F-actin bundles direct the initiation and orientation of lamellipodia through adhesion-based signaling

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Mesenchymal cells such as fibroblasts are weakly polarized and reorient directionality by a lamellipodial branching mechanism that is stabilized by phosphoinositide 3-kinase (PI3K) signaling. However, the mechanisms by which new lamellipodia are initiated and directed are unknown. Using total internal reflection fluorescence microscopy to monitor cytoskeletal and signaling dynamics in migrating cells, we show that peripheral F-actin bundles/filopodia containing fascin-1 serve as templates for formation and orientation of lamellipodia. Accordingly, modulation of fascin-1 expression tunes cell shape, quantified as the number of morphological extensions. Ratiometric imaging reveals that F-actin bundles/filopodia play both structural and signaling roles, as they prime the activation of PI3K signaling mediated by integrins and focal adhesion kinase. Depletion of fascin-1 ablated fibroblast haptotaxis on fibronectin but not platelet-derived growth factor chemotaxis. Based on these findings, we conceptualize haptotactic sensing as an exploration, with F-actin bundles directing and lamellipodia propagating the process and with signaling mediated by adhesions playing the role of integrator.

Introduction

The importance of cell migration in development, immunity, wound repair, and cancer progression has long been appreciated. Unifying these various physiological and pathological contexts is a common design principle: the ability of migrating cells to change or maintain directionality as they monitor their microenvironment for spatial cues (Petrie et al., 2009; Bear and Haugh, 2014). However, different cell types use fundamentally distinct mechanisms to achieve this objective. Whereas amoeboïd cells such as leukocytes exhibit a robustly polarized and excitable cytoskeleton, which only needs to be subtly perturbed by soluble cues to bias cell movement (i.e., in chemotaxis; Xu et al., 2003; Arrieumerlou and Meyer, 2005; Iglesias and Devreotes, 2012), mesenchymal cells such as fibroblasts exhibit weakly polarized migration phenotypes and respond to both chemical and physical cues (Lara Rodriguez and Schneider, 2013; Bear and Haugh, 2014). The latter include spatial gradients of immobilized, adhesive ligand density (haptotaxis) and of mechanical stiffness (durotaxis).

The most prominent cytoskeletal structure that drives cell locomotion is the lamellipod, a broad, fan-shaped protrusion with an F-actin–rich leading edge. The dendritic architecture of the leading-edge F-actin array is formed by integration of the Arp2/3 complex, which nucleates assembly of new actin filaments from existing ones and thus largely controls the rate of actin polymerization that drives lamellipodial protrusion (Rotty et al., 2013). This activity is in turn controlled by a host of signaling molecules, most notably the small GTPase Rac and the phospholipid phosphatidylinositol (3,4,5)-trisphosphate (PIP₃), which cooperate to activate the SCAR–WAVE regulatory complex upstream of Arp2/3 (Lebensohn and Kirschner, 2009). PIP₃ is produced by type I phosphoinositide 3-kinases (PI3Ks) and, like GTP-bound Rac, is focally enriched in protruding lamellipodia (Kraynov et al., 2000; Weiger et al., 2009). The weakly polarized morphology of fibroblasts is typically characterized by multiple lamellipodia, which exhibit intermittent protrusion and signaling and compete with one another

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Abbreviations used in this paper: FMI, forward migration index; FP, fluorescent protein; GPI, glycoposphatidylinositol; MEF, mouse embryonic fibroblast; PDMS, polymethylsiloxane; PI3K, phosphoinositide 3-kinase; PIP₃, phospholipid phosphatidylinositol (3,4,5)-trisphosphate; TIRF, total internal reflection fluorescence.

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to determine the overall direction of migration (Petrie et al., 2009; Weiger et al., 2010).

Previously, we characterized a mechanism by which fibroblasts execute large-scale changes in orientation by extension of nascent lamellipodia, which most often form by bifurcation of the dominant lamellipod; if the two branches successfully propagate to their fullest extent, a 90° turn is achieved (Welf et al., 2012). Our experiments revealed a specific role for PI3K signaling in lamellipodial spreading, which is required to maintain the propagation of the branches, whereas initiation of branching is PI3K independent. Accordingly, we found that increases in local PI3K signaling lag behind the acceleration of protrusion. These findings established the macroscopic morphodynamics of fibroblast migration that allow efficient reorientation of directionality, e.g., in response to external cues, but they also spurred a new set of questions aimed at the subcellular level. How do newly branched lamellipodia form? What determines the distinct directions of lamellipodial extension? Here, we show that F-actin bundles containing fascin-1, which often manifest as filopodia, seed the formation and set the orientations of nascent lamellipodia.

Filopodia are narrow, dynamic, finger-like protrusions with established roles in neuronal communication and development (Teddy and Kulesa, 2004), epithelial cell–cell adhesion (Vasioukhin et al., 2000; Wood et al., 2002), and cell motility, yet the precise contexts in which filopodia affect cell migration are largely unknown. The concept that filopodia generally serve as sensing organelles has been broadly speculated (Ridley et al., 2003; Mattila and Lappalainen, 2008), yet there is scant evidence supporting this notion except in the neuronal context (Davenport et al., 1993; Dent et al., 2011). In fibroblasts, it has been observed that filopodia seed the formation of distinct lamellipodia during the transition from isotropic to anisotropic spreading on an adhesive surface (Guillou et al., 2008), but the functional and mechanistic connections to random or directed locomotion are not yet established.

