Supplementary Notes

1. Cofilin and gelsolin in actin network disassembly

The ADF/cofilin family of proteins have been implicated as possible agents for spatially organized actin network disassembly. They are capable of severing actin filaments and dissociating the branch-forming Arp2/3 complex in vitro. ADF/cofilin proteins also contribute to the disassembly of *Listeria monocytogenes* comet tails, and play a role in the maintenance of the actin monomer pool in cells. Binding of ADF/cofilin to actin is dependent on the nucleotide state of the actin, a property that may serve as a mechanism for temporal (and hence, spatial) regulation for network disassembly. However, cofilin in protruding lamellipodia is concentrated near the leading edge with at most a narrow (< 2 µm) zone of exclusion, and in some cell types cofilin activation has been shown to promote actin assembly by producing new filament ends. It therefore remains unclear how or whether cofilin activity in motile cells can explain the large-scale coordination of assembly and disassembly ranging over tens of micrometers from the front to rear.

Gelsolin-family proteins also sever actin filaments by a different mechanism and can contribute to actin turnover in *Listeria* comet tails and motile cells, but, again, the extent of their contribution and the mechanism of their spatial regulation remain unclear in the context of actin network disassembly in crawling cells.

Our results suggest that, in addition to myosin II–mediated disassembly, there is a second pathway for actin network disassembly that does not depend on myosin II activity but is sensitive to jasplakinolide. It is possible that cofilin or gelsolin activity might play a role in this pathway, although the cell would have to establish a gradient of activity of these proteins over a large (tens of micrometers) distance scale. Although possible mechanisms for the formation of such a gradient have been speculated on, just how the gradient might be coordinated over the whole cell with the actin network is yet to be established. It is also possible that, in the absence of myosin II activity, retraction of the rear is driven by membrane tension (see Note 2, below) and the “swept up” actin filaments are disassembled by cofilin or gelsolin, whose localization of activity would then need not be as stringently restricted to the extreme rear margin.

It has been observed that there is balanced assembly and disassembly of the actin network throughout the lamellipodium in fibroblasts and epithelial cells. The gross disassembly in this context might be driven by the myosin II–independent pathway, although we cannot completely rule out the contribution of myosin II.

2. Roles of nonmuscle myosin II in cell motility

Nonmuscle myosin II is typically localized to the rear of rapidly crawling cells, and compromised myosin II activity results in defects in cell polarization and rear retraction in many types of motile cells. Functions of myosin II contributing to the formation and...
reinforcement of the polarized cell rear may include contraction of the rear cortex\(^{46}\), generation of traction force against the substrate to bring the rear forward\(^{47,48}\), generation of forward-directed hydrodynamic flow\(^{49,50}\), and force-mediated modulation of adhesion complexes\(^{51}\).

As we note in the main text, myosin II activity is required for the generation of inward flow in the rear, perpendicular to the direction of cell motion (Fig. 2, Supplementary Movie 1), and inward traction force (Supplementary Fig. 3, Supplementary Movie 2), consistent with myosin II activity driving contraction of the actin network. The dependence of inward flow on myosin II activity was further confirmed by the observation that calyculin A, which upregulates myosin II activity\(^9\), increased the average inward flow rate in cells (Supplementary Fig. 4).

In some motile cell types, such as neuronal growth cones\(^{13}\), myosin II activity contributes to retrograde flow of the actin network with respect to the substrate. Retrograde flow has also been observed in fish keratocytes from certain species\(^{52,53}\), where its rate is inversely correlated with the rate of leading-edge protrusion. However, in Central American cichlid keratocytes under the conditions of our experiments, retrograde flow of the actin network is very slow, uncorrelated with cell speed, and unaffected by blebbistatin treatment (Supplementary Fig. 5a, b). We also observed that cell speed is poorly correlated with inward flow (Supplementary Fig. 5c). These observations indicate that myosin II activity does not drive retrograde flow in keratocytes under normal conditions.

