Vinculin–actin interaction couples actin retrograde flow to focal adhesions, but is dispensable for focal adhesion growth

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In migrating cells, integrin-based focal adhesions (FAs) assemble in protruding lamellipodia in association with rapid filamentous actin (F-actin) assembly and retrograde flow. How dynamic F-actin is coupled to FA is not known. We analyzed the role of vinculin in integrating F-actin and FA dynamics by vinculin gene disruption in primary fibroblasts. Vinculin slowed F-actin flow in maturing FA to establish a lamellipodium–lamellum border and generate high extracellular matrix (ECM) traction forces. In addition, vinculin promoted nascent FA formation and turnover in lamellipodia and inhibited the frequency and rate of FA maturation. Characterization of a vinculin point mutant that specifically disrupts F-actin binding showed that vinculin–F-actin interaction is critical for these functions. However, FA growth rate correlated with F-actin flow speed independently of vinculin. Thus, vinculin functions as a molecular clutch, organizing leading edge F-actin, generating ECM traction, and promoting FA formation and turnover, but vinculin is dispensable for FA growth.

Introduction

Cell migration is driven by a cycle of cell edge protrusion, ECM adhesion, cell body contraction, and de-adhesion at the cell rear. Coordinating these processes requires integration of forces generated in the F-actin cytoskeleton near the leading cell edge and the formation and disassembly of integrin-based focal adhesions (FA) to the ECM (Choi et al., 2008). Leading edge protrusion is driven by F-actin polymerization in the lamellipodium generating force against the plasma membrane that pushes the leading edge forward and counter-force that pushes lamellipodial F-actin rearward, resulting in retrograde F-actin flow (Ponti et al., 2004). Proteins in nascent FA that indirectly link ECM-bound integrin cytoplasmic tails to F-actin are thought to constitute a “molecular clutch” for “engaging” lamellipodial retrograde F-actin flow (Lin and Forscher, 1995; Chan and Odde, 2008; Gardel et al., 2008; Renkawitz et al., 2009). Engagement of retrograde flow at nascent FA may provide friction that reduces flow velocity and harnesses the force of polymerization to drive membrane protrusion and generate ECM traction forces. Force on nascent FA may drive their maturation, during which they grow and recruit cytosolic proteins, which strengthen their linkage to the cytoskeleton and change their signaling properties (Balaban et al., 2001; Choi et al., 2008;
Vinculin organizes lamellipodia and lamella F-actin and mediates F-actin retrograde flow engagement at FA to generate ECM traction

To determine the role of vinculin in regulating leading edge F-actin dynamics, we imaged F-actin and FA in control and Vcl-KO MEF microinjected with X-rhodamine actin and EGFP-paxillin cDNA by time-lapse spinning disk confocal (SDC) microscopy and analyzed the images by quantitative fluorescent speckle microscopy (qFSM; Ponti et al., 2004). Control MEF exhibited typical organization of leading edge F-actin dynamics (Fig. 1 A), characterized by a lamellipodium with rapid retrograde flow (~0.45 µm/min) in a narrow (~1–3 µm) band along the cell edge adjacent to a broad lamellum region located ~5–15 µm behind the leading edge where F-actin exhibited slower retrograde flow (~0.15 µm/min; Fig. 1, B [arrow] and C; and Video 3). The junction between fast retrograde flow in the lamellipodium and slower flow in the lamellum corresponded to the site of nascent FA, as shown previously (Fig. 1 D and Video 4; Hu et al., 2007). Vcl-KO MEF exhibited a similar organization of F-actin dynamics with fast F-actin retrograde flow in the lamellipodium and slower flow in the lamellum (Fig. 1 B, arrowhead). However, F-actin flow in both lamellipodium and lamellum of Vcl-KO MEF was significantly faster than in the same regions of control cells (Fig. 1 C). Thus, vinculin slows F-actin retrograde flow in the leading edge of migrating cells.

To test whether vinculin modulates F-actin flow locally within FA, we developed algorithms to measure F-actin flow specifically within or outside of segmented FA (Fig. S2 A and computational source code in online supplemental material). This showed a comparable reduction of F-actin flow velocity within nascent FA, compared with lamellipodial areas outside nascent FA, in both control and Vcl-KO MEF (Fig. 1 E), suggesting that vinculin was not required to slow F-actin flow at FA in the nascent state. However, in control MEF, F-actin flow velocity in mature FA was significantly lower than in nascent FA, whereas in Vcl-KO MEF, F-actin flow velocity was similar in nascent and mature FA (Fig. 1 E). Consistent with this finding, control MEF showed a rapid drop of F-actin flow velocity ~1.5 µm behind the leading edge at the site of nascent FA, whereas flow velocity in FA of Vcl-KO MEF dropped more gradually with distance from the leading edge, where FA undergo maturation (Fig. S2 B).

Thus, vinculin is not required to slow F-actin flow at nascent FA in the lamellipodium but is critical for slowing F-actin flow in mature FA and for maintaining a steep flow velocity gradient between lamellipodium and lamellum.