Critical to the assembly and stability of filopodia is the cross-linking of parallel actin filaments. Fascin is one of the major actin-bundling proteins in filopodia, though it has been shown to promote filopodia independent of its cross-linking function (Zanet et al., 2012). Among the three isoforms of fascin, only fascin-1 is broadly expressed in mesenchymal cells. Depletion of fascin-1 or impairment of fascin–actin binding significantly reduces the number of filopodia, whereas the constitutively active (S39A) actin-binding mutant increases the number and mean length of filopodia (Vignjevic et al., 2006; Li et al., 2010).

In this work, we used high-resolution imaging and analysis of subcellular dynamics, combined with directed migration assays, to elucidate a key function of F-actin bundles/filopodia in orchestrating lamellipodia during both random and directed migration of mesenchymal cells. Our results implicate both structural and signaling roles of fascin-containing bundles in this process and thus connect the subcellular and macroscopic morphodynamics of cell locomotion.

Results
Filopodia direct formation of newly branched lamellipodia
To study the formation of nascent lamellipodia, high-resolution total internal reflection fluorescence (TIRF) videos of cells randomly migrating on fibronectin were acquired and analyzed. Owing to the exquisite z resolution of TIRF illumination, adherent filopodia/microspikes of migrating NIH 3T3 fibroblasts were readily visible. We found that such filopodia typically preceded the emergence and oriented the protrusion of new lamellipodia, which most often branch off from existing ones (Fig. 1). Accordingly, spatiotemporal maps of protrusion and retraction activity show that large-scale branching of lamellipodia coincides with protrusion over filopodia (Fig. 1 A and Video 1). A method for identifying and tracking filopodia was devised (Fig. S1), allowing a clearer visualization of these structures in relation to cell shape dynamics. Lamellipodia were also identified automatically, based on a combination of morphological and protrusion criteria, and spatiotemporal overlap of lamellipodia and filopodia was assessed. Of the lamellipodia identified (n = 1,005, in 21 cells analyzed), 72% overlapped with filopodia; 55% of the lamellipodia overlapped with preexisting filopodia (Fig. 1 B).

We next asked whether or not the tendency to initiate protrusion around filopodia is a general property of lamellipodia. To test this, we used photoactivatable Rac (Wu et al., 2009) to locally induce Rac and Arp2/3 activation in NIH 3T3 cells. Although the spots of photoactivation were aimed near and not centered over visibly adhered filopodia, the ensuing protrusions occurred preferentially along those filopodia (Fig. 1 C and Video 2). This protrusion bias was seen 92% of the time (66/72), and PI3K signaling was enriched along the bundle in all but one of those instances. In a related experiment reported previously (Wu et al., 2012), Arp2/3-depleted fibroblasts were microinjected with purified Arp2/3 complex to induce lamellipodial protrusion globally. Analysis of this sequence confirms that the protrusions originated along filopodia before coalescing to form broad lamellipodia (Fig. 1 D).

Expression of the actin-bundling protein fascin-1 tunes cell morphology
To monitor filopod/lamellipod dynamics in better detail, we used fluorescent protein (FP)–tagged fascin-1 as a live-cell marker of F-actin bundles. In FP-fascin–expressing fibroblasts, we observed the same templating of branched protrusions as before, but here, we were not limited by the appearance of filopodia and could readily see F-actin bundles embedded behind the leading edge (Fig. 2 A, Video 3, and Fig. S2 A). The protrusions that emerge around bundles are labeled by FP-Lifeact, indicating F-actin network at the leading edge, characteristic of lamellipodia (Fig. S2 B). Analysis of lamellipodial overlap with F-actin bundles and filopodia in FP-fascin–expressing fibroblasts showed that 84% of the lamellipodia (n = 1,429, from 31 cells) overlapped with preexisting F-actin bundles or filopodia (Fig. 2 B). Conversely, most F-actin bundles/filopodia never overlap with lamellipodia during their lifetime (Fig. S2 C), indicating that emergence of lamellipodia is rare compared with the appearance and disappearance of adherent filopodia.
PI3K signaling colocalizes with F-actin bundles and is enriched in filopodia

Our previous work showed that PI3K signaling locally increases after the onset of protrusion, stabilizing nascent lamellipodia to affect macroscopic changes in cell morphology (Welf et al., 2012). With the present insight that F-actin bundles/filopodia initiate and orient this process, we asked whether or not these structures also harbor PI3K signaling and thus serve as primers for actin polymerization. Consistent with this idea, in NIH 3T3 fibroblasts coexpressing FP-fascin and FP-AktPH (Akt pleckstrin homology domain; a translocation biosensor for 3-phosphoinositides), the two typically colocalize near the leading edge and especially at the bases of filopodia during the early phase of lamellipod formation, when protrusion accelerates (Fig. 3 A and Video 4).

Is PI3K signaling enriched within filopodia as well? This question is not straightforward, given the size of these structures: ∼100 nm in diameter, below the resolution limit and comparable to the depth of TIRF illumination. Therefore, we assessed enrichment of PI3K signaling in filopodia by ratio imaging. The mean TIRF intensity of the FP-AktPH domain was normalized by that of an FP cytoplasmic volume marker; this
Nascent adhesions form along F-actin bundles in tandem with propagation of PI3K signaling

Integrin-mediated adhesion is responsible for attachment of filopodia to ECM (Partridge and Marcantonio, 2006; Galbraith et al., 2007), whereas in lamellipodia, it is established that adhesion complexes foster activation of PI3K and Rac, leading to actin polymerization mediated by the Arp2/3 complex (Chen and Guan, 1994; Cox et al., 2001). To test whether or not adhesions nucleate beneath F-actin bundles with the appropriate timing relative to lamellipod formation, we imaged NIH 3T3 fibroblasts coexpressing FP-fascin and FP-paxillin, a marker of adhesion complexes. Adhesions were found along the length of fascin-containing bundles, with the highest density of paxillin typically at the base (Fig. 4 A and Video 5). Most often, such adhesions at the cell periphery lack zyxin, a marker for mature adhesions and stress fibers (Fig. S4, A and B; Zaidel-Bar et al., 2003; Yoshigi et al., 2005). Small adhesions often appeared at the tips of filopodia followed by adhesion growth along the length of the bundle as the nascent lamellipod protruded over it. In cells coexpressing FP-paxillin and FP-AktPH, the large adhesions present at the bases of filopodia colocalize (overlap) with the emergence of PI3K signaling, during the early phase of lamellipodial protrusion (Fig. 4 B and Video 6). Overlap was detected in 42/49 (86%) of protrusions over actin bundles (Fig. S4 C).

