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

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

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Introduction

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.

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Kuo et al., 2011; Schiller et al., 2011). Slowing of F-actin flow at maturing FA is thought to establish a border between the lamellipodium and the adjacent F-actin structure, the lamellum (Alexandrova et al., 2008; Shemes et al., 2009). In the lamellum, actomyosin powers slow retrograde F-actin flow (Ponti et al., 2004), and forces are transmitted through mature FA to the ECM to drive cell body advance.

Despite extensive evidence for the molecular clutch hypothesis (Lin and Forscher, 1995; Hu et al., 2007; Chan and Odde, 2008; Gardel et al., 2008; Renkawitz et al., 2009), it is unclear which molecules engage F-actin retrograde flow to integrins in FA. Thus, it is not known how F-actin engagement regulates F-actin organization and FA maturation and dynamics. The integrin and F-actin binding protein talin may be part of the molecular clutch, as talin depletion results in excessive retrograde F-actin flow in spreading cells (Zhang et al., 2008). Vinculin is an F-actin and talin binding protein that bears force in FA, strengthens and stabilizes FA, is partially coupled to F-actin movement within FA, and is situated in a layer between integrins and F-actin within FA (Galbraith et al., 2002; Saunders et al., 2006; Hu et al., 2007; Humphries et al., 2007; Dumbauld et al., 2010; Grashoff et al., 2010; Kanchanawong et al., 2010). Thus, vinculin is also a candidate for a molecular clutch component and a mediator of FA maturation. However, the role of vinculin in regulating the organization and dynamics of F-actin at the leading edge in migrating cells has not been addressed. In addition, vinculin has numerous interactors in FA and lamellipodia including paxillin, Arp2/3, and vasodilator-stimulated phosphoprotein (VASP; Carisey and Ballestrem, 2011), and it is unclear whether vinculin regulates F-actin and FA dynamics by direct or indirect interaction with F-actin.

Here we report the effect of vinculin gene (Vcl) disruption on F-actin cytoskeleton and FA dynamics in migrating primary mouse embryonic fibroblasts (MEF). We find that vinculin is critical to coordinate F-actin organization and FA dynamics at the leading edge. Characterization of a vinculin point mutant that specifically disrupts F-actin binding shows that vinculin interaction with F-actin is critical for these functions.

**Results**

We sought to determine the role of vinculin in F-actin and FA organization and dynamics in cells lacking vinculin. To circumvent long-term adaptation of cells to vinculin loss, we generated vinculin-deficient primary MEF by in vitro cre recombinase-mediated excision of essential Vcl sequences in MEF from E13.5 Vcl<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup> embryos (Fig. S1 A; Zemljic-Harpf et al., 2007). Cre-mediated Vcl disruption resulted in complete loss of vinculin protein within 4 d (Fig. S1 B).

Compared with control (Vcl<sup>fl<sub>ox</sub>/fl<sub>ox</sub> adenovirus control) MEF, Vcl knockout (Vcl<sup>-KO</sup>; genotype Vcl<sup>fl<sub>ox</sub>/fl<sub>ox</sub>/adenovirus-cre</sup>) MEF were less spread, displayed narrow lamellae, lobular lamellipodia, and long tails (Fig. S1, C and D; and Video 1) and exhibited an increased random migration velocity (Fig. S1, E and F; and Video 2), all similar to vinculin-deficient clonal MEF and F9 cells (Volberg et al., 1995; Xu et al., 1998a,b; Mierke et al., 2010).

**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<sup>-KO</sup> 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<sup>-KO</sup> 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<sup>-KO</sup> 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<sup>-KO</sup> 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<sup>-KO</sup> 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<sup>-KO</sup> 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<sup>-KO</sup> 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<sup>-KO</sup> 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 regulatory 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 (ΔAB) 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. ΔAB 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 ΔAB (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 ΔAB (1.4 ± 0.2 𝜇M) compared with WT (12 ± 2 𝜇M) vinculin, resulting in an equilibrium shift of F-actin–bound versus unbound ΔAB vinculin (Fig. 3, B and C). To visualize the effect of ΔAB vinculin on F-actin organization in vitro we used fluorescence microscopy and phallolidin...
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 AB 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 AB 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 lamellum (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).