To test whether vinculin mediates coupling of F-actin retrograde flow to the ECM to generate traction at FA, we used high resolution traction force microscopy (TFM; Sabass et al., 2008) to measure ECM traction stresses at individual FA in control and Vcl-KO MEF expressing EGFP-paxillin. As the resolution of our TFM was not sufficient to analyze nascent FA, we restricted our analysis to mature FA. This revealed significantly lower ECM-traction stresses generated by Vcl-KO compared with control FA (Fig. 1, F and G). Together, these results show that vinculin is required to slow F-actin retrograde flow and increase traction forces in maturing FA, suggesting that...
vinculin mediates F-actin flow engagement to the ECM during FA maturation.

Because engagement of F-actin flow at FA is thought to limit the width of the lamellipodium and to establish a border between lamellipodium and lamellum (Ponti et al., 2004; Alexandrova et al., 2008; Shemesh et al., 2009), we sought to test whether vinculin affected the spatial organization of lamellipodium and lamellum. We localized F-actin and the lamellipodial protein cortactin or the lamellum protein phosphoserine19 myosin light chain-2 (pS19MLC2) in control and Vcl-KO MEF (Wu and Parsons, 1993; Ponti et al., 2004; Gupton et al., 2005; Lai et al., 2008). Line scans of staining intensity across the leading edge of control MEF revealed a sharply defined, narrow band of cortactin that colocalized with dense lamellipodial F-actin (Fig. 2, A [arrow], D, and E). In contrast, cortactin staining at the leading edge of Vcl-KO MEF was significantly broader (Fig. 2, A [asterisk], D, and E) with a less defined border (Fig. 2 A, arrowhead). Line scans across the lamellum revealed a sigmoidal gradient with low amounts of pS19MLC2 near the cell edge (Fig. 2, B [asterisk] and C), whereas Vcl-KO MEF displayed a more linear gradient with considerable pS19MLC2 staining near the cell edge (Fig. 2, B [arrowhead] and C). Thus, consistent with the requirement of vinculin to engage F-actin flow at FA, vinculin is required to restrict lamellipodium width and to establish a sharp border that spatially segregates lamellipodium and lamellum.

Isoleucine 997 mutation to alanine (∆AB) perturbs vinculin binding to F-actin

To test whether vinculin regulates leading edge F-actin dynamics and organization through direct interaction with F-actin, we introduced a mutation into the vinculin tail domain that impairs F-actin binding. ∆AB impairs binding of the isolated vinculin tail domain to F-actin, but does not affect tail domain structure or acidic phospholipid binding in vitro (unpublished data). In the context of full-length vinculin ∆AB (Fig. 3 A) significantly reduces vinculin cosedimentation with F-actin in the presence of the vinculin-activating peptide IpaA (Bourdet-Sicard et al., 1999; Fig. 3 B). This corresponds to an ~10-fold decrease in the apparent dissociation constant for ∆AB (1.4 ± 0.2 μM) compared with WT (12 ± 2 μM) vinculin, resulting in an equilibrium shift of F-actin–bound versus unbound ∆AB vinculin (Fig. 3, B and C). To visualize the effect of ∆AB vinculin on F-actin organization in vitro we used fluorescence microscopy and phalloidin...
reducing F-actin flow velocity in Vcl-KO MEF to levels similar to control MEF both in the lamellipodium and within maturing FA in the lamellum (Fig. 4, A, C, and D; and Fig. 1 C). In contrast, add-back of WT or PA-AB vinculin to Vcl-KO MEF only slightly rescued the effects of Vcl-KO on F-actin retrograde flow, showing a decrease in F-actin flow in lamellipodia and maturing FA compared with Vcl-KO, but not to the same extent as add-back of WT vinculin. Immunostaining for cortactin revealed that add-back expression of either WT or PA vinculin in Vcl-KO MEF rescued the effects of vinculin deficiency, exhibiting a narrow cortactin band along the leading edge similar to control MEF (Fig. 4 B, arrows; and Fig. 1 H). In contrast, Vcl-KO MEF expressing WT or PA-AB vinculin exhibited a significantly wider cortactin band with a diffuse border, similar to those in nontransfected Vcl-KO MEF (Fig. 4 B [arrowheads] and E; and Fig. 1 H). Together, these results show that the direct interaction of vinculin with F-actin is necessary to slow F-actin flow in lamellipodia and within mature FA, and to restrict lamellipodium width to define a sharp lamellipodium–lamellum border. However, other activities of vinculin may be required for full inhibition of F-actin flow by vinculin.

