Mechanism of integrin activation by talin and its cooperation with kindlin

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Talin-induced integrin binding to extracellular matrix ligands (integrin activation) is the key step to trigger many fundamental cellular processes including cell adhesion, cell migration, and spreading. Talin is widely known to use its N-terminal head domain (talin-H) to bind and activate integrin, but how talin-H operates in the context of full-length talin and its surrounding remains unknown. Here we show that while being capable of inducing integrin activation, talin-H alone exhibits unexpectedly low potency versus a constitutively activated full-length talin. We find that the large C-terminal rod domain of talin (talin-R), which otherwise masks the integrin binding site on talin-H in inactive talin, dramatically enhances the talin-H potency by dimerizing activated talin and bridging it to the integrin co-activator kindlin-2 via the adaptor protein paxillin. These data provide crucial insight into the mechanism of talin and its cooperation with kindlin to promote potent integrin activation, cell adhesion, and signaling.
The attachment of cells to the extracellular matrix (ECM) is a fundamental cellular process crucial for the development and responses of multicellular organisms, and its dysfunction has been linked to many human disorders such as cancer, stroke, and thrombosis. This cell-attachment process, also called cell–ECM adhesion, is critically controlled by integrins, a class of heterodimeric (α/β) cell-surface receptors discovered three decades ago. Each integrin subunit consists of a large extracellular domain (ectodomain), a single transmembrane domain (TM), and typically, a small cytoplasmic tail (CT). Integrins can adopt different conformations that display different affinities for ligand. The transition from low- to high-affinity ligand-binding state, referred as integrin activation, can be triggered by a distinct process called integrin inside-out signaling where cellular stimulation elicits intracellular signals to perturb the integrin cytoplasmic face, inducing a global conformational change of the receptor and its subsequent high-affinity binding to ECM ligand. Ligand binding to integrins also transmits signals in an “outside-in” manner, leading to the assembly of large integrin-containing complexes involving hundreds of proteins (focal adhesions, FAs) that are linked to actin filaments. These adhesion complexes form signaling hubs that can transmit signals to promote assembly–reassembly–disassembly cycles of FAs, leading to cytoskeleton rearrangement and dynamic adhesion-dependent processes such as cell migration and spreading.

As the first key step to trigger cell– ECM adhesion, integrin activation has been extensively studied for more than two decades, which led to the discovery of talin as a major integrin activator. Talin is a large cytoskeletal protein containing an N-terminal FERM-like head domain (talin-H, 1–405), a linker (406–485), and a C-terminal rod domain (talin-R, 486–2541). Talin-H, which constitutes less than 20% of the molecule, contains four subdomains, F0, F1, F2, and F3, whereas the large talin-R can be subdivided into 13 helical bundle subdomains (R1–R13) followed by a C-terminal dimerization domain (DD) (Fig. 1a). In unstimulated cells, talin is randomly distributed in the cytosol in an autoinhibited conformation where the integrin-binding site on talin-H is masked by a talin-R subdomain (talin-Rins, a class of heterodimeric (406–485)–485). As described above, release of talin-H from its autoinhibitory helix (Fig. 1b) initiates its activation by dimerizing active talin and bridging it with the integrin co-activator kindlin-2 via the adaptor protein paxillin. Mutations disrupting this talin-R-mediated complex network that diminished integrin activation, ultimately led to substantially reduced cell adhesion and spreading. Our study significantly advances the understanding of the terminal step of integrin activation, highlighting a crucial role of talin-R in forming a crucial talin dimer/paxillin/kindlin complex to cluster integrins, thereby promoting spatiotemporal integrin binding to multivalent ligands, focal adhesion formation, and cell adhesion.

Results

A constitutively activated full-length talin protein promotes strong integrin activation. As described above, release of talin autoinhibition enables talin-H to bind and activate integrin (Fig. 1b). In this model, talin-R was assumed not to be involved in integrin activation, meaning that talin-H should have a similar capacity to activate integrins compared with an activated (non-autoinhibited) full-length talin. To rigorously evaluate this model, we designed an activated full-length talin mutant. Instead of single-point mutations that may only partially disrupt talin autoinhibition, we simultaneously mutated three critical autoinhibition interface residues M319, T1767, and E1770 (Fig. 1b, c) based on the crystal structure of the autoinhibitory talin-F2F3/talin-R9 complex (PDBe code 4F7G). Supplementary Fig. 1a shows GST pull-down experiments, confirming our design in that tlnM3 but not talin-WT, binds to integrin-β3 CT. Consistently, tlnM3 eluted earlier than talin-WT in size-exclusion experiment (Supplementary Fig. 1b) and likely due to its extended shape compared with the autoinhibited/inactive talin as also suggested by the recent cryo-EM analysis. Surface plasmon resonance (SPR) experiments indicated an affinity of integrin-β3 CT binding to tlnM3 at KD ~ 14.5 nM (Supplementary Fig. 1d). Under the same experimental conditions, this affinity is slightly higher than that of integrin-β3 CT to talin-H (KD ~ 29.7 nM, Supplementary Fig. 1e) possibly because full-length talin may form dimer that strengthens integrin binding (of note, a talin deletion mutant without C-terminal dimerization domain binds integrin similarly as talin-H with KD ~ 36.0 nM, see Supplementary Fig. 1d). Next, we compared the effect of talin-H and tlnM3 on the ligand binding to prototypic integrin αIIbβ3 stably expressed in Chinese-hamster ovary (CHO) cells—a well-established assay for studying integrin activation. To our surprise, while tlnM3 only slightly increased binding of integrin αIIbβ3 to the monovalent ligand fibronectin repeat 10 (FN10) compared with talin-H alone (Fig. 1d), tlnM3 dramatically increased integrin-αIIbβ3 binding to PAC-1—a well-known activation itself. Rather, kindlin may somehow enhance talin-H-mediated integrin binding to multivalent but not monomeric ligands. The mechanism of how kindlin cooperates with talin-H to promote integrin activation remains highly elusive, despite extensive investigation over the past 15 years. In this study, we set out to investigate these major puzzling issues through combined biochemical, structural, and cell biological approaches. We found that while being capable of activating integrin, talin-H alone exhibits unexpected low potency to induce integrin binding to multivalent ligand compared with a constitutively activated full-length talin. This finding challenges the long-standing talin-H “only” model for integrin activation (Fig. 1b) and prompted us to perform a comprehensive investigation, which unveiled a crucial role of talin-R in regulating talin-H-mediated integrin activation. Specifically, we found that talin-R, which otherwise masks the integrin-binding site on talin-H in inactive talin, dramatically enhances the talin-H-mediated integrin activation by dimerizing active talin and bridging it with the integrin co-activator kindlin-2 via the adaptor protein paxillin. Mutations disrupting this talin-R-mediated complex network that diminished integrin activation, ultimately led to substantially reduced cell adhesion and spreading. Our study significantly advances the understanding of the terminal step of integrin activation, highlighting a crucial role of talin-R in forming a crucial talin dimer/paxillin/kindlin complex to cluster integrins, thereby promoting potent integrin binding to multivalent ligands, focal adhesion formation, and cell adhesion.
Fig. 1 Talin is an intracellular activator of integrin. **a** Domain organization of talin. N-terminal head domain (talin-H) is composed of four subdomains and C-terminal rod domain (talin-R) contains 13 subdomains followed by dimerization domain (DD). Talin-H and talin-R are connected by a flexible linker. **b** A scheme of talin activation and talin-mediated integrin activation. Upon stimulation, inactive talin is released from an autoinhibited state to expose talin-H to bind integrin cytoplasmic tail and trigger conformational change of integrin to bind ligand. The autoinhibitory interface between talin F3 and talin R9 is displayed in the box showing how M319, T1767, and E1770 are involved in the interface and mutated to release the autoinhibitor. **c** Diagram of the various talin variants, including mutation sites to activate talin or disrupt talin dimerization. **d** Left panel: Integrin-activation level reflected by monomeric FN10 binding is not elevated much by constitutively activated full-length talin mutant, (tlnM3) compared with talin-H (tlnH). **p = 0.0081 with 95% confidence interval 0.2727–0.9914 (t-test), N = 3 biologically independent samples. All values are given as mean ± S.E.M. Right panel: FN10 staining was similar between tlnH (orange) and tlnM3 (light green). **e** Left panel: Integrin-activation level reflected by PAC-1 binding is substantially elevated by tlnM3 compared with tlnH. Talin rod (tlnR) alone has no effect on integrin activation. ****p < 0.0001 with 95% confidence interval 5.478–6.260 (t-test), N = 6 biologically independent samples. All values are given as mean ± S.E.M. Right panel: Significantly more PAC-1 staining was induced by intact active talin (tlnM3, light green) in comparison with talin head (tlnH, orange) without normalization. **d, e** Raw data are provided in Source Data file.
ligand-mimetic multivalent antibody that recognizes activated 
albβ3 compared with either talin-H alone (Fig. 1e) or full-length WT talin (Supplementary Fig. 1f). Because expression of talin-R alone had no effect on PAC-1 binding (Fig. 1e), the drastic effect of tlnM3 compared with talin-H on PAC-1 binding (Fig. 1e) suggests a previously unrecognized but crucial role of talin-R in regulating integrin binding to multivalent but not monomeric ligands. As mentioned earlier, multivalency is an important feature of most of physiological integrin ligands.