Fluorescence ratio, measured for each filopodium, was normalized again by the fluorescence ratio of a region near the center of the contact area (see Materials and methods and Fig. S3 for technical notes). PI3K signaling in filopodia is considered significant if the resulting quantity, the enrichment ratio \(E\), is significantly >1. Averaged over the lifetime of each filopodium (identified by the aforementioned segmentation algorithm; Fig. S1), the mean value of \(E = 3.3\), whereas pharmacological inhibition of PI3K yielded a mean value of \(E = 1.1\) (Fig. 3 B). Thus, the ratio imaging approach was validated and indicates that filopodia harbor substantial PI3K signaling.

Having devised a method for detecting PI3K signaling in filopodia, we asked whether or not this activity was present before the emergence of lamellipodia. In time-lapse videos of 27 cells subjected to ratio imaging, we identified those instances when lamellipodia emerged over filopodia without marked pre-existing enrichment of PI3K signaling in the proximal region of the plasma membrane (i.e., as would be indicated by inspection of the FP-AktPH channel alone). In 78% of those instances (151/194), PI3K signaling was enriched in the filopodial structures beforehand and was thereafter elevated in the lamellipod (Fig. 3 C). These findings establish filopodia as both structural guides and signaling hubs that prime the emergence of lamellipodia.
These colocalization experiments suggest that adhesions mediate activation of PI3K signaling and other pathways that promote Arp2/3-based, lamellipodial protrusion around F-actin bundles. To test this hypothesis, we assessed the role of FAK, a known intermediate in integrin-mediated activation of PI3K (Chen and Guan, 1994; Reiske et al., 1999). Pharmacological inhibition of FAK activity, which prevents FAK autophosphorylation of the tyrosine residue that engages type IA PI3Ks, ablated the localization of PI3K signaling to putative lamellipodia (Fig. 4 C and Video 7). Whereas FAK inhibition significantly reduced PI3K localization in the cells plated on fibronectin as before, this was not the case for cells plated on poly-lysine, which promotes integrin-independent adhesion of cell membranes (Fig. 4 C). Inhibition of the Src family of protein tyrosine kinases, which are recruited to adhesion complexes as well as other signaling complexes (Plopper et al., 1995; Cuevas et al., 2001), also ablated localization of PI3K signaling in cells plated on fibronectin, and this treatment partially reduced PI3K signaling in cells plated on poly-lysine (Fig. S4 D).

Arp2/3-driven protrusion amplifies adhesion-based signaling by positive feedback

The observation that photoactivation of Rac induces redistribution of PI3K signaling (Fig. 1 C), by a mechanism that requires actin polymerization (Welf et al., 2012), suggested that the signaling circuit controlling Arp2/3 activation in fibroblasts operates under positive feedback. This is plausible because lamellipodial protrusion results in formation of nascent adhesions that are in transient contact with the dendritic F-actin network (Vicente-Manzanares et al., 2009). Consistent with such a mechanism, after Arp2/3 was pharmacologically inhibited in NIH 3T3 cells coexpressing FP-fascin and FP-AktPH, most of the numerous lamellipodia retracted, in concert with loss of PI3K signaling (Fig. 5 A). FP-paxillin labeling confirmed that small adhesions near the leading edge were also lost after Arp2/3 inhibition (Fig. S5 A) as well as in other contexts in which lamellipodia are impaired: after PI3K inhibition and in fascin-depleted cells (Fig. S5, B and C). The effect of Arp2/3 inhibition on PI3K signaling was not observed in cells plated on poly-lysine (Fig. 5 B), linking the phenomenon to integrin-mediated adhesion. To rule out off-target effects of the drug on PI3K signaling, we performed similar experiments with a different fibroblast line, in which conditional knockout of the Arp2/3 complex was established (Rotty et al., 2015). Whereas uninduced (wild type) cells treated with Arp2/3 inhibitor showed the same ablation of PI3K signaling seen in NIH 3T3 cells, cells treated with tamoxifen to induce Cre-based silencing of Arpc2 showed no marked changes in distribution of PI3K signaling upon Arp2/3 inhibition (Fig. 5 C and Video 8).

A hallmark of positive feedback is the phenomenon of traveling waves. We observed recurring, traveling waves of protrusion/retraction directed along a subset of filopodia (n = 11, in 6 cells analyzed this way; Fig. 5 D and Video 9). In all instances, PI3K signaling was most intense at the base of the structure. We developed an automated analysis to plot the width of protrusions as a kymograph, i.e., as a function of time and position parallel to protrusion. From these kymographs, we estimated a mean wave velocity of ∼3 µm/min (Fig. S5 D).
Fascin-1 depletion blocks fibroblast haptotaxis but not chemotaxis

