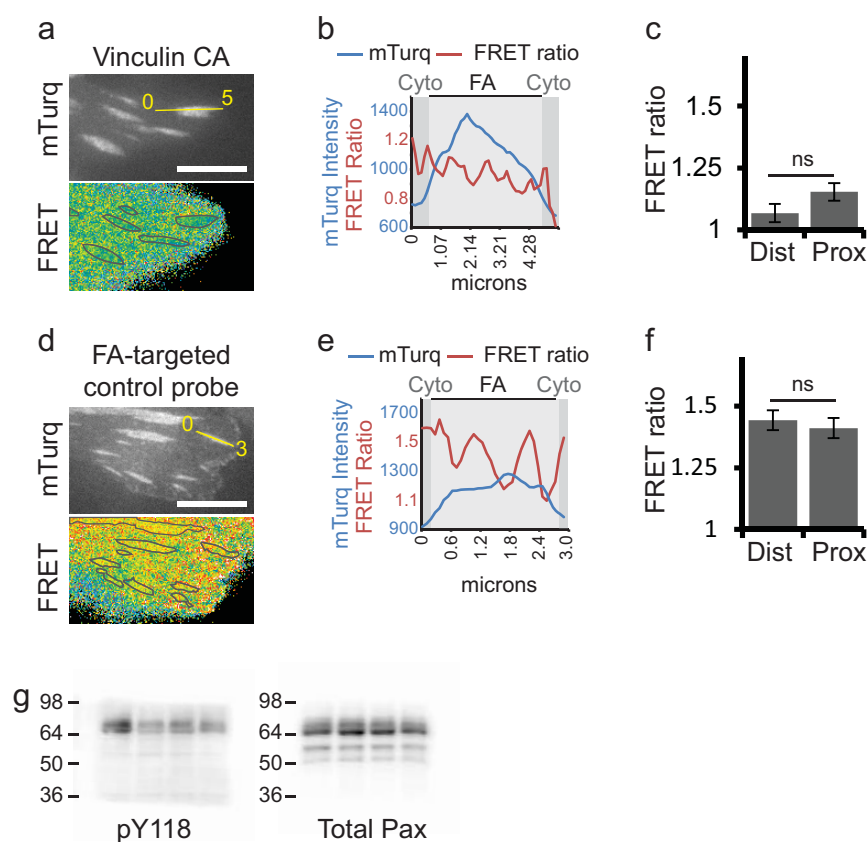
Supplementary Figure 4 Characterization of and controls for the vinculin activation FRET biosensor. **(a)** mTurquoise (mTurq, top row) NeonGreen (NeonGr, middle row) and processed FRET ratio image (bottom row) of HFF cells either expressing mTurquoise fused to NeonGreen by a 10 amino acid linker (Cytosolic control probe), expressing the first 400 amino acids of vinculin (Vcl) fused to mTurquoise and NeonGreen (FA-targeted control probe), co-expressing mTurquoise and NeonGreen, or co-expressing vinculin-mTurquoise and vinculin-NeonGreen. Cartoon schematics of the different FRET probes are displayed beneath the images. Scale bar = 5 micron. In **(a, d)**, The FA mask (grey lines) was created from the mTurq images and superimposed onto FRET ratio images. **(b)** Quantification of the mean FRET ratio value for the constructs described in **(a)** in regions confined within FAs (FA) or in non-FA cytosolic regions (cyto). N = number of cells. **(c)** Scatterplot of the cytoplasmic mTurquoise Intensity vs Cytoplasmic FRET ratio measured

in cells expressing FA-targeted control probe, WT-Vinculin FRET probe, co-expressing mTurquoise and NeonGreen, or co-expressing vinculin-mTurquoise and vinculin-NeonGreen. Each point represents measurements from a single cell. **(d)** mTurquoise (mTurq, top row) and processed FRET ratio image (middle row) of HFF cells expressing wild type (WT) vinculin FRET biosensor (left) or constitutively active (CA, N773/E775A) vinculin FRET biosensor (right). Scale bar = 5 micron. Bottom row: cartoon schematic of the WT vinculin FRET biosensor, numbers refer to amino acid positions in full-length vinculin. **(e)** Quantification of the mean FRET ratio value inside FAs (FA) and outside FAs (Cyto) from n=18 WT-vinculin FRET expressing cells and n=16 CA-vinculin FRET expressing cells. Data in all bar graphs are represented as mean $\pm$ 95% confidence intervals with significance tested with ANOVA test (**** p < 0.00001) followed by Tukey test post-hoc analysis. (* difference is significant at p < 0.05 cutoff, ns: not significant).



