FHOD1 Is Needed for Directed Forces and Adhesion Maturation during Cell Spreading and Migration

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SUMMARY

Matrix adhesions provide critical signals for cell growth or differentiation. They form through a number of distinct steps that follow integrin binding to matrix ligands. In an early step, integrins form clusters that support actin polymerization by an unknown mechanism. This raises the question of how actin polymerization occurs at the integrin clusters. We report here that a major formin in mouse fibroblasts, FHOD1, is recruited to integrin clusters, resulting in actin assembly. Using cell-spreading assays on lipid bilayers, solid substrates, and high-resolution force-sensing pillar arrays, we find that knockdown of FHOD1 impairs spreading, coordinated application of adhesive force, and adhesion maturation. Finally, we show that targeting of FHOD1 to the integrin sites depends on the direct interaction with Src family kinases and is upstream of the activation by Rho kinase. Thus, our findings provide insights into the mechanisms of cell migration with implications for development and disease.

INTRODUCTION

The events following fibroblast binding to and spreading on matrix-coated surfaces can be described by a series of sequential steps (Dubin-Thaler et al., 2008). The earliest events involve the clustering of the integrins to activate adhesion (Jiang et al., 2003). On solid substrates, integrin activation results in rapid spreading and adhesions mature over time through the contraction process (Cai et al., 2010; Giannone et al., 2004). In suspension cells, the binding of soluble ligand to integrins causes activation of Src family kinases (SFKs; Huveneers and Danen, 2009), but the process stalls because subsequent steps involve or depend on surface forces. Recent studies of arginine-glycine-aspartic acid (RGD) ligands attached to mobile lipids with or without barriers to movement show that the initiation of spreading follows actin polymerization from clustered integrins, subsequent recruitment of myosin, and force generation on the clusters (Yu et al., 2011). Actomyosin contractions of integrin clusters to the barriers are important to trigger further spreading by the previously reported pathways (Giannone et al., 2004). This raises the question of how actin polymerization occurs at the integrin clusters and whether it is downstream of SFKs. Since actin filament attachment to RGD-integrin clusters is critical for subsequent steps in the spreading process, we focus here on elucidating the mechanism of actin polymerization following integrin activation.

The ARP2/3 complex (Goley and Welch, 2006; Lai et al., 2008; Svitkina and Borisy, 1999) and several formins are detected in fibroblasts and associate with a range of actin structures, such as filopodia or stress fibers (Campellone and Welch, 2010; Mellor, 2010). Although the function of the ARP2/3 complex was closely linked to cell spreading, knockdown experiments or the use of specific ARP2/3 inhibitors indicate that additional actin assembly factors are involved in spreading (Di Nardo et al., 2005; Nolen et al., 2009; Steffen et al., 2006). In a screening of fibroblast actin assembly factors, we found localization of FHOD1 to early RGD clusters, whereas other prominent fibroblast formins such as mDia1, mDia2, or FMNL3 were not targeted to the integrin sites. Indeed, FHOD1 is an interesting candidate for actin assembly from early integrin sites as it is (1) regulated downstream of SFKs (Koka et al., 2005), even though details of the interaction remained unclear, and (2) FHOD1 has both a barbed end elongation activity and a strong actin bundling activity (Schönichen et al., 2013). Although in mature adhesions actin filaments are bundled by α-actinin and other actin crosslinking proteins to ensure optimal force transmission (Roca-Cusachs et al., 2012, 2013), a combined elongation and bundling activity could guide assembly of contractile structures in the context of early integrin cluster formation.

To analyze a potential role of FHOD1 during early cell spreading, we combined spreading assays on supported lipid bilayers and on rigid substrates, as well as on high-precision force-measuring pillar arrays. Whereas spreading assays on rigid substrates are a well-established model for cell motility,
FHOD1 Targets to Early Integrin Clusters

In order to investigate a potential association of FHOD1 with early adhesions, we employed supported lipid membranes functionalized with RGD peptides as the ligand for integrins. Two-dimensional (2D) mobility of the ligand on supported membranes enabled us to visualize the reorganization of activated integrins and the newly formed actin network associated with integrin clusters in live cells (Yu et al., 2011). FHOD1 is a low-abundance protein, and transfection resulted in a strong overexpression of the FHOD1 signal was observed behind the protruding domain by Rho kinase (ROCK), which results in the release of a consensus sequence in the diaphanous autoregulatory subfamily are known to be activated by phosphorylation of a_DIAPH domain. These were pretreated with and spread in the presence of blebbistatin. As reported previously (Choi et al., 2008), blebbistatin inhibited adhesion maturation and paxillin was found in nascent adhesions around the cell edge (Figure 2E). There, FHOD1 colocalized with paxillin, supporting the model that FHOD1 localization is independent of myosin II. In contrast, this localization was dependent on integrin activation by surface antigens, since no localization close to the cell edge was found when the cells were spread on poly-L-lysine (Figure 2F). Immunostaining with an anti-FHOD1 antibody confirmed the localization pattern to the boundary between lamellum and lamellipodium (Alexandrov et al., 2008). Indeed cotransfection with paxillin showed partial colocalization at nascent and mature focal adhesions (Figure 2D).