Having elucidated the dynamic relationship between fascin-containing F-actin bundles and formation of lamellipodia that affects large-scale changes in cell morphology, we sought to test the functional role of fascin-1 in different modes of directed cell migration. For this, we used IA32 mouse fibroblasts, which exhibit robust tactic migration in response to a gradient either soluble PDGF (chemotaxis) or immobilized fibronectin (haptotaxis) established in a microfluidic chamber (Wu et al., 2012). In this cell line, we confirmed the basic findings that fascin-containing bundles serve as templates for formation of lamellipodia and activation of PI3K signaling (Fig. 6 A and Video 10). As in NIH 3T3 cells, we depleted fascin-1 expression by shRNA interference and achieved rescue in this background by expression of homologous fascin-1 (Fig. 6 B). In PDGF chemotaxis assays, fascin-1 depletion yielded a modest reduction in the mean forward migration index (FMI); however, the difference is not statistically significant (Fig. 6 C). In contrast, fascin-1 depletion ablated haptotaxis, a phenotype that was reversed by rescue of fascin-1 expression (Fig. 6 D). Under both chemotaxis and haptotaxis conditions, metrics of unbiased cell migration efficiency—mean speed and persistence (D/T ratio)—were not significantly affected by fascin-1 depletion. We conclude that fascin-1 performs an important function in directed migration of fibroblasts but in a context-dependent manner.

Discussion

Cooperation of bundled and dendritic F-actin in formation and orientation of lamellipodia

Based on the evidence provided here, together with previous work, we construct the following sequence of processes that link subcellular dynamics to macroscopic changes in cell shape (Fig. 7). (a) F-actin bundles form at the periphery of the cell, often manifest as filopodia. Larger adhesions are typically
FP-AktPH in filopodia is partially attributed to geometric considerations; it is estimated that the entire membrane sheath of a filopodium is within the field of TIRF illumination. Considering also the uncertainties about partitioning of the cytoplasmic FP-AktPH pool in/out of filopodia and the net diffusion of PIP3 from the filopodial sheath to the adjacent region of the plasma membrane, the present data do not allow accurate quantification of the extent of PI3K activity enrichment in these structures. The possibility that the density of PIP3 is enhanced by reduction of its turnover (Schneider et al., 2005), i.e., by regulation of lipid dephosphorylation, must also be considered. If achieved by specific localization of PI3K, our colocalization, FAK inhibition, and poly-lysine control experiments implicate the adhesion complexes that form underneath adherent F-actin bundles/filopodia as both the structural and signaling linchpins.

Our results further suggest that PI3K and other signaling activities associated with F-actin bundles prime a positive feedback loop in which adhesions and FAK/PI3K signaling both promote and respond to Arp2/3-mediated actin polymerization and membrane protrusion (Fig. 5, A–C). Excitability and traveling waves are hallmarks of systems with positive feedback and have been characterized in other migrating cell types (Allard and Mogilner, 2013; Huang et al., 2013). The traveling waves of protrusion and PI3K signaling that we sometimes observe (Fig. 5 D) suggest that there is negative regulation as well. Candidates for such regulation mechanisms include mechanical feedback involving Rho signaling to Myosin II (Guilluy et al., 2011; Welf et al., 2012).
both mechanical and signaling roles. By the same token, deple-
tion of neither fascin-1 nor of the Arp2/3 complex impaired
PDGF chemotaxis, at least not significantly so, suggesting that
mesenchymal cells use diverse, context-dependent mechanisms
for achieving asymmetric force generation (Bear and Haugh,
2014). This does not discount the role of lamellipodia and la-
mellipodial branching in exploratory motility that allows fibro-
blasts to align their direction of migration with a PDGF gradient
(Welf et al., 2012). Furthermore, considering that our PDGF
chemotaxis assay was conducted under near-optimal gradient
directionality, expressed as an angle relative to an external gradient of PDGF, are shown for
control (n = 74) and fascin-1 shRNA (n = 131).
Mean FMI and velocity and persistence (D/T) are displayed ±95% confidence intervals.
(D) Hap-
totaxis assay results are shown for control (n =
128), fascin-1 shRNA (n = 124), and fascin
rescue (n = 57) tracks. Mean FMI and veloc-
ity and persistence (D/T) are displayed ±95%
confidence intervals.
2013) and an incoherent feedforward loop wherein active Rac
both promotes and inhibits the Arp2/3 complex, through WAVE
and Arpin, respectively (Dang et al., 2013).

**The role of fascin-containing bundles in directed cell migration**

Our functional data demonstrate that fascin-1 depletion impairs
lamellipodia formation and haptotactic sensing of fibroblasts on
ECM. Previous work showed that depletion of the Arp2/3 com-
plex ablated dendritic actin arrays associated with lamellipodia
and likewise impaired haptotaxis (Wu et al., 2012). These
results concordantly indicate the importance of actin-based pro-
trusion in ECM haptotaxis, with adhesion complexes serving
both mechanical and signaling roles. By the same token, deple-
tion of neither fascin-1 nor of the Arp2/3 complex impaired
PDGF chemotaxis, at least not significantly so, suggesting that
mesenchymal cells use diverse, context-dependent mechanisms
for achieving asymmetric force generation (Bear and Haugh,
2014). This does not discount the role of lamellipodia and la-
mellipodial branching in exploratory motility that allows fibro-
basts to align their direction of migration with a PDGF gradient
(Welf et al., 2012). Furthermore, considering that our PDGF
chemotaxis assay was conducted under near-optimal gradient
conditions, our results do not exclude the possibility that coor-
dination of bundled and dendritic F-actin plays a more impor-
tant role in sensing suboptimal gradients.
Beyond this level of understanding, our results suggest a more refined hypothesis of mesenchymal taxis. A distinct aspect of haptotaxis is that the cell must actively protrude to encounter immobilized cues, whereas soluble chemoattractants are encountered passively, i.e., by diffusion. The search for adhesive ligands is inherently an exploration, with F-actin bundles apparently directing, and lamellipodia propagating, the process. Our observations of adhesions forming beneath the bundles, even before the emergence of lamellipodia, lead us to speculate that the two F-actin structures dynamically cooperate in the haptotactic sensing of ECM ligands and that integrin-mediated signaling integrates those dynamics. Although this is an appealing model, this mechanism might be only one of multiple ways that fascin influences random and haptotactic cell migration. For example, fascin has been shown to regulate tension in stress fibers and thus the dynamics of mature adhesions (Elkhatib et al., 2014).

