Mena binds 5 integrin directly and modulates 51 function

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Mena binds α5 integrin directly and modulates α5β1 function

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Introduction

The ECM is a mesh of proteins secreted, assembled, and remodeled dynamically by cells that contact it (Wickström et al., 2011; Hyne and Naba, 2012). Fibroblasts are a major source of ECM proteins and regulate ECM homeostasis in tissues and organs (McAnulty, 2007). Cell migration and differentiation are among many processes controlled by the ECM as it engages adhesion receptors and presents matrix-bound growth factors to cell surface receptors. The ECM protein fibronectin (FN) is a ubiquitous component of the interstitial matrix (Singh et al., 2010). Outside the bloodstream, cells typically assemble soluble FN dimers into complex meshworks of fibrils (Schwarzbaue and DeSimone, 2011), which provide a supporting scaffold that delivers multivalent, spatially organized biochemical and mechanical signals that influence cell behavior (Hyne, 2009; Huttenlocher and Horwitz, 2011; Geiger and Yamada, 2011).

Mena is an Ena/VASP family actin regulator with roles in cell migration, chemotaxis, cell-cell adhesion, tumor cell invasion, and metastasis. Although enriched in focal adhesions, Mena has no established function within these structures. We find that Mena forms an adhesion-regulated complex with α5β1 integrin, a fibronectin receptor involved in cell adhesion, motility, fibronectin fibrillogenesis, signaling, and growth factor receptor trafficking. Mena bound directly to the carboxy-terminal portion of the α5 cytoplasmic tail via a 91-residue region containing 13 five-residue “LERER” repeats. In fibroblasts, the Mena-α5 complex was required for “outside-in” α5β1 functions, including normal phosphorylation of FAK and paxillin and formation of fibrillar adhesions. It also supported fibrillogenesis and cell spreading and controlled cell migration speed. Thus, fibroblasts require Mena for multiple α5β1-dependent processes involving bidirectional interactions between the extracellular matrix and cytoplasmic focal adhesion proteins.

The predominant ECM receptors are integrins, a family of heterodimeric transmembrane proteins composed of α and β subunits that link the ECM to the cytoskeleton and transmit signals and mechanical forces bidirectionally across the plasma membrane (Hyne, 2002). Integrins are regulated by clustering and conformational changes triggered either by binding to ECM ligands or by interaction between the intracellular tails of integrin subunits and cytoplasmic proteins (Margadant et al., 2011). The β subunit cytoplasmic tails share significant sequence similarity; several cytoplasmic proteins directly bind most β subunits to regulate integrin activation, trafficking, and signaling (Calderwood, 2004; Moser et al., 2009). In contrast, α integrin subunit tails share a short, conserved membrane-proximal sequence that interacts directly with the β subunit, with proteins that regulate integrin trafficking (Ivaska and Heino, 2011), and with Sharpin, a negative regulator of integrin activation (Rantala et al., 2011). Less is known about the potential unique functions conferred by the distal, divergent cytoplasmic tails of the 18 α subunits.

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Abbreviations used in this paper: CDM, cell-derived matrix; ES, embryonic stem; FA, focal adhesion; FB, fibronectin adhesion; FN, fibronectin; FP, PPP; FX, focal complex; pFAK, phosphorylated FAK; pFAX, phosphorylated protein; pY, phosphorylated tyrosine; RNAI, Rap1-GTP–interacting adapter molecule; VASP, vasodilator–stimulated phosphoprotein.

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αVβ3 and α5β1 are the two major FN receptors (Hynes, 2002). α5β1 is the primary receptor for soluble FN and has a key role in assembling FN into fibrils, though αVβ3 can assemble fibrils in cells that lack α5β1 (Yang et al., 1999). Typically, however, the two receptors exert distinct effects on cell motility, invasion, signaling, and matrix remodeling (Clark et al., 2005; Caswell et al., 2008, 2009; Wickström et al., 2011).

Integrin-based ECM adhesions are complex structures that turn over continually and change their composition and morphology (Geiger and Yamada, 2011). New adhesions form as small integrin-rich punctae at the cell periphery; associated cytoplasmic proteins bound to integrin tails recruit additional signaling, adaptor, or actin-binding proteins (Vicente-Manzanares and Horwitz, 2011). Nascent adhesions enlarge into transient focal complexes (FXs) that mature into focal adhesions (FAs), elongated structures of variable size and composition that are connected to the distal ends of F-actin bundles. In some cell types, including fibroblasts, α5β1 exits FAs, moves toward the cell interior along stress fibers (Pankov et al., 2000), and forms stable fibrillar adhesions (FBSs) that mediate FN fibrillogenesis. FBSs are enriched for FN, α5β1, and tensin (which is absent from FXs and found only weakly in FAs; Pankov et al., 2000; Zamir et al., 2000; Zaidel-Bar et al., 2007). FBSs lack components found in FAs, including phosphorytrosine (pY)-containing proteins, vinculin, FAK, and zyxin. α5β1 drives fibrillogenesis by translocating bound FN out of FAs to FBSs: the movement generates contractile forces on the cytoskeleton and FN, causing conformational changes in FN that align nascent FN fibrils with intracellular actin bundles (Schwarzbauer and DeSimone, 2011).

Enda/vasodilator-stimulated phosphoprotein (VASP) actin-regulatory proteins have diverse roles in cell movement and morphogenesis (Drees and Gertler, 2008; Bear and Gertler, 2009; Homem and Peifer, 2009): they promote formation of longer, less-branched F-actin networks and increase F-actin elongation rates by transferring actin monomer from profilin to the intracellular path (see Fig. 3 A; Zimmermann et al., 2002; Barzik et al., 2005). Each of the three proteins can support many Ena/VASP-dependent cellular functions such as filopodial protrusion (Applewhite et al., 2007; Dent et al., 2007), formation of functional endothelial barriers (Burman et al., 2007), or stimulation of actin-based motility of the intracellular pathogen Listeria monocytogenes (Geese et al., 2002). However, Mena contains the “LERER repeat,” a unique region of unknown function, with 13 repeats of a 5-residue motif within a 91-residue span between the EVH1 domain and proline-rich core (Gertler et al., 1996).

We found that the LERER repeat interacts with the cytoplasmic tail of α5 integrin, and mediates a robust adhesion-modulated interaction between Mena and α5β1 that contributes to key α5β1 functions: FN fibrillogenesis, cell spreading, motility, and activation of adhesion-dependent signaling. We conclude that Mena is involved in both inside-out and outside-in signaling through α5β1.