Since the lipid bilayer experiments suggested that localization of FHOD1 to integrin clusters was independent of myosin-generated forces, we next analyzed the localization of FHOD1 in cells that were pretreated with and spread in the presence of blebbistatin. As reported previously (Choi et al., 2008), blebbistatin inhibited adhesion maturation and paxillin was found in nascent adhesions around the cell edge (Figure 2E). There, FHOD1 colocalized with paxillin, supporting the model that FHOD1 localization is independent of myosin II. In contrast, this localization was dependent on integrin activation by surface antigens, since no localization close to the cell edge was found when the cells were spread on poly-L-lysine (Figure 2F). Immunostaining with an anti-FHOD1 antibody confirmed the localization pattern to the tips of radial actin filaments (Figures 2G and S1A). Interestingly, both transfected as well as endogenous FHOD1 were at least partially detected in a periodic pattern along the actin filaments, which could be related to an actin bundling activity (Schönichen et al., 2013).

We next sought to analyze whether FHOD1 was active during early cell spreading and, thus, could be involved in actin assembly at the early integrin clusters and adhesions. Formins of the FHOD subfamily are known to be activated by phosphorylation of a consensus sequence in the diaphanous autoregulatory domain by Rho kinase (ROCK), which results in the release of the auto-interaction and promotion of actin assembly (Iskra et al., 2013; Takeyama et al., 2008). Using a phosphospecific antibody (Figures 2H and S1C–S1E), we found the peak of FHOD1 activity 3–8 min after cell plating, and thus at times where the majority of cells started to form adhesions at the cell edge.

FHOD1 Knockdown Suppresses Focal Adhesion Formation

To further test whether FHOD1 was required during early cell spreading for the actin assembly from adhesion sites, we designed small hairpin RNA (shRNA) constructs against FHOD1 (Figure S1B). FHOD1 knockdown cells formed filopodia, but failed to form coherent protrusions and spread in a segmented fashion (Figure 3A; Movies S3 and S4). Frequently, protrusions were not stable and collapsed entirely (arrows in Figure 3A). Moreover, the speed of cell spreading was strongly reduced (Figure 3B). Cell area, shape, and F-actin content were restored to normal levels by coexpression of small interfering RNA-resistant (siRNA-resistant) human FHOD1 (Figure S3). Similarly, treatment with the pan-formin inhibitor smiFH2 impaired cell spreading in a dose-dependent manner (Figure S4), thus confirming the requirement for formin family proteins for early cell spreading.

FHOD1 Knockdown Suppresses Focal Adhesion Formation

Since FHOD1 localized to early adhesions and protrusions in knockdown cells were less stable, we tested whether there was a defect in focal adhesion formation. For this we knocked...
down FHOD1 for 72 hr and then additionally transfected the cells with paxillin-GFP and pRuby-Lifeact and imaged the cells for >20 min, starting with the fast spreading phase (P1) to observe the adhesion formation during the protrusion-retraction phase. During the outward spreading, control cells formed nascent adhesions, many of which matured to focal adhesions after the transition to the protrusion-retraction phase (Figures 3C and 3D; Movie S5). Again, FHOD1 knockdown cells formed protrusions that frequently collapsed. Although knockdown cells initially formed nascent adhesions as well, they did not mature and eventually turned over (see kymograph in Figure 3D). Additionally, the actin filament density and organization was reduced in knockdown cells (Figures 3C, S5A, and S5B). We further confirmed a defect in adhesion formation by immunostaining with an anti-paxillin antibody (Figure 3E). Quantification of the adhesion area showed a significant reduction in FHOD1 knockdown cells and a concomitant increase in the fraction of nascent adhesions (Figures 3F and 3G). Again, treatment with the pan-formin inhibitor showed a similar reduction in adhesion size and F-actin density (Figure S5C). Immunostaining for active β1-integrin (9EG7) showed that the FHOD1 knockdown did not affect the integrin activation at the cell edge, suggesting that

Figure 1. FHOD1 Localizes to Early Integrin Clusters

(A) MEF cells were transiently transfected with Ruby-Lifeact and GFP-FHOD1 and then plated onto RGD membrane labeled with Cascade Blue neutravidin. Ribbon-like actin polymerizes from RGD clusters and expands outward. FHOD1 is also enriched at RGD clusters.