Vinculin promotes nascent FA formation and turnover in lamellipodia and slows FA growth in the lamellum

The formation and turnover of nascent FA occurs within protruding lamellipodia, whereas a small fraction of nascent FA that do not disassemble in lamellipodia go on to mature in the

staining (Fig. 3 D). In the absence of IpaA, neither wild-type (WT) nor ΔAB vinculin induced F-actin bundles. In contrast, in the presence of IpaA, WT vinculin induced large F-actin bundles, whereas ΔAB vinculin did not (Fig. 3, D and E). Because F-actin binding may be critical for the release of vinculin head–tail interaction to allow vinculin activation (Bakolitsa et al., 2004), we included additional mutations (N773/E775A) to reduce the affinity of head–tail interaction to partially activate (PA) vinculin (Cohen et al., 2005) in both WT (PA vinculin) and ΔAB (PA-ΔAB vinculin) contexts as controls in our in vivo experiments (Fig. 3 A). F-actin binding experiments confirmed that PA-ΔAB vinculin was still able to be activated by IpaA, despite its decreased affinity for F-actin (Fig. 3, F and G). Importantly, expression of EGFP-tagged WT, PA, ΔAB, and PA-ΔAB vinculin variants in Vcl-KO MEF followed by immunostaining showed that all variants colocalized with paxillin in nascent and mature FA (see Figs. 6 A and S5).
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To test whether loss of vinculin specifically affects nascent FA in the lamellipodium, we analyzed FA size and spatial distribution relative to F-actin structures in control and Vcl-KO MEF by immunofluorescence staining of paxillin as FA marker and phalloidin to label F-actin (Fig. 5 A). Paxillin and F-actin intensity line scans across lamellipodia and lamella revealed high paxillin intensity within the first \( \leq 3 \) µm from the cell edge in the F-actin–dense lamellipodium of control MEF. In contrast, Vcl-KO MEF lacked paxillin in the lamellipodium and instead showed a broader paxillin peak at \( \geq 5–15 \) µm from the cell edge in the lamellum (Fig. 5 B). Quantification of individual FA size showed that Vcl-KO MEF exhibited a slight increase in mean FA size (Fig. 5 C) that was caused by a significant decrease in the fraction of nascent (<0.25 µm\(^2\)) FA and a significant increase in the fraction of midsized (0.25–3 µm\(^2\)) mature FA compared to control MEF.

Vinculin and its F-actin binding activity are required for force-mediated stabilization of mature FA (Humphries et al., 2007; Carisey et al., 2013), but it remains unclear how vinculin and its F-actin binding activity specifically regulate nascent FA formation and turnover in the lamellipodium and the transition from nascent to maturing FA in the lamellum. To answer this question, we characterized the role of vinculin and its F-actin binding activity in FA organization and dynamics in the leading edge.

We first verified that deletion of vinculin had no major effects on FA composition by immunofluorescence analyses of talin, paxillin, FAK, zyxin, VASP, and β1 integrin in Vcl-KO MEF (Fig. S3). This also revealed that control MEF displayed numerous small, peripheral FA along the cell edge and fewer mature FA in the lamellum, whereas peripheral FA were sparse and mature FA were more prominent in Vcl-KO MEF (Fig. 5 A), regardless of the FA protein examined (Fig. S3). This suggests that vinculin may affect both nascent FA formation and FA maturation.

To test whether loss of vinculin specifically affects nascent FA in the lamellipodium, we analyzed FA size and spatial distribution relative to F-actin structures in control and Vcl-KO MEF by immunofluorescence staining of paxillin as FA marker and phalloidin to label F-actin (Fig. 5 A). Paxillin and F-actin intensity line scans across lamellipodia and lamella revealed high paxillin intensity within the first \( \sim 3 \) µm from the cell edge in the F-actin–dense lamellipodium of control MEF. In contrast, Vcl-KO MEF lacked paxillin in the lamellipodium and instead showed a broader paxillin peak at \( \sim 5–15 \) µm from the cell edge in the lamellum (Fig. 5 B). Quantification of individual FA size showed that Vcl-KO MEF exhibited a slight increase in mean FA size (Fig. 5 C) that was caused by a significant decrease in the fraction of nascent (<0.25 µm\(^2\)) FA and a significant increase in the fraction of midsized (0.25–3 µm\(^2\)) mature FA compared to control MEF.