**Talin-R-mediated talin dimerization promotes integrin binding to multivalent ligands.** Talin exists in a monomer–dimer equilibrium at native conditions45,48, and prefers dimerization at higher protein concentration (>3 μM)49 which may be reached in cells with high talin levels up to 50 μM and can be further elevated by subcellular enrichment after recruitment to the plasma membrane. A compact-shaped monomeric structure of autoinhibited talin-WT has been recently solved at lower salt concentration (75 mM) and low protein concentration (~1 μM) favoring the monomeric state18, although a significant dimeric population was also observed under these conditions (see Fig. 2c in Dedden et al. 201945). SPR experiments by immobilizing tlnM3 onto the CM5 chip and then flowing through tlnM3 in 150 mM salt revealed the distinct response curves that validated the intermolecular tlnM3/tlnM3 interaction with K_d ~ 1.82 μM (Supplementary Fig. 2a). This affinity is consistent with the earlier sedimentation studies on talin dimerization (at ~ 3 μM)45, which used higher salt (220 mM NaCl) to mimic the talin-activation status (elongated state)18. Previous studies revealed that talin dimerization is critically mediated by the C-terminal talin-R dimerization domain (DD) (Fig. 1a)50 and its deletion was shown to disrupt the dimerization of full-length talin-WT (see Fig. 2c in Dedden et al. 201945) and affect focal adhesion formation and cell spreading51. Since dimeric talin could bind and bridge two integrins, thereby promoting the formation of integrin microclusters, we wondered if the talin dimerization could account for the enhanced multivalent PAC-1 binding induced especially by full-length activated talin (see the scheme in Fig. 2a). To examine this possibility, we generated a tlnM3 construct lacking the C-terminal DD domain (tlnM3 DDDel as illustrated in Fig. 1c). Remarkably, Fig. 2b, c show that deletion of the talin-R DD domain significantly impaired tlnM3-induced PAC-1 binding to integrin-albβ3. Moreover, a specific point mutation disrupting talin dimerization (R2526G52,53) also significantly impaired tlnM3-induced PAC-1 binding (Supplementary Fig. 2b). To further evaluate the role of talin dimerization in regulating the integrin/ligand interaction, we took advantage of our established talin1 and talin2 double-deficient fibroblast system (talin1/2Δko)52,21. These cells were retrovirally transduced with constructs encoding for C-terminally ypet-tagged full-length talin and talin constructs that either lacked the DD domain or contained the dimerization-disrupting R2526G mutation. Remarkably, integrin binding to the multivalent ligands, fibronectin, vitronectin, and laminin were all reduced by the mutations that blocked talin dimerization (Fig. 2d, Supplementary Figs. 2c, d), indicating for the first time that talin dimerization plays a critical role in controlling integrin binding to multivalent ligands. However, the talin DD deletion mutant and the R2526G mutant only impaired ligand binding partially (~25%) compared with intact tlnM3, and were still significantly more potent than talin-H (Fig. 2b, c, Supplementary Fig. 2b), suggesting the existence of additional factors that control talin-R-mediated integrin binding to multivalent ligands.

**Talin-R bridges talin with kindlin-2 via paxillin to promote integrin binding to multivalent ligands.** Next, we investigated whether talin utilizes talin-R to promote integrin activation by cooperating with other integrin regulators. Given the essential role of kindlins as integrin co-activators, and the fact that kindlins also enhance talin-H-mediated PAC-1 binding but not FN10 monomer binding59, which is similar to the tlnM3 function in Fig. 1d, e, we wondered whether talin-R plays a role in linking talin with kindlin-2—the most widely expressed member of the kindlin family13,36,37. Interestingly, kindlin-2 drastically enhanced the capacity of tlnM3 (Fig. 3a, b) but not tlnWT (Fig. 3c) to induce PAC-1 binding of integrin-albβ3. Of note, this enhancement was even greater than the known effect of kindlin-2 on talin-H-mediated PAC-1 binding to integrin-albβ352,53 (Fig. 3a, b). These data support the hypothesis that talin-R in activated talin enhances the ability of talin-H to cooperate with kindlin-2 to promote potent integrin binding to multivalent ligands. How does talin-R achieve this enhancement? Since both kindlin-2 and tlnM3 bind to integrin-β CT in a nonexclusive manner (Supplementary Fig. 3a) with no detectable interaction between kindlin-2 and tlnM3 (Supplementary Fig. 3b), we wondered whether kindlin-2 and talin-R of tlnM3 are physically linked by a common binding partner, thereby further enhancing talin-dimer-induced microclustering of integrins to more efficiently bind to multivalent ligands. Since paxillin was shown to interact with talin41,53,54 and kindlin55,56 (see also Supplementary Fig. 3b), and all three proteins colocalize in nascent focal adhesions to regulate cell adhesion55,58,59, we speculated that paxillin might physically link activated talin with kindlin-2 to regulate integrin activity. Remarkably, expression of a paxillin-binding defective kindlin-2 mutant G42K/L46E (Fig. 3d) and knockdown of paxillin (Fig. 3e, f) both significantly reduced the synergistic action of tlnM3/kindlin-2 on integrin activation. Furthermore, co-expression of paxillin and tlnM3 resulted in a synergistic increase (~2.5-fold) in PAC-1 binding compared with tlnM3 alone (Fig. 3g) and such synergy was also significantly reduced when a kindlin-2-binding defective paxillin-F577E mutant56 was used (Fig. 3g) or kindlin-2 was knocked down (Fig. 3h, i). By contrast, paxillin (also kindlin-2) had no significant effect on tlnM3-mediated monomeric FN10 binding to integrin-albβ3 (Supplementary Fig. 3c). However, either paxillin or kindlin-2 cooperates with talin to induce potent oligomeric FN10 binding to integrin (Supplementary Fig. 3d), which is similar to the PAC-1-binding data in Fig. 3. Overall, these data provide strong evidence that paxillin links activated talin with kindlin-2 to promote potent integrin binding to multivalent ligands. We note that although a previous study had proposed that paxillin may bridge talin-H (instead of talin-R) and kindlin for promoting integrin activation41, the study did not observe any synergistic effect of integrin activation when paxillin and talin-H were co-expressed vs talin-H alone. Supplementary Fig. 3e also shows that co-expression of paxillin and talin-H induced no synergistic effect on PAC-1 binding compared with the additive effect of expressing talin-H and paxillin separately. The slight enhancement of PAC-1 binding by the paxillin/talin-H co-expression vs talin-H alone was caused by paxillin that can induce a small effect of PAC-1 binding when expressed alone, see Supplementary Fig. 3e. This is in sharp contrast to the dramatic synergistic effect of co-expressing tlnM3 and paxillin (Fig. 3g), strongly supporting our hypothesis that paxillin acts through talin-R to promote potent integrin activation. A question is: if paxillin does not affect talin-H activity by linking talin-H and kindlin-2, how does overexpression of talin-H and kindlin-2 result in some synergy in the integrin binding to PAC-1?13,36,37 (also see Fig. 3a)? One possible mechanism is that overexpression of talin-H, which is known to recruit PIPKl to produce PIP223,24 that in turn binds and activates talin15,17,18, may result in the activation of some endogenous talin, which then triggers
endogenous talin (via talin-R)/paxillin/kindlin pathway. Consistent with this possibility, the talin-H/kindlin-2 synergy to induce PAC-1 binding was significantly reduced when endogenous talin-1 was knocked down (Supplementary Figs. 3f and 3g), demonstrating that endogenous talin is indeed involved in mediating the talin-H/kindlin-2 synergy. Furthermore, while paxillin has no effect in the talin-H activity regulation (Supplementary Fig. 3e), the talin-H/kindlin-2 synergy to induce the PAC-1 binding was significantly reduced by the paxillin-binding defective kindlin-2 mutant G42K/L46E (Supplementary Fig. 3h).
These data provide evidence that activation of endogenous talin/paxillin/kindlin-2 pathway contributes to the known talin-H/kindlin-2 synergy in activating integrin in CHO-A5 system.