(B) Zoom of marked area in (A). Red arrowheads indicate actin ribbon and green arrowheads indicate the intensity maxima of FHOD1-GFP.

(C) A kymograph of the region marked by the red line in (B) shows early colocalization of both FHOD1 and Lifeact with the RGD clusters (see line profile 1). At later time points, Lifeact is localized stronger to the periphery of the cluster, and FHOD1 localizes to the center and the periphery of the cluster (line profile 2). Red arrowheads indicate the outward extending actin and FHOD1; blue asterisks indicate the RGD cluster and the colocalizing FHOD1 and actin; white asterisks indicate the center of the cluster that is depleted from actin.

(D and E) RGD clusters of cells spread for 5 min were stacked and averaged with ImageJ. To analyze the enrichment around the clusters, the average pixel intensity outside a circular ROI (radius = 2.5 µm) was subtracted (D) and the radial profile was calculated for each channel (E). n = 31 RGD clusters of seven cells; for mDia1, mDia2, and FMNL3 localization, see Figure S2.
FHOD1 acted downstream of integrin engagement (Figure 3H). However, both total and active β1-integrin signals were limited to the cell edge, further documenting the defect in adhesion maturation.

**FHOD1 Knockdown Impairs Cell Migration**

Cell spreading assays are commonly used as model system for motility experiments and especially for adhesion formation. The highly reproducible sequence of functional phases had in the past led to many important insights (Döbereiner et al., 2006; Dubin-Thaler et al., 2008; Giannone et al., 2004, 2007). Nevertheless, to confirm that FHOD1’s role in adhesion formation had a broader relevance for cell migration, we performed wound scratch assays in control and FHOD1 knockdown cells (Figures 3I–3L). After application of a wound with a pipette tip, control cells started to migrate quickly toward the center of the wound and the wound was closed within approximately 24 hr. FHOD1 knockdown cells, in contrast, migrated slowly and only one-third of the wound was closed within 24 hr (Figures 3J and 3K). When we fixed the cells after 8 hr, we found that, similar to the spreading assays, FHOD1 knockdown cells lacked strong, polarized actin filaments and adhesions were frequently limited to the periphery and had a dot-like appearance (Figure 3L). Within the first 50 μm from the leading edge (i.e., approximately the first row of cells), the cell area occupied by adhesions was reduced from 17.0% ± 1.3 to 6.7% ± 0.4.
stress (vector component perpendicular to the cell edge) was model in Figure 5A). As a result, the forces were not directed that myosins were pulling from multiple directions (see working of FHOD1.

is noteworthy that the maximum pillar displacement values inward traction was lost (average traction stress; Figure 4E). It enabled nondisruptive live imaging of localized traction forces on sub-micron polydimethylsiloxane (PDMS) pillar arrays that organizing the actin for efficient force coupling at the adhesions. Therefore, we plated cells in the force coupling at the adhesion. Therefore, we plated cells in the force coupling at the adhesions downstream of SFKs. Furthermore, it was instrumental in organizing the actin for efficient force coupling at the adhesions and thus facilitated continued cluster growth.

FHOD1 Interaction with SFKs Is Necessary for Its Activation
Recent findings showed that the SFK inhibitor PP2 inhibited actin polymerization from early integrin clusters (Yu et al., 2011). Moreover, it was shown that FHOD1 was regulated downstream of SFKs, since treatment with the Src inhibitor PP2 abolished the FHOD1-induced transcription of the skeletal actin promoter (Koka et al., 2005). If FHOD1 played a role in the actin organization at integrin clusters, SFKs were possibly involved in the targeting of FHOD1 to integrin sites. Therefore, we analyzed targeting of FHOD1 to RGD-integrin clusters in PP2-treated fibroblasts. As reported previously (Yu et al., 2011), the PP2 treatment reduced growth of the integrin clusters. Furthermore, no ribbon-like polymerization of actin was detected and enrichment of FHOD1 around the clusters was reduced (Figure S6).

FHOD1 contains a conserved YEEI sequence on its N terminus (YEEI) that, when phosphorylated, constitutes a strong Src homology 2 (SH2) binding motif (Songyang et al., 1993; Figure 7A). Additionally, the poly-proline stretch of the FH1 domain is a putative SH3 binding site (Jia et al., 2005; Roskoski, 2004). Indeed, we confirmed tyrosine phosphorylation of immunoprecipitated full-length FHOD1, which was absent in a tyrosine to phenylalanine mutant (Y99F) and also lost after deletion of the poly-proline region (Δpoly-Pro; Figure 7B). Furthermore, SFKs coprecipitated with FHOD1, but the binding was reduced in case of the Y99F mutation and absent in the case of the Δpoly-proline mutation. Moreover, both mutations led to a loss of FHOD1 activity, as measured by Thr1141 phosphorylation, thus indicating that the interaction with SFKs via the poly-proline region resulted in tyrosine (Y99) phosphorylation, stronger SFK binding, and downstream activation of FHOD1.

