**FRET imaging and statistical signal processing reveal positive and negative feedback loops regulating the morphology of randomly migrating HT-1080 cells**

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Accepted 18 January 2012

Journal of Cell Science 125, 2381–2392

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doi: 10.1242/jcs.096859

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**Summary**

Cell migration plays an important role in many physiological processes. Rho GTPases (Rac1, Cdc42, RhoA) and phosphatidylinositols have been extensively studied in directional cell migration. However, it remains unclear how Rho GTPases and phosphatidylinositols regulate random cell migration in space and time. We have attempted to address this issue using fluorescence resonance energy transfer (FRET) imaging and statistical signal processing. First, we acquired time-lapse images of random migration of HT-1080 fibrosarcoma cells expressing FRET biosensors of Rho GTPases and phosphatidyl inositols. We developed an image-processing algorithm to extract FRET values and velocities at the leading edge of migrating cells. Auto- and cross-correlation analysis suggested the involvement of feedback regulations among Rac1, phosphatidyl inositols and membrane protrusions. To verify the feedback regulations, we employed an acute inhibition of the signaling pathway with pharmaceutical inhibitors. The inhibition of actin polymerization decreased Rac1 activity, indicating the presence of positive feedback from actin polymerization to Rac1. Furthermore, treatment with PI3-kinase inhibitor induced an adaptation of Rac1 activity, i.e. a transient reduction of Rac1 activity followed by recovery to the basal level. In silico modeling that reproduced the adaptation predicted the existence of a negative feedback loop from Rac1 to actin polymerization. Finally, we identified MLCK as the probable controlling factor in the negative feedback. These findings quantitatively demonstrate positive and negative feedback loops that involve actin, Rac1 and MLCK, and account for the ordered patterns of membrane dynamics observed in randomly migrating cells.

**Key words:** Rac1, FRET, Feedback, Adaptation

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**Introduction**

Cell migration is an essential process in a wide variety of biological phenomena, such as embryonic development, wound repair, immune surveillance, tumor cell invasion and metastasis in mammalian cells (Lauffenburger and Horwitz, 1996). Extracellular cues elicit the various intracellular responses in the organization of both the actin and the microtubule cytoskeletons. It is widely accepted that the major driving force of migration is the extension of a leading edge protrusion or lamellipodium, cell body contraction, and detachment of adhesions at the cell rear (Rajakulendran et al., 2009). All these steps involve the assembly, disassembly and reorganization of the actin cytoskeleton, which are coordinated both in space and time (Mitchison and Cramer, 1996; Rajakulendran et al., 2009).

The molecular mechanisms of cell migration have been extensively studied in tissue culture cells. Rho GTPases and lipid kinases have been identified as key regulators of cell migration. The best-characterized function of Rho GTPases is in the regulation of actin dynamics: RhoA increases the actomyosin contractility, whereas Rac1 and Cdc42 induce the polymerization of actin to form lamellipodial and filopodial protrusions, respectively (Ridley, 2001). Phosphatidyl inositols, which are composed of a D-myoinositol-1-phosphate linked through its phosphate group to diacylglycerol, are phosphorylated and dephosphorylated by phosphatidylinositol kinases and phosphatases, generating distinct phosphoinositide species (Saarikangas et al., 2010). Among them, phosphatidylinositol 3,4,5-trisphosphate \([\text{PtdIns}(3,4,5)_\text{P}_3]\) and phosphatidylinositol bisphosphates \([\text{PtdIns}(3,4)_\text{P}_2\text{ and PtdIns}(4,5)_\text{P}_2]\) regulate reorganization of the actin cytoskeleton by providing a membrane-binding platform for a variety of proteins, including guanine nucleotide exchange factors (GEFs) for Rho GTPases and actin filament nucleating proteins (Saarikangas et al., 2010). With the advent of a myriad of fluorescent proteins, genetically encoded biosensors have been increasingly used to visualize the activities of Rho GTPases and phosphatidylinositols (Aoki et al., 2008; Miyawaki, 2003). In particular, live-cell imaging with biosensors based on the principle of Förster (or fluorescence) resonance energy transfer (FRET) have uncovered the spatiotemporal dynamics of these molecules in migrating cells (Itoh et al., 2002; Kurokawa and Matsuda, 2005; Nishioka et al., 2008).