Figure 3. \( \Delta AB \) perturbs vinculin binding to F-actin. (A) Point mutations introduced into full-length vinculin to analyze the role of vinculin–F-actin binding. PA, N773A/E775A point mutations in the vinculin head domain resulting in partial activation of WT vinculin; \( \Delta AB \), I997A mutation in the vinculin tail domain perturbing F-actin binding/bundling of WT vinculin; PA-\( \Delta AB \), N773A/E775A/I997A point mutations resulting in partial activation of \( \Delta AB \) vinculin. cDNAs were expressed as EGFP fusion constructs. (B) SDS-PAGE of supernatant and pellet after high-speed cosedimentation of WT and \( \Delta AB \) vinculin with F-actin at indicated concentrations in the absence or presence of activating peptide IpaA. S, supernatant; P, pellet. (C) Densitometric quantification of F-actin cosedimentations shown in B. Data were fit for single site, saturation binding. Error bars show SEM. (D) Fluorescence micrographs of actin filaments polymerized in the absence or presence of IpaA and WT or \( \Delta AB \) vinculin. Bar, 10 µm. (E) Bar diagram of F-actin bundling induced by WT and \( \Delta AB \) vinculin in the absence or presence of IpaA. n = 1 (WT – IpaA), n = 1 (WT + IpaA), n = 3 (\( \Delta AB \) – IpaA), and n = 3 (\( \Delta AB \) + IpaA). Error bars show standard deviation. (F) SDS-PAGE of supernatant (S) and pellet (P) after high-speed cosedimentation of WT, PA, and PA-\( \Delta AB \) vinculin with actin at indicated concentrations in the absence or presence of IpaA. (G) Densitometric quantification of F-actin cosedimentations shown in F. Data were fit for single site, saturation binding. Error bars show SEM.
Figure 4. Vinculin–F-actin binding restricts lamellipodium width and limits F-actin flow velocity in lamellipodium and mature FA. (A) qFSM of Vcl-KO MEF expressing mApple-actin and the indicated EGFP-vinculin cDNA. (left to right) SDC-FSM images of F-actin (Bar, 5 μm); F-actin flow maps (Bar, 2 μm/min); and F-actin speed maps (μm/min). 5-s frame rate. (B) Cortactin immunofluorescence (purple) staining of Vcl-KO MEF expressing the indicated EGFP-vinculin cDNA. Note distinct, narrow cortactin band in WT- and PA-vinculin (arrows) expressing Vcl-KO MEF and wider, diffuse cortactin band in nontransfected, ΔAB-, and PA-ΔAB-vinculin (arrowheads) expressing Vcl-KO MEF. Bars, 2 μm. (C) Box and whisker plot of mean F-actin flow velocities in protruding lamellipodia of Vcl-KO MEF expressing the indicated EGFP-vinculin cDNA, data calculated from qFSM F-actin speed maps; n = 70 (nontransfected), n = 120 (WT), n = 35 (PA), n = 110 (ΔAB), and n = 45 (PA-ΔAB) time points during protrusion [7–22 cells/condition; means indicated; *, P < 0.01, Mann-Whitney U test]. (D) Box and whisker plot of mean F-actin flow velocities within mature FA in Vcl-KO MEF expressing the indicated EGFP-vinculin cDNA; n = 41 (nontransfected), n = 32 (WT), n = 22 (PA), n = 30 (ΔAB), and n = 22 (PA-ΔAB) FA of 7–16 cells/condition. EGFP-vinculin mutants were used as FA markers [EGFP-paxillin, nontransfected] and F-actin flow was measured on kymographs; means indicated; *, P < 0.02, Student’s t test. (E) Box and whisker plot of lamellipodium [LP] width at lpwm/2 of cortactin line scans, placed through the lamellipodium at regular intervals; n = 145 (nontransfected), 160 (WT), 150 (PA), 195 (ΔAB), and 195 (PA-ΔAB) scans from 29–39 cells/condition; means indicated; *, P < 0.001, Mann-Whitney U test.

With control MEF (Fig. 5 D and Fig. S4). Thus, vinculin promotes nascent FA in the lamellipodium and inhibits mature FA in the lamellum.

To test how vinculin altered the balance between nascent and mature FA, we analyzed FA dynamics in cells coexpressing EGFP-paxillin to label FA and mApple-actin to label lamellipodia by time-lapse total internal reflection fluorescence (TIRF) microscopy (Fig. 5 E and Video 7). This revealed that lamellipodial protrusion in control MEF was accompanied by a high density of nascent FA formation (FA formed per micrometer squared protrusion), whereas nascent FA formation density in Vcl-KO MEF was strongly reduced (Fig. 5, E and F). Because mature FA arise by growth of nascent FA (Choi et al., 2008), we reasoned that the increase in mature FA in cells lacking vinculin could result from reduced nascent FA disassembly and increased maturation frequency. To test this, we quantified the percentage of nascent FA that did not disassemble within the lamellipodium and went on to elongate in the lamellum. In control cells, most nascent FA disassembled and only 19% matured, whereas in Vcl-KO MEF, although fewer nascent FA were formed, a larger fraction (22%) of them matured (Fig. 5 G). To test whether vinculin also regulated the growth of FA after the onset of maturation, we measured FA growth rate in control and Vcl-KO MEF expressing EGFP-paxillin as an FA marker and microinjected with X-rhodamine actin using kymograph analyses of SDC time-lapse sequences (Fig. 5 H). This revealed that FA in Vcl-KO MEF elongated significantly faster than FA in control MEF (Fig. 5 I and Video 8). Neither assembly time (time to maximal paxillin fluorescence intensity) nor lifetime of nascent FA were significantly affected by vinculin loss (unpublished data). Together, these results demonstrate that vinculin promotes the formation and turnover of nascent FA in the lamellipodium, inhibits the transition of nascent FA to mature FA, and slows the rate of maturation in the lamellum.