Continued motility of keratocytes in the absence of inward network flow and inward traction force generation suggests that motor-driven contraction of the cytoskeleton may not be the sole driving force for retraction of the trailing edge. This is consistent with the idea that membrane tension can drive rear retraction\(^{54}\). Additionally, network disassembly itself may drive rear retraction, as has been demonstrated in nematode sperm motility\(^{55}\). Theoretical modeling supports the idea that actin network disassembly can be sufficient for retraction in actin-based movement as well\(^{56–58}\).

3. Computation of F-actin assembly/disassembly maps

3a. Alternative methods for assembly/disassembly map computation

While the steady-state approach to computing F-actin assembly and disassembly maps described in Methods (Fig. 1e, 2e, Supplementary Fig. 2b) directly implements mass conservation of compressible materials, the method brings with it a numerical requirement that can be difficult to satisfy for complex flow fields and low signal-to-noise ratio image data: the support of the filters for flow field and fluorescence images need to be matched with the length scale of the divergence operator. To ensure that our parameter settings were valid, we tested the results from this algorithm against two alternative methods which do not require parameter adjustments to flow field and noise conditions (Supplementary Fig. 2c, d).

Both of these alternative approaches depended on local measurements of net turnover made between frame pairs (denoted here \(i\) and \(i+1\)); we created the final steady-state turnover maps by averaging these maps over the length of the movie. For both approaches, we created a tiled array of small measurement windows (11 \(\times\) 11 pixels) across the cell and resampled the flow
field on the same $11 \times 11$ pixel grid. In the first method (Supplementary Fig. 2c), we multiplied the average density, $q_i$, by the window area, $A_i$, to get a measure of the local mass, $I_i$. $q_i$ was found by averaging the interpolated intensity values at the window corners in frame $i$. We then transformed the edges of the window according to the velocity vector field to estimate the position of the corresponding window in frame $i+1$. We calculated $A_{i+1}$ and $q_{i+1}$ using the transformed window corners and their intensities from frame $i+1$. Normalized net assembly was then simply $I_{i+1} - I_i$ divided by the area of the window in the first frame, $A_i$. Turnover values were subsequently interpolated to every pixel in the cell. In the second method (Supplementary Fig. 2d), rather than using intensities at the window corners, we estimated the local mass, $I_i$, by summing the window pixel intensities in frame $i$. We summed the frame $i+1$ intensities of all pixels whose centers were within the velocity-transformed window to get $I_{i+1}$. As in the first method, the per-pixel net assembly was $(I_{i+1} - I_i) / A_i$ and these values were interpolated to each pixel in the cell. Both of these alternative methods yielded results similar to the steady-state divergence method over a number of tested cells (compare Supplementary Fig. 2c, d to 2b).

3b. Characteristics of the assembly/disassembly computation

The F-actin speckle labeling and microscopy methods applied in this study were optimized to reveal information about the system without perturbing it (except in the case of pharmalogical manipulations). These compromises, however, place certain constraints on what information about assembly and disassembly can be obtained from the analysis (see Methods for details) when applied to these data. Therefore, in interpreting the assembly/disassembly measurements, we have been careful to draw conclusions only from those aspects of the measurements which are within the limits of what is observable in this system, and which are reported consistently by the different approaches (see Methods and above; Supplementary Fig. 2b–d). Specifically, speckled labeling of the actin network (enabling reliable movement tracking) with very small amounts of fluorescent phalloidin and minimal fluorescence excitation illumination (to avoid perturbing the live cells) limit the spatial and temporal resolution of actin density observation; hence the spatial smoothing and measurement windows employed in the calculations. In our interpretation we restrict our consideration to characteristics of the assembly and disassembly spatial distributions which cover multiple micrometers and are sustained over several frames. Likewise, the use of a probe which binds specifically to F-actin enables reliable observation of F-actin movement and disassembly, but compromises the resolution of F-actin assembly measurement. The delay between actin filament elongation and binding of the phalloidin probe to the newly exposed filament length results in blurring of the assembly reported at the leading edge. For disassembly, we mainly consider the areas of the cell where we can measure it confidently, including the trailing edge and foci flanking the cell body. We avoid statements about the area under the cell body itself, however. In this area the accumulation of F-actin (which itself can be measured as described in the Methods) obscures the observation of actin movement. Also, in contrast to the rest of the cell, which has a depth of approximately 200 nm and therefore fits within the optical section of the microscope, the cell body is up to several micrometers deep; thus decrease in fluorescence can indicate either loss of F-actin or movement out of the plane of observation. Finally, we do not report absolute numbers for assembly or disassembly, which would depend on the total amount of actin in a given cell and the amount of phalloidin taken up by that cell upon electroporation; instead we consider the spatial
distribution of relative assembly and disassembly within a cell; in before/after figures we show measurements made in the same cell before and after pharmacological manipulation.
Supplementary Figure 1. Schematic diagram of results and model for myosin II–driven actin network disassembly.

