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Protrusion and Actin Assembly are Coupled to the Organization of Lamellar Contractile Structures

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Abstract

Directed cell migration requires continuous cycles of protrusion of the leading edge and contraction to pull up the cell rear. How these spatially distributed processes are coordinated to maintain a state of persistent protrusion remains unknown. During wound healing responses of epithelial sheets, cells along the wound-edge display two distinct morphologies: ‘Leader cells’ exhibit persistent edge protrusions, while the greater majority of ‘follower cells’ randomly cycle between protrusion and retraction. Here, we exploit the heterogeneity in cell morphodynamic behaviors to deduce the requirements in terms of cytoskeleton dynamics for persistent and sporadic protrusion events. We used quantitative Fluorescent Speckle Microscopy (qFSM) to compare rates of F-actin assembly and flow relative to the local protrusion and retraction dynamics of the leading edge. Persistently protruding cells are characterized by contractile actomyosin structures that align with the direction of migration, with converging F-actin flows interpenetrating over a wide band in the lamella. Conversely, non-persistent protruders have their actomyosin structures aligned perpendicular to the axis of migration, and are characterized by prominent F-actin retrograde flows that end into transverse arcs. Analysis of F-actin kinetics in the lamellipodia showed that leader cells have threefold higher assembly rates when compared to followers. To further investigate a putative relationship between actomyosin contraction and F-actin assembly, myosin II was inhibited by blebbistatin. Treated cells at the wound edge adopted a homogeneously persistent protrusion behavior, with rates matching those of leader cells. Surprisingly, we found that disintegration of actomyosin structures led to a significant decrease in F-actin assembly. Our data suggests that persistent protrusion in these cells is achieved by a reduction in overall F-actin retrograde flow, with lower assembly rates now sufficient to propel forward the leading edge. Based on our data we propose that differences in the protrusion persistence of leaders and followers originate in the distinct actomyosin contraction modules that differentially regulate leading edge protrusion-promoting F-actin assembly, and retraction-promoting retrograde flow.

Keywords

Protrusion; Contraction; speckles; cell motility; wound-healing; correlation

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Introduction

Cell protrusion is driven by assembly of actin filaments (F-actin) at the leading edge. Addition of monomers at barbed ends subadjacent to the plasma membrane is thought to produce forces that can overcome membrane tension causing edge advancement [1]. However, other factors may also contribute to the protrusion process. Importantly, polymerizing filaments need to be anchored to the extracellular domain via the formation of adhesions [2,3]. Otherwise, assembly merely leads to rearward translocation of the growing network, a process known as retrograde flow [4–6]. Retrograde flow can also be generated by contraction of the actin network, driven by myosin II motors [7–9]. Hence, contraction appears to antagonize the effect of filament assembly by generating forces that favor edge retraction [10]. Indeed in neuronal growth cone motility, global and regional variations of retrograde flow activity are established regulators of protrusion efficiency [11,12]. How these variations contribute to the protrusion control in other cell types is still unclear.

Besides its putative role as an antagonist to actin filament assembly and protrusion, actomyosin contractility is thought to be responsible for the generation of cell traction and the advancement of the cell rear [13–16]. Thus, persistent cell migration requires a tight coordination of actin filament assembly at the front and actomyosin contraction throughout the cell [17].

In an ideal model efficient cell migration is initiated by actin filament assembly at the front with concurrent reduction of retrograde flow via down-regulation of contractile activity and/or increased adhesion. Upon stabilization of the newly formed protrusion by adhesions, the contractile activity is upregulated to pull forward the cell rear with minimal front retraction. Upon completion of the contraction events the filament assembly at the front is reinitiated.

Work over the past years has shed light on the spatial distribution of these processes. There is consensus that actin filament assembly is concentrated in the lamellipodium actin network, where the actin nucleator Arp2/3 and the actin severing and depolymerization factor cofilin, maintain a rapid treadmilling of actin filaments. Lamellipodium treadmilling is accompanied by fast retrograde flow, which is independent of the activity of myosin II motors. Myosin II-driven retrograde flow is a characteristic of the lamella, a second, structurally and molecularly different actin network behind the lamellipodium [Ponti, 2004 #89]. At the front of the lamella actin filaments engage with adhesion complexes that generate the traction required for cell advancement driven by lamellar contraction. The precise modes of interaction between lamellipodium and lamella remain elusive. Lamellipodium actin filaments may flow into the lamella network [18] or they may disassemble completely followed by de novo assembly of lamella filaments. In the first scenario the mechanical coupling of lamellipodium and lamella would be relatively rigid. In a second scenario the two networks would be decoupled. As a third, intermediate possibility, we have proposed that lamellipodium and lamella filaments partially overlap to form a transient mechanical link that is regulated by cofilin, among other factors [5,19]. At the base of the lamella, retrograde flows merge with anterograde flows of actin filaments in a highly contractile region known as the convergence zone [8,20].

How the contractile machineries of the lamella are dynamically coordinated over many micrometers with the assembly of lamellipodium and lamella filaments at the front is unknown. To begin to address this question we have developed new analytical methods, which allowed us, on the basis of quantitative Fluorescent Speckle Microscopy (qFSM) [21], to define the relationships between retrograde flow (assembly- and contraction-dependent), F-actin assembly, and cell edge dynamics in a heterogeneous population of persistently and sporadically protruding cells. By high-resolution fluorescence imaging and qFSM, we have identified characteristic differences between the architectural features of the actomyosin

structures in the convergence zones of persistently and sporadically protruding cells. Together, our data suggests that the requirement for persistent epithelial cell migration depend on the mechanical link between the convergence zone and nascent adhesions at the lamellipodium/lamella interface, which controls via mechanotransduction, the rate of F-actin assembly in the lamellipodia.