Morphological polarization (i.e. a clear distinction between cell front and rear) is required for cell migration to enable cells to turn intracellularly generated forces into net cell body translocations (Lauffenburger and Horwitz, 1996). It has been suggested that intrinsic mechanisms of intracellular signaling systems with their kinetic fluctuations or noise suffice to activate...
this front–rear polarization during the locomotion of eukaryotic motile cells. For example, spatially homogenous stimulation with a chemoattractant, at least for neutrophils, induces a front–rear polarity with a change in filamentous actin (F-actin) distribution from azimuthal symmetry around the cell rim to concentration within a particular region (Coates et al., 1992; Lauffenburger and Horwitz, 1996). In addition, even in the absence of concentration gradients of external stimuli, ordered patterns of cell shape are observed in Dictyostelium, flies and mammalian cells, such as directional migration, oscillation behavior of membranes and laterally traveling waves along the cell boundary (Döbereiner et al., 2006; Machacek and Danuser, 2006; Maeda et al., 2008; Weiner et al., 2007). The Turing’s reaction–diffusion model is one of the most supported models for the formation of ordered patterns in migrating cells (Gierer and Meinhardt, 1972; Kondo and Miura, 2010; Otsuji et al., 2010). On the basis of a framework in the reaction–diffusion model, self-enhancing positive and negative feedback regulations play a pivotal role in the autonomous emergence of an ordered pattern. It is suggested that PtdIns(3,4,5)P_3, Rac1 and the actin cytoskeleton are part of a positive feedback loop that can initiate polarization (Peyrollier et al., 2000; Srinivasan et al., 2003; Wang et al., 2002; Weiner et al., 2002). However, contrary to these models, rapid activation of endogenous Rac1 proteins triggered effective actin polymerization but failed to activate phosphoinositide 3-kinase (PI3K) to generate PtdIns(3,4,5)P_3 or induce cell polarization (Inoue and Meyer, 2008). Furthermore, the molecular mechanisms of such negative feedback loops remain largely unknown. We have attempted to identify molecular mechanisms of positive and negative feedback loops underlying the random migration of mammalian cells using FRET imaging with statistical signal processing.

**Results**

**Time-lapse imaging of Rho GTPases, phosphatidylinositols and F-actin in randomly migrating HT-1080 cells**

We aimed to elucidate the molecular networks of Rho GTPases and phosphatidylinositols in randomly migrating mammalian cells by time-lapse imaging. We used human HT-1080 fibrosarcoma cells, which exhibit a high motility and invasiveness, as a model system of spontaneous random migration (Hoffinan, 2005; Itoh et al., 2002). All experiments presented below employed time-lapse imaging of HT-1080 cells 1 hour after seeding on collagen-coated glass-bottomed dishes to induce spontaneous migration. The HT-1080 cells expressing biosensors were imaged every 1 minute for 2 hours in order to minimize phototoxicity and photobleaching.

We first examined the activity of the Rho GTPases Rac1, Cdc42 and RhoA in migrating HT-1080 cells using the FRET biosensors Raichu–Rac1, Raichu–Cdc42 and Raichu–RhoA, respectively. As reported previously (Itoh et al., 2002; Kraynov et al., 2000; Nalbant et al., 2004), Rac1 and Cdc42 were locally activated at lamellipodia and membrane ruffles at the leading edge, and inactivated at the retracting tail of migrating cells.