The distribution of steady-state actin network flow (yellow arrows), net assembly (purple), and net disassembly (green) is mapped onto the left-hand side of a canonical keratocyte shape. On the right-hand side, the distribution and organization of F-actin (gray) and myosin II (red) is depicted.

Near the front, where F-actin assembly dominates, the dendritic actin network has little myosin II. Toward the middle of the lamellipodium, myosin II contractile activity has begun to reorient actin filaments to a more parallel organization, and at the rear the F-actin and myosin II have coalesced into dense foci on either side of the cell body along with dense parallel bundles. These configurations allow myosin II to efficiently exert mechanical force and break actin filaments, giving rise to a pattern of net network disassembly that closely resembles the pattern of myosin II localization. The slow incorporation of myosin II filaments into the lamellipodial network and the gradual increase in myosin II efficiency due to actin rearrangement and myosin II thick filament formation provide a slow timing mechanism that enables long-range steady-state network treadmilling over the entire front-to-rear axis of the cell. When myosin II is inhibited, a second, unidentified, jasplakinolide-sensitive network disassembling activity that is normally concentrated near the cell body is sufficient for network treadmilling, albeit with an accumulation of excess F-actin at the rear margins where it is gathered in by the retracting plasma membrane.
**Supplementary Figure 2. Computation of the pattern of net assembly and disassembly.**

**a**, Schematic diagram of assembly/disassembly computation. Regions of the cell where the divergence of the F-actin flow vector field is negative (left) or positive (center) must include net disassembly or net assembly, respectively, at steady state. In regions where the divergence is zero (right), assembly and disassembly are balanced. The full calculation shown in Fig. 1e and 2e also takes into account local variations in actin network density \( \rho \) (see Methods).

**b–d**, Alternative approaches to computing the pattern of net assembly and disassembly give comparable results. The results of three methods of assembly/disassembly computation are shown for the same 2 min movie (2 s frame intervals) of an unperturbed keratocyte (see Methods and Supplementary Note 3a for full description).

**b**, A steady-state net turnover map was calculated by taking the divergence of the average smoothed velocity field (resampled per-pixel) weighted by the average smoothed intensity (similar to ref. 9). This is the method used to obtain the images displayed in Fig. 1e and 2e and described in the Methods.

**c, d**, The results from two alternative methods based on local measurements of net assembly/disassembly between consecutive frame pairs (denoted here \( i \) and \( i+1 \)). For both of these frame-by-frame approaches, a tiled array of small measurement windows (11 × 11 pixels) was created across the cell and the flow field was resampled on the same 11 × 11 pixel grid. The steady-state turnover maps shown here were created by averaging these maps over the length of the movie (see Supplementary Note 3a).

**c**, The local F-actin mass, \( I_i \), was estimated by multiplying the average density, \( \rho_i \), by the window area, \( A_i \). Normalized net assembly was then simply \((I_{i+1} - I_i)\) divided by the area of the window in the first frame, \( A_i \). These values were interpolated to every pixel in the cell.