Vinculin activation and F-actin binding promote nascent FA formation and turnover, whereas F-actin binding slows FA growth

To determine the role of vinculin–F-actin binding in FA formation and maturation, we analyzed FA in lamellipodia of Vcl-KO MEF expressing WT, PA, ΔAB, or PA-ΔAB EGFP-tagged vinculin variants by immunofluorescence staining of paxillin and cortactin (Fig. 6 A). Line scans across the leading edge of Vcl-KO MEF expressing WT or PA vinculin showed that add-back of these vinculin variants rescued the effects of vinculin loss, as indicated by a peak of paxillin intensity within the cortactin-rich lamellipodium and low paxillin levels in the lamellum (Fig. 6 B),
similar to controls (Fig. 5 B). In contrast, Vcl-KO MEF expressing ΔAB or PA-ΔAB vinculin lacked the peak of paxillin in the lamellipodium, but exhibited a broad peak of paxillin across the lamellum comparable to nontransfected Vcl-KO MEF (Fig. 6 B). Quantification of individual FA area revealed that add-back of WT vinculin in Vcl-KO cells fully rescued the effects of Vcl-KO, decreasing the mean FA size to values similar to control (Fig. 6 C and Fig. 5 C) by gain of nascent FA and loss of midsized and large FA (Fig. 6 D). In contrast, add-back of either PA-ΔAB vinculin or the vinculin head domain lacking the F-actin binding tail (Vh; amino acids 1–821) to Vcl-KO MEF did not rescue the effects of loss of vinculin on FA size, with FAs exhibiting a significantly larger size compared with add-back of WT vinculin and size distribution that was indistinguishable from Vcl-KO MEF (Fig. 6 C and D). This is consistent with the notion that activated vinculin promotes enlarged FA (Humphries et al., 2007; Carisey et al., 2013), but further suggests that F-actin binding in the tail of activated vinculin is required for limiting FA size. Add-back of PA or ΔAB vinculin to Vcl-KO partially rescued the effects of vinculin deficiency, reducing FA size via gain of nascent FA and loss of midsized FA, but not to the same extent as add-back of WT vinculin (Fig. 6 C and D). Together, these data demonstrate that both vinculin activation and F-actin binding are required for promoting nascent FA and reducing mature FA.

To determine how activation and F-actin binding of vinculin regulated the balance between nascent and mature FA, we imaged FA dynamics in Vcl-KO MEF expressing WT, PA, ΔAB, or PA-ΔAB EGFP-vinculin to label FA and mApple-actin to label lamellipodia by TIRF microscopy (Fig. 7 A and Videos 9 and 10). We first confirmed the suitability of EGFP-tagged vinculin mutants as markers for the analysis of nascent FA assembly and maturation by cotransfecting Vcl-KO MEF with EGFP-vinculin variants (WT, PA, ΔAB, or PA-ΔAB) and mApple-paxillin and testing the colocalization of paxillin and FA marker paxillin; means indicated; *, P < 0.001, Student’s t test. (G) Maturation fraction among 55 (control) and 65 (Vcl-KO) nascent FA (6 cells/genotype); means indicated; *, P < 0.001, Student’s t test. (H) SDC fluorescence time-lapse images of FA growth in control and Vcl-KO MEF; FA marker EGFP-paxillin; 10-s frame rate. Red line shows line scan positioning parallel to the long axis of FA for kymograph analyses (right panels) of FA growth (and local F-actin flow velocity; see Fig. 8 A). D, distance; T, time. Bar, 2 µm. (I) Box and whisker plot of FA (paxillin) growth rate in n = 10 (control) and n = 11 (Vcl-KO) MEF (6–10 FA/cell); means indicated; *, P < 0.001, Student’s t test.
in control (Fig. 7, B and C; Video 9; and Fig. 5, F and G). In contrast, add-back of either ΔAB or PA-ΔAB vinculin variants to Vcl-KO MEF did not rescue the effects of Vcl-KO on either formation density or maturing fraction of nascent FA (Fig. 7, B and C; and Video 10). Analysis of the growth rate of maturing FA on SDC time-lapse series of Vcl-KO MEF expressing EGFP-tagged WT, PA, ΔAB, or PA-ΔAB vinculin, or EGFP-paxillin (nonrescued Vcl-KO MEF) to label FA and mApple-actin showed that add-back expression of either WT or PA vinculin in Vcl-KO MEF rescued the effects of vinculin deficiency, restoring the slow FA growth rate seen in control MEF (Fig. 7 D and Fig. 5, H and I). In contrast, add-back of ΔAB or PA-ΔAB vinculin to Vcl-KO MEF did not fully rescue the effects of vinculin loss on FA growth rate, but reduced it to levels significantly lower than in Vcl-KO MEF. Thus, vinculin activation and binding to F-actin are both required to promote the formation and turnover of a high density of nascent FA during lamellipodial protrusion, whereas vinculin–F-actin binding and activation are required but not sufficient for slowing the rate of FA growth during maturation in the lamellum.
Recent studies have shown that overexpression of activated vinculin or vinculin lacking the F-actin binding tail domain promote contractility-independent FA hypertrophy and stabilization (Humphries et al., 2007; Carisey et al., 2013), suggesting that vinculin activation and F-actin binding may promote FA maturation. Our finding that vinculin–F-actin binding inhibits the transition of nascent FA to maturation and slows FA growth appears somewhat contradictory to these data. To determine the requirement for the vinculin–F-actin interaction and vinculin activation in FA stabilization, we imaged Vcl-KO MEF expressing GFP-tagged WT, PA, ΔAB, PA-ΔAB vinculin, or isolated Vh by confocal microscopy and quantified FA persistence as the fraction of initial FA that did not disassemble after 1 h of imaging (Fig. 7 E).