![Fig. 1. Spatiotemporal activity maps of signaling molecules in randomly migrating HT-1080 cells.](https://example.com/image)

**Fig. 1.** Spatiotemporal activity maps of signaling molecules in randomly migrating HT-1080 cells. HT-1080 cells transfected with the plasmid encoding the indicated biosensors were imaged for CFP and FRET every 1 minute. FRET/CFP ratio ranges in intensity modulated display (IMD) mode, which associates color hue with emission ratio value and the intensity of each hue with the source image brightness, are shown at the right of each image. Scale bars: 10 μm. (A–F,H) Montage images showing the spontaneous migration of HT-1080 cells expressing the biosensors Raichu–Rac1 (A), Raichu–Cdc42 (B), Raichu–RhoA (C), Pippi–PtdIns(3,4,5)P_3 [PI(3,4,5)P_3], (D), Pippi–PtdIns(3,4)P_2 (E), Pippi–PtdIns(4,5)P_2 (F) and Raichu–PAK–Rho (H). (G) Montage images showing the ratio of Lifeact–EGFP to ERed–NES.
correlation function (ACF) in Fig. 2D has a wave-like pattern, demonstrating the occurrence of the lateral membrane wave along the cell boundary. With the temporal ACF (ΔWindow no.=0, dashed line in Fig. 2D), three parameters, Δ0, Δmin and Δmax, were defined as shown in Fig. 2E. The pattern of the ACF map and threshold criteria of Δ0 value (Δ0>30 min) were utilized to classify the pattern of morphological dynamics into four categories: wave-like, oscillatory, directional, and non-classifiable mode (Maeda et al., 2008) (see Materials and Methods for more details; Fig. 2F; supplementary material Movie 8). Almost half of HT-1080 cells demonstrated the wave-like mode of migration (Table 1). Furthermore, the velocity of the lateral membrane wave, \( V_L \), was calculated as 4.2±2.1 μm/minute by dividing the length of the cell periphery by Δmax. These results suggested the existence of an intrinsic signaling network that established an ordered pattern of morphological dynamics without external stimuli. Notably, the expression of FRET biosensors per se did not substantially perturb the mode of cell migration in HT-1080 cells (supplementary material Fig. S2). Therefore, in the following study, we examined a molecular mechanism for generating wave-like motion in randomly migrating HT-1080 cells.

**Quantification of correlation and time delay between morphological change and signaling by cross-correlation analysis**

To identify a hierarchical connection among Rho GTPases, phosphatidylinositol and morphological change, we used a cross-correlation analysis and characterized their temporal correlation (Machacek et al., 2009; Tsukada et al., 2008). The cross-correlation function (CCF) between edge velocity (Fig. 3A) and Rac1 activity (Fig. 3B) was calculated with Rac1 activity being fixed as described in the Materials and Methods (Fig. 3C). Temporal CCF was also extracted (Fig. 3C; dotted line) to analyze the lag time of signaling and morphological change (Fig. 3D; supplementary material Fig. S3; Table S1). The positive peak value of the cross correlation coefficient was obtained with a time delay of −2.2 minutes [95% confidence interval (CI), −3.0 to −1.4 minutes] from Rac1 activity, indicating that the edge velocity was increased before the increase in Rac1 activity and vice versa (Fig. 3E). However, taking the interval of image acquisition (every 1 minute) into account, the Cde42 activity was slightly delayed or nearly synchronized with morphological change (time delay of −1.3 minutes, 95% CI, −2.2 to −0.5 minutes; supplementary material Fig. S4A). Rac1 activity did not demonstrate any correlation with edge velocity (peak value of 0.082; supplementary material Fig. S4B). This was probably because the high Rac1 activity at both protrusion and retraction during cell migration marginally contributed to the overall correlation, leading to an offset of the net correlation coefficient value (Kurokawa and Matsuda, 2005). Furthermore, F-actin accumulation was coupled to the edge velocity with a time delay of −0.25 minutes (95% CI, −1.5 to 1.0 minute; Fig. 3F). We time-lapse imaged Rac1 activity and F-actin accumulation every 30 seconds in migrating HT-1080 cells, demonstrating the time delay values of −2.6 minutes (95% CI, −3.3 to −1.5 minutes) and −0.9 minutes (95% CI, −1.4 to −0.4 minutes) for Rac1 activity and F-actin, respectively (supplementary material Fig. S5, Table S1). These values are consistent with those obtained by imaging with the time interval.
of 1 minute. Of note, we previously demonstrated that EGF-induced Rac1 activation preceded the lamellipodial extension in Cos1 cells (Kurokawa et al., 2004). Thus, the role of Rac1 appears to be different in growth factor-induced and stochastic membrane protrusions.