RESULTS

Identification of Leader and Follower Cells

To study the requirements for persistent cell protrusion we chose to analyze the migratory response of PtK1 cells undergoing wound repair. Similar to other epithelial cell types [22, 23], select PtK1 cells, referred to as leader cells, developed persistent protrusions in response to a wound inflicted on a confluent cell monolayer (Figure 1A). Time lapse imaging showed that wound edge cells required 1–2 hours to reestablish adhesive contacts to the glass substrate, and cells were seen to crawl toward the site of injury soon thereafter. Leader cells were visible 2–3 hours after wounding, residing at the tip of newly formed cell mounds flanked on either side by follower cells. Leader cells maintained their ‘lead’ position over many hours or until the wound was fully closed. They exhibited an extended lamella and a broad leading edge that rarely retracted (Figure 1B; Supplemental Data, Video 1). In contrast, follower cells were characterized by compacted lamellae and sporadic protrusions that randomly alternated with phases of edge retraction.

Previous motility studies involving PtK1 cells have focused on islands composed of 2 – 8 cells [19,24,25]. Island cells for the most part did not exhibit directed motility (Figure 1C). Instead, cell morphologies were varied. In some instances they resembled follower cells in that their leading edge would randomly cycle between protrusion and retraction. Other island cells exhibited phases of persistent protrusion, although they were short-lived relative to leader cells (minutes instead of hours).

Analysis of Leading Edge Activity

To analyze the different protrusion behaviors of these cell types, time-lapse images were acquired at 10 second intervals, over an observation window of 7–10 minutes. Edge movements were tracked along the entire cell edge with submicron resolution via automated edge detection software [26] (Figure 1D; Supplemental Data, Video 2). Superimposed edge positions indicate that in leader cells most sectors move forward over 10 minutes. This results in substantial net advancement of the entire cell edge. In contrast, over the same period individual boundary sectors in follower and island cells advance and retract in quasi-random cycles. Thus, the average cell position remained largely unchanged.

To quantify these observations we computed the ‘mean protrusion rate’, i.e. the total net displacement of the cell edge divided by the duration of the observation period, with the average obtained from $n = 4$ to 8 cells (Figure 1E, left panel; see Exp. Procedures). Leaders had two- to three-fold higher protrusion rates than follower and island cells. The same cell averaging procedure was applied to determine the ‘protrusion persistence,’ i.e. the time ratio an individual edge sector spends in advancement versus in retraction (see Exp. Procedures). Leader cells exhibited approximately three-fold higher persistence than followers (Figure 1E, center panel; p -value < 0.05). They also showed slightly faster ‘instantaneous protrusion velocities,’ i.e. the average of all (positive) protrusion velocities of all reporter windows (p -value < 0.05 between leaders and followers). However, the instantaneous retraction velocities were indistinguishable between the three cell types (Figure 1E, right panel; p -value > 0.3). Together, these analyses suggest that leader cells protrude efficiently because they advance more coordinately in time and space, and, to lesser extent, because they advance faster.

Cross-Correlation Analysis of Leading Edge and F-actin Dynamics

The instantaneous protrusion velocity is determined by the difference in the rates of F-actin assembly and retrograde flow at the leading edge [27]. The latter depends on how the propulsive forces at the plasma membrane are balanced by adhesion formation [28] and may also depend on the mechanical coupling between the lamellipodium and lamella [19], defining the degree of contraction force transmission to the leading edge [29]. The protrusion persistence is largely determined by the time scales over which the assembly-to-flow ratio varies. Therefore, to understand the differences in protrusion efficiency between cells, it was crucial to determine from independent measurements the relationships between edge movement, rates of filament assembly, and rates of retrograde flow.

To achieve this, we constructed activity maps on the basis of reporter windows (Figure 2A; windows 2–4 μm in width, 1–2 μm in depth) aligned along the entire cell edge (see Exp. Procedures). Within each window we determined the edge protrusion/retraction rates and *locally* compared it to the activity maps of F-actin flow and turnover (Figure 2B). The strength of the relationship was determined by the magnitude of the pairwise cross-correlation between activity maps, including time-shifts to account for differences in the relative timing of activities (see Exp. Procedures)[30]. Correlation functions extracted from individual reporter windows were then averaged along the cell edge and subsequently over multiple cells. Underlying this procedure is the assumption that the protrusion and retraction states may be heterogeneous along the cell edge (and over time), but the relationships between edge movement, filament assembly and flow would be preserved.

Using this analysis, leader cells showed significant correlation between protrusion and assembly at -20 s. This behavior was consistent amongst leaders (Figure 2C; top row – left panel), resulting in a strong averaged correlation profile (Figure 2C; top row – right panel). The time shift of -20 s indicates that maximal F-actin assembly lags behind the fastest forward edge motion by ~ 20 s [28]. In contrast, cross-correlation between protrusion and retrograde flow (Figure 2C; mid row) resulted in a negative correlation score, reaching a minimum at -40 s. Therefore, 40 s after the maximal protrusion activity, retrograde flow of F-actin transiently speeds up. Consistent with this time course, the cross-correlation between F-actin retrograde flow and F-actin turnover led to a negative peak at $+20$ s (Figure 2C, bottom row), indicating that the transient increase in retrograde flow is preceded by increased assembly by ~ 20 s. Thus, in a leader cell, edge advancement is initiated by increased assembly of actin filaments, which reaches a maximum ~ 20 s after cell undergoes fast protrusion. As F-actin reaches the state of maximal assembly, filaments begin to slide rearward, possibly because propulsion forces exceed the force levels sustained by substrate adhesion [28]. The fastest flow rates occur ~ 20 s after maximal F-actin assembly. This event terminates a protrusion cycle, which lasts $100 - 120$ s (derived from the temporal autocorrelation of the protrusion activity map).