Results

Relocalization of Mena to mitochondrial recruits α5

While investigating Ena/VASP- and integrin-mediated neuritogenesis (Gupton and Gertler, 2010), we observed that artificially relocalized Ena/VASP influenced α5β1 subcellular distribution. We depleted Ena/VASP from their normal locations and sequestered them on the mitochondrial surface by expressing a construct containing multiple EVH1-binding sites fused to a mitochondrial-targeting motif (FP4-Mito; Bear et al., 2000). FP4-Mito expression phenocopies defects that arise from loss of Ena/VASP function in fibroblasts, endothelial cells, neurons, and in Drosophila melanogaster, where transgenic expression of FP4-Mito phenocopies axon guidance and epithelial defects observed in Ena mutants (Bear et al., 2002; Dent et al., 2007; Burman et al., 2007; Gates et al., 2007). Despite redistribution of Ena/VASP proteins to the mitochondrial surface by FP4-Mito, localization of known Ena/VASP-binding partners such as the FA proteins zyxin and vinculin is unaffected, and no defects are evident when FP4-Mito is expressed in Ena/VASP-deficient cells (Bear et al., 2000).

Primary fibroblasts transfected with GFP-tagged FP4-Mito, and stained with anti-Mena and anti-α5 antibodies, exhibited the expected redistribution of Mena (not depicted); however, α5 integrin, which localizes to the lamellipodium, remained at the cell periphery, and only the α5 integrin was seen to colocalize with transfected Mena. These findings illustrate that Ena/VASP function in fibroblasts is dependent on α5 integrin.

The three vertebrate Ena/VASP proteins Mena, VASP, and EVL share conserved domains (Gertler et al., 1996), including: (a) an N-terminal EVH1 domain that binds to proteins that typically contain one or more EVH1-binding sites with an optimal core consensus motif of “FPFFFF” (FP4; Ball et al., 2002); however, unconventional EVH1 ligands have been identified (Boëda et al., 2007). (b) A proline-rich center, containing
Figure 1. **FP4-Mito recruits α5 integrin to the mitochondrial surface.** (A) Anti-α5 staining in wild-type primary fibroblasts (top) or in cells expressing FP4-Mito (bottom). Phalloidin staining shows F-actin distribution. (B) MV07 cells expressing GFP-Mena transiently transfected with mCherry-FP4-Mito (red) and stained for indicated adhesion component (green). (C) Pearson’s coefficients of colocalization of adhesion proteins with FP4-Mito; *, P < 0.05 compared with a shuffled image. Bars, 10 µm.
Expression of the control construct “DP4-Mito,” which cannot bind Ena/VASP, failed to recruit Ena/VASP proteins to mitochondria, and had no effect on α5 localization (Fig. S1 A). These data were confirmed by Western blot analysis of mitochondria isolated from NIH3T3 cells (Fig. S1 B).

To determine if Ena/VASP could recruit other integrins or FA components to the mitochondrial surface, we used immunostaining of cells expressing FP4-Mito: both Mena and α5 were significantly redistributed to the mitochondrial surface (Fig 1, B and C), as was a fraction of the β1 integrin pool (likely by association with α5); however, we saw no significant relocalization of αv- and α6-integrins and zyxin (Fig. 1, B and C). Therefore, Ena/VASP-dependent α5β1 recruitment to mitochondria via FP4-Mito is specific and does not affect other integrins or FA proteins tested.

It is possible that such recruitment to mitochondria occurs by capture of α5β1-containing vesicles by Ena/VASP, in which case the cytoplasmic tails of α5β1 may remain accessible to bind the mitochondrial-tethered Ena/VASP proteins directly or indirectly. To determine whether such vesicle capture occurs during a particular stage of trafficking, FP4-Mito–expressing cells were immunostained for markers of vesicle populations involved in α5β1 trafficking pathways (Caswell et al., 2009; Margadant et al., 2011): EEA1, an early endosomal marker; Rab7, for vesicles containing activated β1 integrins (Arjoner et al., 2012); and Rab11, which decorates α5β1-containing vesicles as they pass through the perinuclear recycling compartment (Margadant et al., 2011). None of the markers were enriched on the α5β1-coated mitochondria of FP4-Mito expressing cells (Fig. S1 D).

We used the FP4-Mito assay to examine the Ena/VASP–α5 integrin interaction in MV177 cells, derived from Mena/VASP double null embryos that express only trace levels of EVL (Bear et al., 2000); expression of FP4-Mito in MV177 cells failed to relocalize α5 to mitochondria (Fig. 2 A), except when GFP-Mena (but not EVL or VASP) was coexpressed (Fig. 2, A and B). To determine if endogenous Mena forms complexes with α5, we immunoprecipitated α5 from NIH3T3 cell lysates followed by Western blot analysis (Fig. 2 C and Fig. S1 C). As expected, β1 was enriched in the immunoprecipitates, as was Mena; however, neither paxillin nor p34 (a component of the Arp2/3 complex) were detected (Fig. 2 C). Therefore, Mena is present in specific complexes with α5 integrin.

The LERER repeat mediates Mena–α5 interaction

We next transfected FP4-Mito into cells that express a series of characterized GFP-tagged Mena deletion mutants, and stained them for α5 to map the regions in Mena (Fig. 3 A) required to interact with α5 (Loureiro et al., 2002). As expected, the GFP-tagged EVH1 domain of Mena was recruited to FP4-Mito–labeled mitochondria, though α5 localization was unaffected (Fig. 3, C and D), which indicates that interaction with α5 requires additional Mena sequences. A mutant lacking the proline-rich region of Mena (MenaPro) co-recruited α5 integrin to mitochondria, whereas α5 distribution was unchanged in a mutant lacking the LERER repeat (MenaΔLERER; Fig. 3, C and D); this indicates that the LERER repeat (LERER is the consensus motif repeated within this region; Fig. 3 B), but not Mena’s proline-rich central core, is required to recruit α5 to FP4-Mito–labeled mitochondria. GFP-Mena, but not GFP-MenaΔLERER, could be detected in Western blot analysis of α5 immunoprecipitates from MV177 cells that express intact GFP-Mena or GFP-MenaΔLERER (Fig. 3 E). We conclude that the LERER repeat is necessary for complex formation between Mena and α5 integrin.

α5 binds directly to the LERER repeat

Because the LERER repeat is necessary to detect the Mena–α5 complex, we asked whether it is sufficient to mediate the interaction. We expressed a GFP-LERER fusion in MV177 cells that also express mCherry-Mena. GFP-LERER was enriched significantly in peripheral FAs that contain both α5 and mCherry-Mena, but was weak/undetectable in adhesions containing only α5 or mCherry-Mena (Fig. 4, A and E); thus, targeting of GFP-LERER to α5-containing adhesions may arise because of association with the LERER repeat in Mena. The LERER repeat is predicted to form a coiled-coil structure that might dimerize or oligomerize (Fig. S2 E). To test whether intact Mena is required for GFP-LERER to localize to α5-containing adhesions, we next expressed the construct in parental MV177 cells. We found that the GFP-LERER signal is diffuse, with no significant colocalization with α5 (Fig. 4 E), although it was present in some FA-like structures present along F-actin (Fig. 4 A, bottom).