Given the potential indirect effect in the CHO system due to protein overexpression, we decided to underpin the role of the talin/paxillin/kindlin-2 complex on regulating integrin activity and function again by using the talin-1 and talin-2 double-deficient fibroblast system (talin\textsuperscript{1/2\textsubscript{dko}}). We retrovirally expressed ypet-tagged talin-H, tlnWT, and tlnM3 and sorted for cells with similar ypet levels (Supplementary Fig. 3), which also showed comparable integrin-surface levels, and then analyzed cell adhesion on fibronectin and vitronectin, as well as cell spreading on fibronectin. As expected, talin-H was unable to fully promote cell adhesion and completely failed to induce cell spreading likely due to the absence of talin-R to connect with paxillin and kindlin as well as actin filaments (Fig. 4a, b). By contrast, however, tlnWT and tlnM3 expression induced very similar cell adhesion and spreading (Fig. 4a, b). We note that the retrovirally expressed tlnWT becomes activated by the endogenous activation machinery and does not require activating mutations, which is different from the CHO model. Next, we compared talin-H- and talin-WT-dependent cell adhesion in the presence of kindlin-2 and upon CRISPR/Cas9-mediated kindlin-2 gene deletion (Fig. 4c), which revealed that a synergistic effect of talin and kindlin-2 on cell adhesion can only be found in cells expressing full-length talin-WT but not talin-H (Fig. 4d). This observation is consistent with the above CHO cell-based data that the talin and kindlin-2 cooperativity on cell adhesion requires the presence of talin-R. We then reexpressed mCherry-tagged kindlin-2 WT and paxillin-binding deficient kindlin-2 G42K/L46E mutant in kindlin-2-deficient fibroblasts that express talin-WT, sorted cells with similar mCherry expression, and then analyzed cell adhesion on fibronectin-coated surfaces. In line with the above CHO data, expression of the paxillin-binding deficient kindlin-2 G42K/L46E mutant (Fig. 4e) showed significantly reduced cell adhesion compared with cells expressing kindlin-2 WT (Fig. 4f).

Moreover, siRNA knockdown of paxillin also led to reduced cell adhesion in kindlin-2 WT-expressing cells but did not further reduce adhesion in kindlin-2 G42K/L46E-expressing cells (Fig. 4f). We also knocked out kindlin-2 in mouse embryonic fibroblasts (MEFs) and observed significantly reduced adhesion of cells expressing the paxillin-binding defective kindlin-2 G42K/L46E mutant compared with cells that were rescued with wild-type kindlin-2 (Supplementary Fig. 4a and 4b). Moreover, only expression of paxillin-WT but not the kindlin-binding deficient paxillin-F577E mutant in paxillin siRNA-mediated knockdown MEFs induced cell adhesion, indicating again the critical importance of the paxillin–kindlin-2 interaction (Supplementary Fig. 4c and 4d). Overall, these data collectively showed that disruption of paxillin–kindlin interaction impairs ligand binding and cell adhesion, which is fully consistent with the above CHO cell-based data and also with the previously observed defects of focal adhesion formation, cell spreading, and migration induced by the disruption of this interaction\textsuperscript{60}.

Molecular insight into the binding of paxillin to talin. Paxillin is a multidomain adaptor containing five LD motifs at its N-terminus followed by four tandem double zinc-finger LIM domains\textsuperscript{60} (Supplementary Fig. 5a). To further elucidate the mechanism of how paxillin binds to talin for regulating integrin activation, we undertook detailed structure-based mapping of the interaction. We first purified full-length paxillin, tlnM3, and talin-WT, and performed GST-based pull-down assays. Interestingly, purified paxillin exhibits stronger binding to tlnM3 than talin-WT in these pull-down assays (Fig. 5a). We next performed pull-down experiments using GST-paxillin and various talin fragments. Supplementary Fig. 5b shows that talin R1–R4 is a major fragment to bind to paxillin. Consistently, talin1–1047 containing only talin-H and talin R1–R4 (deleting the C-terminal 1048–2541 containing the dimerization domain and autoinhibitory talin-R9, see Fig. 1b) still activates integrin much more potently than talin-H (Supplementary Fig. 5c), consistent with the crucial role of talin-R1–R4 to mediate the talin/paxillin interaction in connection with kindlin. Among other talin-R fragments, talin-R9–R12 also displayed binding to paxillin, but this interaction was much weaker than talin-R1–R4 (Supplementary Fig. 5b). Paxillin also binds to talin-H but to a significantly lesser extent than talin-R1–R9 or talin-R1–R4 (Supplementary Figs. 5b and 5d). The dominant role of talin-R1–R4 in binding to paxillin was further confirmed by the quantitative SPR experiments (see below). On the paxillin side, deletion-based binding analysis revealed that paxillin N-terminal 1–160 containing LD1 and LD2 (Supplementary Fig. 5a) plays a major role in binding to talin (Fig. 5b and Supplementary Fig. 5d). Consistently, deletion of paxillin 1–160 almost completely abolished the paxillin synergy with tlnM3 to induce PAC-1 binding (Supplementary Fig. 5e). On the other hand, expression of paxillin 1–160, which does not contain the major kindlin-2-binding site located in paxillin LIM4 domain (Supplementary Fig. 5a) (see Zhu et al. 2019\textsuperscript{56}), also hardly showed any synergistic effect on tlnM3-induced PAC-1 binding (Supplementary Fig. 5e). These data provide further evidence that full-length paxillin is required to act as a link between activated talin (primarily via paxillin N-terminal 1–160) and kindlin-2 (via paxillin C-terminal LIM4 domain) to form the talin/paxillin/kindlin axis and promote high-affinity integrin binding to multivalent ligands.

To further characterize the binding mode between talin and paxillin in more detail, we prepared individual \textsuperscript{15}N-labeled talin-H subdomains and talin-R1–R13 subdomains and examined their binding to paxillin 1–160 using NMR-based heteronuclear single quantum correlation (HSQC) experiments. The strength of the binding can be roughly estimated by the extent of the chemical shift changes with and without the binding partner. In talin-R, R1, R5, R6, R7, R9, R10, and R12 had no chemical shift changes (not shown), whereas R2, R3, R4, R8, R11, and R13 had chemical shift changes upon addition of paxillin 1–160. Among these paxillin-binding subdomains, R2, R8, and R11 showed significantly bigger chemical shift changes by paxillin (Fig. 5c, Supplementary Figs. 6a and 6b) than R3 (Supplementary Fig. 6c) and R4 (Supplementary Fig. 6d), respectively. R13 had very tiny/
almost negligible chemical shift changes by paxillin (Supplementary Fig. 6e). In talin-H, only talin-F3 exhibited chemical shift changes upon addition of paxillin 1–160 (Supplementary Fig. 6f). Table S1 provides the summary of the HSQC-based binding results for all talin subdomains. Quantitative SPR experiments on those subdomains that showed significant chemical shift changes by paxillin 1–160 revealed that paxillin 1–160 binds talin-R2 at $K_D \sim 2.21 \mu M$ (Supplementary Fig. 7a), to talin-R8 at $K_D \sim 5.37 \mu M$ (Supplementary Fig. 7b), to R11 at $K_D \sim 12.7 \mu M$ (Supplementary Fig. 7c), and to F3 at $K_D \sim 3.51 \mu M$ (Supplementary Fig. 7d). As expected from the pull-down data (Supplementary Figs. 5b and 5d), talinR1–R4 as the dominant paxillin-binding core exhibited strong affinity to paxillin 1–160 at $K_D \sim 153 \text{nM}$ (Supplementary Fig. 7e). It is important to note that talin-R1–R4 binding to paxillin 1–160 fits very well in 1:1 ratio by two-state model, suggesting that talin-R2, as the main binding module, may cooperate with R3 or R4 to bind paxillin via a multisite binding mode. As a comparison, talin-F3, talin-R8,
and talin-R11, of which each has an isolated binding site, binds much weaker than talin-R1–R4. Interestingly, despite the presence of multiple domains/sites within paxillin and talin, SPR experiments revealed that binding of full-length paxillin to tlnM3 also fitted well with a 1:1 model at K_D ~ 33.1 nM (Supplementary Fig. 7f), indicating that multiple sites in one paxillin may bind to multiple sites in one talin, resulting in 1:1 binding. This is reminiscent of talin/vinculin binding in 1:1 ratio, despite that there are 11 vinculin-binding sites on talin-R18. Nevertheless, talin-R1–R4 clearly contributes most significantly to the tlnM3 binding to paxillin.