Follower and island cells displayed distinctly different timing relationships. Overall, the significance of the correlation functions was weaker than with leaders, owing to increased cell-to-cell variation (Figure 2D, E; left columns). This suggested that protrusion in these cells is more complex, possibly the result of convoluted contributions from multiple mechanisms. Indeed, both cell types exhibited a transient increase in filament assembly during protrusion, albeit without the time delay found in leaders (Figure 2D, E; top row). Furthermore, they showed a significant positive correlation score at zero time lag between protrusion and retrograde flow (Figure 2D, E; mid row), but no correlation between retrograde flow and assembly was observed (Figure 2D, E; bottom row). This indicates that during a protrusion event, retrograde flow is transiently decreased, thereby supporting forward propulsion of the cell edge by growing F-actin. The modulation of retrograde flow at the leading edge is

independent of F-actin assembly, and therefore unrelated to the assembly-mediated modulation of retrograde flow seen in leader cells.

Characterization of Lamellar Actomyosin Structures

We hypothesized that the less persistent protrusions of follower and island cells is driven by two weakly synchronized processes, F-actin assembly in the lamellipodium and variations in retrograde flow which depend on F-actin contraction of the lamella [31]. On the other hand, these processes in leader cells may be mechanically isolated by adhesion formation near the cell edge. To test this possibility we characterized the organization of the contractile and adhesion systems in these three cell types by immunolocalization of F-actin, myosin II, and paxillin. In leader cells, long actomyosin bundles were organized parallel to the direction of migration. We refer to these structures as longitudinal bundles (Figure 3A; yellow regions) [32,33]. Myosin II staining was absent from the utmost lamellipodia [5] and gradually accumulated in the lamella co-localizing with F-actin bundles. Numerous paxillin-containing sites were present at the cell edge (Figure 3A; arrowhead) with an elongated appearance marking the ends of longitudinal bundles (Figure 3A; arrows). This suggests that adhesions are under posterior-anterior tension [34]. In contrast, follower and the majority of island cells were characterized by thick bundles with dense myosin II staining arranged perpendicular to the direction of migration (Figure 3B, c; blue regions). We refer to these structures as transverse actomyosin arcs [35]. Paxillin staining revealed numerous adhesion sites that appeared less regular in size and orientation than those observed in leaders (Figure 3B, C; arrowheads). Interestingly, a small minority of island cells resembled the morphology of leader cells with an expanded lamella, array of longitudinal bundles, and elongated adhesion sites (Figure 3D; arrowheads). These observations suggest that the organization of F-actin structures in the lamella and the dynamics of F-actin assembly and protrusion at the leading edge are tightly coupled and thus may be functionally related.

Characterization of Lamellar Contraction Modes

To further characterize the contractility of lamellar actomyosin structures we imaged and tracked F-actin speckle movements. Leader and island cells with high protrusion persistence displayed large areas of converging F-actin flow, with anterograde flow distal from the cell edge outpacing proximal retrograde flow (Figure 4A; Supplemental Data, Video 3). Peaks in fluorescence intensity of eGFP-myosin II were observed in this convergence zone (Figure 4A; highlighted region) [36, 37]. Closer inspection of speckle motion by specialized single particle tracking methods (see Exp. Procedures) indicated that the apparent zero net speeds of F-actin speckles reported by coarser-grained flow analyses result from mutual cancellation of interdigitated anti-parallel movements of individual speckles. This is likely the product of thin F-actin fibers being cross-linked by myosin II [38]. Conversely, non-persistently protruding cells displayed retrograde flow of F-actin throughout the narrow lamella, but lacked significant anterograde flow. Multi-wavelength time-lapse images of F-actin and eGFP-myosin II indicate that retrograde flow of actomyosin filaments were continuously compressed into a gradually denser network of transverse actomyosin arcs [38](Figure 4B; Supplemental Data, Video 4). Some follower and island cells exhibited a third contraction mode, in which retrograde and anterograde flows slide past one another in a shear-like fashion without interdigitated speckle movements (Figure 4C). From these experiments we conclude that persistent protruders show a strong degree of posterior - anterior connectivity established by contractile actomyosin structures that span the entire zone of the lamella and cell body, while non-persistent protruders lack this alignment of the contractile machinery with the direction of protrusion.

Myosin II-Inhibited Cells Exhibit Persistent Protrusions

Several reports have shown that inhibition of myosin II results in faster protrusion and motility rates [39,40], suggesting a negative role of actomyosin contraction in promoting cell protrusion. Moreover, it was argued that decreased myosin II contractility activates Rac1 [40] which may promote heightened F-actin assembly and leading to the enhanced protrusion rates. Both data seem inconsistent with our proposal that myosin II-mediated posterior - anterior connections may be required for persistent protrusion. To address this controversy we blocked myosin II function by blebbistatin [41]. After two hours of treatment with 50 μ M blebbistatin, all wound edge cells exhibited persistent protrusions (Figure 5A). Immunolocalization studies confirmed the disintegration of actomyosin structures (Supplemental Data, Figure S1A) [33, 42]. Paxillin staining revealed that elongated or larger adhesion complexes were replaced by tiny adhesion sites at the utmost edge, which were sufficient to sustain cell-substrate contacts during cell migration (Supplemental Data, Figure S1A; arrowheads) [25]. Edge tracking revealed efficient protrusion activity for all cells at the wound edge (Supplemental Data Figure S1B, left panel). Analysis of mean protrusion, protrusion persistence, and instantaneous edge velocity were statistically indistinguishable from leader cells ($p > 0.3$; Figure 5B). Yet, as expected, myosin-inhibition abolished both retrograde and anterograde flows in the lamella, but preserved lamellipodial flow (Supplemental Data Figure S1B, mid and right panels) [5]. Thus, the apparently identical protrusion behavior of leader and myosin-disrupted cells originated from profoundly distinct F-actin dynamics.