Cross-correlation analysis revealed that PtdIns(3,4,5)P3 accumulation was almost synchronized with membrane protrusion (time delay of 2.1 ± 1.7 minutes, 95% CI, 2.0 to 2.2 minutes; Fig. 3G). The downstream metabolite, PtdIns(4,5)P2, demonstrated a time delay of 1.6 minutes in comparison with the edge velocity (95% CI, 1.6 to 1.8 minutes; supplementary material Fig. S4C). PtdIns(4,5)P2 accumulation was delayed 1.4 minutes (95% CI, 1.3 to 1.5 minutes; supplementary material Fig. S4D). In migrating MDCK cells, phosphatidyl inositol 5-kinase and PLC-γ, but not PI3K, mainly contribute to the distribution of PtdIns(4,5)P2 at

Table 1. Classification of migration mode based on the auto-correlation analysis

<table>
<thead>
<tr>
<th>Mode</th>
<th>Wave-like</th>
<th>Oscillatory</th>
<th>Directional</th>
<th>Unclassifiable</th>
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<tbody>
<tr>
<td>Percent (%)</td>
<td>42</td>
<td>6</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td>Length (µm)</td>
<td>124±35.4</td>
<td>126±23.9</td>
<td>126±31.7</td>
<td>129±23.1</td>
</tr>
<tr>
<td>Δt0 (minute)</td>
<td>10.7±7.54</td>
<td>16.8±8.6</td>
<td>48±22.1</td>
<td>39.6±36.6</td>
</tr>
<tr>
<td>Δtmin (minute)</td>
<td>19.9±14.8</td>
<td>14.3±3.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Δtmax (minute)</td>
<td>39.8±24.9</td>
<td>29.0±9.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>fL (µm/minute)</td>
<td>4.2±2.1</td>
<td>4.4±1.0</td>
<td>—</td>
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</tr>
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More than forty cells were analyzed and classified as described in Materials and Methods. Values are means ± s.d.
control FRET biosensor did not show any direct correlation with edge velocity (Fig. 3H).

In brief, the cross-correlation analysis showed a sequential relationship between signaling molecules and morphological change in the following temporal order: (1) PtdIns(3,4,5)P_3 accumulation and morphological change (polymerized actin localization); (2) PtdIns(3,4)P_2 accumulation and Cdc42 activation; and (3) Rac1 activation.

**Perturbation analysis of the signaling pathway**

Rac1 induces actin reorganization and, consequently, lamellipodial formation (Hall, 1998). According to this scheme, the delay in Rac1 activation shown in Fig. 3 implies a positive feedback regulation from actin polymerization to Rac1 in migrating HT-1080 cells. To explore this assumption, we examined the effects of the F-actin depolymerizers latrunculin B and cytochalasin D on PtdIns(3,4,5)P_3 accumulation and Rac1 activity. Latrunculin B (Fig. 4A,B) and cytochalasin D (supplementary material Fig. S6A,B) treatment did not induce any change in the level of PtdIns(3,4,5)P_3, but were associated with a decrease in Rac1 activity (Fig. 4C). These results indicated that actin polymerization activated Rac1 by a positive feedback mechanism. Cdc42 activity was not suppressed by latrunculin B or cytochalasin D treatment (data not shown), although the reason for this remains unknown. Therefore, we focused on only Rac1 activity in the following study. Next, we investigated another positive feedback mechanism from Rac1 to PI3K (Aoki et al., 2007; Weiner et al., 2002) by the inducible translocation technique (Inoue et al., 2005). Rapamycin triggers heterodimerization formation of a Lyn N-terminal sequence-tagged FRB (LDR) with an FKBP-fused protein. Upon rapamycin treatment, FKBP-Tiam1 was translocated to the plasma membrane, and consequently activated Rac1 within 2 minutes, followed by the formation of lamellipodia (Fig. 4E,G). However, rapid activation of Rac1 did not increase the level of PtdIns(3,4,5)P_3 (Fig. 4F,H). These findings demonstrated that the positive feedback pathway consisting of Rac1 and PI3K did not contribute to the signaling network in migrating HT-1080 cells as reported in HL-60 neutrophils (Inoue and Meyer, 2008).