The binding of paxillin to talin-F3 in the talin-H is interesting since such binding competes with the autoinhibitory R9 binding to talin-F3 (Supplementary Fig. 8a), providing a basis for understanding why paxillin preferably binds to activated talin (Fig. 5a). More interestingly, such binding competes with the talin-F3 binding to integrin-β CT when we used higher protein concentration in the HSQC experiments (Supplementary Fig. 8b), indicating that the paxillin- and integrin-binding sites on talin-F3 overlap. However, given the modest affinity of paxillin binding to talin-F3 (K_D ~ 3.51 μM, Supplementary Fig. 7f), significant competition between integrin-β CT and talin-H in PIT2-enriched membrane media41, which was also reflected in our SPR tests where integrin-β CT was immobilized to CM5 chip to bind talin-H, Supplementary Fig. 1) would readily replace paxillin from talin-F3 when paxillin is recruited by talin bound to both membrane and integrin. In other words, paxillin may temporarily stabilize the activated talin by binding to talin-H, but once talin-H binds to integrin-β CT, paxillin is dissociated from talin-H. Indeed, paxillin had very little effect on talin-H-mediated integrin activation (Supplementary Fig. 3e). On the other hand, paxillin had drastic effect (Fig. 3g) on tlnM3 apparently via the talin-R/paxillin-kin2/2 pathway in combination with the dimerization effect (see more detailed analysis below). Our data suggest that the engagement of talin-R with paxillin is crucial for spatial clustering of integrins by linking talin to kindlin in addition to talin dimerization, thereby leading to potent integrin binding to multivalent ligands. To further examine this hypothesis, we decided to perform more detailed structural mapping studies to identify specific paxillin sites on talin-R, so that we can perform more definitive mutation-based functional analysis.

Previous structural analysis indicated that paxillin LD motifs bind to talin-R8 that forms a helical bundle similar to other paxillin LD-binding modules54. We therefore synthesized paxillin LD1 and LD2 peptides (Supplementary Fig. 9a) that are present in paxillin 1–160 fragment and examined their binding to talin R2, R8, and R11 using HSQC. The LD peptides induced similar chemical shift perturbation spectra, but less extensive chemical shift changes, on talin-R2, R8, and R11 as compared with paxillin 1–160. To illustrate this more clearly, we plotted residue-based chemical shift changes of talin R2 and R8 by LD peptides and paxillin 1–160 (Supplementary Fig. 9b and 9c, respectively) (BMRB codes for the chemical shift assignments of talin R2 and R8 are 17350 and 19339, respectively). The perturbed residues that are surface-exposed are highlighted on the structures of talin-R2 (Supplementary Fig. 9d) and talin-R8 (Supplementary Fig. 9e), respectively, which reveal potentially similar LD-binding site in these domains. To further elucidate the LD-binding mode, we built a HADDOCK-based structure model of talin-R2 bound to LD2 by using chemical shift changes and the conserved interface residues in LD motif as constraints (Supplementary Fig. 9b showed that LD2 binds more tightly to R2 than LD1, see details in the method section and Supplementary Table 3 for statistics). Figure 5d shows the best-calculated model where the helix in LD2 binds to a hydrophobic groove formed by helices a1 and a2 of talin R2. Important interfacial interactions are well conserved in talin-R2 as compared with those of known structure of talin-R8 (Supplementary Fig. 9f) bound to DLEC1-LD peptide54 (PDB code 5FZT) (Supplementary Fig. 9a), which includes the hydrogen bond between D146 of LD2 and K687 of R2, and a couple of hydrophobic packing pairs: LD2–L152/R2 A680 and A710, LD2–L148/R2 T707, and LD2–L145/R2-I703. Structure-based alignment of R8 with R11 shows that R11 can also use the conserved surface to bind LD motif (Supplementary Fig. 9g), as consistent with the NMR- (Supplementary Fig. 6b) and SPR- (Supplementary Fig. 7c) binding data. These analyses illustrated how LD motifs bind similarly to these helical bundle domains in talin-R. A key question is: given the 1:1 binding between paxillin and tlnM3 (Supplementary Fig. 7f), how do the LD motifs in paxillin engage with multiple talin subdomains via a multisite binding mode? To address this question, we focused on paxillin 1–160 (<18 kDa) and talin-R1–R4 (~70 kDa) for NMR-based binding studies since they play major role in the paxillin/talin interaction as indicated by the above studies. Supplementary Fig. 10a shows that 1H-15N HSQC of paxillin 1–160 exhibits a narrow spectral window, indicating that it is largely unstructured. However, titration of unlabeled talin-R1–R4 induced chemical shift changes or line broadening of a dozen residues (Supplementary Fig. 10a). The perturbed residues mostly belong to LD1
and LD2 (Supplementary Fig. 10a) since different set of perturbed signals disappeared upon deletion of N-terminal LD1 region (Supplementary Fig. 10b) or C-terminal LD2 region (Supplementary Fig. 10c). Representative peaks from LD1 and LD2 are selected to show their perturbations during talin-R1–R4 binding at four different ratios (Supplementary Fig. 10d). Remarkably, Alphafold262 was able to predict a model in which LD2, which binds tighter to R2 than LD1 (Supplementary Fig. 9b), chooses to bind talin-R2, whereas LD1 is bound to talin-R3 that was shown to weakly bind to paxillin 1–160 (Supplementary Fig. 6c and Table 1). Thus, it is possible that paxillin uses multiple LD motifs and surrounding regions that are spaced within hundreds of residues to engage with multiple talin subdomains, leading to stronger binding. While the atomic details of such multisite binding of the entire paxillin/tlnM3 complex (~700 kDa as a dimer) remain to be determined, Supplementary Fig. 10e at least provided some insight into how such multisite binding may occur through the interaction between paxillin 1–160 and talin-R1–R4, which represents the core unit of the paxillin/tlnM3 complex as elucidated above.

Based on the conserved LD-binding surfaces in talin-R2 (Fig. 5d and Supplementary Fig. 9d), R8 (Supplementary Fig. 9e and 9f), and
R11 (Supplementary Fig. 9g), we designed LD-binding defective mutations in talin-R2, R8, and R11 as talin-R2 A680E/K687E (AKEE) (Fig. 5e), R8 A1537E/K1544E (AKEE) (Supplementary Fig. 11a), and R11 T2126E/K2133E (TKEE) (Supplementary Fig. 11b). We confirmed that all these mutations drastically reduced the binding of these subdomains to paladin-1–160 as summarized in Fig. 5f, Supplementary Fig. 11c and 11d, respectively. We then generated these specific paladin-binding defective mutants in talin-M3, talin-M3–R2-mut (A680E/K687E, AKEE), talin-M3–R2-R11mut (A680E/K687E/T2126E/K2133E, AKEE/TKEE), and talin-M3–R2-R8–R11mut (A680E/K687E/A1537E/K1544E/T2126E/K2133E) (illustrated in Fig. 6a) to examine the effect of the talin/paladin cooperativity in inducing integrin activation. The combined mutations of talin-R2, R8, and R11 had the strongest effect when co-expressed with paladin-WT (Fig. 6b). Remarkably, when testing talin-M3–R2–R8–R11 mutant with a kindlin-2-binding defective paladin mutant (F577E)36, the PAC-1 binding was further reduced to almost the level of talin-M3 alone (Fig. 6b). These data provide strong structure-based evidence that paladin binding to talin-R is crucial for promoting integrin activation via a talin/paladin/kindlin-2 complex.