Vinculin–F-actin binding restricts lamellipodium width and slows F-actin flow in mature FA

To test whether vinculin regulates leading edge F-actin by direct F-actin binding, we performed SDC microscopy of F-actin dynamics and immunostaining of cortactin in Vcl-KO MEF expressing EGFP-tagged WT, PA, ΔAB, or PA-ΔAB vinculin and monomeric Apple (mApple)–actin (Fig. 4 A and Videos 5 and 6). qFSM and kymograph analysis showed that add-back expression of WT or PA vinculin rescued the effects of Vcl-KO, 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 ΔAB or PA-ΔAB 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 ΔAB or PA-ΔAB 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 establishes a lamellipodium–lamellum border

(A) Cortactin immunofluorescence (purple) and Alexa Fluor 488–phalloidin staining (green) of control and Vcl-KO MEF. Note wider lamellipodium in Vcl-KO (asterisk) and diffuse lamellipodium–lamellum border (arrowhead) compared to control MEF (arrow). Bar, 5 µm. (B) pS19MLC2 immunofluorescence (purple) and Alexa Fluor 488–phalloidin staining (green) of control and Vcl-KO MEF. Note low amount of pS19MLC2 in the distal lamellum of control (asterisk) and abundant pS19MLC2 in the distal lamellum of Vcl-KO (arrowhead) MEF. Bar, 10 µm. (C) pS19MLC2 and Alexa Fluor 488–phalloidin fluorescence intensity distribution along line scans perpendicular to the leading edge (mean of 25 cells/condition). (D) Cortactin fluorescence intensity distribution along line scans, placed perpendicularly to the cell edge through the lamellipodium (mean of 60 scans/condition). Width of the distribution at half maximal intensity (I_max/2) indicated. (E) Box and whisker plot of lamellipodium width at I_max/2 of cortactin line scans, placed through the lamellipodium at 15–20-µm intervals along the leading edge; n = 219 (control) and 243 (Vcl-KO) scans of 25 cells/genotype; means indicated; *, P < 0.0001, Mann-Whitney U test.
Vinculin–actin interaction regulates actin and adhesion dynamics.

Thievessen et al. (2016) showed 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 knockout MEF (Vcl−/− MEF) by immunofluorescence staining of paxillin as FA marker and phalloidin to label F-actin (Fig. 5A). Paxillin and F-actin intensity line scans across lamellipodia and lamellum revealed high paxillin intensity within the first ~3 µm from the cell edge in the F-actin–dense lamellipodium of control MEF. In contrast, Vcl−/− MEF lacked paxillin in the lamellipodium and instead showed a broader paxillin peak at ~5–15 µm from the cell edge in the lamellum (Fig. 5B). Quantification of individual FA size showed that Vcl−/− MEF exhibited a slight increase in mean FA size (Fig. 5C) that was caused by a significant decrease in the fraction of nascent (<0.25 µm²) FA and a significant increase in the fraction of midsized (0.25–3 µm²) mature FA compared to control MEF.