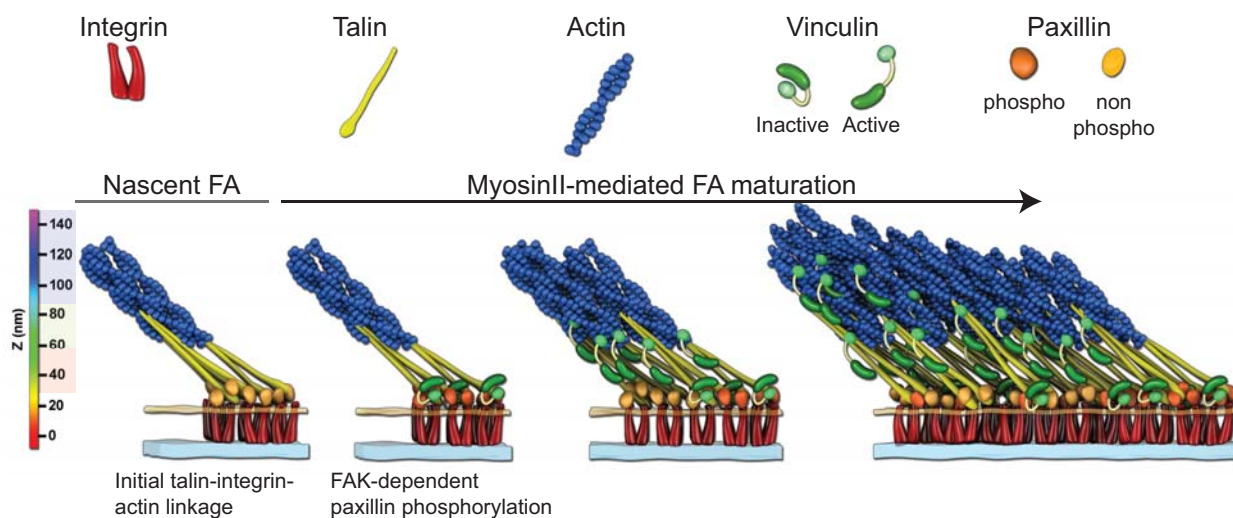
Supplementary Figure 5 Validation of paxillin knockdown and immunoprecipitation experiments, and the effects of paxillin overexpression on vinculin nanoscale localization. **(a)** Western blots of Paxillin (top), Hic-5 (middle) or tubulin (loading control, bottom) protein in lysates of WT HFFs (WT) versus HFFs coexpressing paxillin and Hic-5 siRNA for 48 hours (KD). **(b)** Western blots from figure 5d with molecular size markers labeled. **(c)** Independent co-immunoprecipitation displayed as a replicate experiment of figure 5d and S5b. In (b, c) samples are from HFFs expressing vinculin siRNA for 72hrs and additionally expressing GPF (1), WT-Vinculin-GFP (2), CA(N773/E775A)-Vinculin-GFP (3), A50I-Vinculin-GFP (4), or CA-A50I-Vinculin-GFP (5). **(d, e)** Representative iPALM renderings from HFF cells expressing WT-vinculin-tdEos (Vcl) and WT-paxillin-mCerulean (d) or WT-vinculin-tdEos (Vcl) and WT-paxillin-mCerulean in a paxillin/hic5 siRNA (Pxn KD) background (e). In (d, e) the colourscale represents Z-position (nm),

FAs oriented with the distal tip facing up, scale bar = 1 micron. Histograms of the Z-position of the molecules within individual FAs (white boxes in d, e) are displayed next to the colourscale. **(f)** Mean of Z-median measurements of molecules in individual FAs. **(g)** Averaged Z-position frequency histograms of molecules within FAs. Solid line, mean frequency; Shaded region, bootstrapped 95% confidence about the mean. Significance tested with two-sample KS test. **(h)** Mean fraction of molecules localized to each of the three FA layers. Colouring in (g, h) used to highlight the three FA layers as in Fig. 1. Graphs in (f-h) represent measurements of n=110 FA from 5 WT cells coexpressing WT-vinculin-N-tdEos and WT-paxillin-mCerulean and n=111 FA from 5 PxnKD cells coexpressing WT-vinculin-N-tdEos and WT-paxillin-mCerulean. Data in all bar graphs represented as mean $\pm$ 95% bootstrapped confidence intervals with significance tested by one-way ANOVA.