Can the LERER repeat bind directly to the α5 cytoplasmic tail? Like other Ena/VASP proteins, Mena forms stable tetramers through a coiled-coil sequence at the C terminus of the EVH2 domain (Zimmermann et al., 2002; Barzik et al., 2005). Reasoning that tetramerization could affect binding to the α5 tail, we generated constructs to express a His-tagged fusion of the LERER repeat to the Mena EVH2 domain (His-LERER-EVH2) or to the EVH2 domain alone (His-EVH2). Purified His-LERER-EVH2 or His-EVH2 proteins were mixed with purified GST fused to the GST-α5 cytoplasmic tail (GST-α5 tail), or to GST alone, immobilized on glutathione beads (see Coomassie-stained gels of purified proteins in Fig. S2, A–C). After incubation, GST and GST-α5 beads containing bound His-LERER-EVH2 or His-EVH2 were recovered, along with aliquots of unbound protein from the supernatant, and analyzed by Western blotting with anti-His antibodies. His-LERER-EVH2 (Fig. 4 B, top) but not His-EVH2 (Fig. 4 B, bottom) was detected in the fraction bound by GST-α5; neither protein was detected in the GST-bound fraction. When a fusion of the LERER repeat alone to the His tag (His-LERER) was used in the assay, His-LERER was detected in the fraction bound to GST-α5 but not to GST alone (Fig. 4 C). Therefore, the LERER repeat binds directly to the α5 tail.

Next, we delineated sequences within the α5 tail that bind Mena. A GST-α5 tail construct lacking the five C-terminal residues failed to capture His-LERER (Fig. 4 C). We asked whether the free C-terminal end of the α5 tail is required to bind Mena by expressing a full-length α5 expression construct.
Figure 2. **Mena associates with α5, recruiting it to FP4-Mito–decorated mitochondria.** (A) MV<sup>07</sup> cells expressing mCherry-FP4-Mito alone (green), or GFP-tagged Mena, VASP, or EVL, and stained with anti-α5 (red). Bar, 10 μm. (B) Pearson’s coefficients of colocalization of α5 with FP4-Mito. *, P < 0.05 from MV<sup>07</sup> cells. (C) Western blot analyses of anti-α5 immunoprecipitates from NIH3T3 cell lysates probed with anti-α5, -β1, -Mena, -Paxillin, and -p34 subunit of Arp2/3. “Lysate,” 5% of total protein used for immunoprecipitation; “IgG,” control antibody; blank lanes prevent carryover.
that contains GFP fused to its the α5 C terminus (α5-GFP; Laukaitis et al., 2001). In NIH3T3 cells cotransfected with α5-GFP and FP4-Mito, α5-GFP was not enriched significantly on the mitochondrial surface, whereas endogenous α5 was clearly recruited to FP4-Mito–decorated mitochondria (Fig. 4, D and E). To determine whether the GFP tag interfered with α5 recruitment in other fibroblast lines, FP4-Mito and α5-GFP were cotransfected into Rat2 fibroblasts and into primary mouse embryonic fibroblasts (MEFs); neither cell type showed significant recruitment of α5-GFP to mitochondria (Fig. S2 D). Although precise details of the interaction remain to be determined, these results indicate that the LERER repeat of Mena binds directly to α5 through an interaction that requires the C-terminal portion of α5.
The fraction of the ventral cell surface that contained α5 or paxillin was similar in MV D7 and GFP-Mena LERER cells, whereas in cells expressing GFP-Mena, the area of α5-positive adhesions was almost double relative to that of paxillin (Fig. 5 B). Surface levels of α5 were similar in adherent cells of both lines, as seen via FACS analyses with anti-α5 antibodies (Fig. 5 D) and via ELISA measurements of biotinylated α5 integrin (not shown), which indicates that altered distribution of α5 likely does not derive from defects in trafficking α5 to the cell surface, or maintaining it there. Thus, the LERER repeat is necessary for Mena-dependent formation or maintenance of α5-positive central FBs, normally a large fraction of the total area with α5-positive adhesions.

To confirm these results in another cell type, we isolated primary subdermal fibroblasts from perinatal V ASP-null mice that were homozygous for a conditional Mena allele (MenaFlox), and examined the formation of α5-positive FBs in culture. To excise the MenaFlox allele, cells were infected with adenovirus that expressed either GFP-Cre recombinase or GFP alone (Fig. 6, A and C). In GFP-infected control fibroblasts, Mena and α5 colocalized at the leading edge and in peripheral FAs, whereas the cell center displayed robust α5 signal (typical of FBs), but little, if any, GFP-Mena (Fig. 5, A and C). When endogenous Mena was localized by immunofluorescence in fibroblasts transiently transfected with GFP-tensin (a major component of FBs; Zamir et al., 2000), we found only weak overlap of Mena with tensin in central FBs (Fig. 5 E).

Mena's LERER repeat modulates subcellular distribution of α5

Mena and α5β1 levels vary dynamically within cell–matrix adhesions as they mature during cell spreading and migration (Zaidel-Bar et al., 2003). We explored whether the Mena–α5 interaction influences the distribution of either molecule to the different types of adhesions. In fibroblasts cultured on FN, α5β1 is in nascent FXs, FAs, and FBs. In MV D7 cells that express GFP-Mena, Mena, α5, and paxillin colocalized extensively in peripheral FAs, whereas the cell center displayed robust α5 signal (typical of FBs), but little, if any, GFP-Mena (Fig. 5, A and C). When endogenous Mena was localized by immunofluorescence in fibroblasts transiently transfected with GFP-tensin (a major component of FBs; Zamir et al., 2000), we found only weak overlap of Mena with tensin in central FBs (Fig. 5 E).

Parental MV D7 cells contained peripheral FAs with α5 and paxillin, but lacked prominent FB-like α5 signal. Similarly, MV D7 cells expressing GFP-MenaLERER contained α5, paxillin, and GFP-MenaLERER within peripheral FAs, but lacked α5-positive FBs in the cell center (Fig. 5, A and C).