Fascin expression has been implicated in cancer progression and associated with poor prognoses in many cancers, making it a promising therapeutic target or biomarker (Machesky and Li, 2010; Arjonen et al., 2011). Fascin is associated with the epithelial-to-mesenchymal transition (Li et al., 2014) and is thus linked to acquisition of tumor cell invasiveness. Our results implicate fascin and F-actin bundles in exploratory morphodynamics that are generally important for directed cell migration, haptotaxis especially. ECM gradients are encountered during collective fibroblast invasion of wounds and likewise during tumor cell invasion of the surrounding matrix, and it will be interesting to investigate the relative roles of haptotactic and chemotactic cues in those invasion processes (Lara Rodriguez and Schneider, 2013; Bear and Haugh, 2014). Another important aspect of haptotaxis in need of characterization is how it is affected by the structure of ECM in 3D versus 2D and contact guidance of cell migration that is a manifestation of ECM remodeling by mesenchymal cells (Even-Ram and Yamada, 2005).
method with the addition of 5 μg/ml polybrene. Cells expressing shRNAs were selected using 2 μg/ml puromycin and, in the case of cotransfection, 60 μg/ml hygromycin.

Arpc2 fibroblasts were generated as previously described (Rotty et al., 2015). In brief, adult tail fibroblasts were harvested from a conditional knockout mouse with exon 8 of the gene encoding the p34 subunit of the Arp2/3 complex (Arpc2) flanked by LoxP sites in an Ink4a/Arf-/ background. Knockout of p34 was induced by subculturing cells in cell culture media supplemented with 2 μg/ml puromycin and 2 μM tamoxifen for 5 d. The cells were used for up to 10 d thereafter.

Live-cell TIRF microscopy
Prism-based TIRF microscopy was performed as previously described (Schneider and Haugh, 2004, 2005; Johnson and Haugh, 2013). The microscope is equipped with a polychromatic microscope (Axioskop 2 FS; Carl Zeiss) equipped with 40×, 0.8 NA and 60×, 1.0 NA Achroplan water dipping objectives (Carl Zeiss) and a charge-coupled device camera (ORCA ER; Hamamatsu Photonics). Coverslips or glass-bottom dishes (MatTek Corporation) were coated with poly-l-lysine (70–150 kD; 1 mg/ml; Sigma Aldrich). Cells were briefly trypsinized and reseeded in imaging buffer (20 mM Hepes, 125 mM NaCl, 5 mM KCl, 1.5 mM MgCl2, 1.5 mM CaCl2, 10 mM glucose, and 2 mg/ml fatty acid-free BSA, pH 7.4) supplemented with 1% PEG and either 1% (NIH 3T3s) or 10% FBS (IA32s). The cells, transiently transfected 1 d before the experiment, were allowed to spread for 2 h (NIH 3T3s) or overnight (IA32s). FFs were excited using the following lasers: 442 nm (TFP), 488 nm (EGFP), and 561 nm (mCherry and tdTomato); emission filters were 480/40, 515/30, and 630/60 nm, respectively. Photoactivation of Rac was performed using a mercury arc lamp as previously described (Welf et al., 2012). In brief, a 50-W mercury arc lamp passed through a 436/20-nm excitation filter and focused to create a small spot. Diffuse light was blocked, and the spot was imaged using fluorescent dextran. A threshold was applied to this image to determine the area of excitation. Image acquisition was programmed using MetaMorph software (Universal Imaging). The frequency of image acquisition varied from 1–12 frames/min (most commonly, 2 frames/min); acquisition for the protrusion/demotion wave experiments (Fig. 5 D) was consistently at the upper end of the range (6 or 12 frames/min). All experiments were performed at 37°C. Image (National Institutes of Health) was used for basic image preparation for presentation to crop, convert, adjust contrast, or smooth images for clarity as described in detail previously (Johnson and Haugh, 2013).

Morphodynamic analysis
All image analysis was performed using MATLAB (MathWorks). Images were segmented either automatically by k-means clustering or manually via thresholding. Spatiotemporal mapping and the identification of morphological extensions were performed as described in detail previously (Welf et al., 2012; Johnson and Haugh, 2013). In brief, protrusion/retraction for the spatiotemporal maps was calculated by the difference in overlap between consecutive frames. The angle of each protruded/retracted pixel relative to the cell centroid is determined, and these are binned according to 1° increments. The sum of the pixel counts in each bin for the time intervals that range to a velocity, which is plotted as a function of angle and time and smoothed with a moving mean filter. To identify morphological extensions, the distances of all pixels on the cell contour from the centroid are first measured; portions of the contour that exceeded the moving mean distance taken over 100-pixel windows by ≥0.5 μm for >13-μm contour length, and which contained ≥50 pixels in the extended region, were designated as extensions. These parameters were set so that the structures thus identified are clearly extended from the cell body and exclude filopodia (at this resolution, 0.25 μm/pixel, even very long filopodia will not contain 50 pixels above the threshold).