The results of auto-correlation analysis, which showed a wave-like pattern of membrane dynamics, prompted us to investigate a negative feedback loop involving Rac1, phosphatidyl inositols and morphological change. We found that treatment with a PI3K inhibitor, LY294002, induced an immediate and transient decrease in Rac1 activity to below the basal level, and suppressed lamellipodial protrusions (Fig. 4J,L). Interestingly, Rac1 activity and the morphological repression returned to the basal levels within 20 minutes even though PtdIns(3,4,5)P_3 was sustained at low levels for at least 30 minutes after treatment with LY294002 (Fig. 4I,K). We confirmed these findings using another PI3K inhibitor, PIK-93 (Knight et al., 2006) (supplementary material Fig. S6C,D).

**Prediction of a network topology consisting of PI3K, Rac1 and F-actin**

The transient suppression of Rac1 by PI3K inhibition might be caused by adaptation, i.e. the ability of the signaling network to reset itself after responding to a stimulus. A recent study revealed that an adaptation emerges from only two major topologies of a
Fig. 4. Perturbation analysis of PI3K–Rac1–Actin signaling by chemical inhibitors. (A–D) HT-1080 cells expressing Pippi–PtdIns(3,4,5)P_3 (A) or Raichu–Rac1 (B) were treated with 100 nM latrunculin B, and imaged every 1 minute. FRET images at the indicated time points after latrunculin B addition are shown in IMD mode. Ratio ranges in IMD mode are shown at the right of each image. Scale bars: 10 μm. (C,D) FRET/CFP ratios of Pippi–PtdIns(3,4,5)P_3 (C) and Raichu–Rac1 (D) for the individual cells (gray lines) were expressed by measuring the increase over the basal activity, which was averaged over 10 minutes before latrunculin B addition. Red lines indicate the average FRET/CFP ratio; n = 4 (C), n = 9 (D). (E–H) HT-1080 cells expressing LDR and FKBP–Tiam1 with Raichu–Rac1 (E) and Pippi–PtdIns(3,4,5)P_3 (F) were treated with 100 nM rapamycin, and imaged every 1 minute. FRET images are shown in E and F. Scale bar: 10 μm. (G,H) FRET/CFP ratios of Raichu–Rac1 (G) and Pippi–PtdIns(3,4,5)P_3 (H) for the individual cells (gray lines) and their average (red) are shown as described for C and D; n = 10 (G), n = 7 (H). (I–L) HT-1080 cells expressing Raichu–Rac1 (I) and Pippi–PtdIns(3,4,5)P_3 (J) were treated with 20 μM LY294002, and imaged every 1 minute. FRET images are shown in I and J. Scale bars: 10 μm. (K,L) FRET/CFP ratios of Raichu–Rac1 (K) and Pippi–PtdIns(3,4,5)P_3 (L) for the individual cells (gray lines) and their average (red lines) are shown as described for C and D; n = 9 (K), n = 10 (L).
signaling network: a negative feedback loop with a buffering node and an incoherent feed forward loop with a proportioner node (Ma et al., 2009). This prompted us to explore possible kinetic models of PI3K, Rac1 and actin polymerization modules by considering the following two constraints: (1) the inhibition of actin polymerization induces a sustained decrease in Rac1 activity, and (2) Rac1 activity and actin polymerization adapt to PI3K inhibition. Because Rac1 activation did not affect PI3K, we set PI3K as an input. Actin polymerization was set as an output for the membrane protrusion. In preliminary simulations, Rac1 was located between PI3K and actin polymerization (supplementary material Fig. S7). None of the models derived from this topology could fulfill the aforementioned constraints because actin polymerization cannot positively regulate Rac1.