In blebbistatin-treated cells, correlation analysis between edge protrusion and F-actin assembly showed a peak at -20 seconds (Figure 5C, top row), similar to leader cells (Figure 5D; top row). However, the correlation peak was decreased and barely significant at a 95% confidence level. Correlation between edge protrusion and F-actin retrograde flow revealed two significant extrema (Figure 5D, middle row): i) A negative peak at -50 s indicates transient increases in flow after maximal edge protrusions. Together with the negative correlation between flow and F-actin turnover, this suggests that like in leader cells protrusion is limited by the force levels adhesion sites sustain against F-actin propulsion at the leading edge [28]. ii) A positive correlation peak at $+10\sim 20$ s indicates that edge protrusion is further correlated with transient reduction of retrograde flow, a characteristic feature of follower and island cells. Since retrograde flow in blebbistatin-treated cells is independent of lamellar contraction, these flow reductions are likely associated with spontaneous increases in the coupling of lamellipodium F-actin to the substrate. Overall, the mechanism of protrusion in blebbistatin-treated cells relied weakly on F-actin assembly and on the contraction-independent modulation of retrograde flow, establishing a phenotype in between leader and follower cells. This seemed incompatible with the idea that inhibition of myosin II would result in heightened F-actin assembly.

Comparison of Relative F-actin Assembly Rates in Migrating Cells

To confirm this notion, we used quantitative fluorescent speckle microscopy to compare rates of F-actin assembly among different cell types (Figure 5E). Blebbistatin-treated cells had three-fold lower assembly rates when compared to leaders. Follower and island cells also exhibited higher assembly rates than drug-treated cells. Thus, the surprisingly similar protrusion persistence of leader and contraction-inhibited cells is ascribed to different states of F-actin dynamics at the leading edge. In leader cells, the F-actin network grows with an overall surplus over contraction-driven retrograde flow (Figure 5F). Protrusion rates during edge advancement are largely determined by F-actin assembly, while during phases of edge pausing or retraction, assembly rates temporarily fall below the rates of F-actin retrograde flow. In follower and island cells, rates of retrograde transport are similar to those in leader cells, but the levels of filament assembly are reduced so that the magnitudes of network growth and net retrograde flow are similar, thus explaining the frequent switching between protrusion and retraction. In

contraction-inhibited cells, repressed assembly rates are sufficient to drive continued edge advancement as a result of low or no retrograde flow in the lamella.

Discussion

Our study provides a detailed characterization of the intricate balance between F-actin assembly and retrograde flow that is required for persistent protrusion of epithelial cells during a wound healing response. The selection of this cell model allowed us to analyze the differences in the relations between assembly and flow that yield different protrusion persistence in leader and follower cells. Furthermore, these cells exhibit very heterogeneous protrusion behaviors along the cell edge and over the allotted observation time. We exploited this heterogeneity to identify locally the temporal coordination between assembly, flow and protrusion behaviors and to derive from it the requirements for cell edge movement. Together, these data defined precise functional linkages between F-actin dynamics and protrusion events.

Our data can be summarized in a network of synergistic and antagonistic mechanical cues (Fig. 6). The thickness of the connections indicates the importance of the relations between the cues. In leader cells protrusion is driven by high levels of F-actin assembly and nascent adhesion formation. The latter blocks the effects of strong lamellar contraction on retrograde flow, which otherwise would be a major inhibitor of protrusion. Transient increase in filament assembly and/or decrease in adhesion formation generate moderate increases in retrograde flow that temporarily reduce protrusion efficiency. In follower and island cells the levels of filament assembly and adhesion formation are lower, resulting in higher influence of lamellar contraction on retrograde flow. In these cells the edge advances only sporadically when transient increases in adhesion formation generate moderate decreases in retrograde flow, sufficient for the filament assembly to temporarily propel the edge forward. As soon as the adhesions get weaker, higher rates of retrograde flow cause renewed retraction of the cell edge. Hence, these cells exhibit a characteristic instability in protrusions with very low net rates of edge advancement.

In blebbistatin-treated cells the levels of filament assembly and adhesion formation overall are also much lower than in leader cells. However, due to the absence of lamellar contraction, retrograde flow is concentrated to the lamellipodium and reduced to a level where low rates of filament assembly are sufficient to drive protrusion at the same magnitude as in leader cells. Sporadic flow increases, accompanied by protrusion decreases, are observed when the rate of adhesion assembly transiently decreases and/or the rate of filament assembly increases. Therefore, these cells exhibit positive correlation between filament assembly and protrusion and delayed negative correlation between retrograde flow and protrusion like in leader cells, as well as showing correlation between retrograde flow and protrusion like in follower cells.

Our model makes assumptions for two additional interactions. First, it posits that lamellar contraction promotes the formation of nascent adhesions via a mechanical signal. Thus, the contraction machinery positively influences the rates of protrusion. Second, the model posits that nascent adhesions are hubs for mechanotransduction, where contractile forces and forces balancing assembly-mediated propulsion are converted into chemical signals, which in turn control again the rates of filament assembly and adhesion formation.