The fraction of the ventral cell surface that contained α5 or paxillin was similar in MV D7 and GFP-MenaLERER cells, whereas in cells expressing GFP-Mena, the area of α5-positive adhesions was almost double relative to that of paxillin (Fig. 5 B). Surface levels of α5 were similar in adherent cells of both lines, as seen via FACS analyses with anti-α5 antibodies (Fig. 5 D) and via ELISA measurements of biotinylated α5 integrin (not shown), which indicates that altered distribution of α5 likely does not derive from defects in trafficking α5 to the cell surface, or maintaining it there. Thus, the LERER repeat is necessary for Mena-dependent formation or maintenance of α5-positive central FBs, normally a large fraction of the total area with α5-positive adhesions.
Figure 5. Distribution of α5 to FBs requires Mena. (A) MV<sup>D7</sup> cells (top) and MV<sup>D7</sup> cells expressing GFP-Mena (middle) or GFP-MenaΔLERER (bottom), plated on FN and stained for α5 and paxillin. Arrow, FBs in central region; arrowheads, FAs with peripheral paxillin. Bar, 10 µm. (B) Mean fraction of total cell area containing α5- or paxillin-positive ventral adhesions in MV<sup>D7</sup> cells, and in MV<sup>D7</sup> cells expressing GFP-Mena– or MenaΔLERER (**, P < 0.01).
peripheral FAs; and α5, but not Mena, was also present in central FBs (Fig. 6 C). In Mena-deficient cells, α5 localized to the leading edge and in peripheral FAs, but not in central FB-like adhesions (Fig. 6 C). Therefore, central FB-like α5 adhesions are lost when Mena is absent in primary fibroblasts and MV577 cells.

To test the effects of α5 deletion on Mena, primary subdermal fibroblasts isolated from perinatal mice homozygous for an α5Floxed allele (van der Flier et al., 2010) were infected with Cre-expressing or control adenovirus (Fig. 6, A and D).

Reduced α5 levels led to concomitant loss of Mena protein, but VASP levels were unaffected by the α5 deletion, indicating that the effect was specific to Mena and not to all Ena/VASP proteins. To determine whether loss of Mena results from reduced mRNA levels, we used quantitative reverse transcription PCR to analyze Cre-treated and control fibroblasts. We found that Mena mRNA levels were unaffected by α5 deletion (Fig. 6 B). Therefore, elimination of α5 in primary fibroblasts reduces Mena protein levels posttranscriptionally.

(C) Pearson’s coefficients of colocalization of indicated proteins. *, P < 0.05 from MV577 cells. (D) Surface α5 levels in MV577 cells and MV577 cells expressing GFP-Mena and GFP-MenaΔERER. Cells were incubated with antibody to detect surface-exposed α5 by FACS analysis. Expression levels were normalized to fluorescence of GFP-expressing MV577 cells, and averaged over three experiments. Error bars indicate mean ± SEM. P < 0.05. (E) Rat2 fibroblasts were transfected with GFP-tensin and stained for Mena. Bar, 15 μm.
We used established assays for α5β1 function in fibroblasts to test the hypothesis that adhesion-driven dynamics of the Mena–α5 complex have functional consequences. Fibroblast spreading on FN initiates the binding of integrins to FN, and rapid formation of actin polymerization–driven, adhesion-independent membrane extensions, followed by a distinct phase during which adhesions form dynamically and provide the traction required for further spreading (Zhang et al., 2008).

Adhesion to FN increases the amount of Mena in complex with α5

The activation state of integrins often modulates their interactions with their cytosolic binding partners. To determine whether the Mena–α5 interaction is sensitive to α5β1 activation, we immunoprecipitated α5 from adherent, suspended, and spreading cells. 30 min after plating cells on FN, significantly more Mena was in complex with α5 (Fig. 7 A) compared with adherent cells in steady-state conditions. In contrast, the amount of Mena in complex with α5 was reduced in suspended cells.

We examined cell spreading on FN by measuring the area of MV D7, MV D7+GFP-Mena, or MV D7+GFP-Mena ΔLERER 30 min after plating on FN. Fluorescence was photobleached (rectangles), and recovery was imaged over the indicated times. (D) The t 1/2 recovery of mCherry-zyxin or GFP-Mena of cells plated for 30 min on FN or laminin [LN]. **, P < 0.01. (E) Percentage of total FRAP. Error bars indicate mean ± SEM.
cells 30 min after plating on FN-coated coverslips (Fig. 7 B and Fig. S3 A). MV^D7 cells expressing GFP-Mena were significantly more spread (P < 0.01) compared with both MV^D7 cells and MV^D7+GFP-Mena^LERER cells, which spread equivalently. Therefore, adhesion-induced increases in the α5–Mena complex correlate with increased spreading on FN, supporting the possibility that direct interaction between α5 and Mena is required for optimal cell spreading.

Cell spreading requires actin polymerization, which is likely triggered by integrin-mediated signaling (Zhang et al., 2008). We tested whether Mena-dependent regulation of actin polymerization influences the effects of the α5–Mena complex on spreading by examining the spreading of MV^D7 cells expressing a Mena mutant that lacks its F-actin binding site (Mena^ΔFAB, a motif required for Ena/VASP-dependent effects on actin polymerization). We observed that Mena^ΔFAB supports cell spreading to the same extent as Mena (Fig. S3 B). Spreading cells that express Mena^ΔFAB appeared to elaborate numerous filopodia-like protrusions, which is reminiscent of cells spreading in an anisotropic manner, whereas Mena-expressing cells appeared more like cells spreading in an isotropic mode (Fig. S3 A). Furthermore, the filopodia-like structures elaborated by GFP-Mena^ΔFAB cells exhibited GFP signal along the entire shaft rather than being concentrated at the distal tip of filopodia, as is typical of Mena (Dent et al., 2007). Although the mechanism underlying Mena^ΔFAB-dependent cell spreading remain to be determined, it is clear that Mena function in α5β1-dependent adhesion plays a more critical role in early cell spreading than does Mena-dependent actin polymerization.

As fibroblasts attach to and spread on FN, Mena localizes to the leading edge and to nascent β1-positive peripheral adhesions (Zhang et al., 2008). To determine if the adhesion-dependent increase in Mena interaction with α5 affects Mena stability in FAs during spreading, we used FRAP analysis to measure the recovery dynamics after photobleaching of GFP-Mena or GFP-Mena^ΔLERER in nascent, peripheral adhesions in cells plated for 30 min on FN (Fig. 7, C–E). The t_1/2 of FRAP was significantly greater for GFP-Mena than GFP-Mena^ΔLERER (18.9 ± 1.4 s vs. 11.9 ± 1.6 s, P < 0.01), but the overall percentage of FRAP was unchanged (Fig. 7 E). In contrast, the t_1/2 of FRAP of the FA component zyxin did not vary among the three cell types (Fig. 7 D). Zyxin binds Mena directly (Drees et al., 2000) and helps localize it to FAs (Hoffman et al., 2006), and we thus conclude that expression of the GFP-Mena^ΔLERER mutant does not induce a general perturbation of FA protein dynamics. Interestingly, the t_1/2 of FRAP of Mena and GFP-Mena^ΔLERER was equivalent 24 h after plating on FN (unpublished data). When plated for 30 min on laminin (LN), an ECM protein bound by a distinct set of integrins, the dynamics of both Mena and GFP-Mena^ΔLERER were equivalent to those observed for Mena^ΔLERER in cells plated for 30 min on FN. Collectively, these data indicate that FN binding by α5β1 during cell spreading reduces the turnover of Mena, and is dependent on its LERER repeat, which mediates direct binding to α5. Because tagging α5 with a fluorescent protein blocks interaction with Mena (Fig. 4), we were unable to ascertain how binding Mena affects the dynamics of α5 integrin.