FA persistence in Vcl-KO MEF was strongly reduced compared with Vcl-KO MEF expressing WT vinculin (Fig. 7 E), consistent with the role of vinculin in stabilizing FA. In addition, add-back expression of either PA vinculin or Vh also rescued the effects of vinculin loss, promoting increased FA persistence comparable to levels in Vcl-KO MEF expressing WT vinculin (Fig. 7 E), indicating that activation of vinculin is critical to FA stabilization. In line with this, add-back of PA-ΔAB vinculin to Vcl-KO MEF rescued the effects of vinculin, whereas add-back of ΔAB vinculin did not (Fig. 7 E). Interestingly, neither PA nor PA-ΔAB vinculin or Vh increased FA persistence beyond levels seen in WT vinculin-expressing cells, suggesting that additional mechanisms besides vinculin activation and F-actin binding determine FA stability. Together, these data show that vinculin activation is critical for FA stabilization and that F-actin binding is dispensable for FA stabilization when vinculin is activated. This suggests that a key role of the vinculin–F-actin interaction in stabilizing FA may be to facilitate vinculin activation or to keep it in an activated state. Together with our previous finding that F-actin binding of PA-vinculin was required to fully restore the growth rate of maturing FA in Vcl-KO MEF (Fig. 7 D), these results suggest that vinculin modulates FA maturation and stabilization by different mechanisms.

**Vinculin regulates FA growth rate through effects on F-actin flow**

Our finding that FA grow fast (Fig. 5, H and I) and yet exert low ECM traction (Fig. 1, F and G) in cells lacking vinculin appears at odds with the controversial notion that FA maturation and
Control and Vcl-KO MEF showed indistinguishable linear correlations between FA growth and F-actin flow rates within FA (Fig. 8, B and C). Remarkably, analysis of Vcl-KO MEF coexpressing mApple-actin and either WT, PA, ΔAB, or PA-ΔAB vinculin or EGFP-paxillin (Fig. 8 D, Vcl-KO nontransfected) showed that FA growth rate was linearly related to local F-actin flow velocity, with FA growing at about half the speed of F-actin flow regardless of the presence or absence of vinculin or of the growth are force dependent (Balaban et al., 2001; Oakes et al., 2012). However, our observation that rates of FA growth and F-actin flow rates both increase in vinculin-deficient cells (Fig. 8 C and Fig. 5, H and I) suggests that growth rate of FA and F-actin flow velocity may be linked. To test this, we plotted FA growth and F-actin flow rates within individual FA measured by kymograph analyses of SDC time-lapse sequences of X-rhodamine actin and EGFP-paxillin in control and Vcl-KO MEF (Fig. 8 A).
vinculin mutant expressed (Fig. 8, E and F). Thus, FA growth correlates with F-actin flow rate, independent of vinculin, suggesting that vinculin may attenuate FA growth indirectly by effects on F-actin flow.

Discussion

Our results show for the first time that the FA protein vinculin regulates leading edge F-actin organization and dynamics. Our characterization of a single amino acid substitution in vinculin that specifically disrupts F-actin binding allows the first test of the role of F-actin binding in vinculin functions. We show that the vinculin–F-actin interaction is required to attenuate F-actin retrograde flow in the lamellipodium and for functionally delineating the protrusive lamellipodium from the contractile lamellum (Ponti et al., 2004; Hu et al., 2007; Alexandrova et al., 2008; Shemesh et al., 2009). We demonstrate that through F-actin binding vinculin slows leading edge F-actin retrograde flow at the onset of FA maturation. Together with our demonstration that vinculin promotes traction force at FA, this suggests that vinculin mediates conversion of forces generated in the cytoskeleton that drive retrograde flow into traction force on the ECM during FA maturation. These findings support previous studies showing that vinculin bears force between its head and tail domain (Grashoff et al., 2010) and that the vinculin tail domain associates with F-actin in cells (Humphries et al., 2007). Collectively, these studies implicate vinculin as a component of the molecular clutch, and suggest that vinculin exerts its role in determining the architecture of leading edge F-actin by engaging F-actin flow to the ECM at maturing FA.