Evidence for the assumption of a contraction-dependent formation of nascent adhesions comes from both the literature and our own experiments. Positive cross-talk between contractile forces and adhesion formation has been a long-established phenomenon [34,43]. Recently more focused data have emerged that explain the force-sensitive assembly of adhesion complexes on a mechanistic level [44–47]. Our own data provides several correlative lines of evidence for such cross-talk. Paxillin-staining indicated distinct adhesion morphology and distribution for leader, follower and contraction-inhibited cells (Fig. 3 and Fig. S1). In leader cells,

numerous elongated adhesions align with the direction of migration. Co-staining of F-actin and paxillin indicates that these adhesions are directly linked via thin actin fibers to the convergence zone at the base of the lamella. Consistent with observations by Riveline et al., [48] and our analysis of anti-parallel speckle flow in the convergence zone and lamella, we suggest that the formation of these adhesions at the cell edge are mediated by long-range anterior-posterior transduction of contraction forces to the cell edge via longitudinal bundles. In follower cells, where longitudinal bundles are largely absent, paxillin staining displays much less polarity in the adhesions. Thus, we propose that force transduction to the cell edge is less efficient and less directed, preventing a persistent, force-induced assembly of adhesions that counteract retrograde flow. This model is corroborated by the observation that blebbistatin-treated cells exhibit small and non-polarized adhesions (Supplemental Data, Figure S1A; arrowheads).

Evidence for links between force-stimulated adhesion formation and chemical signaling has been established by a large body of work, especially for signals related to the activation of the family of small RhoGTPases [46,49,50]. The mechanisms of such mechanotransduction are still largely elusive; but, it is clear that both Rac1 and RhoA are activated at the leading edge [51,52], and it is conceivable that these signals feedback into pathways that regulate adhesion formation [53,54]. Both signaling molecules are also upstream of F-actin nucleators [55]. Relations between contractility and RhoGTPase signaling have also been described. Most prominently, recent evidence has shown that inhibition of actomyosin contraction leads to increased Rac1 activation [40,54]. However, these relationships have been established only on a global scale. To determine the coupling between contraction forces and signaling at the spatial and temporal scales relevant to the regulation of cell protrusion, it will be necessary to simultaneously measure contraction events and signal activation at second and sub-micron resolutions. Our model would predict that signaling activities are selectively elevated after contraction events. While the basic technologies for the analysis of such correlations are now available [52,56] significant experimental hurdles have to be overcome to monitor contraction forces and signaling at the required resolution. Alternatively, high-resolution laser ablation technology is becoming available with which it should be possible to disrupt selectively longitudinal F-actin bundles that connect contraction events to adhesions. Based on our model, significant reduction in RhoGTPase signaling is expected at adhesion sites in which cut F-actin bundles terminate.

Our current data provide indirect evidence for a link between contraction and signaling. The front-coupled contraction forces in leader cells are accompanied by high rates of filament assembly, which likely requires elevated signaling activity at the cell edge. The less-directed transduction of contraction forces to adhesions in follower cells is accompanied by lower rates of F-actin assembly. Finally, abrogation of contraction by blebbistatin treatment drops the assembly rate approximately threefold. Therefore, we propose that the level of lamellar contraction and the structural efficiency of posterior-anterior force transduction to adhesions at the cell front critically determine the level of F-actin assembly in the lamellipodium.

Our observation of reduced F-actin assembly after blebbistatin treatment seems to contradict the notions of elevated Rac1 activity under blocked myosin II contraction [40] and of Rac1 being a promoter of F-actin polymerization [57]. We interpret our data as an indication that in the absence of contraction, Rac1 may inhibit a Rac1-independent pathway of F-actin nucleation. Given some biochemical evidence that Rac1 antagonizes RhoA, [58,59] it is tempting to speculate that the elevated Rac1 levels oppress a RhoA-mediated assembly pathway. Whether such a Rac1-independent assembly pathway significantly contributes to protrusion in leader cells with an intact contractile system, and whether intrinsic variations in the contractile activity yield temporally alternating levels of contribution between Rac1-dependent and Rac1-independent assembly pathways, again will require concurrent

measurements of actin assembly rates and signaling activities at the scale of protrusion regulation.

In conclusion, this study illuminates via *local* measurements of F-actin dynamics the subtleties of a spatiotemporal balance between lamellipodial F-actin assembly and the dichotomy of pro- and contra-protrusion cues mediated by different actomyosin contractile structures in the lamella. It sets the stage for future analyses where the interdependences of contraction, assembly, and signaling events are resolved by simultaneous recording of these parameters across space and time.

EXPERIMENTAL PROCEDURES

Cell Culture and Imaging Conditions

PtK1 cells were cultured in Ham's F12 media (GIBCO), supplemented with 10% FBS, 0.1 mg/ml streptomycin, 100 U/ml penicillin, 25 mM HEPES, 1 mM sodium pyruvate, 2 mM of L-glutamine and buffered to a pH of 7.2 (with KOH). Oxyrase (Oxyrase Inc.) was added to media (1.0 U per ml) just prior to imaging to reduce photobleaching. Cells were grown on acid-washed glass coverslips from Corning (#1-1/2 coverslips) for a minimum of two days before imaging, or until it reached complete confluency. Confluent monolayers of PtK1 cells were wounded by dragging a p1000 sized micropipette tip across the cell surface to create a scratch approximately 2 mm wide. Leader, follower and island cells were microinjected with X-rhodamine conjugated actin using an Eppendorf Transjector 5246 (Eppendorf Inc.) at a concentration of 0.5 mg/ml and as described in [24]. Full length myosin regulatory light chain (mRLC) was expressed in BL21(DE3) E. coli and purified on a Talon metal affinity resin (Clontech Laboratories) according to the manufacturer's instructions. mRLC cDNA with EcoRI and BamHI restriction sites was cloned into a pHAT2 vector containing a histidine tag followed by an enhanced GFP (eGFP) sequence (kind gift from Torsten Wittmann, UCSF). Purified eGFP-mRLC was co-microinjected with X-rhodamine-conjugated actin at 1.0 and 0.5 mg/ml, respectively. S-(−)-enantiomer of blebbistatin was purchased from Toronto Research Chemicals. Cells were treated with 50 μM of blebbistatin for 2 hours prior to imaging.