To further support the relevance of the talin/paladin/kindlin-2 complex for integrin activation and function in cells, we again used the talin1/2dko fibroblast system19,21. These cells were retrovirally transduced with constructs encoding for C-terminally ypet-tagged talin fusion proteins, which carried the above-mentioned mutations in the talin R2 (AKEE) and the talin R11 domains (TKEE), as well as combined mutations (AKEE/TKEE). Cells expressing identical ypet levels that match endogenous talin protein levels of control cells (talin1/2/3) were sorted and used for the experiments (Supplementary Fig. 12a). Moreover, all cell groups showed comparable kindlin-2, paladin, as well as integrin-surface levels as confirmed by Western blotting and flow cytometry (Supplementary Fig. 12a and 12b). We then tested whether the mutations within the talin R2 and R11 domains affect paladin binding to the cell by performing anti-GFP-immunoprecipitation experiments. Consistent with the GST-pulldown and NMR experiments, both mutants showed reduced binding to paladin with the talin R2 AKEE mutation being most disruptive (Fig. 6c). Importantly, these talin mutations also significantly reduced the amount of coprecipitated kindlin-2 (Fig. 6c), providing strong cell-based evidence that talin is connected to kindlin through paladin. However, such ternary complex, although detectable by Co-IP, was too dynamic to be co-eluted directly with size-exclusion column. It is conceivable that within cells, the talin/paladin/kindlin-2 complex is further strengthened by interactions with the plasma membrane, as both talin and kindlin-2 interact with membrane lipids42,43,65. The size of the ternary complex (~800 kDa as mediated by dimeric talinM3, ~540 kDa) is also too large to be characterized by NMR. However, considering that kindlin-2 F0 represents the major domain in binding to paladin at C-terminal LIM46, we examined 12N-labeled kindlin-2 F0 binding to paladin in the absence and presence of talin-R1–R4 (binds to paladin at 1–160). Supplementary Fig. 12c shows that while addition of paladin to 12N-labeled kindlin-2 F0 caused line broadening of many residues in kindlin-2 F0 due to its interaction with paladin (~70 kDa), addition of talin-R-R4 (~70 kDa) caused additional line broadening of many perturbed residues in kindlin-2 F0 apparently due to the talin-R1–R4-binding (to paladin) induced size increase, thus providing strong evidence for the ternary complex formation. Next, we investigated whether a disruption of the talin/paladin/kindlin-2 complex affects cell adhesion, focal adhesion formation, and cell spreading, which are initiated upon the integrin/ligand interaction. To this end, we first plated the cells on the multivalent integrin ligands commonly existing in the extracellular matrix (fibronectin, vitronectin, and laminin) and found that while talin1/2dko cells expressing ypet as a control that hardly adhered to the substrata, the R2 and R2/R11 double-mutant cells showed an average 30–40% reduction in cell adhesion, whereas the R11 mutant showed a smaller adhesion defect compared with cells expressing WT talin (Fig. 6d). We then plated the cells on FN and measured cell spreading for 4 h. While ypet-transduced talin1/2dko cells remained roundish and did not spread over time, in comparison with WT talin, both paladin-binding mutants displayed a significant spreading defect, which became even more prominent, when both sites were mutated (Fig. 6e). Consistent with our adhesion data, the R2 mutant revealed a more pronounced spreading defect compared with the R11 mutant (Fig. 6e). Next, we characterized the adhesion structures of the different cell lines by immunofluorescence staining (Supplementary Fig. 12d). Interestingly, while we observed a similar focal adhesion (FA) size between the groups, their number and the relative FA area relative to the total cell area were also strongly reduced (Fig. 6f). To further test whether impaired paladin binding affects the localization of talin to focal adhesions, we measured the intensity of ypet fluorescence within FAs in relation to the total cellular ypet fluorescence intensity. Our measurements showed significantly reduced localization of paladin-binding defective talin in FAs, suggesting that paladin–talin interaction is crucial for recruiting/maintaining talin within FAs (Fig. 6g and Supplementary Fig. 12d). Moreover, we also detected significantly less kindlin-2 in FAs (Fig. 6g and Supplementary Fig. 12d), further supporting the hypothesis that talin via paladin maintains/stabilizes kindlin-2 within FAs or vice versa. In addition, we found that the number of active β1 integrins within FAs (indicated by 9EG7 staining) was significantly reduced in cells expressing the talin R2 AKEE mutant (Fig. 6g). These data provide...
strong evidence that paxillin binding stabilizes talin as well as kindlin-2 within focal adhesion, and thus promotes integrin activation. Overall, a disruption in the talin–paxillin interaction causes reduced talin/paxillin/kindlin complex in FAs, resulting in reduced integrin activation and impaired cell adhesion and spreading.

A talin-mediated supramolecular machinery orchestrates potent integrin activation. The above data identified two critical talin-R-dependent pathways to promote talin-H-induced integrin activation: (i) talin-R-mediated talin dimerization and (ii) talin-R-mediated talin–paxillin–kindlin-2 complexation. How do these two pathways cooperate? We show that disruption of the talin dimerization significantly reduced the ability of tlnM3 to activate integrin (Fig. 7a), but disruption of both dimerization and paxillin binding led to further significant reduction in integrin activity (Fig. 7a and Supplementary Fig. 13a). Furthermore, the synergistic effect of tlnM3 and kindlin-2 on integrin activity was also reduced by the disruption of the talin dimerization, and even
more substantially reduced when both pathways were disrupted (Fig. 7b, Supplementary Fig. 13b and 13c). These data clearly demonstrate the existence of a supramolecular machinery involving dimerized talin and talin/paxillin/kindlin-2 linkage to promote potent integrin activation and cell adhesion.

Discussion

Unlike many transmembrane receptors such as GPCRs, ion transporters, cytokine receptors, and receptor tyrosine kinases, which are activated by binding to extracellular ligands, the ECM ligand binding of integrins can be induced by intracellular cues. Such unique inside-out activation process prevents spontaneous or unbalanced ligand binding to integrin, allowing tight control of many adhesion-dependent physiological responses such as platelet aggregation, leukocyte adhesion, muscle contraction, and tissue regeneration. While talin-H is well known to induce integrin-α/β CT separation that in turn triggers conformational change of the ectodomain, which is widely thought as the final step of integrin activation (Fig. 1b), our studies here revealed for the first time that talin-R also plays a crucial role in this process by dramatically enhancing the talin-H activity. Mechanistically, we showed that talin-R connects active talin dimer (via paxillin) to kindlin to form a vital integrin-activation complex to promote microclustering of integrins to potently bind to multivalent ligands that are characteristic of natural integrin ligands. Talin-R thus has a dual role in regulating the integrin activity in a spatiotemporal manner: (a) it acts as a negative regulator by masking the integrin-binding site on talin-H in the autoinhibited state of talin to prevent integrin activation; (b) upon the conformational activation of talin by PIP2, it then acts as a positive regulator by forming a vital membrane-associated integrin-activation complex.

Our detailed molecular mapping studies revealed that N- and C-termini of paxillin bind talin and kindlin, respectively, providing a basis for understanding how paxillin gets recruited to integrin-containing nascent adhesions by engaging with both talin and kindlin. Based on these analyses, we propose a significantly updated model of integrin-activation process (Fig. 7c): upon talin activation, talin-H of the activated talin disrupts the integrin-α/β CT interaction, triggering an inside-out conformational change of the receptor, which allows its initial contacts with ligands as indicated by its binding to monovalent fibronectin FN10 repeat. Meanwhile, the activated talin, which is dimerized via talin-R, clusters two talin-H-activated integrin molecules. Such clustering is further strengthened by the talin/paxillin/kindlin complex, thereby leading to preassembled active integrin microcluster to potently bind to multivalent ligand (Fig. 7c). This model is strongly supported by our extensive functional data in both CHO cells and fibroblasts that are associated with both β1 and β3 integrins. Although further studies need to be performed, other integrins likely function via the same mechanism since talin, paxillin, and kindlin are broadly expressed. It is conceptually important to emphasize here that the integrin dimer/paxillin/kindlin complex-induced integrin microclustering is part of the integrin inside-out signaling process that triggers integrin high-affinity and stable integrin-ligand interactions, but is fundamentally different from post-ligand-induced cell-adhesion events such as focal adhesion formation, cell spreading, and migration that are associated with integrin macroclustering (avidity regulation). Such microclusters of activated integrins are advantageous for forming multiple contacts with ligands leading to an immediate increase in integrin-ligand-binding strength, which may be particularly important for the rapid formation of firm adhesions, such as those required for leukocyte-endothelial interactions to withstand the shear forces of flowing blood. The integrin microclusters connected by the talin/paxillin/kindlin complex also provide a key platform for transitioning integrin inside-out activation to outside-in signaling, resulting in the assembly of nascent adhesion complexes that interact with other adapter and signaling proteins, such as ILK, FAK, and actin filaments to ensure growth and assembly of the large focal adhesion complex (Fig. 7c). How this complex process is regulated remains to be determined, but may depend on the stoichiometry of talin, kindlin, and paxillin in different cell types. Paxillin has been shown to be one of the first proteins present at sites where focal adhesions form and talin and kindlins are expressed at similar levels in various cell types. So variations in paxillin-expression levels or its local enrichment at the membrane may be critical for regulating the formation of the complex and focal adhesion assembly. Additional mechanisms may exist to fine-tune the talin dimer/paxillin/kindlin-mediated integrin microcluster formation and subsequent focal adhesion formation. For example, several recent studies observed a small degree of kindlin oligomerization under certain experimental conditions such as low-salt concentration. Such kindlin oligomerization was thought to also contribute to the talin-H/kindlin synergy. However, other studies reported a monomeric form for kindlin-2/kindlin-3 in buffers mimicking physiological conditions, and kindlin inhibits integrin activation. Thus, the role of kindlin oligomerization in general and in the context of the talin/paxillin/kindlin complex remains to be further investigated. It is important to emphasize that integrin microclustering in our model is facilitated by talin and kindlin binding to the β-CTs of two different integrins. While talin and kindlin may bind to the β-CT of the same integrin in a nonexclusive manner, it remains to be further investigated how such binding affects activation of single-integrin molecule as co-expression of thnM3 and K2 had only a small effect on the
integrin binding to monomeric ligand compared with tlnM3 alone (Supplementary Fig. 3c). It also remains to be determined if any weak talin/kindlin interaction exists beyond the detection limit of common methods, or is strengthened by unknown cellular factors such as post-translational modifications, which may regulate the integrin microclustering. However, a recent single-molecule imaging study found that talin and kindlin-2 are actually closely spaced within ~20-nm distance in association with active β1 integrins⁶⁹, supporting our finding that the two proteins do not directly associate with each other, but are bridged by the mediator paxillin to cluster different integrins.