Although our findings support the notion that vinculin participates in linking F-actin flow to FA, we observed a partial rescue (50–70%) of F-actin and FA dynamics in Vcl-KO MEF by F-actin binding-deficient vinculin. Thus, vinculin may also regulate F-actin dynamics by mechanisms independent of direct F-actin binding. Other vinculin binding partners that could mediate effects on F-actin dynamics include F-actin regulatory proteins such as Arp2/3 (DeMali et al., 2002) or VASP (Brindle et al., 1996) or partners such as paxillin that regulate signaling to Rho-GTPases (Turner et al., 1990; Deakin and Turner, 2008; Carisey et al., 2013). Residual F-actin binding of ∆AB or PA-∆AB vinculin could also be responsible for the partial rescue, despite the strong reduction in vinculin–F-actin binding observed in vitro. Alternatively, changes in F-actin dynamics produced by vinculin loss could be a result of secondary effects of the highly curved leading edge and lobular lamellipodial morphology observed in the absence of vinculin. However, we found that leading edge lamellipodial curvature and F-actin flow velocity were not correlated, independent of the presence of vinculin or its interaction with F-actin (unpublished data).

Our characterization of an F-actin binding point mutant also allowed us to tease out distinct roles for F-actin binding and vinculin activation in regulating FA dynamics. We found that both vinculin activation and F-actin binding are required for promoting the formation of a high density of nascent FA during lamellipodial protrusion, for inhibiting the transition of nascent to mature FA by stimulating nascent FA disassembly, and for slowing FA growth rate during maturation. However, we uncovered distinct roles for vinculin activation and F-actin binding in FA stabilization. Previous findings showed that loss of vinculin resulted in small FA (Saunders et al., 2006), whereas constitutively activated vinculin (T12) or vinculin head induced enlarged, stable FA throughout the ventral cell surface (Humphries et al., 2007; Carisey et al., 2013), demonstrating vinculin’s role in FA stabilization. We found that although F-actin binding was required for FA stabilization in the absence of vinculin pre-activation, preactivation of vinculin relieved the requirement of F-actin binding in promoting FA stabilization (Fig. 7 E). This suggests that vinculin binding to F-actin stabilizes FA by facilitating vinculin activation, rather than through the transmission of cytoskeletal forces to integrins, in agreement with the model of Carisey et al. (2013). Furthermore, our demonstration that vinculin inhibits FA maturation but promotes FA stabilization reveals that FA maturation and stabilization are mechanistically distinct processes.

By examining the rate of F-actin retrograde flow within growing FA, we surprisingly found that FA growth rate during maturation correlates with local F-actin flow speed, independent of vinculin or its activation or F-actin binding activities. Furthermore, our data reveal that vinculin promotes strong force transmission but slows growth of FA. This contradicts the notion that FA grow and mature in response to force (Balaban et al., 2001). However, this notion has been challenged by recent findings that suggest FA growth is force independent but F-actin dependent (Oakes et al., 2012). This agrees with our current finding that FA growth does not correlate with force but depends on the speed of F-actin flow. Together with our previous demonstration that F-actin flow rate dictates ECM traction stress at FA (Gardel et al., 2008), our findings strengthen the notion that the velocity of retrograde F-actin flow is a major regulator of FA function. We propose that in addition to direct regulation by interaction with FA proteins, vinculin also regulates FA dynamics by an indirect mechanism, i.e., by engaging retrograde F-actin flow to FA. Slowing F-actin flow rate by vinculin engagement of F-actin to FA could in turn reduce tension on ECM-bound integrins in nascent FA to promote their disassembly and turnover and could also reduce the F-actin flow-dependent growth rate of FA (Fig. 8 G). Together, our results show that in addition to vinculin’s well-established role in FAs vinculin also contributes to cell migration through regulation of leading edge F-actin organization and dynamics and highlight the importance of interdependent feedback between F-actin and FA in leading edge processes.