Analysis of lamellipodium/filopod overlap
To identify putative filopodia in each cell, the area containing the cell was cropped and binarized by a manually set threshold. This threshold was determined by studying a low value and iteratively increasing it until either the cell outline becomes sharp or filopodia begin to be lost; at that point, the threshold is returned to the lowest value that gives a reasonably crisp cell outline. A 7-pixel-wide (1.75 μm at 40×), square top-hat filter was applied to the binary cell mask to segment filopodia. Pixels were filled in between segmented structures within a few pixels of each other to “heal” discontinuities. Then, regions below a certain area cutoff (<15 pixels at 40×) were removed; by subtracting the resulting mask of segmented filopodia from the whole-cell mask, a mask of the rest of the cell was also generated. The first several frames of each cell were checked to ensure reasonably accurate segmentation. A sensitivity analysis was performed on a few cells, varying the chosen threshold and the constant top-hat filter size. Increasing or decreasing the threshold by as much as 20% of the chosen value or varying the filter size by ±1 pixel altered the mean number of filopodia identified by no more than 15% across all frames for the cells tested. Errors in segmentation are primarily attributable to fragmentation of a single filopod into multiple regions or merging of multiple filopodia into a single region.

Based on the morphodynamic characteristics of lamellipodia as seen in cell migration videos, and as analyzed using protrusion maps (Machacek and Danuser, 2006), we defined these structures in NIH 3T3 cells as continuous regions showing estimated protrusion velocity > 1.25 μm/min and spanning ≥8° for ≥1 min. This is performed using the mask excluding filopodia; therefore, the pixels associated with the identified lamellipodia and filopodia cannot overlap. Rather, we define the regions as “overlapping” if they contain pixels in the same angular bin or in adjacent bins (for the same frame of the video or in adjacent frames).

For the FP-Fascin–expressing cells, submembraneous bundles were included in addition to the putative filopodia. Those bundles were segmented from the FP-Fascin channel by applying a 1-μm top-hat filter and taking the highest k-means bin of that image. Bundles not within 5 μm of the cell edge were excluded. At least in protruding lamellipodia, this would exclude regions associated with stress fibers, for example.

Ratiometric analysis of PI3K signaling in filopodia
The volume marker (TFP) channel was used to make masks of the putative filopodia as described in a previous section. For each filopod, the base was identified as the end connected or closest to the cell body. Tracking of filopodia across frames of a video was performed as follows. If the base of a filopod was within a few pixels of one found in the previous two frames, it was counted as the same; otherwise, it was counted as a new filopod. Filopodia that appeared for fewer than three frames were considered non-adherent and removed. The aforementioned mask of the cell excluding filopodia was eroded by 5 μm to identify a central region of the cell. The mean, background-subtracted TIRF intensities, F, from the TFP and AktPH channels were calculated for both the filopodia and the central region. For each filopod, the enrichment ratio, E, was calculated and averaged over its lifetime.

A value of E > 1 is interpreted to mean that AktPH is enriched in the filopod relative to a cytosolic volume marker.

Quantification of change in PI3K signaling pattern
To quantify the change in PI3K signaling in response to FAK inhibition, we first identified morphological extensions as described under Morphodynamic analysis. Subtracting these regions from the whole-cell mask yields a mask of the cell body. The mean AktPH intensity in the cell body region was used as the background level, which was subtracted from the highest AktPH mean intensity among the morphological extensions. The net intensity thus calculated, averaged over five frames beginning 5 min after adding FAK inhibitor, was compared as a ratio to the net intensity for the five-frame mean just before the inhibitor was added (after/before). Complete ablation of localization (AktPH intensity becomes spatially uniform) by the inhibitor returns a value close to 0; whereas no change in the localization returns a value of 1.

Analysis of PI3K signaling/adhesion colocalization
To quantify overlap enrichment of PI3K signaling with adhesions at the base of filopodia, we visually determined when protrusion over an actin bundle first began and then located where increasing FP-AktPH recruitment was first visible. We next identified the most intense pixels in the associated FP-AktPH image by k-means segmentation, using a bin size of 4; if there was no local region of FP-AktPH enrichment, the protrusion was scored as having no overlap. The corresponding paxillin channel was likewise segmented, and size cutoffs were applied to filter out noise. The hotspot was then checked for overlap with all adhesions (the two highest bins were 3 and 4; ≥15 contiguous pixels in size) and those containing the highest intensity of FP-paxillin labeling (bin 4 only; ≥7 contiguous pixels in size). A segmented example is shown in Fig. 5 A C.
Chemotaxis and haptotaxis assays

Microfluidic devices were prepared as previously described [Wu et al., 2012]. In brief, transparency masks were printed using a high-resolution printer (FINELINE Imaging), and the pattern for the chamber was fabricated on 4-inch silicon wafers by a two-step photolithography process. The silicon wafer was exposed to silane overnight after developing and after baking. Poly(methylsilazane) (PDMS) was then poured on the wafer and cured overnight at 70°C. Individual PDMS devices were then cut out from the wafer, and ports were punched out. The devices were washed with water and then with ethanol, blow dried, and plasma cleaned. The PDMS device was placed into contact with a glass dish bottom immediately, ensuring that an irreversible seal was formed. To establish a PDGF gradient, 27.5-gauge needles were attached to gas tight 100-µl Hamilton glass syringes (81020, 1710 TIL 100 µ SYR) and connected via tubing to the entrance ports of the device. Both syringes were filled with DMEM, and the source was supplemented with 120 ng/ml PDGF and 1–5 µg/ml TRITC/Cy5-dextran to visualize the gradient. Using a syringe pump, the flow rate was set to 20 nl/min, and a stable gradient was established within 30 min. Haptotactic gradients were formed by the addition of 250 µg/ml Cy5-labeled fibronectin to the source channel. After washing with sterile PBS, the gradient of immobilized fibronectin was visualized by epifluorescence before seeding cells in the culture chamber.