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−/− 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−/− MEF (Fig. 5A), 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−/− MEF by immunofluorescence staining of paxillin as FA marker and phalloidin to label F-actin (Fig. 5A). Paxillin and F-actin intensity line scans across lamellipodia and lamellum revealed high paxillin intensity within the first ~3 µm from the cell edge in the F-actin–dense lamellipodium of control MEF. In contrast, Vcl−/− MEF lacked paxillin in the lamellipodium and instead showed a broader paxillin peak at ~5–15 µm from the cell edge in the lamellum (Fig. 5B). Quantification of individual FA size showed that Vcl−/− MEF exhibited a slight increase in mean FA size (Fig. 5C) that was caused by a significant decrease in the fraction of nascent (<0.25 µm²) FA and a significant increase in the fraction of midsized (0.25–3 µm²) mature FA compared to control MEF.
expressing EGFP-paxillin as an FA marker and microinjected vinculin also regulated the growth of FA after the onset of 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 maturing 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), 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 maturing FA, and slows the rate of maturation in the lamellum.
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 turnover in lamellipodia and slowed FA growth in the lamellum. (A) Paxillin immunofluorescence and Alexa Fluor 488-phalloidin staining of F-actin in control and Vcl-KO MEF. Note abundant nascent FA in control (arrows) but not Vcl-KO (arrowhead) lamellipodia and prominent FA in the distal lamellum of Vcl-KO but not control MEF (asterisks). Bars, 10 µm. (B) Paxillin and F-actin (phalloidin) intensity distribution along line scans perpendicular to the leading edge (mean of 25 cells/condition). (C) Box and whisker plot of mean FA size in WT and Vcl-KO MEF expressing EGFP-paxillin, showing FA at t = 0 s (green) and t = 92 s (purple). Lamellipodia borders outlined based on coexpressed mApple-actin (Video 7). Note the high density of newly formed nascent FA in control compared to Vcl-KO MEF (asterisks) and the high number of nascent FA undergoing maturation in Vcl-KO compared to control MEF (arrows). 2 s frame rate. Bar, 2 µm. (D) Box and whisker plot of FA size distribution in n = 10 (control) and n = 10 (Vcl-KO) MEF; FA marker paxillin; means indicated; *, P < 0.05, Student’s t test. (E) TIRF micrographs of live control and Vcl-KO MEF expressing EGFP-paxillin, showing FA at t = 0 s (green) and t = 92 s (purple). Lamellipodia borders outlined based on coexpressed mApple-actin (Video 7).
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.

Figure 6. Vinculin activation and F-actin binding are required for promoting nascent FA and reducing mature FA. (A) SDC fluorescence micrographs of Vcl-KO MEF expressing the indicated EGFP-vinculin cDNA, immunofluorescence-stained for cortactin and paxillin. Bars, 2 µm. (B) Paxillin intensity distribution along line scans perpendicular to the leading edge of Vcl-KO MEF expressing the indicated EGFP-vinculin cDNA [mean of 20 cells/condition]. (C) Box and whisker-plot of mean FA size in Vcl-KO MEF expressing the indicated EGFP-vinculin cDNA and immunofluorescence stained for paxillin as FA marker; n = 11 [nontransfected], n = 9 [WT], n = 9 (PA), n = 12 (ΔAB), n = 10 (PA-ΔAB), and n = 17 (Vh) cells/condition; means indicated; *, P < 0.05, Student’s t test. (D) Box and whisker plot of FA size distribution in Vcl-KO MEF expressing the indicated EGFP-vinculin cDNA and immunofluorescence stained for paxillin as FA marker; n = 11 [nontransfected], n = 9 [WT], n = 9 (PA), n = 12 (ΔAB), n = 10 (PA-ΔAB), and n = 17 (Vh) cells/condition; means indicated; *, P < 0.05, Student’s t test.
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, ∆AB, or PA-∆AB 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. 1 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).