Mena concentrates α5 and increases signaling within FAs

To determine whether Mena affects the amount of α5 within adhesions and signaling downstream of α5β1, we used immunofluorescence to measure the amount of α5, FAK phosphorylated at tyrosine 397 (pFAK397), paxillin phosphorylated at residue 118 (pPAX118), and global tyrosine phosphorylation (pY) specifically in Mena or Mena^ΔLERER containing peripheral adhesions in MV^D7 cells (Fig. 8 A and Fig. S4). The signal intensity of α5 in GFP-Mena–positive FAs was significantly higher than in GFP-Mena^ΔLERER FAs (Fig. 8 B), which indicates that FAs containing Mena capable of binding α5 have higher concentrations of α5. Significantly higher levels of pFAK397 (Fig. 8 C), pPAX118 (Fig. 8 D), and pY (not depicted, P < 0.001) were observed in GFP-Mena–containing adhesions compared with GFP-Mena^ΔLERER FAs. No significant differences were observed (by either immunofluorescence or Western blotting) in levels of phosphor-FAK (pFAK), phosphor-paxillin (pPax), or pY throughout the whole cell between MV^D7 cells expressing GFP-Mena versus GFP-Mena^ΔLERER (unpublished data); this indicates that differences in α5 and downstream signaling are spatially restricted to Mena-containing adhesions. Despite their ability to promote cell spreading, GFP-Mena^ΔFAB adhesions also contained lower levels of pFAK397 compared with GFP-Mena adhesions (Fig. S4), which suggests that the F-actin binding as well as α5 binding capabilities of Mena are required for normal FAK activation.

The Mena-α5 interaction is required for normal FN fibrillogenesis

During fibrillogenesis, α5β1 is attached to FN as it moves centripetally along stress fibers, forming FBs and generating the required tension (Pankov et al., 2000; Danen et al., 2002). Because central α5β1-positive FBs are absent in MV^D7 and Mena^ΔLERER cells (Fig. 5), and pFAK is reduced (Fig. 8), we asked whether Mena–α5 and Mena–F-actin binding are required for α5β1-dependent FN fibrillogenesis. Parental MV^D7 cells and MV^D7 cells expressing GFP-Mena, GFP-Mena^ΔLERER, GFP-Mena^ΔFAB, or GFP-VASP (negative control) were plated overnight on vitronectin, then FN was added to the media for 4 h and cells were fixed and stained to identify FN fibrils (Fig. 9 and Fig. S5). MV^D7+GFP-Mena cells generated typical FN fibrils aligned with stress fibers and FBs, whereas parental MV^D7 cells and MV^D7 cells expressing either GFP-Mena^ΔLERER or GFP-VASP (Fig. 9 and Fig. S5) formed significantly less fibrillar FN, which suggests that the interaction between Mena and α5 is critical for efficient fibrillogenesis. Surprisingly, Mena^ΔFAB partially, but significantly, rescued fibrillogenesis (Fig. 9).

The Mena-α5 interaction influences cell motility

Because Mena and α5β1 exert context-dependent effects on cell motility, we explored how disrupting their interaction influences cell migration on FN. MV^D7 cells exhibit a hypermotile phenotype, migrating twice as fast as MV^D7 cells that express levels of GFP-Mena that are typical for fibroblasts (Bear et al., 2000),
Figure 8.  
Mena–α5 interaction modulates α5 density in adhesions and adhesion signaling. (A) MVΔ7 cells and MVΔ7 cells expressing GFP-Mena or GFP-MenaΔLERER were plated on FN, then stained for α5 and FAK phosphorylated at Tyr397 (pFAK397). Bar, 10 µm. (B) Mean intensities of α5 immunofluorescence. (C and D) pFAK397 (C) and the ratio of pPaxillin/Paxillin (D) were measured in Mena and MenaΔLERER-containing adhesions: α5 intensity, pFAK levels, and the ratio of pPax118/Paxillin were significantly increased in Mena compared with MenaΔLERER-containing adhesions. ***, P < 0.001.
Discussion

Cell motility is a highly regulated, dynamic process that requires continual remodeling of the cytoskeleton as well as cell–cell and cell–matrix adhesions. Involvement of Ena/VASP in these processes has been demonstrated in a wide range of systems. Although Ena/VASP influences cellular protrusion dynamics by regulating actin polymerization through a mechanism of emerging focus (Bear and Gertler, 2009; Hansen and Mullins, 2010), how Ena/VASP affects adhesion is not well understood. Here we identify a direct connection between Mena and α5, and document that it is required for fibroblast spreading on FN, fibronectin (FN) fibrillogenesis. We also show that the Mena–α5 interaction affects cell motility in 2D motility assays on planar FN, and in 3D assays in cell-derived matrix (CDM; Cukierman et al., 2001), a FN environment, and obtained results similar to those for the 2D migration assay on FN.

Figure 9. Mena–α5 interaction, but not Mena–F-actin interaction, is necessary for normal fibrillogenesis. [A] MVΔ7 cells and MVΔ7 cells expressing GFP-Mena, GFP-MenaΔLERER, or GFP-MenaΔFAB were plated on vitronectin over night, and incubated with 10 µg/ml of fluorescently tagged FN for 4 h before fixation. Bar, 10 µm. [B] Percentage of cell area containing FN fibrils. [C] Total amount of FN within fibrils per cell. **, P < 0.01; ***, P < 0.001.
The Mena–α5 interaction requires the last 5 of the 28 residue α5 cytoplasmic tail, and is blocked by tagging the tail at its C terminus. Mena binds to α5 via the LERER repeat, a region spanning 91 or 121 amino acids with 13 or 15 repeats of the five-residue LERER motif in mouse and human, respectively. Whether each repeat binds an α5 tail is unknown, but multiple α5 tails could bind LERER repeats within each subunit of a Mena tetramer, raising the possibility that Mena clusters α5β1, thereby strengthening FN binding by increased avidity. Mena promotes actin polymerization in cell protrusions (Bear and Gertler, 2009), FAs, and sarcomeric units along F-actin bundles attached to FAs of endothelial cells (Furman et al., 2007). Given Mena’s role in actin polymerization, it was surprising that MenaΔFAB, which does not bind F-actin or regulate actin dynamics, supports significant levels of FN fibrillogenesis; this suggests that Mena’s role in this process can be, in part, uncoupled from its effects on actin dynamics. Mena may also link indirectly to the actin cytoskeleton by association with other FA components that bind F-actin. Direct Mena F-actin interaction is required to mediate α5β1 outside-in signaling that regulates pFAK397 levels.