To further investigate the interaction with SFKs, we used mouse fibroblast cells deficient in Src, Yes, and Fyn (SYF cells; Klinghoffer et al., 1999), as well as SYF cells that had Src reintroduced by retroviral transduction or that were stably transfected with either Yes or Fyn. Interestingly, coprecipitation of the SFKs and partial restoration of tyrosine and Thr1141 phosphorylation were detected with all three kinases, but only Src restored the phosphorylation to the control levels (Figure 7C). However, due to the retroviral transduction, as opposed to the stable transfection, Src levels exceeded those of the other kinases.

If SFKs were upstream of FHOD1 activation, knockdown of FHOD1 should not affect the spreading phenotype of SYF cells. In line with previous results (Cary et al., 2002; Klinghoffer et al., 1999; Kostic and Sheetz, 2006; von Wichert et al., 2003), SYF cells showed aberrant spreading behavior and reduced adhesion formation. Thus, the cells exhibited several similarities with FHOD1 knockdown cells, such as reduced cell area (Figures 7D and 7E), segmented cell morphology (Figure 7F), reduced adhesion area, and an increased number of small adhesions after 30 min of spreading (not yet measured; Figure 7G). FHOD1 knockdown in SYF cells, however, did not result in an aggravation of any of the spreading defects, indicating that FHOD1 was downstream of SFKs.

Targeting to Adhesion Sites Is Controlled by SFKs
Because both the Y99F and the Δpoly-Pro lacked Thr1141 phosphorylation (and hence were inactive) and FHOD1 binding to RGD clusters was reduced after PP2 treatment, we...
Figure 3. FHOD1 Knockdown Disrupts Cell Spreading
(A and B) FHOD1 knockdown cells spread more slowly. (A) MEF cells were transfected with control shRNA (upper panel) or FHOD1 shRNA plasmids (lower panels) for 72 hr, plated on fibronectin-coated coverslips, identified for knockdown by means of their GFP fluorescence, and imaged with a DIC microscope in intervals of 1 s. Arrows indicate a collapsing protrusion in a knockdown cell. (B) Spread area was determined with ImageJ and is shown as the average over n = 10 cells; error bars indicate SE; nonlinear regression and comparison between the fitted curves suggests a significant difference with p < 0.0001.
(C and D) MEF cells were transfected with control shRNA (upper panel) or FHOD1 shRNA plasmids. After 72 hr, cells were additionally transfected with paxillin-GFP and pRuby-Lifeact and imaged by TIRF microscopy in intervals of 3 s. Whereas most nascent adhesions mature to focal adhesions (see (D) for the kymographs of the regions marked by the red lines in (C)), the nascent adhesions in the knockdown cells do not mature and remain small adhesions that eventually turn over.
(E) Immunostaining of cells after 30 min spreading confirms a reduced adhesion area and adhesion maturation in FHOD1 knockdown cells.
(F) Quantification of the adhesion area as fraction of the whole cell area; n = 26 for both conditions.
(G) The decreased adhesion area is a result of reduced adhesion maturation. Single adhesions (n = 2,072 and n = 1,158 from ten control and FHOD1 shRNA-transfected cells, respectively) were measured with ImageJ and grouped into three categories (<0.2 μm², 0.2–1 μm², and >1 μm²; error bars: SEM). FHOD1 knockdown cells have a significantly higher number of nascent (<0.2 μm²) adhesions (two-way ANOVA and Bonferroni posttest: p < 0.001).

(legend continued on next page)
hypothesized that the interaction with SFKs was necessary for correct targeting to the integrins and subsequent activation by ROCK. Indeed, the typical localization pattern of FHOD1 with paxillin was lost in the SYF cells and was partially restored in all three SYF add-back cell lines (Figure 8A). Also, localization of the Y99F mutant to the adhesions was reduced and no overlap between FHOD1-Δpoly-Pro-GFP and paxillin was found (Figures 8B and 8D), whereas a constitutive inactive mutant of the ROCK phosphorylation sites (FHOD1-3A) still localized to the adhesions. Colocalization with actin decreased both with FHOD1-3A and with the Δpoly-proline mutation, which was presumably due to a loss of actin filaments in these cells (Figures 8C and 8E). Indeed, transfection with FHOD1-Δpoly-Pro-GFP induced a phenotype similar to a FHOD1 knockdown. Cells were depleted of actin filaments and lacked strong adhesions. In lipid bilayer experiments, the depletion of actin filaments and lack of strong adhesions. In lipid bilayer experiments, the