Figure 8. **FA growth rate linearly correlates with local F-actin flow velocity in FA, independently of vinculin.** [A] Kymographs through the long axis of control and Vcl-KO MEF showing FA growth rate (EGFP-paxillin, green) and local F-actin flow velocity (X-rhodamine actin, purple) of individual FA. Distal end of FA at top and proximal end at bottom. SDC time-lapses, 10-s frame rate; D, distance; T, time. [B] Plot of FA growth rate versus local F-actin flow velocity for individual FA in control and Vcl-KO MEF; n = 85 (control) and n = 102 (Vcl-KO) FA. [C] Box and whisker plot of the ratio of FA growth rate and local F-actin flow velocity for individual FA in control and Vcl-KO MEF; n = 85 (control) and n = 102 (Vcl-KO) FA; means indicated; Student’s t test. [D] Kymographs through the long axis of FA in Vcl-KO MEF expressing the indicated EGFP-vinculin cDNA showing FA growth (EGFP-paxillin, nontransfected/EGFP-vinculin variants, green) and local F-actin flow velocity (mApple-actin, purple) of individual FA. SDC time-lapses; 5-s frame rate; D, distance; T, time. [E] Plot of FA growth rate versus local F-actin flow velocity for individual FA in Vcl-KO MEF expressing the indicated EGFP-vinculin cDNA; n = 41 [nontransfected, EGFP-paxillin], n = 32 (WT), n = 22 (PA), n = 30 (∆AB), and n = 22 (PA-∆AB) FA of 9–16 cells/condition. [F] Box and whisker plot of the ratio of FA growth rate and local F-actin flow velocity for individual FA in Vcl-KO MEF expressing the indicated EGFP-vinculin cDNA; n = 41 [nontransfected, EGFP-paxillin], n = 32 (WT), n = 22 (PA), n = 30 (∆AB), and n = 22 (PA-∆AB) FA of 9–16 cells/condition; means indicated; Student’s t test. [G] Model for the role of vinculin in FA maturation. Reduced traction stress at FA lacking vinculin is associated with an increased local F-actin flow velocity and a corresponding increase in FA growth rate. We propose that vinculin regulates FA maturation by an indirect mechanism and by engaging retrograde F-actin flow to FA, thus limiting F-actin flow velocity–dependent growth of FA.
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 ΔAB or PA-ΔAB 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/Bl6 background and PCR genotyped for loxP-modified Vinculin (Vclfl/fl; 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
Fluorescence intensity. The system was also used for TIRF microscopy of EGFP-paxillin and mApple-actin during leading edge protrusion and after cell detachment via trypsin perfusion. The same system was used with a 60×/1.2 NA Plan Apo violet-corrected objective and EGFP images were captured every 10 s. 

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, 3x 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), anti-FAK (1:250; Invitrogen); rat monoclonal anti-phosphorylated myosin light chain-2 (1:50); Cell Signaling Technology). Rabbit polyclonal anti-α-actinin (1:100), anti-Vasp (1:100), anti-p519 myosin light chain-2 (1:50; Cell Signaling Technology), Rabbit polyclonal anti-α-actinin (1:100,000) was provided by M. Becker (Huntsman Cancer Institute, Salt Lake City, UT). Anti-rabbit, -mouse, and -rat fluorophore conjugates (1:50) were obtained from Jackson ImmunoResearch Laboratories, Inc.

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 p519MLC2. Fluorescence intensity distributions of paxillin, Alexa Fluor 488-phalloidin, and p519MLC2 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 (≈0.1 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.

Nascent FA assembly time and lifetime. EGFP-paxillin fluorescence intensity within nascent FA was recorded and local background was subtracted over time. Time elapsed between the onset of intensity increase and intensity maximum was defined as assembly time. FA growth. SDC time-lapses of EGFP-paxillin expressing MEF were analyzed via kymographs aligned along the axis of adhesion elongation, and growth rate was calculated as elongation at the proximal FA tip over time.

Local F-actin flow velocity within maturing FA. The same kymograph used to determine the growth of individual FA was applied to the X-hodamine/mApple channel to determine F-actin flow within the FA at the given time.

Lamellipodium width. Cortactin immunofluorescence intensity distributions along line scans (perpendicular to leading edge through the lamellipodium; 1.2-µm wide), placed at 15–30-µm intervals along the leading cell edge, were recorded in epifluorescence images. Extracellular background was subtracted, and the width of the intensity distribution at FWHM was defined as lamellipodium width.