Therefore, four models of possible networks with an additional ‘node’ were constructed to reproduce the experimental results obtained by cross-correlation and perturbation analyses (Fig. 5A). In these models, we assumed that actin polymerization corresponded to the edge velocity. The numerical simulation in each proposed model reproduced the effect of actin polymerization inhibitors and PI3K inhibitors on the change in Rac1 activity – namely, sustained decrease and adaptation, respectively (Fig. 5B, Model A; supplementary material Figs S8–S10 Models B–D, Tables S2–S5).

Role of MLCK as a buffering node among PI3K, F-actin and Rac1 signaling modules

To distinguish these four models, we searched for candidate molecules that might act as nodes in the signaling scheme. In particular, we focused on negative regulators for actin reorganization and Rac1 signaling, and found that myosin light chain kinase (MLCK) and/or its target signaling played a role in a buffering node of the network. To visualize MLCK accumulation, the high molecular mass isoform of human MLCK1 tagged with GFP at the N-terminus, hereafter referred to as EGFP–Long-MLCK, was expressed in HT-1080 cells (Poperechnaya et al., 2000). EGFP–Long-MLCK demonstrated clear accumulation at not only the retracting tail, but also the leading edge of migrating HT-1080 cells (Fig. 6A; supplementary material Movie 9). The temporal CCF demonstrated a positive peak of correlation efficiency at the time delay of approximately −4.0 minutes (95% CI, −4.9 to −3.1 minutes; Fig. 6B, arrow). Furthermore, the negative correlation coefficient peak was obtained at the positive time delay of approximately 0.5 minutes (95% CI, −0.8 to 1.8 minutes; Fig. 6B, arrowhead). These delay timing values did not correlate with the expression level of EGFP–Long-MLCK, negating the artificial effect of over-expression of Long-MLCK on the migration in HT-1080 cells (supplementary material Fig. S11). To validate the relationship between MLCK and Rac1, we examined whether rapid activation of Rac1 induced translocation of MLCK to the periphery of the membrane. As expected, rapamycin-induced heterodimerization between LDR and FKBP–Tiam1-activated Rac1, which in turn increased the level of localization of EGFP–Long-MLCK at the peripheral membrane (Fig. 6C, arrows) with a delay of 4.6 minutes (Fig. 6D). In addition, treatment with an MLCK inhibitor, ML-7, induced a rapid increase in Rac1 activity (Fig. 6E,F). Thus, a reaction between Rac1 and MLCK could be the buffering node, which was postulated in Model A (Fig. 5). Finally, we examined the involvement of Rho-associated protein kinase (ROCK), which is well known to regulate myosin contractility (Totsukawa et al., 2004). ROCK inhibitor, Y27632, was added to the medium of HT-1080 cells expressing Raichu–Rac1 to determine whether Y27632 increased Rac1 activity as ML-7 does. Unexpectedly, however, Y27632 had little effect on Rac1 activity, although Y27632 induced typical morphological changes in ROCK inhibition: long protrusions and a sickle-cell shape (supplementary material Fig. S12). These results strongly supported the negative feedback loop in Model A, in which the MLCK pathway acted as a buffering node for the negative feedback.
In this study, we have revealed a signaling network comprising positive and negative feedback regulations between PI3K, actin polymerization, Rac1 and MLCK in randomly migrating HT-1080 cells (Fig. 7A). On the basis of the auto- and cross-correlation analyses, we can summarize the relative delay timing in comparison to edge velocity change (Fig. 7B) and the process of signaling and membrane protrusion and retraction as follows (Fig. 7C). In the first phase of protrusion, the edge velocity takes a positive value, indicating membrane extension and actin polymerization (0 minutes). Simultaneously or slightly after membrane protrusion, PtdIns(3,4,5)P3 accumulates at the tip of the protrusion. In the second phase (2 minutes), Rac1 activity increases. In the third phase (4 minutes), MLCK accumulates at the tip of the leading edge in a Rac1-dependent manner, and consequently decelerates the edge velocity through actin reorganization. According to the results of the auto-correlation analysis, the lateral membrane wave revolves around the cell periphery with a periodicity of 40 minutes. Thus, the edge velocity, Rac1 activity and MLCK localization reach a peak at 10, 12 and 14 minutes, respectively. Meanwhile, the edge position of the cell periphery is described by integrating the edge velocity value, and peaks at 20 minutes after the start of the increase in edge velocity (Fig. 7C, gray line). The latter half of the process, i.e. retraction, represents the same sequential process in which the edge velocity and molecular activities show negative and low values, respectively.