In primary fibroblasts that normally express both α5 and Mena, acute depletion of α5 causes a reduction in Mena levels either by blocking Mena translation or inducing its degradation. Consistent with this idea, integrins and FA proteins form complexes with the mRNA translation machinery (de Hoog et al., 2004; Humphries et al., 2009), and adhesion to FN triggers α5β1-dependent translation (Gorrini et al., 2005; Chung and Kim, 2008). FA proteins are also regulated by proteolytic enzymes (Franco and Huttenlocher, 2005) and by ubiquitin-mediated proteasome degradation (Huang et al., 2009). Mena and α5 are also normally expressed in cells that lack the other (e.g., cortical neurons contain Mena but lack α5; unpublished data), which suggests that cells expressing both proteins have specific regulatory mechanisms for coordinating levels of Mena with α5.

The Mena–α5 complex is also regulated by, and necessary for, outside-in signaling by α5β1. Mena–α5 complex formation is driven by adhesion to FN. Mena binding to α5 also causes formation of FAs with higher concentrations of α5: this may reflect enhanced α5β1 clustering and binding to FN via increased avidity, though further work is needed to test this possibility. Mena binding to α5 is also necessary for signaling downstream of α5β1, as indicated by reductions in pFAK397, pPAX118, and global pY in adhesions that contain GFP-MenaΔLERER relative to those containing GFP-Mena. Based on these findings, we propose that Mena is a key modulator of α5β1-mediated bidirectional signaling between ECM and the actin cytoskeleton.

In addition to these roles in inside-out regulation of α5β1, the Mena–α5 complex is also regulated by, and necessary for, outside-in signaling by α5β1. Mena–α5 complex formation is driven by adhesion to FN. Mena binding to α5 also causes formation of FAs with higher concentrations of α5; this may reflect enhanced α5β1 clustering and binding to FN via increased avidity, though further work is needed to test this possibility. Mena binding to α5 is also necessary for signaling downstream of α5β1, as indicated by reductions in pFAK397, pPAX118, and global pY in adhesions that contain GFP-MenaΔLERER relative to those containing GFP-Mena. Based on these findings, we propose that Mena is a key modulator of α5β1-mediated bidirectional signaling between ECM and the actin cytoskeleton.
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"We show that rescue of the MVD7 hypermotile phenotype by GFP-Mena requires the LERER repeat; we have also reported that GFP-Mena and GFP-MenaLERER rescue the MVD7 hypermotility phenotype equivalently, as do GFP-VASP or GFP-EVL (Loureiro et al., 2002). We verify that GFP-MenaLERER is expressed stably, with a subcellular distribution similar to that of GFP-Mena (see also Loureiro et al., 2002). The divergent results may derive from differences in methods and reagents (including FN) used in the earlier study, or the use of cells adapted to CO2-independent media as opposed to the current enclosed environmental chamber that we used for live-cell imaging. Our current sample size is also much larger: 372 MVD7 cells expressing GFP-MenaLERER from four separate 12-h time-lapse movies were analyzed, compared with 22 cells from two separate 4-h experiments in the older study.

Why is the LERER repeat required for Mena to rescue MVD7 cell spreading and motility? The interaction with α5β1 potentially allows Mena to influence cell motility through a variety of mechanisms, including modulation of adhesion strength and changes in outside-in signaling that affect other components of the motility machinery. Additionally, Ena/VASP deficiency reduces cellular capacity to generate actin-driven protrusive forces that drive lamellipodial and filopodial extension and propulsion of the intracellular pathogen Listeria monocytogenes, even though the actin networks formed during these processes are organized differently. Expression of Mena, VASP, or EVL rescues the actin polymerization–dependent phenotypes arising from deficiency of Ena/VASP in MVD7 cells or in primary neurons from triple Mena/VASP/EVL-null embryos (Loureiro et al., 2002; Geese et al., 2002; Appelwhite et al., 2007; Dent et al., 2007). In general, Ena/VASP activity produces longer, sparsely branched filament networks; in the absence of stabilizing interconnections, these increasingly buckle against the membrane as they elongate because of their inherent flexibility (Mogilner and Oster, 2003). By coupling its stimulatory effect on barbed end elongation with its ability to bind and potentially cluster α5β1, Mena could present activated but unbound integrins right at the tips of lamellipodia and filopodia, which is consistent with the proposed “sticky fingers” mechanism for haptotaxis (Galbraith et al., 2007). In addition, through its role in FN remodeling, Mena may help form the interstitial fibrillar network that serves both as a migration substrate as well as a template that organizes growth factors and other ECM components into spatially organized cues. These cues elicit complex, coordinated responses (Hynes and Naba, 2012) when touched by the sticky fingers of cells in transit.

Recently, both α5β1 (Caswell et al., 2008; Valastyan et al., 2009; Muller et al., 2009) and Mena (Philippar et al., 2008; Robinson et al., 2009; Roussos et al., 2011a) have been implicated in breast cancer invasion and metastasis through effects on EGFR (Gertler and Condeelis, 2011). During tumor progression, changes in alternative splicing produce additional, functionally distinct Mena protein isoforms coexpressed with the canonical isoform. Menaα5V, a Mena isoform expressed in..."
a subpopulation of highly invasive, motile, and chemotactic tumor cells (Goswami et al., 2009), has been detected in breast cancer patients with invasive ductal carcinomas (Roussos et al., 2011b). Mena(1) expression promotes tumor cell invasion and metastasis by a mechanism involving increased tumor cell sensitivity to EGF (Philippart et al. 2008; Roussos et al., 2011a).

Interestingly, EGFR is sometimes found in complexes with sensitivity to EGF (Philippar et al. 2008; Roussos et al., 2011a). α5β1-RCP association with EGFR leads to coordinated recycling that targets α5β1 and EGFR to the front of cells, promotes 3D invasion, and dysregulates signaling downstream of both receptors. The potential functional and biochemical links between Mena(1) and EGFR during tumor progression are an important topic for further investigation.