In summary, our studies significantly advance the understanding of integrin activation through spatiotemporal regulation of talin by paxillin that further links talin dimer with kindlin into a supramolecular complex. We have found that talin-R is extremely important for such paxillin-mediated talin/kindlin linkage. The architecture of this talin/paxillin/kindlin complex machinery is not only important for promoting potent integrin activation
but also for triggering rapid post-ligand cell-adhesion events such as focal adhesion assembly, signaling, cell spreading, and migration. Future studies are needed to further investigate how this machinery is regulated under physiological and pathological conditions. Given the critical role of integrin activation in these responses, our findings may be highly valuable for guiding future development of diagnostics and therapeutics to treat many integrin-dependent diseases.

**Methods**

**Plasmid constructs and mutagenesis.** The following constructs for bacterial expression were used in this study: mouse full-length talin subcloned in a pET28T vector, human full-length paxillin subcloned in a pGST-1 vector, and mouse kindlin-2 subcloned in a pET28-SUMO vector. Talin subdomains are subcloned into pHis-1 vector or pET28T vector according to solubility and digestion sites. Paxillin fragments are subcloned into pGST-1 vector. Fibronectin repeat 10 (1541–1639, FN10) was subcloned into pET28a and pGST-1 vectors, respectively. The following constructs for mammalian expression were used in this study: human full-length paxillin (1-605) subcloned into pmsgCherry-c1 vector and human full-length kindlin-2 (1-680) subcloned into pmCherry-c1 vector. Mouse full-length talin (1–2541), talin head (1–433), and talin rod (434–2541) subcloned into pEGFP-c1 vector were obtained originally from Addgene. Construct mutagenesis was conducted by using QuickChange lightning site-directed or Multi site-directed mutagenesis Kit (Agilent Technologies). Mutated constructs generated in this study include tlnM3 (M193A, T1767L, and E177K), talinR2mut (A680E, K687E), talin8R8mut (A1537E, K1545E), talin R11mut (T2126E, K2133E), and talinD60Δ (Q2483stop). Note that pET28T is a modified pET28a vector where the thrombin-cleavage site is substituted with a TEV protease cleavage site. Primers used in this study are listed in Supplementary Table 4.

**Protein expression and purification.** Recombinant proteins with fusion tag (6xHis tag or GST tag) were expressed in Escherichia coli BL21 (DE3) strain (New England Biolabs). Typically, bacteria were initially grown in 50 ml of Lysogeny broth (LB) medium and then amplified in 2 L of LB at 37 °C. The culture was induced by 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20 °C or room temperature (RT) for overnight when it reached an A600 of 0.6. The pellet was then collected and suspended in buffer and frozen at −80 °C. For protein purification, the pellet was lysed by incubation with lysosome and sonication. After high-speed centrifugation, the supernatant was subjected to affinity-column purification by using either nickel or GST gravity column. Gel filtration was always performed in the final step by using either Superdex-75 (16/60) or Superdex-200 (16/60, GE Healthcare). Note that Superose-6 (10/300) was used for full-length talin purification.

Purified protein was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDN PAGE). The fusion tag (6x His tag or GST tag) was removed by TEV protease during protein purification when applied for NMR analysis. Isotope-labeled (15N) proteins were achieved by employing minimal medium with 15NH4Cl. Protein concentration was measured by absorbance at 280 nm or Pierce BCA protein assay kit (Thermo Fisher Scientific).

His-FN10 and GST-FN10 were purified using affinity columns and GST tag was removed by TEV protease. Both His-FN10 and GST-cleaved FN10 were further purified by size-exclusion column, which both eluted at the monomer position (<17kD according to protein marker on Superdex-75 increase 10/300 GL). Purified proteins were then subject to labeling in DPBS using EZ-link Sulfo-NHS-Biotin (Catalog#21217, Fisher) on ice for 2 h. Biotin-labeled FN10s were purified again through FPLC (Superdex-75 increase 10/300 GL) and the spectrum was overlaid with the FPLC profile of the labeling. confirming that after labeling, the His-FN10 (His-FN10-biotin) was oligomerized, eluting at ~600 kD position, while FN10 (without tag) with biotin labeling (FN10-biotin) was still eluted at ~17kD corresponding to monomer. This biotin-induced oligomer of His-FN10 was then used as multivalent ligand control for mono-FN10 in our avidation-binding assay to better characterize the talin–paxillin–kindlin-induced integrin activation.

**NMR experiments.** 2D-HSQC experiments were performed on Bruker 600-MHz NMR spectrometer with Bruker NMR Topspin software (version 4.0.6) at 25 °C (except for the titration experiments shown in Supplementary Fig. 12c, 5 °C was applied to retain homogeneous solution). Samples were generally made of 50 mM N-acetylated protein and the desired ratio of unlabeled binding partner proteins or peptides in a buffer containing 50 mM NaH2PO4/Na2HPO4 (pH 6.8), 30 mM NaCl, and 2 mM DTT (1:1-dithiothreitol) or 0.5 mM TCEP (tris(2-carboxyethyl)phosphine) in the case of full-length paxillin. In total, 1024 and 256 data points were collected in proton and nitrogen dimensions, respectively. In total, 32 scans were used to achieve sufficient signal-to-noise ratio. DSS was used to reference 2H chemical shifts and indirectly reference 15N. Standard sine window function, zero filling, and Fourier transformation were used to process the data, and the spectra were visualized via NMRPipe70 (version 9.2) and NMRFAM-SPAARKY71 (version 3). Amide nitrogen and proton chemical shifts of residues in talin-R2 and talin-R8 were assigned according to the published BMRB database with access code 17350 and 19339, respectively. Chemical shift change (Δδam); [HN, N] = [(HN, N) − (HN, N)free]/(HN, N)free, where W HN and W N are weighting factors based on the gyromagnetic ratios of 2H and 15N (W HN = 1 and W N = 0.154) and Δδ (p.p.m.) = 6 bound – 6 free.

**Docking simulations.** Models of the talin R2 and paxillin-LD2 complex were calculated from molecular docking simulations using the HADDOCK webserver (version 2.4, wemnr.science.uu.nl/haddock2.4/)72,73. Talin R2 (655–159) structure extracted from the crystal structure of human-paxillin-LD2 (141–159) structure extracted from the crystal structure of human-paxillin-LD2 complex in complex with FAK fragment (PDB code: 4XGZ), were used as starting structures. Docking restraints were derived from the chemical shift perturbations observed during paxillin 1–160 NMR titration. “Active” residues, which are mostly perturbed and solvent-accessible residues that likely take place in the binding interface, included A674, S677, A681, K687, Q708, T709, A717, S711, and T714 in talin R2. The classic interacting LD motif residues L145, D146, L148, and L152 were considered “active” in paxillin LD2. “Passive” residues, which
are solvent-exposed neighbors to “active” residues, were defined automatically by HADDOCK server. Ambiguous interaction restraints (AIRs) were defined between the active residues of talin R2 and paxillin LD2. Eleven clusters with at least 4 structures in each cluster were generated and included total 147 structures out of the final 200 calculated from HADDOCK server, which represents 73% of the water-restrained models. The statistics of each cluster is summarized in Supplementary Table 3. Overlaying all the models in the first two clusters that have the best Haddock score and contain the most structures shows a converged ensemble with heavy atoms RMSD 0.31 ± 0.11 Å and 0.26 ± 0.10 Å, respectively. Examining the representative model in each of these two clusters shows that paxillin-LD2 structures are similar but with opposite direction with respect to talin R2 domain. This is likely due to the highly symmetrically positioned Leu residues in the sequence of LD motif (Supplementary Fig. 9a), and the docking program cannot specify the interacting residue groups without unambiguous distance constraints. Examining the remaining clusters reveals complex structures more or less similar to cluster 1 or 2.