**Fig. 6. Identification of MLCK as a buffering node of the negative feedback loop.** (A) HT-1080 cells expressing the long isoform of MLCK fused with EGFP (EGFP–Long-MLCK) and ERed-NES as a cytoplasmic reference were imaged every 1 minute. EGFP and ratio images at the indicated time points are shown. Ratio ranges in IMD mode are shown at the right of each image. Scale bars: 10 μm. (B) Temporal cross-correlation between edge velocity and Long-MLCK localization are shown for individual cells (gray lines). The red line indicates average temporal cross-correlation function; n=6. (C,D) HT-1080 cells expressing LDR and FKBP–Tiam1 with EGFP–Long-MLCK and ERed-NES were treated with 100 nM rapamycin, and imaged every 1 minute. The ratio images are shown in C. Arrowheads indicate the accumulation of EGFP–Long-MLCK after rapamycin treatment. Scale bars: 10 μm. (D) The mean EGFP–MLCK/ERed-NES ratios at the cell periphery (red and green circles) and the FRET/CFP ratios of Raichu–Rac1 over the whole cell (blue circles) from measurements of the relative increase compared with the reference value at 0 minutes of rapamycin addition. FKBP–Tiam1 and FKBP were used as Rac1 activator (blue and red) and control (green), respectively. Blue and red lines are the curves fitted with a single exponential function, and τrac1 and τMLCK are their time constants. Bars represent the standard deviation; n=5 (Rac1), n=6 (MLCK) and n=5 (control). (E,F) HT-1080 cells expressing Raichu–Rac1 were treated with 20 μM ML-7, an MLCK inhibitor, and imaged every 1 minute. FRET images are shown in Fig. 1A. Scale bar: 10 μm. (F) FRET/CFP ratios of Raichu–Rac1 for the individual cells (gray lines) and their average (red line) are shown as described in Fig. 4D (n=5).
Arai et al. have found that self-organized waves of Dictyostelium cells in the presence of latrunculin A, and they have developed a reaction diffusion model that recapitulates the relaxation oscillator of phosphatidylinositol dynamics with positive and negative feedback loops (Arai et al., 2010). This model differs from the present models of HT-1080 cells in terms of the signaling hubs: phosphatidylinositol sols play a central role in the positive and negative regulations in Dictyostelium cells, whereas Rac1 acts as a signaling hub for two feedback loops, as observed in HT-1080 cells. Of note, the velocity of the lateral membrane wave in this study, ~4 μm/minutes (Table 1), was comparable with those in mouse embryonic fibroblasts (2.4 μm/ minutes), fly cells (0.72 μm/minutes), T cells (0.33 μm/minutes) (Döbereiner et al., 2006) and Dictyostelium cells (2–4 μm/ minutes as roughly calculated from Arai et al. (Arai et al., 2010), implying the presence of general molecular mechanisms underlying the random migration of eukaryotic cells.