Supplementary Figure 6 Spatial characterization of FRET biosensors and temporal characterization of paxillin phosphorylation during myosin-II dependent FA maturation. **(a,d)** mTurquoise (mTurq, top) and processed mTurquoise/NeonGreen FRET ratio images (FRET, bottom) of a protruding region of an HFF cell expressing the CA(N773/E775A)-vinculin FRET biosensor (a) or the first 400 amino acids of vinculin (Vcl) fused to mTurquoise and NeonGreen (FA-targeted control probe, d). The FA mask (grey lines) was created from the mTurq image and superimposed onto FRET ratio image. Scale bar = 5 microns. **(b,e)** Values for mTurquoise intensity

(blue line) and FRET ratio (red line) along the 5µm long yellow line in (a,d). Dark grey shaded areas are in the cytosolic (cyto) regions adjacent to the FA (FA, light grey shaded area) **(c, f)** Quantification of the mean FRET ratio of the distal (Dist) 1/3 and proximal (Prox) 1/3 of FA linescans. Data represented as mean± standard error of measurements of n=19 FA from 5 CA-vinculin FRET cells (c) or n=18 FA from 7 control probe cells (f). Significance tested with one way ANOVA. In (c, f), "Dist" refers to the end of the FA facing the leading edge, and "Prox" refers to the end of the FA facing the cell center. **(c)** Uncropped western blots from figure 8r with molecular size markers labeled.



Supplementary Figure 7 Model of vinculin activation and nanoscale positioning during FA formation and maturation. Speculative model for the role of vinculin-protein interactions in vinculin nanoscale localization and function during focal adhesion maturation. See text for details.

	HFF Zcentre (nm)	U2OS Zcentre (nm) ⁴
Paxillin	48.6 ± 9	46.2 ± 7
Talin-N	41.2 ± 11	42.8 ± 4
Talin-C	74.8 ± 11	76.7 ± 11
Actin	95.7 ± 13	96.9 ± 15
Vinculin-N	54.0 ± 14	53.7 ± 6

Supplementary Table 1 Comparison of FA protein vertical distribution in HFFs vs U2OS cells. Vertical distributions of proteins in individual FAs in HFFs were determined from iPALM imaging and analysis of fluorescently-labeled proteins. The Z_{centre} (relative to the coverslip) and width parameter (σ_{vert}) of the vertical distribution of molecules in individual FAs was

determined from Gaussian fits. Actin monomers were labeled with mEos2, and other FA proteins were labeled with tdEos on either the N-terminus (-N) or C-terminus (-C). Measurements represent the average $\pm$ the standard deviation. U2OS Z_{centre} and σ_{vert} were previously published¹ and reprinted for comparison.