Microscopy

Live-cell imaging experiments were acquired on a multispectral spinning-disk confocal microscope based on a Nikon TE-2000E platform, equipped with a perfect focus system (Nikon PFS) to automatically correct for focus drift. The confocal scanner head utilized was a customized Perkin Elmer Ultraview RS, with appropriate filter sets supplied by Semrock or Chroma Technology. Images were acquired on a CoolSNAP HQ² camera (Photometrics, Roper Scientific, Inc.), with acquisition settings controlled via MetaMorph version 7.0 (MDS Analytical Technologies). Electronically controlled filter wheels/shutters (Lamda 10-2; Sutter Instruments) and linear position feedback encoded stages (MS-2000; Applied Scientific Instrumentation, Inc.) allowed for automated multi-position imaging. Laser power was supplied by a Coherent Innova 300. All live-cell speckle studies used a Plan Apo 100×/1.40 phase oil objective (Nikon). Wound migration studies used a Plan Fluor 20×/0.50 phase objective.

Wide-field epifluorescence images were acquired on an inverted Axiovert 200M (Zeiss), equipped with an Orca II-ER camera (Hamamatsu Photonics), electronically controlled filter wheels/shutters and an automated stage (as described above). Acquisition settings were controlled via MetaMorph 7, with illumination supplied by a 100W mercury arc lamp. Fixed-cell samples were prepared as described in [60]. Alexa 488 labeled phalloidin (Invitrogen) was used to label F-actin, monoclonal Myosin-IIA antibody (Covance Research Products) was used to target endogenous myosin II, and polyclonal pY118 antibody (Biosource) was used to label

paxillin. Appropriate secondary antibodies were obtained from Jackson ImmunoResearch Labs. Plan Apo 100 $\times$ /1.4 DIC oil objectives (Zeiss) provided up to 9 images per panel (Figure 3), and were then 'photomerged' (function in Adobe Photoshop CS2) to create a panoramic image.

Quantitative Fluorescent Speckle Microscopy

Measurement of F-actin dynamics in relation to edge morphodynamics was determined via quantitative Fluorescent Speckle Microscopy (qFSM). 'Speckles' are the product of low concentration incorporation of microinjected, fluorescently labeled actin monomers (X-rhodamine) into the F-actin cytoskeleton network. When imaged with high numerical aperture optics, stochastic intensity profiles of closely clustered fluorophores (3 to 7) results in the appearance of speckles[61]. Speckle trajectories and relative rates of assembly and disassembly were determined by algorithms reviewed in [21].

Cell Edge Tracking

Cell edges were detected via an intensity-based segmentation of the speckle signal in micro-injected cells. Cell edge protrusion and retraction were tracked using the mechanical model of edge evolution described in [26].

Mapping of Cell Protrusion and F-actin Dynamic Activities

To probe the local activities of edge movements and subadjacent F-actin dynamics, protrusion rates, speckle flow and kinetic scores were sampled in reporter windows tracking the cell edge (Supplemental Data, Video 2). The window sizes were selected to provide sufficient averaging of the stochastic speckle signal, while maintaining adequate spatial resolution of edge dynamics. Sizes ranging from 2–4 μm in width and 1–2 μm in depth captured multiple F-actin turnover scores and flow measurements per frame. These dimensions matched the length scale over which the autocorrelation of the protrusion and retraction activities decays to insignificant values, i.e. the protrusion and retraction states of the cell edge in neighboring probing windows are statistically independent. For visualization and further processing of the spatiotemporal relationships between sampled parameters the edge velocity and the rates of F-actin flow and assembly/disassembly were copied into activity maps and color-coded as illustrated in (Figure 2A).

Cross-Correlation Analysis

To quantify the relationships between edge movements and F-actin dynamics, their activity maps were cross-correlated[61]. The cross-correlation score indicates how much two activities, e.g. protrusion and filament assembly, fluctuate jointly in time and space. A positive cross-correlation value means, when one parameter is high the other is also high; and when one parameter is low the other is also low. Conversely, a negative cross correlation value means that one parameter is high when the other is low and vice versa. The cross-correlation is approximately zero when the two parameters are modulated independently. This holds true also when only one parameter is modulated significantly while the other is approximately constant.

To detect potential delays of one activity relative to another activity, cross-correlation scores were calculated for different time shifts (referred to as time lags) between activity maps. We use the notation 'A* vs. B' to indicate that A is the reference parameter relative to which the activity map of parameter B is shifted. A positive correlation value at a positive time lag means the activity of the parameter B systematically increases or decreases *before* the activity of parameter A increases or decreases. A positive correlation at a negative time lag means that

the activity of parameter B increases or decreases *after* the activity of parameter A increases or decreases.