**d**, The local F-actin mass, \( I_i \), was estimated by summing the window pixel intensities. Normalized net assembly was computed and interpolated as in panel **c**.
Supplementary Figure 3. Inward traction force generation requires myosin II activity.

A keratocyte on a deformable gelatin substrate, before (a) and after (b) treatment with 50 µM blebbistatin. As keratocytes (phase contrast, left) crawl over a layer of 2.5% gelatin, they pinch the gelatin inward, perpendicular to the direction of cell motion, observed via the gathering of quantum dots coating the gelatin into wrinkled “wings” under and behind the cell (center). This deformation dissipates within a few minutes of changing the medium to one containing 50 µM blebbistatin. The keratocyte no longer deforms the gelatin, though it continues to crawl over it. An overlay of the phase contrast (magenta) and fluorescence (green) images is shown (right); contrast is enhanced in the overlay images. Compare Supplementary Movie S2.
Supplementary Figure 4. Calyculin A treatment increases inward flow only in the rear.

Actin network flow before (left) and ~3 min after (right) treatment with 25 nM calyculin A, a general phosphatase inhibitor that results in net activation of myosin II contractile activity⁹.

a, Map of the component of flow perpendicular to the direction of cell motion (“perpendicular flow”), as determined by FSM.

b, Time-averaged magnitude and direction of network flow in five cell regions. Inward flow is confined to the rear left and rear right regions, with the entire front lamellipodial region behaving as a coherent unit with actin network in the cell frame of reference only along the axis of cell locomotion.

c, Time series of perpendicular flow averaged over the regions indicated in panel b, over 1 min periods.

d, Average and standard deviation of inward perpendicular flow (solid lines in panel c) over the 1 min period. The characteristic inward flow in the cell rear is slightly increased ($p = 2 \times 10^{-7}$ by $t$-test) by promotion of myosin II activity with this small dose of calyculin A, consistent with myosin II driving inward flow in the rear.
Supplementary Figure 5. Myosin II activity does not drive retrograde flow in the lamellipodium, and inward flow rate shows only a weak correlation with cell speed.

**a.** Retrograde flow in the keratocyte lamellipodium (rearward F-actin movement relative to the substrate in the laboratory frame of reference) is very slow, is not correlated with cell speed, and is not affected by blebbistatin treatment. Cell speed and retrograde flow is plotted for 23 untreated cells (open circles) and 8 cells treated with 50 µM blebbistatin (closed triangles). Horizontal bars indicate the standard deviation in cell speed over a time series; vertical bars indicate the standard deviation in retrograde flow. The small variation in retrograde flow (inset) appears uncorrelated with cell speed.

**b.** Data from panel **a** plotted in the cell frame of reference, on the same scale. Treatment with blebbistatin does not alter the relationship of retrograde flow to cell speed. Dotted lines in panels **a** and **b** indicate linear fits (for the combined population) to the rearward flow as a function of cell speed.

**c.** The relationship of inward perpendicular flow in the rear left and right regions to cell speed in a population of 23 untreated cells. Average inward flow and cell speed over a time series are plotted for each cell. Bars indicate standard deviation over the time series. There is no strong correlation ($r = 0.3$) between whole cell speed and inward actin network flow rate.
Supplementary Figure 6. Actin network disassembly in the rear of detergent-extracted keratocyte cytoskeletons is reproducibly ATP-dependent and blebbistatin-sensitive.

As in Fig. 4, ATP was added to detergent-extracted, phalloidin-labeled keratocytes untreated \((n = 6)\) or treated with 50 µM blebbistatin \((n = 5)\) for 30 min prior to extraction. Loss of phalloidin fluorescence intensity over the whole cell in the first 30 s following exchange with buffer containing (or, as a control, not containing; \(n = 7\)) ATP is shown. Addition of ATP resulted in 4.4-fold greater loss of intensity than the mock control (double asterisk, \(p < 0.01\) by \(t\)-test). Blebbistatin pretreatment significantly suppressed the effect of ATP (single asterisk, \(p < 0.05\) by \(t\)-test). Error bars indicate mean ± s.d. Intensities for each cell were computed as the average over 3 timepoints (at 10 s intervals) centered at 10 s before and 30 s after addition of ATP or buffer.
Supplementary Movie Captions

Supplementary Movie 1. Myosin II inhibition alters actin network flow.