Co-cultures of control and fascin shRNA-containing cells were used in each chamber. The control cells were dyed using CellTracker green (Life Technologies), with the exception of the rescue experiments, in which the rescue cells expressed EGFP-tagged human fascin-1 (isolated by fluorescence-activated cell sorting). Differential interference contrast images were acquired every 10 min for 12–24 h using an X81 (Olympus), VivaView FL (Olympus), or Biostation (Nikon) microscope, each equipped with motorized stages and a 20× S Apochromat, 0.75 NA or a 20× Plan Fluor DL, 0.5 NA objective. All experiments were performed at 37°C in a humidified environment.

Cells were tracked using the manual tracking plugin (Ibiud) in ImageJ. Intervals during which cells were touching or dividing were excluded. The cell centroid tracks were analyzed in MATLAB to calculate FMI and speed and persistence (D/T ratio) and to construct wind-rose plots. Each wind-rose plot shows a histogram of the angles between endpoints of each track, smoothed by a moving mean. The FMI is defined as the total translocation in the direction of the gradient divided by the overall path length [Foxman et al., 1999]. The D/T ratio is defined as the Euclidian distance between endpoints divided by the overall path length.

Online supplemental material

Fig. S1 illustrates the method for segmentation of adherent filopodia. Fig. S2 includes an additional example of fascin-containing bundles templating branch formation, images of Lifeact-labeled F-actin during formation of lamellipodia over actin bundles, quantification of the fates of individual filopodia and persistence (D/T ratio) and to construct wind-rose plots. Each wind-rose plot shows a histogram of the angles between endpoints of each track, smoothed by a moving mean. The FMI is defined as the total translocation in the direction of the gradient divided by the overall path length [Foxman et al., 1999]. The D/T ratio is defined as the Euclidian distance between endpoints divided by the overall path length.

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References


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Supplemental material

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Figure S1. **Automated identification of filopodia.** (A) Putative filopodia are segmented (red; cell mask shown in yellow) by top-hat filtering of TIRF images. These structures may be tracked based on frame-to-frame overlap/proximity. (B) The structures thus identified tend to be enriched in FP-tagged fascin-1. (C) The putative filopodia are labeled by FP-tagged Myosin X at their tips. Bars, 10 µm.
Figure S2.  **F-actin bundles direct bifurcation of lamellipodia.** (A, left) Additional example from the cohort of cells analyzed in Fig. 2 A; TIRF montage of an NIH 3T3 fibroblast expressing EGFP-fascin (inverted grayscale), with prominent F-actin bundles marked by red arrows. Bar, 10 µm. (right) Corresponding spatiotemporal map of protrusion and retraction. The initiation of lamellipodial branching apparent in the latter two panels of the montage at left manifests on the map as an upside-down U shape (roughly, between 115° and 140° relative to the centroid starting at ~8 min in this time course). (B) TIRF montage of an NIH 3T3 coexpressing tdTomato (Td-Tom)-Lifeact and EGFP-fascin, illustrating F-actin organization in nascent lamellipodia and bundles. Bar, 5 µm. (C) Analysis of the percentages of filopodia that (from top to bottom): appear before an overlapping lamellipod; appear after, and persistent after, an overlapping lamellipod; appear after, and disappear during the lifetime of, an overlapping lamellipod; or appear and disappear with no overlap with lamellipodia. (D) Quantification of cell speed, by cell centroid tracking (sampled every 12 min) for control (n = 21) and fascin-overexpressing (OE; n = 31) cells. The data are presented as means ± 95% confidence interval.
Figure S3. Calculation of the enrichment ratio for filopodia. (A) Putative filopodia were identified from volume marker (TFP) images as described under Materials and methods and Fig. S1. The mask associated with filopodia is dilated, and the mean intensity of each filopodial region is computed along with that of the cell interior. The ratio of those intensities (filopod/interior) is recorded. This procedure is then repeated for each region of pixels in the other channel, the mCherry-AktPH biosensor, for example. In this case, the enrichment ratio, $E$ [the filopod/interior ratio of AktPH normalized by that of the volume marker] is used to assess the presence of PI3K signaling in the filopodia. Bar, 10 µm. (B) The value of $E$ reflects both the extent of biosensor translocation (from the cytosol to the membrane) and the filopod geometry, effects that are not readily decoupled. Using a phosphatidylinositol (GPI)-anchored FP to uniformly label the outer leaflet of the plasma membrane, we estimated the relative enrichment of plasma membrane area relative to the volume of a filopodium. With FP-GPI in place of FP-AktPH, the mean value of $E$ was 4.7 ($n = 798$ filopodia from nine cells; error bars indicate ±95% confidence interval), indicating a significant effect of geometry. Put another way, the ratio of AktPH/volume marker TIRF should not be used to compare relative phosphoinositide densities in filopodia versus elsewhere in the contact area.