Materials and methods

Western blotting/immunoprecipitation

Standard procedures were used for protein electrophoresis, Western blotting, and immunoprecipitation. Western blots were developed with HRP-tagged secondary antibodies and ECL reagent (GE Healthcare). For α5 integrin immunoprecipitation, cells were lysed with intermittent agitation for 20 min at 4°C in CSK buffer (Humphries et al., 2009) and passed through a 23.5-gauge needle; the supernatant was saved after spinning for 13 min at 21,000 g. Lysates were precleared with protein A beads for 2 h, incubated with an α5 integrin antibody (1928; Millipore) for 2 h at 4°C, and then captured with BSA-blocked protein A beads for 2 h. Beads were washed three times in lysis buffer, and proteins were eluted in sample buffer. Western blots were probed with antibodies to α5 integrin (sc-166681; Santa Cruz Biotechnology, Inc.), β1 integrin (ab1950; Abcam), vinculin (Sigma-Aldrich), p34 (07-227; EMD Millipore), and VASP polyclonal (Lanier et al., 1999). Function-blocking α5 antibody IIIG2 was purchased from the Developmental Studies Hybridoma Bank and used at 20 μg/ml.

Mitochondrial purification

Mitochrondria were isolated from NIH3T3 cells that expressed either FP4-Mito or FP4-Mito, with use of paramagnetic beads conjugated to an antibody specific for mitochondrial protein Tom34 (as per the manufacturer’s instructions; Milheny Biotech).

Binding assays

GST-α5 constructs and His-tagged variants of the LERER repeat region were expressed and purified from Escherichia coli. 10 nM of α5 integrin cytoplasmic tail was immobilized on Glutathione beads and incubated at 4°C for 1 h, with 200 nM HisLERER variants at constant agitation in PBS with 0.1% Triton X-100 and 2 mM βME. Beads were washed three times, and proteins were eluted in sample buffer and assayed by Western blotting.

Microscopy

Cells were fixed for 20 min in 4% paraformaldehyde in PBS buffer warmed to 37°C; they were permeabilized in 0.2% Triton X-100 and blocked in 10% donkey serum. Primary antibodies used for immunofluorescence include α5 integrin (1928; Millipore), integrin α (P5/2) (ab25247; Abcam), integrin α (RMW7) (ab63490; Abcam), integrin α (GoH3) (ab105669; Abcam), vinculin (Sigma-Aldrich), Mena, GFP (JL-8; Takara Bio Inc.), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 2118; Signal Transduction Laboratories), porin (A-21317; Molecular Probes), tubulin (DM1A), His tag (H1029; Sigma-Aldrich), pTyr (4G10; EMD Millipore), and VASP polyclonal (Lanier et al., 1999). Function-blocking α5 antibody IIIG2 was purchased from the Developmental Studies Hybridoma Bank and used at 20 μg/ml.

Imaging assay

Cell masks of cell area were made by thresholding phalloidin images. Subsequently, thresholding was done to even up adhesion structures between cells within these masks, and the intensity and area of these regions were measured. For analysis of photobleaching data, images were first corrected for overall photobleaching, and the integrated fluorescence intensity (Fr) inside a region that was smaller than the original bleached region by 4 pixels in x and y in each image was measured in the prebleach and recovery image series. Calculation of the t1/2 for recovery and the percent fluorescence recovery was performed as described previously (Bulinski et al., 2001). In brief, cellular background was subtracted from these data, and the decay of fluorescence (photo-bleaching) over the same time period in an unbleached portion of a different adhesion was fitted with an exponential decay curve [F(t) = F0e(-kt)], in which F(t) is the fluorescence at any time, F0 is the initial fluorescence, and k is the fluorescence decay constant. Decay-corrected FA fluorescence was plotted against time of recovery and fitted to an exponential recovery curve: F = Fr + [F0(1 - e(-kt))], in which F0 is the fluorescence at the time of bleaching, and Fr is the fluorescence at t = ∞ (that is, fluorescence completely recovered). This equation was used to determine kFr. The t1/2 was calculated as ln(2)/kFr and the percent recovery was calculated as [F(t = ∞) - F(t) / Fr] × 100.

Pearson’s coefficients of colocalization were calculated using the Intensity Correlation Analysis Plugin available for ImageJ.

Statistical analysis

The paired Student’s t test was used for statistical analyses of experiments with two conditions. In the cases of three or more conditions, analysis of variance (ANOVA) was used with the least significant difference post hoc test. Significant differences are indicated throughout as: * P < 0.05; ** P < 0.01; and *** P < 0.001.

Cell culture and plasmids

Coverslips were coated with 10 μg/ml bovine FN (Sigma-Aldrich) for 2 h at 37°C. Primary meningeal fibroblasts were cultured with cortical neurons, isolated from embryonic day 14.5 mice as described previously (Dent et al., 2007). In brief, cortices were microdissected, trypsinized for 30 min at 37°C, pelleted, and plated and maintained in neurobasal medium (Gibco) supplemented with B27 and l-Glutamine at 37°C, 5% CO2. Perinatal subdermal fibroblasts were isolated from postnatal day 1 mice that harbored either floxed α5 integrin (van der Flier et al., 2010) or floxed Mena. Pups were washed in PBS, placed in 1% iodine for 1 min, following with 70% EtOH for 1 min, and washed twice in PBS. After decapitation, the skin was removed. Dermis and epidermis were separated by placing in...
Eagle’s minimal essential medium supplemented with 2.4 U/ml dispase (Roche) at 4°C overnight. Dermis was then separated and digested in 0.1% collagenase I + II in DME with 10% FBS for 30 min 37°C. They were then passed through a 70-mm filter, pelleted, and plated.

NIH3T3 cells, Rat2 cells, and perinatal fibroblasts were cultured in DME supplemented with 10% fetal bovine serum and maintained at 37°C, 5% CO2. Parental MVA5 cells and MVA5 cells expressing tagged Mena and Mena mutants were maintained at 32°C, 5% CO2 in DME supplemented with α-Glutamine, penicillin and streptomycin, 15% fetal bovine serum, and 50 U/ml interferon-γ (I-4777; Sigma-Aldrich; Bear et al., 2000). mCherry-Fp4-Mito, pMSCV-GFP-LERER, and pGFP-a5 integrin were introduced into MVA5 cells with use of Lonza nucleofection according to the manufacturer’s instructions. pMSCV-GFP-LERER, pMSCV-GFP-MenaΔLERER, pGEX-GST-a5 cytoplasmic tail, pGEX-GST-a5 cytoplasmic tail ΔCOOH, pQE80L-His-LERER, pQE80L-His-LERER-CoC, pQE80L-His-LERER-EVH2, and pQE80L-His-EVH2 were cloned according to standard cloning procedures with N-terminal tags. N-terminally tagged mCherry-Fp4-Mito has been described previously (Bear et al., 2000). N-terminally tagged GFP- tensin (full length, chicken) was a gift from K. Yamada (National Institutes of Health, Bethesda, MD) and was introduced into Rat2 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s directions. GFP-a5 integrin (Laukaitis et al., 2001) was purchased from Addgene.