**AlphaFold2 structure prediction.** The model of talin R1–R4 and paxillin 1–160 complex was generated via the publicly available Google ColabFold notebook (github.com/sokrypton/ColabFold) that was modified from the DeepMind’s original notebook. The input includes the primary protein sequence and multiple-sequence alignment from MSeq2 without any templates. Amber-relaxation option is selected to optimize side-chain bond geometry. Structures
were visualized in PyMOL (version 2.4.1) [Schrodinger, L. L. C. The PyMOL Molecular Graphics System (2010)].

**GST pulldown.** A volume of ~25 μg of purified GST or GST-fused protein was immobilized on 15 μl glutathione-Sepharose 4B resin (beads) via incubation on a rotator for 1.5 at 4 °C in the binding buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM TCEP, and 0.01 or 0.1% NP40 (nonyl phenoxypolyethoxylethanol), and supplemented with Complete EDTA-free Protease Inhibitor (Roche). The desired amount of prey protein was then added in and incubated with beads for another 2 at 4 °C. After that, the beads were washed by 500 μl binding buffer three times and subjected to denaturation by adding 30 μl 2 × SDS loading buffer and boiling for 5 min. After centrifuging at high speed, supernatants were resolved by SDS-PAGE or western blotting. All the pull-down experiments were performed at least twice independently.

**Western blotting and antibodies.** Western blotting was performed using standard protocol. Briefly, samples resolved by SDS-PAGE were transferred onto 0.45-μm polyvinylidenefluoride (PVDF) membrane (MilliporeSigma). The membrane was blocked with 5% nonfat milk in TBST buffer 1 h at RT. After that, the membrane was incubated with primary antibody at 4 °C overnight and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at RT for 1 h. The blots were detected by Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). The following primary antibodies were used in this study: GAPDH (D61H16), Rabbit mAb (Catalog# 5174, Cell Signaling Technology), Anti-Actin (Ab-1) mouse mAb (Catalog# CP01, MilliporeSigma), GST Tag Anti-Body (Catalog# 7074S, Cell Signaling Technology), Anti-Actin (Ab-1) mouse mAb (Catalog# CP01, MilliporeSigma), GST Tag Anti-Body (Catalog# 7074S, Cell Signaling Technology), and anti-mouse IgG, HRP-linked Antibody (Catalog# 7074S, Cell Signaling Technology), and anti-mouse IgG, HRP-linked Antibody (Catalog# 7075S, Cell Signaling Technology). Primary antibodies were used at 1:1000 dilution or 1:500 (paxillin and talin 8d4 antibody), dilution, and secondary antibodies were used at 1:3000 dilution.

**Surface plasmon resonance (SPR).** SPR studies were performed using BIACore S200 (GE Healthcare) instrument in 50 mM sodium phosphate (pH 6.8), 150 mM NaCl, 0.05% Tween 20, 0.03% BSA, and 0.05% NaN₃. GST or GST-integrin beta3CT or paxillin 1-160 or talinM2 were covalently coupled to series S CM5 sensor chips via amine coupling where 10 μg/ml purified proteins in 10 mM sodium acetate (pH 4.5 or 5.0) were injected over the activated chip surface to reach a general density of 400 response units (RU) (talinM2 is immobilized 1000 or 10000RU for different tests). A series of concentrations of talin subdomains (0, 750 nM, 1.5 μM, 3 μM, 6 μM, and 12 μM) or talinM2 or talinM3 (0, 1.96 nM, 3.91 μM, 7.82 μM, 15.63 μM, 31.25 μM, 62.5 μM, and 125 μM) or paxillin (0, 7.8 μM, 15.6 μM, 31.2 μM, 62.5 μM, 125 μM, 250 μM) were prepared and injected and incubated at 20 μL/min over the surface where paxillin or integrin or talin was immobilized. All experiments were performed at 25 °C. Each test was repeated. Data were analyzed using BIAEvaluation software 1.1 (GE Healthcare) with 1:1 fitter or bivalent fitting with other biological data. All the sensorgram traces were subtracted by the reference-channel response and the zero-concentration response during data analysis.

**Size-exclusion analysis.** In all, 100 μl purified protein samples were injected into Superose-6 10/300 GL balanced with 25 mM Tris-HCl (pH 7.5) and 150 mM NaCl, and running at the flow rate of 0.5 ml/min. Elution fractions were collected as 0.5 ml per tube after 0.2CV after injection and subject to SDS-PAGE analysis.

**Integrin-activation assay.** CHO-A5 cells stably expressing integrin aIIβ3 were transfected with pEFGP-c1 constructs (talin) together with mCherry-1 constructs (paxillin or kindlin-2) using PEI (Polyethylenimine). In the case of protein knockdown, cells were pretreated with siRNA purchased from Sigma (universal negative control #1, NC, Catalog#Sc0010) or siRNA duplexes (Catalog#VC30002) following the manufacturer's instructions. Sequences of used primers are listed in Supplementary Table 4. For production of viral particles, HEK293 cells were cotransfected with the respective pLCX vector and the packaging plasmid pCI-Eco using Lipofectamine 2000 (Thermo Fisher Scientific). Virus containing HEK cell supernatants was supplemented with 5 μg/ml polybrene and transferred to talin1/2dKO cells in five infection cycles.

**Generation of cell lines and cell culture conditions.** Murine talin1 and -2 double-knockout fibroblasts (talin1/2dKO)19-22 were retrovirally transduced with pLPCX expression construct containing YFP alone, C-terminally YFP-tagged talin-1 cDNA (talin WT), YFP-tagged talin head only, YFP-tagged paxillin- binding defective talin-1 mutants (AD600, RO260), RNP input (T212E, K2133E), and R211mut, or YFP-tagged talin-1 dimerization mutants (DDeel) following the manufacturer’s instructions. Sequences of used primers are listed in Supplementary Table 4. For production of viral particles, HEK293 cells were cotransfected with the respective pLCX vector and the packaging plasmid pCI-Eco using Lipofectamine 2000 (Thermo Fisher Scientific). Virus containing HEK cell supernatants was supplemented with 5 μg/ml polybrene and transferred to talin1/2dKO cells in five infection cycles.

**Kindlin-2 was knocked out using the CRISPR/Cas9 system in talin1/2dKO fibroblasts rescued with talin H and talin WT expression constructs. Cells were electroporated with RNP complexes by the Neon Transfection system (Thermo Fisher Scientific) following the manufacturer’s protocols. Mouse anti-talin, mouse anti-GAPDH, goat anti-rabbit-HRP, and goat anti-mouse-HRP (Catalog# 115-035-003, Jackson ImmunoResearch Laboratories) antibodies following standard protocols. Mouse anti-talin, mouse anti-GAPDH, goat anti-rabbit-HRP, and goat anti-mouse HRP antibodies were used at a dilution of 1:20,000. Mouse anti-rabbit anti-talin, anti-integrin β3, anti-integrin αIIb anti-α×mAb (Catalog# 115-035-144, Jackson ImmunoResearch Laboratories), and goat anti-mouse-HRP (Catalog# 115-035-003, Jackson ImmunoResearch Laboratories) antibodies following standard protocols. Mouse anti-talin, mouse anti-GAPDH, goat anti-rabbit-HRP, and goat anti-mouse HRP antibodies were used at a dilution of 1:50,000, 1:1,000, and 1:20000, respectively. Cells were cultured under standard conditions in DMEM GlutaMAX (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and nonessential amino acids (all from Thermo Fisher Scientific). Mouse embryonic fibroblast (MEF) cell line (ATCC®CRL-1191) and the MEK-derived Kindlin-2-knockout cell line (clone3, kindly provided by Dr. Huan Liu53) were maintained in the DMEM supplemented with 10% fetal bovine serum. Cells were transfected with Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific) following the manufacturer’s instructions and analyzed after 1–2 days.