Experimental conditions	siRNA (if used)	N cells	N FA	N molecules	Average Z_{MEDIAN} (nm)	Z_{MEDIAN} 95% CI (nm)	$Z_{\text{Centre}} \pm \text{stdev}$ (nm)	$\sigma_{\text{vert}} \pm \text{stdev}$ (nm)
CAAX-tdEos	-	5	27	7.23E+05	40.2	38.3 - 42.2	29.9 ± 6.0	10.9 ± 2.3
Actin -mEos2	-	8	56	1.14E+06	94.5	91.6 - 97.3	95.7 ± 12.6	23.0 ± 8.5
Vinculin-N-tdEos	-	8	115	1.95E+06	61.5	60.1 - 62.8	54.0 ± 13.9	20.1 ± 6.9
PaxillinWT-tdEos	-	7	120	2.14E+06	54.9	53.5 - 56.3	48.6 ± 9.1	16.0 ± 5.6
PaxillinWT-tdEos	Vinculin siRNA	6	117	1.85E+06	56.3	54.4 - 58.4	50.08 ± 10.9	13.8 ± 4.7
Actin -mEos2	Vinculin siRNA	6	50	4.51E+05	94.4	92.2 - 96.7	93.2 ± 10.2	22.7 ± 6.7
Talin-N-tdEos	-	7	75	2.45E+06	55.3	52.5 - 58.5	41.2 ± 10.5	14.3 ± 5.6
Talin-N-tdEos	Vinculin siRNA	5	107	2.64E+06	55.1	53.4 - 56.8	44.4 ± 10.5	15.7 ± 5.4
Talin-C-tdEos	-	5	95	2.44E+06	78.0	76.0 - 80.0	74.8 ± 10.9	19.6 ± 6.8
Talin-C-tdEos	Vinculin siRNA	5	66	2.19E+06	69.1	66.3 - 71.9	63.5 ± 13.7	20.2 ± 7.2
Talin-C-tdEos + VinculinWT-mCer	Vinculin siRNA	5	79	2.95E+06	88.8	86.1 - 91.6	88.3 ± 17.6	28.3 ± 11.9
Vinculin-C-tdEos	-	5	137	3574039	73.0	71.9 - 74.1	71.4 ± 9.2	26.2 ± 7.9
Vinculin N773/E775A-N-tdEos	-	8	82	2.22E+06	67.4	65.0 - 69.8	63.4 ± 12.5	22.8 ± 7.0
Vinculin T12-N-tdEos	-	6	103	4.35E+06	77.2	74.9 - 79.4	75.6 ± 13.9	24.6 ± 6.0
Vinculin N773/E775A-C-tdEos	-	6	148	6.32E+06	74.0	72.1 - 75.9	69.9 ± 11.6	24.2 ± 9.0
Vinculin-N-tdEos	Vinculin siRNA	7	133	1.48E+06	65.9	64.2 - 67.5	62.0 ± 11.5	20.8 ± 5.9
VinculinA50I-N-tdEos	Vinculin siRNA	7	102	1.84E+06	61.7	60.3 - 63.0	51.4 ± 8.7	22.5 ± 6.5
Vinculin A50I/N773/E775A-N-tdEos	-	6	118	2.68E+06	59.5	56.7 - 62.3	53.3 ± 16.0	14.4 ± 6.9
Vinculin-N-tdEos	Paxillin + Hic5 siRNA	6	109	2.90E+06	69.8	68.0 - 71.6	64.1 ± 9.4	15.7 ± 6.4
Paxillin Y31/118E-tdEos	-	6	150	5.12E+06	57.5	56.0 - 59.0	50.0 ± 10.8	17.2 ± 5.7
Paxillin Y31/118F-tdEos	-	6	152	7.54E+06	54.9	53.4 - 56.3	51.4 ± 9.7	14.2 ± 4.4
Paxillin E151Q-tdEos	-	8	79	1.31E+06	53.0	51.4 - 54.5	42.5 ± 10.4	17.1 ± 5.0
Vinculin-N-tdEos + Paxillin-mCer	-	5	110	1.18E+06	65.7	63.9 - 67.4	59.7 ± 14.0	18.1 ± 12.0
Vinculin-N-tdEos + Paxillin-mCer	Paxillin + Hic5 siRNA	5	111	1.59E+06	67.5	65.7 - 69.4	62.7 ± 12.7	20.6 ± 6.1
Vinculin-N-tdEos + Paxillin Y31/118E-mCer	-	8	92	6.29E+05	61.7	59.7 - 63.8	57.0 ± 10.8	20.2 ± 7.3
Vinculin-N-tdEos + Paxillin Y31/118F-mCer	Paxillin + Hic5 siRNA	6	117	2.59E+06	72.7	70.4 - 75.1	69.6 ± 14.6	19.2 ± 6.4
Vinculin-N-tdEos + Paxillin E151Q-mCer	Paxillin + Hic5 siRNA	6	175	1.03E+07	75.0	73.8 - 76.3	72.0 ± 9.3	20.9 ± 5.8
Vinculin V1001A-N-tdEos	Vinculin siRNA	8	182	3.70E+06	66.4	64.6 - 68.2	62.9 ± 13.5	19.5 ± 8.7
Vinculin I997A-N-tdEos	Vinculin siRNA	6	155	2.34E+06	67.9	66.0 - 69.8	61.3 ± 12.5	17.1 ± 6.2
Vinculin-N-tdEos, 1min blebbistatin washout	-	6	61	1.60E+05	57.4	54.3 - 60.5	44.5 ± 18.0	18.8 ± 9.9
Vinculin-N-tdEos, 5min blebbistatin washout	-	7	52	281064.00	75.6	71.1 - 80.1	70.1 ± 20.0	21.1 ± 9.0