SDC-FSM of X-hodamine actin/mApple-actin and EGFP-paxillin/EGFP-vinculin mutants. F-actin flow analysis was performed using fsmCenter software (MatLab; Mathworks) as described previously (Danuser and Waterman-Storer, 2006). Mean F-actin flow rates in lamellipodia and lamellae were quantified from F-actin speed maps of leading edge protrusion phases. Lamellipodia and lamellae areas were manually outlined for each frame based on FSM F-actin images. Lamellipodia were defined as the region of fast F-actin flow abutting the cell edge and lamellae were defined as the region just proximal to the lamellipodia. Automated FA tracking and grouping of F-actin flow velocities based on their proximity to FA was based on a custom-written FA detection and tracking algorithm (see the computational source code in the online supplemental material). In brief, EGFP-paxillin images of control and Vcl-KO MEF were filtered using a fourth order steerable filter (Jacob and Unser, 2004) and local maxima were extracted. An anisotropic Gaussian model was then fitted to each local maximum. A goodness-of-fit test on the residual of each fit and a Student's t-test on each estimated model parameter were performed to remove outliers (significance level P = 0.05). Detected features were then tracked using uTrack (Jaqaman et al., 2008), with gap closing and linear motion estimation modes enabled. Individual tracks were then grouped together to reconstruct FA over time. Grouping was achieved in a graph-matching procedure that globally maximized a pairwise score function computed between tracks. The score function evaluated the relative proximity of the two tracks and their alignment. Alignment was measured according to the orientation of the features provided by the fitting step and the main direction of the tracks. Pairs of tracks were discarded if their mean pairwise distance was >400 nm within the first 2 µm from the cell edge or >1.5 µm elsewhere. F-actin speed was then assigned to each detected FA by gathering all speckles lying within the vicinity of each adhesion. FA vicinity at each frame was defined as the set of pixels falling into a 5-pixel (335 nm) radius around the detected adhesion. Lamellipodium-lamellum border for automated, region-specific F-actin speed measurements outside of segmented adhesions was defined based on lamellipodium width measurements shown in Fig. 2 E and was set at a distance of 1.7 µm from the leading edge as a common cutoff for both control and Vcl-KO MEF.

F-actin cosedimentation assay

F-actin binding/bundling properties of vinculin variants were assessed using an adapted F-actin cosedimentation assay (Shen et al., 2011). In brief, G-actin purified from rabbit muscle acetone powder (Pel-Freez Biologicals) was polymerized with an equal volume of 2X actin polymerization buffer (20 mM Tris, 200 mM KCl, 5 mM MgCl₂, and 4 mM diithiothreitol, pH 7.5) at RT for 1 h. The reported F-actin concentration was calculated using the concentration of G-actin before polymerization. The samples were centrifuged at high speed (184,200 g) on a TLA 100 rotor (Beckman Coulter) for 30 min at 25°C. For bundling assays, 200-µl samples in actin polymerization buffer containing 20 µM of actin, 10 µM of vinculin protein, and 100 µM of IpaA peptide (Ac-NNJYKAAKDVTTTSLSKVKIKNIN-NH₂) were incubated at RT for 1 h. The reported F-actin concentration was calculated using the concentration of G-actin before polymerization. The samples were centrifuged at high speed (184,200 g) on a TLA 100 rotor (Beckman Coulter) for 30 min at 25°C. For bundling assays, 200-µl samples in actin polymerization buffer containing 20 µM of actin, 10 µM of vinculin protein, and 100 µM of IpaA peptide were incubated at RT for 1 h. Samples containing F-actin bundles were obtained by careful extraction of the supernatant after low speed centrifugation (5,000 g). The pellet was resuspended in 200 µl of 0.1% SDS buffer (0.1% SDS, 25 mM glycine, and 25 mM Tris, pH 8.3). Actin and vinculin protein in supernatant and solubilized pellet were SDS-PAGE separated (15%) and densitometrically quantified using Image software. Vinculin binding percentages were calculated using the following formula:

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\% \text{ vinculin bound} = \frac{\text{vinculin}_{\text{pellet}}}{\text{vinculin}_{\text{pellet}} + \text{vinculin}_{\text{supernant}}} 
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References


Vinculin-actin interaction regulates actin and adhesion dynamics. • Thievessen et al.