Figure 4. FHOD1 Knockdown Abolishes Inward-Directed Traction Stress

Control shRNA and FHOD1-shRNA-transfected cells were plated on PDMS pillar arrays (d = 0.5 µm; h = 1.1 µm) and imaged on a bright field microscope for ≥15 min, from the initiation of spreading, with one frame per second. (A) Force maps indicate a loss of directed inward traction after FHOD1 knockdown. Red arrows, pillar displacements; yellow line, cell edge; yellow arrows, protrusion direction. (B–E) Angle and magnitude of pillar displacements fluctuate in FHOD1 knockdown cells (see example curves with color-coded displacement angles in B), and pillar displacement angles are widely distributed (C). As a result, the maximum inward stress per pillar is reduced as well (p < 0.0001) (D) and there is a striking loss of the overall mean inward stress (E). The two data sets are statistically different (nonlinear regression and comparison of fits: p < 0.0001). In (C)–(E), n = 280 and 326 pillars from five cells each for the control and the FHOD1 knockdown, respectively. Displacement angles are all displacements over noise level (i.e., 10 nm) for 3 min after first contact with the cell edge (control, n = 35,196 events; FHOD1-shRNA, n = 36,872 events).

DISCUSSION

In the present study, we combine the supported lipid bilayer system with high-resolution, force-sensing pillar arrays and

See also Figure S3 for the siRNA rescue and Figures S4 and S5 for the effects of the formin inhibitor smiFH2 on cell spreading and adhesion maturation, respectively.

(H) Integrin activation at the cell edge is not affected by the FHOD1 knockdown. Green, total β1-integrin (12G10); red, activated β1-integrin (9EG7).
(I) FHOD1-shRNA cells are closing wounds more slowly.
(J) Percentage of initial wound. Error bars = SEM.
(K) Speed of wound closure in µm/hr. Error bars: range, n = 10
(L) FHOD1 knockdown cells are forming only small adhesions at the cell edge during wound closure. Cells were fixed after 8 hr and stained for paxillin and F-actin. See also Figure S3 for the siRNA rescue and Figures S4 and S5 for the effects of the formin inhibitor smiFH2 on cell spreading and adhesion maturation, respectively.
well-established cell spreading and migration assays on solid substrates to study actin assembly from early integrin clusters and subsequent adhesion formation. Importantly, since supported bilayers provide an environment in which there is no in-plane resistance to movement and thus provide an important contrast to planar glass substrates (Yu et al., 2011), we are able to characterize steps during adhesion formation that are prior to myosin activity. Sub-micron elastomer pillars, on the other hand, allow us to analyze localized traction stress with high precision. Using these methods, we show here that the formin family protein FHOD1 is critical for actin assembly from integrin clusters, inward-directed traction stress, as well as cell spreading and adhesion maturation. This is consistent with the hypothesis that the early spreading involves actin polymerization from ligand-bound integrin clusters to enable myosin-dependent cluster growth and further spreading through lamellipodial extension (Yu et al., 2011).

Furthermore, our results show that FHOD1 is required to form actin structures that allow effective coupling of myosin traction forces to the adhesion. Without FHOD1, forces on adhesion sites are present only as short bursts and not directed. Since there are other actin assembly proteins present at adhesions (e.g., VASP; Worth et al., 2010) or close to adhesions (e.g., ARP2/3), actin filaments are still formed in the absence of FHOD1, albeit to lower extent. However, without FHOD1 there is a lack of actin organization. This is in agreement with an actin bundling function, which was reported previously (Schönichen et al., 2013). Indeed, in lipid bilayer experiments, a part of the GFP-tagged FHOD1 moved outward from the integrin clusters, together with the polymerizing actin. Moreover, we also detected FHOD1 in a periodic pattern on fibronectin-coated glass, which could further indicate such bundling activity.

While the in vitro work by Schönichen et al. (2013) found that FHOD1 lacks actin nucleation activity and only displays weak filament elongation activity, other studies reported that FHOD1 enhanced actin polymerization in cyto (Gasteier et al., 2003, 2005; Koka et al., 2005; Watanabe et al., 1999), suggesting that FHOD1 might elongate previously nucleated filaments. Similarly, some of our results—especially the decreased actin polymerization in cyto—point toward an active actin elongation by FHOD1 during early spreading.

Independent of a bundling or elongation activity, our data show clearly that FHOD1 is targeted to integrin adhesions downstream of SFKs. Subsequently, it appears to be activated by ROCK to enable actin assembly from integrin clusters and cell spreading. This is in support of the proposed model that formin localization and activation are separate phenomena (Ramalingam et al., 2010).