Actin network flow in a cell before and after addition of 50 µM blebbistatin. Left: Alexa Fluor 546 phalloidin fluorescence with raw flow velocities in the laboratory frame of reference. Right, top: fluorescence with raw flow measurements in the cell frame of reference. Right, middle: resampled flow velocity field in the cell frame of reference. Right, bottom: perpendicular flow component. Movie is at 20 × real time. The characteristic inward movement of the actin network decreases as blebbistatin takes effect, and after several minutes is indistinguishable from noise. Compare Figure 2.

Supplementary Movie 2. Inward traction force generation requires myosin II activity.

A keratocyte on a deformable gelatin substrate, before and after treatment with 50 µM blebbistatin. As keratocytes (phase contrast, left) crawl over a layer of 2.5% gelatin, they pinch the gelatin inward, perpendicular to the direction of cell motion, observed via the gathering of quantum dots coating the gelatin into wrinkled “wings” under and behind the cell (center). This deformation does not dissipate immediately upon changing the medium to one containing 50 µM blebbistatin. Within a few minutes, however, the keratocyte no longer deforms the gelatin, though it continues to crawl over it. An overlay of the phase contrast (magenta) and fluorescence (green) movies is shown (right). Movie is at 48 × real time. Compare Figure S3.

Supplementary Movie 3. Jasplakinolide halts actin dynamics of cells in which myosin II is inhibited.

Actin network flow in a cell in 50 µM blebbistatin (left) before and after additional treatment with 1 µM jasplakinolide. A separate cell before and after treatment with jasplakinolide alone is shown (right) for comparison. Far left and right: Alexa Fluor 546 phalloidin fluorescence (top) and phase contrast (bottom) movies of the cells in the laboratory frame of reference. Center left and right: raw actin network velocity measurements (top) and flow magnitude (bottom) in the cell frame of reference. Movie is at 20 × real time. The combination of blebbistatin and jasplakinolide immobilize the actin network, an effect that is not achieved by blebbistatin or jasplakinolide alone. Compare Figure 3.

Supplementary Movie 4. Actin network disassembly in the rear of detergent-extracted keratocyte cytoskeletons is ATP-dependent and blebbistatin-sensitive, consistent with a direct role for myosin II in this process.

a–d, ATP triggers an acute loss of actin network in the rear region of the cell, where myosin II is localized (compare Fig. 1f). a, A detergent-extracted and phalloidin-labeled keratocyte cytoskeletonb. The same cytoskeleton at the indicated time point. 1 mM ATP was added at t = 0. c, Overlay of initial frame (a, cyan) and frame at the indicated time point (b, yellow); regions with more, less, or equal intensity relative to t = 0 appear yellow, cyan, or white, respectively. d, Time evolution of fluorescence intensities (normalized at t = 0) in the indicated regions. Time points for a mock buffer wash (chevron) and ATP addition (black arrowhead) are indicated.
e–h, In a cell treated with 50 µM blebbistatin for 30 min prior to extraction, addition of ATP does not induce a loss of actin network. There is a slow loss of fluorescence due to photobleaching or background dissociation.

i–l, The F-actin severing protein villin rapidly disassembles the lamellipodial actin network, demonstrating that this part of the cytoskeleton is not protected against a general disassembling activity. 0.1 µM GST-villin was added instead of ATP (arrow in l).

m–t, Addition of GST-villin (arrows in p, t) in addition to ATP (arrowheads in p, t), in either order, results in complete, rapid disassembly of the actin network.

Compare Figure 4.
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