Supplementary Table 2 Summary of iPALM experiments. Summary of all iPALM experiments mentioned in the results. Fluorescently labeled proteins were expressed in HFFs for iPALM imaging and analysis. iPALM measurements were performed on either tdEos or mEos2 tagged proteins. FA proteins were labeled on either the N-terminus (-N) or the C-terminus (-C). mCerulean(mCer) labeled proteins were coexpressed with Vinculin-N-tdEos for some experiments. For each condition, the fluorescent proteins expressed and any pharmacological perturbation is listed in the “Experimental conditions.” Any siRNA targets are also indicated. Total

number of cells (N cells), number of individual FAs (N FA), and the number of FA-associated molecules (N molecules) measured for each experimental condition are shown. The Z coordinates of FA-associated molecules were quantified by determining the median Z-position at each FA (Z_{MEDIAN}). Data was bootstrapped 10,000 to compute the 95% confidence intervals (CI) of the mean Z_{MEDIAN} . In addition the Zcentre and width parameter (σ_{vert}) were determined from Gaussian fits (data represent mean $\pm$ standard deviation, see methods for analysis details). Thus, we determined both the Z-median and Z-centre for individual FAs in multiple cells.

Oligo_Name	Sequence
AgeI forward	GCG CTA CCG GTC GCC ACC ATG CCC GTC TTC CAC ACG CGC ACC
BspEI reverse	TCT GAT CCG GAG CCA CTG GTA CCG TCG ACC TGA TAC CAT GGG GTC TTT CTG ACC CAG CGC
KpnI forward	GCG CAT AGG TAC CAT GGT GAG CAA GGG CGA GGA GCT GTT CAC C
XhoI reverse	TCT CGA CTC GAG TTA CTT GTA CAG CTC GTC CAT GCC GAG AGT GAT CCC
NotI forward	GCG CAT AGC GGC CGC ATG GTG AGC AAG GGC GAG GAG GAT AAC ATG GC
NotI reverse	GAC AGC GCG GCC GCC CTT GTA CAG CTC GTC CAT GCC CAT CAC ATC GGT AAA G
HindIII forward	GCG CAT AAG CTT CGA TGG TGA GCA AGG GCG AGG AGC TGT TCA CC
KpnI reverse	GGC CCG CGG TAC CTC ATG CAA CTT TCC TTG CTT CAG ACA TAA TTC CTC GA
EcoRI reverse	GAC AGC GAA TTC GAC TTG TAC AGC TCG TCC ATG CCG AGA GTG ATC CC
NheI forward	GCG AGG GCT AGC CAC CAT GGT GAG CAA GGG CGA GGA GGA TAA CAT GGC CTC TCT CC
AgeI reverse	ACC GCA CCG GTG GGG ATC TGA GTC CGG ACT TGT ACA GCT CGT CCA TGC CCA T
CA-Vinculin (tdEos)	CTA AGA GGG AGG TGG AGG CTT CCG CGG ATC CCA AGT TCC GTG
CA-Vinculin (FRET + GFP)	GCT TGT GGC AAA ACG GGA GGT TGA AGC TTC AGC AGA CCC TAA ATT CAG
T12-Vinculin (tdEos)	CTC ACA TAC CTG TAA GAG GTT GGT TGC AAT CGC TGC AGC TGT GCA CTG CTT GGC AAC CTC CTT G
A50I-Vinculin (tdEos)	CGC CGT GCA GGC GAT CGT CAG CAA CCT C
A50I-Vinculin (FRET + GFP)	CGG CCG TGC AGG CCA TCG TCA GCA ACC TGG T
V1001-Vinculin (tdEos)	CAG CAT GGT GGC CTT CGC TGT GGA CAG GAT TTT
V1001-Vinculin (FRET)	CCC AGC ATG GTA GCT TTC GCT GTG GAA AGA ATT TTG A
PaxillinY31E (tdEos + mCer)	GTT TCT AAC AGA GGAAAC GCC TGA ATC CTA CCC AAC TGG AAA C
PaxillinY118E (tdEos + mCer)	AGT GAG GAG GAA CAC GTG GAA AGC TTC CCA AAC AAG CAG
PaxillinY31F (tdEos + mCer)	CTA ACA GAG GAA ACG CCT TTC TCC T
PaxillinY118F (tdEos + mCer)	GTG AGG AGG AAC ACG TGT TCA GCT TCC CAA ACA AGC
PaxillinE151Q (tdEos + mCer)	GGC TTC TCC TGC AAC TGA ATG CTG TTC AAC ATA ATC CCC CC
Vinculin siRNA1	GGAAGAAAUACAGAAUCAUU
Vinculin siRNA2	CCAGAUGAGUAAAGGAGUAUU

Supplementary Table 3 DNA sequences of cloning primers and custom siRNA oligos. The name of the primer or siRNA oligo (as described in Methods) and the accompanying 5'â3' DNA sequences are displayed.