Although it has been shown previously that FHOD1 is activated by ROCK (Hannemann et al., 2008; Schulte et al., 2008; Takeya et al., 2008) and regulated downstream of SFKs (Koka et al., 2005), details of the mechanism remained unclear.
confirm here that the activation is downstream of SFKs in several ways: (1) abolishing the interaction by incubation with PP2, (2) using SYF cells, or (3) mutation of the interaction sites all reduce activation of FHOD1. In contrast, a constitutive inactive mutant (FHOD1-3A; Takeya et al., 2008) can still localize to the adhesion regions. Furthermore, binding of Src to the poly-proline region, followed by phosphorylation of a YEEI sequence on the N terminus, enables the subsequent activation. FH1-SH3 domain interactions have been reported previously for yeast and trematode formins (Kamei et al., 1998; Quack et al., 2009), and a similar mechanism has been detected also for other proteins, such as p130Cas, where the interaction between the poly-proline region and the Src SH3 domain serves to activate Src and results in subsequent phosphorylation of p130Cas (Bibbins et al., 1993; Pellicena and Miller, 2001; Pellicena et al., 1998). The interaction with SFKs might bring FHOD1 in close proximity to ROCK at the membrane (Riento and Ridley, 2003) and thus enable the activation. During cell spreading, this results in a peak of FHOD1 activity at a time when the majority of cells form new adhesions all around the cell edge. At later time points during spreading (and also migration) nascent adhesions are formed as well, but only a small portion of the edge moves out at any time (not all around the cell edge and in a large fraction of the spreading cells). Therefore, FHOD1 phosphorylation returns to the baseline levels in spread cells. Rapid and transient phosphorylation of FHOD1 after receptor engagement was also found in other cell types, i.e., after collagen-related peptide stimulation in human platelets (Thomas et al., 2011) and could mark a universal event in the stimulation of actin polymerization upon integrin clustering.

Our results show that only Src can efficiently phosphorylate FHOD1 at Y99 to enable the downstream activation by
ROCK. However, in Yes and Fyn add-back cells, the expression levels of the respective SFKs are lower than in wild-type cells and thus the SFK activity might not be sufficient for a full recovery of FHOD1 phosphorylation on a cellular level, but rather only lead to localized effects. Indeed, our results suggest that add back of Yes or Fyn is sufficient to restore the targeting at least partially. Nevertheless, we cannot exclude that these effects on adhesion targeting are only due to a redundancy between the SFKs (Lowell and Soriano, 1996).

![Figure 7. FHOD1 Is Phosphorylated by SFKs](image)

(A) Schematic of FHOD1 and the putative SH2 and SH3 binding sites and alignment of human (H. sapiens), mouse (M. musculus), and rat (R. norvegicus) FHOD1 sequences.

(B) Immunoprecipitated wild-type FHOD1, but not the Y99F or the poly-proline deletion (FHOD1-Δpoly-proline) construct, shows a band when probed with an anti-phospho-tyrosine antibody. Similarly, a band with the phospho-Thr1141 antibody can be detected only on wild-type FHOD1, and interaction with SFKs (anti-pSFK) is reduced or abolished for FHOD1-Y99F and FHOD1-Δpoly-proline, respectively. n = 3, error bars indicate SD.

(C) Tyrosine phosphorylation and Thr1141 phosphorylation are reduced in SYF cells, but can be restored by Src; n = 3, error bars indicate SD. Incubation with Src, Fyn, or pSFK antibodies suggests interaction with Src, Yes, and Fyn.

(D-G) SYF cells spread more slowly (D and E) and have irregular cell shapes (D and F). However, the phenotype is not aggravated by FHOD1 knockdown (error bars: range). Similarly, SYF cells have a reduced adhesion area and more immature adhesions after 30 min spreading (G), irrespective of FHOD1 levels. For p values from Student’s t test (E and F) or two-way ANOVA with Bonferroni posttests (G): error bars, SEM; *p < 0.05, **p < 0.005, ***p < 0.001; ns, not significant. Results from t tests between SYF shRNA control and SYF FHOD1 shRNA are shown in red.

See also Figure S6 for PP2 treatment.
Together, our study shows that FHOD1 is a critical actin assembly factor at the early integrin clusters during cell spreading and migration, and it triggers a cascade of events that eventually produces longer term adhesions through the polymerization and bundling of actin filaments. Although several formins that were previously studied in fibroblasts participate in adhesion formation and/or maturation (Goode and Eck, 2007; Gupton et al., 2007; Yamana et al., 2006), our study shows specific targeting of a formin to integrin adhesions. Furthermore, our data show that this targeting depends on integrin-ligand engagement, but not myosin contractility. This could be important since recent findings have highlighted the role of mammalian and yeast formins as mechanosensors (Courtemanche et al., 2013; Higashida et al., 2013; Jégo et al., 2013). In these studies, formin activity was enhanced in presence of profilin by a pulling force on tethered mDia1 or Bni1p in flow chambers and on membrane-bound mDia1 in cells. Although it remains to be seen if the same applies to FHOD1, a force-dependent regulation of an adhesion-localized actin assembly factor would provide a direct link between integrin mechanosensing and actin-driven cell protrusion.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**