Cross-correlation scores sampled in individual windows were averaged for all reporter windows along the leading edge. This yielded the cross-correlation profile for a single cell (see left columns of Figure 2C – E). Since the correlation was computed locally per reporter window, the coupling between two activities could be detected despite the heterogeneous states of activities along the cell edge. To determine the significance of the coupling between two activities, average cross-correlation profiles were computed by spline-fitting the cross-correlation scores obtained from individual cells ($n = 4$ to 8). The variance of the spline fit and hence the uncertainty of the average cross-correlation profile was calculated by a non-parametric bootstrapping [62]: From the residuals of the spline fit, 2000 bootstrap samples were taken to reconstruct 2000 cross-correlation samples. From this sample pool, the local variation of the spline and the variation in the location of the correlation maximum (as an estimate of the time lag) were inferred. The 95% confidence interval (right columns of Figure 2C – E; red lines) was obtained in each location as the interval containing 95% of the bootstrapped spline samples. Correlation maxima are significant with 95% confidence if the confidence interval at the position of the maximum does not include the zero correlation level.

Determination of Antiparallel (Interdigitated) Actin Flow: Modification of Single Particle Tracking

The convergence zone in leader cells was characterized by a narrow strip of zero flow speed (Figure 4A), suggesting that retrograde and anterograde flowing F-actin structures collide and disassemble. This picture would contradict the immunofluorescence data indicating longitudinal bundles spanning the entire lamella and reaching the cell body behind the convergence zone (Figure 3A). Visual inspection of speckle motion in the convergence zone suggested that retrograde and anterograde flows interpenetrate (Supplemental Data, Video 3), a characteristic missed by the regional filtering of speckle trajectories necessary for noise reduction in the flow analysis [63]. To test the possibility of interpenetrating flows, we applied a speckle tracking method specifically designed to capture dense antiparallel flows required to detect tubulin flux in mitotic spindles [64]. In brief, this tracking method predicts from the track history of an individual particle the most probable particle position in the next time point. The search for the corresponding particle in this time point is then limited to the predicted position. Because of the extrapolation, a particle moving against the direction of neighboring particles will be found again in the next time point, whereas without extrapolation some of the neighboring particles will likely be confused as better links.

Relative Measurements of Net F-actin Assembly

Net F-actin assembly per cell was calculated by averaging the assembly scores (excluding disassembly scores) sampled in a $2\text{ }\mu\text{m}$ -wide band following the lamellipodium over the total observed time (8.5 minutes). These values were then averaged across the total number of cells analyzed. Error bars indicate standard error of the mean of individual cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

qFSM	quantitative fluorescent speckle microscopy
F-actin	Actin filaments

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Figure 1. Identification of leaders and followers during wound healing of PtK1 cells

(A) Time progression of wound healing. Three hours after wounding, triangularly-shaped cell mounds form, at the tip of which leader cells (arrowheads) develop their characteristic protrusive behavior. Scale bar = 200 μm . (B) Zoom-up of cell mound with one leader cell and flanking follower cells. Scale bar = 100 μm . (C) Island cells imaged 2 days after plating. Over an observation window of 8 hours, cells rarely display directed motility. Scale bar = 200 μm . (D) Edge evolution of leader (left), follower (middle), and island cells (right panel). Color encodes time point of edge position. Bar: 10 μm . (E) Quantification of edge movement. Mean protrusion: average net displacements of ~ 1.5 μm -wide edge sectors over the time period of an image series. Protrusion persistence: mean time ratio edge sectors spend in a state of

protrusion vs. in a state of retraction. Instantaneous velocities of protrusion (dark blue) and retraction (light blue): average frame-to-frame displacements of edge sectors. Statistics were generated from $n = 7$ island cells, $n = 8$ leader cells, and $n = 4$ follower cells. Error bars indicate standard error of mean values for individual cells. The difference in protrusion persistence between leaders and followers is statistically insignificant due to the large cell-to-cell variation in the behavior of followers.

Figure 2. Relationships between actin filament dynamics in lamellipodium and protrusion/retraction activity

(A) Construction of activity maps of protrusion and F-actin dynamics. Reporter windows of $\sim 2 \times 3 \mu\text{m}$ in depth and width (yellow boxes) tracked the position of the cell edge. Mean values of edge velocity, F-actin flow, and F-actin turnover rates as measured by FSM were pasted into a space-time matrix and color-coded for visual presentation. (B) Activity maps for leader, follower and island cells. Top row: Edge protrusion velocity. Warm colors indicate protrusion, cool colors indicate retraction. Leader cells exhibit high protrusion activity throughout the entire map. Follower and island cells exhibit transient, less persistent bursts of protrusion (arrowheads). Mid row: Activity map of F-actin retrograde flow. Note: flow velocities have

the opposite sign of protrusion velocities and thus are only negative. Bottom row: F-actin turnover. Assembly in red colors, disassembly in green colors. (C – E) Cross-correlation analysis of relationships between parameters of F-actin dynamics and cell edge velocities. Left column: Correlation profiles for individual cells (thin lines) and for cell population (thick lines). Right column: Average correlation profiles for cell population (blue line) with 95% confidence interval calculated by bootstrap analysis. Asterisk (‘*’) indicates reference activity. Open green diamonds indicate correlation score data points from individual cells. Correlation values for a specific time shift are statistically significant if the zero correlation level is outside the confidence band. Significant correlation maxima (or minima) occurring in the negative time (‘After’) indicate that variations in the second activity are co-modulated but lag variations in the reference activity.