Figure S4. TIRF imaging of adhesion dynamics. (A) Two-color TIRF imaging of zyxin and paxillin in NIH 3T3 cells confirms that mature, zyxin-containing adhesions are found some distance behind the leading edge. Arrowheads indicate where adhesions form at the base of or beneath filopodia. (B) Two-color TIRF imaging of fascin and zyxin in NIH 3T3 cells confirms that zyxin-containing adhesions are typically located behind F-actin bundles at the periphery. White arrowheads show filopod proceeding lamellipod formation, devoid of zyxin localization. Red arrowheads show formation of zyxin-positive adhesions farther back in the cell. (C, left) Example of adhesion and PI3K signaling segmentation (k-means) for the protrusion shown in Fig. 4 B. Adhesions are outlined in yellow and the region of high PI3K signaling is outlined in magenta. All adhesions are regions that include pixels in the top two of 4 bins. High paxillin adhesions are defined as regions with pixels segmented in the highest bin only. (right) Fractions of protrusions over F-actin bundles with emergence of local PI3K signaling overlapping the two categories of adhesions (49 protrusion events in 13 cells, same cohort as in Fig. 4 B). (D) TIRF images of FP-AktPH-expressing NIH 3T3 cells, plated on either fibronectin or poly-lysine, before and after inhibition of Src-family kinases (PP2; 10 µM). The images are representative of 13 cells viewed for fibronectin and 19 cells viewed for poly-lysine. Bars: (A, B, and D) 10 µm; (C) 5 µm.
Figure S5. Imaging interplay between protrusion and adhesion dynamics. (A) Two-color TIRF imaging of Lifeact and paxillin before and after inhibition of Arp2/3 complex by 100 µM CK666. The arrows show where small adhesions were lost after collapse of lamellipodia. Td-Tom, td-Tomato. (B) Same as A but with inhibition (Inhib) of PI3K (1 µM PI3K-α inhibitor IV). (C) Two-color TIRF imaging of AktPH or Lifeact biosensor with tagged paxillin in cells depleted of fascin-1. (D, left) Kymograph of protrusion width during periodic protrusion waves as in Fig. 5 D. Lines are drawn through the waves and used to calculate the velocity of each wave. (right) Histogram indicating velocities of protrusion dilation waves (n = 48), estimated from kymograph as shown on the left. These were taken from high-resolution videos of 11 recurrent protrusions observed in six different cells. Bars: (A and C) 20 µm; (B) 10 µm.
Video 1. **Time-lapse video of filopodia directing nascent lamellipodia, as depicted in Fig. 1 A.** TIRF microscopy images of an NIH 3T3 cell expressing EGFP-AktPH (pseudocolored) were acquired every 15 s for 132 min. Time stamp is shown in minutes, seconds, and milliseconds. Bar, 20 µm.

Video 2. **Time-lapse video of PI3K signaling and protrusion during photoactivation of Rac near filopodia, as depicted in Fig. 1 C.** TIRF microscopy images of an NIH 3T3 cell coexpressing mCerulean-PA-Rac and mCherry-AktPH (pseudocolored) were acquired every minute for 79 min. Time stamp shows hours and minutes. The red spots indicate the timing and area of photoactivation. Bar, 20 µm.

Video 3. **Time-lapse video of F-actin bundles directing lamellipodial protrusion, as depicted in Fig. 2 A.** TIRF microscopy images of an NIH 3T3 cell expressing EGFP-fascin (inverted grayscale) were acquired every 30 s for 21 min. Time stamp shows minutes and seconds. Bar, 10 µm.

Video 4. **Time-lapse video of PI3K signaling and fascin localization during protrusion over an F-actin bundle, as depicted in Fig. 3 A.** TIRF microscopy images of an NIH 3T3 cell coexpressing mCherry-AktPH (left, pseudocolored) and EGFP-fascin (right, inverted grayscale) were acquired every 30 s for 67 min. Time stamp is shown in hours, minutes, and seconds. Bar, 10 µm.

Video 5. **Time-lapse video of adhesion dynamics during protrusion over F-actin bundles, as depicted in Fig. 4 A.** TIRF microscopy images of an NIH 3T3 cell expressing mCherry-Fascin (left, grayscale) and EGFP-paxillin (center, pseudocolored) were acquired every 30 s for 20 min. Time is given in minutes and seconds. An overlay of the two channels is shown at right. Bar, 5 µm.

Video 6. **Time-lapse video showing adhesion formation and PI3K signaling during protrusion over actin bundles, as depicted in Fig. 4 B.** TIRF microscopy images of an NIH 3T3 cell expressing mCherry-AktPH (left) and EGFP-paxillin (right) were acquired every 30 s for 10 min. Time is given in minutes and seconds. An overlay of the two channels is shown at right. Bar, 5 µm.

Video 7. **Time-lapse video showing ablation of localized PI3K signaling by FAK inhibition in cells plated on fibronectin but not on poly-lysine, as depicted in Fig. 4 C.** TIRF microscopy images of NIH 3T3 cells expressing FP-AktPH (pseudocolored), plated on either fibronectin (left) or poly-lysine (right), were acquired every 30 s for 17 min in separate experiments. At the indicated time, FAK activity was inhibited by addition of 10 µM FAK inhibitor II. Time is given in minutes and seconds. Bar, 20 µm.
Video 8. Time-lapse video showing ablation of localized PI3K signaling by Arp2/3 inhibition in uninduced cells, but not in cells with tamoxifen-induced knockout of Arpc2, as depicted in Fig. 5 C. Fibroblasts with conditional knockout of the Arpc2 (p34) subunit of the Arp2/3 complex, transfected with FP-AktPH (pseudocolored), were either uninduced (top) or tamoxifen-induced (bottom). TIRF images were acquired every 30 s for 19 min in separate experiments. At the indicated time, the Arp2/3 complex was inhibited by addition of 100 µM CK666. Time is given in minutes and seconds. Bar, 20 µm.

Video 9. Time-lapse video of protrusion dilation waves, as depicted in Fig. 5 D. TIRF microscopy images were of an NIH 3T3 cell expressing mCherry-AktPH (pseudocolored) were acquired every 10 s for 30 min. Time is given in minutes and seconds. Bar, 10 µm.

Video 10. Time-lapse video of multiple F-actin bundles directing protrusions that coalesce to form a broad lamellipod, as depicted in Fig. 6 A. TIRF microscopy images of an IA32 MEF coexpressing EGFP-fascin (inverted grayscale) and mCherry-AktPH (pseudocolored) were acquired every 30 s for 21 min. Time is given in minutes and seconds. Bar, 10 µm.