RPTPα−/− mouse embryonic fibroblast (MEF) cells and SYF cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin to 70–80% confluency and passed the day before the experiment. If not indicated otherwise, cells were transfected for 24 hr with an AMAXA nucleofector using the MEF transfection KIT (Lonza) according to the manufacturer’s instructions. SYF+Src cells were obtained from ATCC; SYF+Fyn and SYF+Yes add-back cells were a kind gift from Dr. Yasuhiro Sawada.

**Microscopy**

Differential interference contrast (DIC) microscopy and bright field time-lapse imaging were performed on an Olympus IX-70 inverted microscope maintained at 37°C using a 100× numerical aperture (N.A.) 1.40, 60× N.A. 1.40, or a 20× N.A. 0.80 oil objective (all Olympus), a CoolSNAP HQ charge-coupled device (CCD) camera (Photometrics), and micro-manager or MetaMorph microscopy software (Molecular Devices).

Total internal reflection fluorescence (TIRF) images and time-lapse micrographs were taken using an Olympus IX81 fluorescence microscope maintained at 37°C with a 60× N.A. 1.45 objective and a Cool Snap FX cooled CCD camera (Photometrics) controlled by SimplePCI software (Compix). Confocal microscopy was performed on a Zeiss LSM700 laser-scanning confocal microscope using a 63× N.A. 1.40 objective (Zeiss) or an Olympus Fluoview FV500 laser-scanning confocal microscope using a 60× N.A. 1.40 objective (Olympus).

**Spreading Assays**

Cells were spread on human plasma full-length pure fibronectin-coated (10 μg/ml; Roche) silanized cover glasses or, for western blotting and immunoprecipitation assays, on fibronectin-coated tissue culture dishes. Cells were trypsinized, washed with soybean trypsin inhibitor, centrifuged, and precipitated in Ringer medium (150 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 20 mM HEPES, and 2 g/L D-Glucose at pH 7.4) for 30 min prior to cell lysis. Cells were plated and imaged by time-lapse microscopy (DIC or TIRF), or fixed with 4% paraformaldehyde/PBS and stained for confocal microscopy. For western blotting and communoprecipitation assays, cells were lysed in 20 mM Tris, 137 mM NaCl, 1% glycerol, and 1% NP40 at pH 8.0, including protease and phosphatase inhibitor cocktails (Complete EDTA-free Protease Inhibitor Cocktail Tablets and PhosSTOP Phosphatase Inhibitor Cocktail Tablets, both Roche) on ice, snap frozen, re thawed, and cleared from cell debris by centrifugation. In case of smFH2 (Sigma), blebbistatin (Sigma), or Y-27632 (Tocris) treatment, indicated doses of drugs were present during the preincubation as well as the spreading experiment. For FHOD1-GFP immunoprecipitations, we used the Crosslink immunoprecipitation kit (Pierce) and monoclonal mouse anti-GFP antibody (Roche).

**Analysis of Edge Velocities**

Spread area was calculated with the “Analyze Particles” function of ImageJ, after using the “Fill Edges” function and thresholding to binarize the cells. Where necessary, “Close,” “Fill Holes,” and “Remove Outliers” functions were used to receive a coherent mask of the cell. Outlines of the measured cells were added to the original image series with the “Image Calculator” function as a control. Spread phases were identified after plotting the logarithm of the area versus the logarithm of the time (Dubin-Thaler et al., 2008; see also Figure S1A). Average edge velocities were calculated as the slope of the radius of a circle with the measured cell area over the time.

**Wound Healing Assays**

Cells were transfected with FHOD1 shRNA or Control shRNA plasmids and cultured for 96 hr in the presence of 150 μg/ml zeocin. Subsequently, cells were plated to confluency on fibronectin-coated tissue culture, or if intended for immunofluorescence, on fibronectin-coated glass-bottom dishes (MatTek). After 12 hr, a wound was applied with a pipette tip, cells were washed twice with fresh full medium, and marked areas were imaged every 8 hr with an Olympus IX81 fluorescence microscope maintained at 37°C with a 10× N.A. 0.3 objective (Olympus). The (paxillin) adhesion area was measured with ImageJ within a 50 μm wide region of interest (ROI), after thresholding and using the “Analyze Particle” function. Regions with a strong, diffuse cytoplasmic paxillin signal were excluded from the analysis.