FN fibrillogenesis

FN-depleted medium was prepared as described previously (Pankov and Momchilova, 2009). In brief, 10 ml of gelatin Serum SeraLab AS 48 was washed with sterile PBS three times. After removing the third wash, 10 ml of FBS was added, rocked for 30 min at room temperature, collected, aliquoted, and stored at −20°C. FN was fluorescently labeled with 549-NHS ester from Thermo Fisher Scientific (46407), as directed by the manufacturer. MVA5 cells were seeded on coverslips coated with 10 µg/ml vitronectin from Sigma-Aldrich (V9881) and allowed to adhere overnight. Medium was replaced with FN-depleted growth medium containing 10 µg/ml fluorescently labeled FN and incubated at 32°C for 4 h. Cells were then fixed and immunostained.

Motility analysis

MVA5 cells were stained with 1 µM CMFDA (Invitrogen) and seeded overnight in growth medium at 2,000 cells/cm² on FN (10 µg/ml)-coated coverslips. Media was replenished directly before imaging to facilitate addition of 10 µg/ml of a5 blocking antibody (SH1027, MERS, BD) where applicable. 2D migration was quantified by recording cell centroid position, a 5% CO2, 37°C environment, chamber fluorescent light source, and 10× Plan-Fluar objective lens. Resulting images were semiautomatically tracked using Imaris software (Bitplane, Inc.). A custom Matlab (Mathworks) script was used to calculate migration parameters and create wind-rose plots. Cell speed is reported for the final 6 h of the experiment to ensure steady state.

CDM

CDM were prepared as directed by King and Parsons (2011). In brief, perinatal dermal fibroblasts (Lonza) were seeded at 50,000 cells/cm² on coverslips coated with cross-linked gelatin for 10 d in FGM2 media (Lonza) supplemented with 5 µg/ml FN and 35 µg/ml ascorbic acid. Cells were extracted with 20 mM Na4H4O + 0.5% Triton X-100, and CDMs were washed extensively and kept at 4°C in PBS + penicillin/streptomycin. Matrix containing coverslips were placed in 12-well tissue culture plates and adhered with epoxy (3M). Fibroblasts (MVA5 variants; MVA5, MVA5-MenaΔLERER) were dyed with 1 µM CMFDA, then seeded on the CDM-containing coverslips at 2,500 cells/cm² and allowed to spread for 4–6 h before imaging. Coverslips were imaged every 15 min for 6 h at 37°C and 5% CO2, at least eight images were captured per coverslip and four coverslips were imaged per condition.

a5 integrin surface levels

For assessment of a5 integrin surface levels, MVA5 fibroblasts were incubated on ice in 1% BSA, 2 mM EDTA in PBS with biotinylated a5 integrin antibody (557446; BD) or biotinylated rat IgG (012-060-003; Jackson ImmunoResearch Laboratories, Inc.) for 30 min. Cells were washed and incubated for 30 min on ice with APC streptavidin (554067; BD) and propidium iodide. Cells were washed, resuspended, and directly analyzed on a flow cytometer (FACSCalibur; BD). Biotinylation and analysis of surface levels of a5 integrin was performed as described previously (Caswell et al., 2008). In brief, cells were starved for 1 h, washed with PBS, and labeled with sulfo-NHS-Biotin (Thermo Fisher Scientific) for 30 min at 4 °C. Cells were extensively washed. Alexa Fluor 680-streptavidin was added to the plate 1 h at 4°C, washed, and developed using an Odyssey imaging system (Licor).

Generation of MenaLOXED mice

A targeting vector was generated using pGKNeoF212DTA (Addgene). The construct contained a 1.1 kb short arm from intronic sequence 5′ to exon 2 of Mena with a loxp site at the end proximal to exon 2. Adjacent to this lox site is a PGK-Neo resistance cassette flanked by FRT recombination sites. Next to this, a long arm consisting of a sequence containing Mena exon2 flanked by a lox site followed by an addition 5 kb of intronic sequence 3′ to exon 2. A PGK-DTA cassette for negative selection was inserted outside of the short arm. The linearized targeting vector was electropropated into R1 embryonic stem (ES) cells. More than 1,000 G418-resistant ES colonies were picked and screened for homologous recombination by PCR. Five clones were identified, and homologous recombination was confirmed by Southern blotting. Standard methods were used to inject the targeted ES cells into blastocysts, generate chimeric animals, and finally identify germline transmission of the targeted allele (Kwiatkowski et al., 2007). The PGK-Neo cassette was excised by crossing to a transgenic “Fliper” that expresses FLP recombinase. The resulting allele, MenaLOXED contains LoxP recombination sites flanking exon 2 of Mena. Introduction of CRE recombinase by transgene or in cultured primary cells causes excision of exon 2 generating a protein null allele.

Description of a5 FLOXED mice

A conditional a5 integrin targeting vector containing a thymidine kinase (TK) negative-selection cassette, an FRT-flanked PGK-neo cassette, and the 253-bp exon 1 of a5 integrin flanked by loxP sites was electroporated into R1 ES cells. Parental MVA5 cells and MVA5 cells expressing tagged Mena and Mena mutants were maintained at 32°C, 5% CO2 in DME. a5 integrin targeting vector containing a thymidine kinase (TK) negative-selection cassette, an FRT-flanked PGK-neo cassette, and the 253-bp exon 1 of a5 integrin flanked by loxP sites was electroporated into R1 ES cells. These cells were selected and screened for correct recombination and single integration. The PGK-neo cassette was removed by transient expression of Flip recombinase. Two karyotyped, correctly targeted ES cell clones (2H2 and 3G3) gave germine transmission and identical results. Cremedicated excision of exon 1 was confirmed by PCR genotyping and Southern blotting (van der Flier et al., 2010). Standard methods were used to inject the targeted ES cells into blastocysts, generate chimeric animals, and finally identify germine transmission of the targeted allele. The resulting allele, a5FLOXED, contains LoxP recombination sites flanking exon 1. Introduction of CRE recombinase by transgene or in cultured primary cells causes excision of exon 1, generating a protein-null allele.

Online supplemental material

Fig. S1 shows that FP4-Mito, but not DP4Mito, expression recruits Mena and a5. Fig. S2 is a supplement to Fig. 4 to show that the LERER repeat region binds a5, requires the C-terminal amino acids, and likely forms a coiled coil structure. Fig. S3 shows that a5, but not F-actin, binding is required for Mena to mediate efficient cell spreading. Fig. S4 shows that F-actin binding is required for high pFAK397 levels. Fig. S5 shows that FN fibrillogenesis in MVA5 cells is rescued by GFP-Mena but not GFP-VASP. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201202079/DC1.

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