Figure 3. Immunolocalization of F-actin, myosin-II and adhesions in PtK1 cells

(A) Leader cell at the tip of a cell mound (inset). Left panel: Phalloidin staining of F-actin. Longitudinal bundles are highlighted in yellow. Right panel: Magnified region (see inset left panel) shows numerous F-actin longitudinal bundles via phalloidin staining (red); a general increase in myosin II localization at larger distances from the cell edge (green); and progression from assembly of tiny adhesion sites at the edge (arrowheads) to elongated adhesions (arrows) near the termination sites of longitudinal bundles (paxillin staining in cyan). (B) Follower cell in the flanking of a cell mound (inset). Top panel: Phalloidin staining of F-actin. Transverse arcs are highlighted in purple. Lower panels: F-actin (red) and myosin II staining (green) show co-localization of myosin filaments in transverse arcs. Paxillin staining (cyan) shows sporadic

distribution of adhesions (arrowheads). **(C)** Island cell. Top panel: Phalloidin staining of F-actin. The majority of cells adopt the structure of followers with transverse arcs (purple regions; see also inset). Lower panels: F-actin staining (red). Arrow highlights the transformation of transverse arcs into longitudinal bundles in areas of sporadic polarization and protrusion. Myosin II staining (green) show accumulation of myosin filaments in transverse arcs. Paxillin staining shows sporadic distribution of adhesions that vary in size and shape (arrowheads). **(D)** Polarized island cells adopt the morphology of leader cells. Top panel: Phalloidin staining. Longitudinal bundles are highlighted by yellow regions. Purple regions indicate transverse arcs in flanking, non-polarized island cells. Lower panels: F-actin staining (red) shows longitudinal bundles spanning the lamella of a polarized island cell. Myosin II staining (green) gradually increases towards the middle of the lamella. Paxillin staining (cyan) shows distribution of elongated adhesions (arrows). All scale bars are 20 μm .

Figure 4. Modes of F-actin contraction

(A) Interpenetrating contraction of F-actin anterior and posterior to the convergence zone in leader cells; eGFP-mRLC localization increases from the edge; region with highest intensity highlighted by yellow area (left panel). Instantaneous F-actin flow at one selected frame (mid panel). Vectors indicate direction and speed of flow; region of highest eGFP-mRLC intensity is highlighted as a white, transparent area. Speed of F-actin flow averaged over 100 s (right panel). Inset: Classification of speckle motion in convergence zone (zero average flow speed). Green and red squares highlight speckles moving retrogradely and anterogradely, respectively. (B) Compression and bundling into transverse arcs of F-actin anterior and posterior to the convergence zone in follower/island cells. eGFP-mRLC distribution (left panel); Instantaneous F-actin flow (mid panel); speed of F-actin flow averaged over 100 s (right). (C) Shear contraction between F-actin anterior and posterior to convergence zone. eGFP-mRLC distribution (left panel); Instantaneous F-actin flow (mid panel); speed of F-actin flow (right); speed of F-actin flow averaged over 100 s (right). Scale bars in (a - c) = 10 μm . Units of speed maps in (a - c): $\mu\text{m}/\text{min}$.

Figure 5. Relationships between edge movement, lamellipodial F-actin dynamics, and lamellar contraction probed during the perturbation of myosin-II

(A) Phase image of wound edge cells treated with 50 μM blebbistatin (B) Quantification of edge movement. Comparison between leader (blue) and blebbistatin-treated (red) cells. None of the parameters shows significant differences (p -values > 0.3 for all cases). (C) Relationships between actin filament dynamics in lamellipodium and protrusion/retraction activity inferred by cross-correlation profiles (see Fig. 2 for explanation of panels) (D) Comparison of averaged cross-correlation profiles of leader and blebbistatin-treated cells. Top row: Correlation between protrusion and turnover shows increased dependency of protrusion in leader cells on F-actin assembly than in blebbistatin-treated cells (correlation score: 0.22 vs. 0.08 in leader cells). Mid

row: Correlation between protrusion and flow shows increased dependency of protrusion in blebbistatin-treated cells on transient slow down of F-actin flow shortly before and during protrusions (correlation score: 0.12 vs. 0.03 in leader cells). Both cell types display significant negative correlation scores (−0.18 vs. −0.16) between protrusion and retrograde flow 40 s after protrusion. Last row: Correlation between retrograde flow and turnover shows lower coordination between these parameters in blebbistatin-treated cells (correlation score: −0.08 vs. −0.14 in leader cells, both scores are significant). **(E)** Net F-actin assembly rate at the leading edge normalized to assembly rates in leader cells. P-value between blebbistatin-treated and leader cell < 0.05. **(F)** Contributions of F-actin assembly and retrograde flow to protrusion activity. **(G)** Relationships between lamellipodial F-actin dynamics and lamellar contraction. The model predicts that different levels of F-actin assembly are related to differential transmission of contractile forces from the lamella to the lamellipodium.

Figure 6. Integrated model of cell protrusion

Protrusion efficiency depends on an interconnected network of mechanical and chemical cues that govern F-actin contraction, assembly, and adhesion strength. Thick and thin lines indicate the relative influences among cues. The model rests on two key assumptions: i) Contraction forces mechanically stimulate the formation of nascent adhesions (red dotted arrow). ii) Force-stimulated assembly of nascent adhesion stabilizes membrane advancements, and reduces the antagonistic effect of retrograde flow on protrusion. Nascent adhesions may also serve as hubs for mechano-transduction that activate F-actin assembly through the recruitment of signaling proteins to adhesion sites (blue arrow). In leader cells, the contraction and nascent adhesion formation at the cell front are tightly coupled via longitudinal F-actin bundles. Thus, the increased influence of contraction on retrograde flow is compensated by stronger adhesions and elevated F-actin assembly rates. In follower and island cells the coupling of contraction to adhesion formation is weaker, and absent in contraction-inhibited cells. As a result, in both cell types the F-actin assembly rates are lower than in leader cells; yet, as lamellar retrograde flow in contraction-inhibited cells is essentially stalled, these reduced assembly rates are still sufficient to generate a persistent protrusion.