**Traction Force Measurements**

Pillar arrays (1.1 μm in height, 0.5 μm diameter, 1 μm center to center, k = 13.9 N/μm) coated with 10 μg/ml of fibronectin (Invitrogen) were prepared as described previously (Ghassemi et al., 2012). Briefly, cells were spread on pillar arrays and bright field movies were taken as described above with a frame rate of one frame per second. Displacements were measured with ImageJ, using the NanoTracking plugin.

**Bead Displacement Analysis**

Silica microspheres 1 μm in diameter (Bangs Laboratories) were activated with cyanogen bromide and covalently labeled with fibronectin according to the manufacturer’s instructions (TechNote 205). Cells (Control and FHOD1 shRNA transfected for 4 days) and beads were added onto fibronectin-coated coverslips. Beads were placed at the edges of cells with an optical trap, using a 2 W diode pumped 1,064 nm laser (CrystalLaser) until they were fixed on the cell surface (i.e., the optical trap force was unable to produce a detectable movement of the bead) and visualized with a 100× N.A. 1.3 objective on an inverted Axiovert 100 TV microscope, equipped with Nomarski optics. After thresholding, beads were tracked with ImageJ (Fiji) using the MTrack2 plugin.

**Functionalyzed Supported Lipid Bilayer Membrane**

RGD peptide, a biotinylated peptide of cyclo (Arg-Gly-Asp-D-Phe-Lys(biotin-polyethylene glycol-polyethylene glycol)), was purchased from Peptides International (3697-PI). Both 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (16:0 biotinyl-Cap-PE) were purchased from Avanti Polar Lipids. Cascade Blue neutravidin was purchased from Invitrogen. Detailed preparation methods were previously described (Yu et al., 2011). In brief, small lipid vesicles (0.4 mol% of biotinyl-Cap-PE and 99.6 mol% of DOPC) were made by sonication and then used to deposit onto glass cover glass under aqueous condition with 150 mM PBS in room temperature. Neutravidin serves as the link between biotinyl-Cap-PE and biotinylated RGD peptide. A total of 1 μg/mL of fluorescently labeled neutravidin (Cascade Blue) was added onto supported lipid membranes for 30 min incubation. After washing off excess neutravidin, 1 μg/mL of biotinylated RGD was added to neutravidin-coated supported membranes for another 30 min. Excess RGD was removed by serial solvent exchange, 25 mL of 150 mM PBS in each chamber, and then 15 mL of serum-free DMEM. In general, the 2D diffusion coefficient of RGD-supported membrane is measured as high as 2.5 μm²/s.

Developmental Cell
FHOD1 at Early Adhesions Drives Cell Spreading
RGD Cluster Analysis
A tiff stack of RGD clusters from cells that were spread for 5 min was created and the average image calculated with ImageJ. A circular ROI with a radius of 2.5 μm was drawn around the center of the clusters. To analyze the enrichment of proteins around the clusters, we subtracted the average pixel intensity outside the ROI from each channel. The resulting image was used to quantify the integrated intensity of each channel and create radial profiles with ImageJ, using the Radial Profile Plot plugin. The data are presented as the radial profile of the average over all clusters for a certain condition, as well as the mean integrated intensities (±SD) of the average clusters from single cell.

Immunofluorescence
Immunofluorescence staining was carried out as described previously (Ishikawa et al., 2010). Briefly, fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min, or 0.02% Triton for 5 min for the integrin staining, blocked with MAXblock blocking medium (Active Motif) according to the manufacturer’s instructions, and stained with the primary antibody mix in immunostaining buffer (1% BSA, 20 mM Tris-base, 155 mM NaCl, 2 mM EGTA, and 2 mM MgCl2, pH 7.5) for 1 h under shaking at room temperature. Cells were washed three times with PBS and incubated with the secondary antibody mix, containing Phalloidin (Alexa Fluor 546 or Alexa Fluor 633-Phalloidin; Invitrogen) where indicated. After washing three times with PBS, cells were mounted in 0.1 M Tris-HCl/glycerol (3:7) and 50 mg/ml N-propyl-gallate, pH 9.5.

Statistical Testing
In the current study, two-tailed Student’s t test was used for comparison between two groups. Data sets were tested for normal distribution using the Shapiro-Wilk test. If not stated otherwise, all box plots are median (central line), upper and lower quartile (box), ±1.5 × interquartile range (whiskers), with outliers displayed separately. Two-way ANOVA and Bonferroni posttests were calculated with Graphpad Prism 5.

Image Processing
Original digital images obtained were assembled to the figures and labeled using InDesign or Illustrator (Adobe). Only linear contrast adjustments were used and were always applied to the entire image.

Supplemental Experimental Procedures
Additional experimental procedures can be found in the Supplemental Experimental Procedures, including the antibodies, sequences of the siRNAs, and primers used in this work.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, six figures, and eight movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.11.003.

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