**Cells were lysed and characterized by western blotting using mouse anti-GFP (human-derived monoclonal antibody) or YFP (human-derived YFP) as a hybridized protein tag, generated at the Max-Planck-Institute of Biochemistry, Munich, Germany), mouse anti-talin (Catalog# T2387, Sigma-Aldrich), rabbit anti-talin (H-300, sc-15336, Santa Cruz), mouse anti-paxillin (Catalog# 610051, BD Transduction Laboratories), mouse anti-kindlin-2 (Catalog# MAB2617, MilliporeSigma), Anti-GFP (G5.1) XP antibody (Catalog# 2956, Cell Signaling Technology), and anti-mouse-IgG, HRP-linked Antibody (Catalog# A11121, Abcam) as secondary antibodies included Anti-rabbit IgG, HRP-linked Antibody (Catalog# 7074S, Cell Signaling Technology), and anti-mouse IgG, HRP-linked Antibody (Catalog# 7075S, Cell Signaling Technology). Primary antibodies were used at 1:1000 dilution or 1:500 (paxillin and talin 8d4 antibody), dilution, and secondary antibodies were used at 1:3000 dilution.

**FACS DIVA** Software (version 9.0). Live and positively transfected cells gated using FlowJo software (version 10) for beta3 (AP-3) and PAC-1 binding analysis (Cell-Tracker™Dimensions in Supplementary Fig. 14). WT vs knockout were comparable based on the fluorescence gating of GFP or mCherry tags. Knockdown efficiency was validated at least twice before and after overexpression by western blot. Values for PAC-1 binding were reported as median fluorescence intensities and the data were normalized to vector control and integrin-expression level detected by AP-3. Integrin-activation levels triggered by protein expression are presented by the means ± S.E.M. For mononeric FN10-biotin and multimeric His-FN10-biotin binding to cells, the proteins were incubated with cells at a concentration of 10 μg/μl at RT for 30 min and followed by 1:200 APC-streptavidin (Catalog#554067, BD Biosciences) staining.

**Statistical significance for integrin-activation data was generated by unpaired t-tests using PRISM8 (GraphPad software).**
washed with PBS and subsequently treated with 0.5 mM DSP (dithiobis(succinimidyl propionate), Thermo Fisher Scientific) dissolved in PBS for 10 min to cross-link integrins. The reaction was stopped by adding 50 mM Tris (pH 7.5) for 10 min and washing with PBS. The cells were lysed in MPER buffer (Thermo Fisher Scientific) containing protease and phosphatase inhibitors (Roche Diagnostics). About 1 mg of protein per sample was used for immunoprecipitation experiments using the mPACS GFP Isolation Kit (Miltenyi) following the manufacturer’s instructions. Recovered immunoprecipitated proteins and loading control samples were analyzed by SDS-PAGE and Western blotting.

Adhesion and spreading assays. For adhesion assays, poly(styrol) flat-bottom 96-well microplates (Greiner Bio-One, Frickenhausen, Germany) were coated with 10 μg/ml laminin (Sigma-Aldrich), 5 μg/ml fibronectin (Sigma-Aldrich), or 5 μg/ml vitronectin (Sigma-Aldrich). Cells (Köln, Germany) in coating buffer (20 mM Tris-HCl, pH 9.0, 150 mM NaCl, and 2 mM MgCl2) overnight at 4°C. After blocking with 3% bovine serum albumin in PBS to 30 min at room temperature, 4 × 10^4 cells in DMEM containing 1% FBS and 2 mM EDTA were seeded per well, incubated for 30 min (20 min for MEF paxillin knockdown rescue), and washed with PBS. Adherent cells were fixed with 4% paraformaldehyde (PFA) for 15 min and stained with 5 mg/ml crystal violet in 2% ethanol for 30 min. After washing, the remaining crystal violet was dissolved in 2% SDS and quantified by measuring absorbance at 595 nm using a microplate reader (Tecan, Männedorf, Switzerland). In the case of MEF adhesion, stained cells were imaged with 4x lens under bright field on inverted microscope with LAS X software (version 3.7.4) and cell numbers were quantified by ImageJ software. All experiments were performed in quadruplicates.

For spreading analysis, cells were plated on fibronectin (5 μg/ml)-coated dishes and phase-contrast pictures were taken using an EVOS™ FL Auto life cell imaging system (Thermo Fisher Scientific) at indicated time points after seeding the cells. Cell-spread area of 30 cells per group at each time point was measured using ImageJ software (US National Institutes of Health). Statistical significance for adhesion and spreading data was tested by One-way ANOVA followed by Tukey’s or Sidak’s multiple-comparison test using PRISM9 (GraphPad software).

Flow cytometry. Intact-surface expression was determined by FACS using a CytoFLEX LX flow cytometer (Beckman Coulter). Cells were stained and analyzed in PBS supplemented with 2% FBS and 0.05% EDTA. Cells were incubated with Alexa Fluor 647-conjugated anti-integrin β1 (Catalog# 563153), anti-integrin β3 (Catalog# 563053), anti-integrin α5 (Catalog# 564312, all three BD Biosciences), or anti-integrin α6 (Catalog# 17-0495-82, Thermo Fisher Scientific) antibodies or with biotinylated anti-integrin αV antibody (Catalog# 104103, Biolegend) and subsequently with Cy5-stained streptavidin (Catalog# 016-170-084, Jackson ImmunoResearch Laboratories). All antibodies were used at a dilution of 1:200. Data were analyzed using FlowJo software (version 10).

Immunostaining and FA analysis. Cells were cultured on fibronectin-coated glass coverslips (Thermo Fisher Scientific) for 4 h and fixed for 10 min with 4% PFA. For active β3 integrin staining, cells were incubated for 30 min with rat anti-active β3 integrin antibody (clone 9E7G; Catalog# 553715, BD Biosciences) diluted 1:100 on ice prior to fixation. The following antibodies were used for further staining following standard protocols: Mouse anti-paxillin (Catalog# 610051, BD Transduc- tion Laboratories), rabbit anti-paxillin (Catalog# ab32084, abcam), rabbit anti-total β1 integrin (described in Azimuth et al. 2013), and mouse anti-kindlin-2 (Cat- alog# MAB2617, Merck) as primary antibodies. Goat anti-mouse-Alexa Fluor 546 and goat anti-rabbit Alexa Fluor 647 (Catalog# A11003 and A21214, Thermo Fisher Scientific) as secondary antibodies. All antibodies were used at a dilution of 1:300, except rabbit anti-total β1 integrin (1:5000), Phalloidin–Alexa Fluor 647 was used at a dilution of 1:100 (Catalog# A12280, Thermo Fisher Scientific). Coverslips were acquired using a Leica TCS SP8 X confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with 63× (numerical aperture 1.40) oil objective lens and Leica ConfoScan software (LAS AF, version 3.0). All pictures were processed with ImageJ software (version 1.53k). FA area was defined by paxillin staining and quantified using ImageJ software. For quantitative analysis of talin recruitment to FAs, the total yptel signal intensity within the FA area was normalized to the total yptel fluorescence of the whole cell. Statistical significance was tested by One-way ANOVA followed by Tukey’s multiple-comparison test using PRISM9 software.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Source Data are provided with this paper. HADDOCK docking structure (for talin2/RPL2 complex) is available in ModelArchive (modelarchive.org) with the accession code ma-fuh85. AlphaFold prediction structure (for talinR1–R4/paxillin1–160 complex) is available in ModelArchive (modelarchive.org) with the accession code ma-25bs5. Referenced structures are available in Protein Data Bank with code 4FG7, 5FZ7, 1SI8, and 4XGZ. Referenced chemical shift assignments are available in BMRB chemical shift statistics with code 17350 and 19339. All data supporting the findings of this study are available from the corresponding authors qiunji@cyrc.org and m.moser@tu-muenchen.de upon reasonable request.

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Author contributions
F.L., Z.B., T.B., J.Y., Q.Y., and J.L. performed the experimental studies. E.F.P. was involved in data interpretation. F.L., L.Z., T.B., J.Y., E.F.P., Q.J., and M.M were involved in writing the paper. Q.J. and M.M. supervised the work and involved in data interpretation. F.L., L.Z., T.B., J.Y., E.F.P., Q.J., and M.M were involved in writing the paper. Q.J. and M.M. supervised the work and finalized the paper.

Competing interests
The authors declare no competing interests.

Additional information
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