Materials and methods

Isolation of primary MEF and Vcl disruption

Animals were maintained according to guidelines approved by the National Heart, Lung and Blood Institute Animal Care and Use Committee. Mice were kept on a C57/B16 background and PCR genotyped for loxP-modified Vinculin [Vclfl; Zemljic-Harpf et al., 2007]. E13.5 embryos from Vclfl/fl x Vclfl/fl timed matings were dissected and decapitated, and internal organs were removed. Tissue was cut into pieces and incubated 3x for 10 min in 0.25 mg/ml Trypsin/EDTA (Life Technologies). Single cells were transferred into DMEM/20% FBS after each incubation. Pooled suspensions were passed through 100-µm nylon mesh, and cells were pelleted (5 min at 1,200 rpm) and plated (DMEM/20% FBS) on cell culture dishes. Non-adherent cells
Nuclei were manually tracked (MetaMorph) and fluorescence intensity of beads (40 nm; Invitrogen). Image triplets of EGFP and the two bead colors were acquired every 10 s using a 100×/1.49 NA Apo TIRF objective and a 150 nm depth of field. Long-term time-lapse phase-contrast imaging of single MEF migrating on FN-coated coverslips was performed on an inverted microscope (TE300; Nikon) using a 10×/0.25 NA Plan objective and 0.52 NA condenser. Images were taken every 10 min for 12 h. Differential interference contrast imaging of MEF migrating on FN-coated coverslips was performed on the same system using a 60×/1.49 NA Apo TIRF objective and 0.85 NA condenser. Paxillin immunofluorescence microscopy for FA size quantifications were acquired on the TIRF/SDC system described above (Shin et al., 2010) using 100× or 60×/1.49 NA Apo TIRF objective lenses or an SP5 laser scanning microscope system using a 100×/1.46 NA Plan Apo objective lens (LAS AF acquisition software; Leica). Epifluorescence images of cells stained for paxillin, cortactin, pS199/MLC2 or MLC2, and/or Alexa Fluor 488–phalloidin staining were acquired on either the TIRF/SDC or the TE300-based Epifluorescence system described above using a 100×/1.49 NA Apo TIRF objective. All functions on the TIRF, SDC, and epifluorescence microscope systems were controlled using MetaMorph software (Molecular Devices); temperature was maintained at 37°C (airstream incubator; Nevtek), and images were acquired using a cooled charge-coupled device (CoolSNAP-HQ2; Photometrics) operated in 14-bit readout mode. Laser scanning confocal microscopy of EGFP-tagged vinculin for FA persistence quantification was performed on an SP5 microscope system (LAS AF acquisition software) at 37°C (whole stage incubation chamber; Leica) using a 63×/1.4 NA Plan Apo objective lens at a frame rate of 2 min. All live cell experiments were performed using Phenol red–free DMEM containing 5% FCS, 20 mM Heps, and 10 U/ml oxyrase as imaging medium.

Immunofluorescence
Cells were fixed in 4% paraformaldehyde/PBS, permeabilized 5 min in 0.5% Triton X-100/PBS, and blocked (2% BSA and 0.02% Triton X-100/PBS) for 1 h at RT, followed by overnight incubation (4°C) with primary antibodies, 3× washing (PBS), and secondary antibody incubation (1 h at RT), before mounting in fluorescent mounting medium (Dako) or PBS (TIRF microscopy). Alexa Fluor 488–phalloidin (1:400, Invitrogen) was included in the secondary antibody solution where indicated. Antibodies used were as follows: monoclonal anti-vinculin (1:250), anti-talin (1:200) and polyclonal rabbit anti-vinculin (1:100; Sigma-Aldrich); monoclonal anti-phospho-(Y727) (1:100; Invitrogen); monoclonal anti-phospho-(Y811) (1:100; BD); monoclonal anti-phospho-(T784) (1:100; Invitrogen); rat monoclonal anti-β integrin (9EG7; 1:100); rabbit polyclonal anti-β integrin (1:100; Invitrogen); monoclonal anti-β integrin (1:100; Sigma-Aldrich); monoclonal anti-paxillin (1:100; BD); monoclonal anti-MAP-2K (1:100); rabbit polyclonal anti-cdc42 (1:100; Invitrogen); and anti-mouse– and –rabbit HRP conjugates (1:10,000; Jackson Immunoresearch Laboratories, Inc.).

Image analysis
Cell area. Cells in phase-contrast images were manually outlined (MetaMorph) and the segmented area was determined.

Cell migration velocity. Nuclei were manually tracked (MetaMorph) and instantaneous velocities (displacement per time between consecutive frames) were determined and averaged for each cell.

Spatial distribution of FA, F-actin, and pS199/MLC2. Fluorescence intensity distributions of paxillin, Alexa Fluor 488–phalloidin, and pS199/MLC2 were recorded along line scans (4–8-μm wide, orthogonal to leading edge); leading edge position was defined by increase of Alexa Fluor 488–phalloidin intensity above extracellular background and extracellular background was subtracted. Paxillin images were additionally background flattened (7×-pixel kernel) to compensate for cytoplasmic background.

FA size distribution. TIRF or SDC or laser scanning confocal images of paxillin immunofluorescence stainings were manually thresholded, binarized, and morphologically filtered (“open-close,” 2-pixel kernel) to include small FA. Segmented area of thresholded regions was then determined (MetaMorph).

Nascent FA formation density. TIRF image sequences of MEF expressing EGFP-paxillin and mApple-actin during leading edge protrusion were analyzed for the number of newly assembling nascent FA within the boundaries of the protruding lamellipodium. Diffraction-limited (0–0.1 μm²)
EGFP-paxillin fluorescence intensity maxima that were stable for >6-8 s were counted as nascent FA, whereas short-lived (2-4 s) diffusion-limited intensity maxima were excluded. The leading edge in the region of interest was outlined on each frame in the mApple-actin image to normalize adhesion counts per lamellipodial area gained.

Statistical analysis

Normally distributed data were analyzed using a two-tailed Student’s t test, unequal variance, and significance value specific for each analysis (0.05 if not mentioned). Non-normally distributed data were analyzed using a Mann-Whitney U test with significance value specific for each analysis.

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References


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