One of the key findings in this study was that a negative feedback loop from Rac1 to MLCK enables the system of actin reorganization to adapt to external perturbations. We assumed that GTP–Rac1 recruits MLCK to the edge of membrane protrusions either directly or indirectly, leading to the retraction of the peripheral plasma membrane by increasing the actomyosin contractility. Consistent with this model, MLCK localizes at the leading edge of lamellipodia in mouse embryonic fibroblasts, and regulates the periodic lamellipodial contractions (Giannone et al., 2004). The long MLCK directly binds to actin filaments in vitro and in interphase cells, and these bindings are mediated by five DXRXXL motifs (Poperechnaya et al., 2000; Smith et al., 2002). The inhibition of ROCK by Y27632 did not alter Rac1 activity (supplementary material Fig. S12), suggesting two possible molecular mechanisms of the negative feedback. First, the negative feedback from MLCK to Rac1 was mediated through myosin that was regulated by MLCK, but not ROCK. There are many reports showing spatial and functional discrepancies of myosin regulations between MLCK and ROCK (Totsukawa et al., 2004). Second, myosin-independent MLCK activity mediates the negative feedback from MLCK to Rac1. Consistent with this hypothesis, myosin-independent effect of MLCK on migration of mammalian cells has been reported (Kudryashov et al., 1999; Niggli et al., 2006). In disagreement with the negative feedback model, the rapid and global activation of Rac1 by FKBP–Tiam1 and LDR upon rapamycin stimulation resulted in global lamellipodial extensions (Fig. 4E,F). This discrepancy is presumably attributable to the difference of strength and duration in Rac1 activation between randomly migrating cells and cells expressing FKBP–Tiam1. Robust and sustained activation of Rac1 by the FKBP–Tiam1 translocation system might cause inactivation of MLCK through phosphorylation by PAK, which is a downstream effector of Rac1 (Sanders et al., 1999). However, it has been reported that PAK directly phosphorylates Ser19 of MLC and induces an increase in actomyosin contraction in endothelial cells (Chew et al., 1998; Zeng et al., 2000). Therefore, future studies will be needed to evaluate the spatial and temporal regulation of MLCK activity in migrating cells.

Evidence of a pathway from the actin cytoskeleton to Rac1 was provided by cross-correlation analysis (Fig. 3) and by the latrunculin-B-induced decrease of Rac1 activity (Fig. 4). In this context, we should note that Rac1 induces actin polymerization and formation of lamellipodia (Hall, 1998) (Fig. 4E,F). These
data indicate the existence of a positive feedback loop between the actin cytoskeleton and Rac1. Although it remains unknown how the actin cytoskeleton regulates Rac1 activity, we can adduce several mechanisms of the positive feedback loop. Some GEFs acting on Rac1, such as FGDs, Frabin (Nakanishi and Takai, 2008) and Asf1 (Sagara et al., 2009), interact with F-actin directly or indirectly. These GEFs are candidate molecules for a role in the positive feedback. Furthermore, focal complexes and adhesions induced by Rac1 at lamellipodia recruit the Cas–Crk–DOCK signaling complex (Côté and Vuori, 2007; Meller et al., 2005) and lead to the further activation of Rac1 in a manner consistent with the positive feedback. Such positive feedback regulation between PtdIns(3,4,5)P3, Rac1 and actin has been well characterized in Dictyostelium cells, neutrophils and PC12 cells (Aoki et al., 2007; Iijima et al., 2002; Merlot and Firtel, 2003; Weiner et al., 2002). In contrast to these earlier works, PtdIns(3,4,5)P3 was not involved in the positive feedback in the present model (Fig. 7). Consistent with this, Inoue et al. have shown with HL-60 neutrophils that rapid activation of endogenous Rac1 proteins triggers effective actin polymerization in the absence of PI3K activation and increase in PtdIns(3,4,5)P3 (Inoue and Meyer, 2008).

Cross-correlation analyses have been applied to understand how Rho GTPases coordinate the morphodynamics (Machacek et al., 2009; Tsukada et al., 2008). Tsukada et al. have demonstrated, using the edge evolution tracking (EET) method, that the change in cell edge velocity precedes the activation of Rac1 and Cdc42, with a time delay of ~6 and 8 minutes, respectively (Tsukada et al., 2008). By contrast, Machacek et al. have shown with a sophisticated imaging processing technique that Cdc42 and Rac1 are activated ~40 seconds after the increase in the edge velocity (Machacek et al., 2009). Differences in the values of the time delay between these studies and our study, 2.2 minutes for Rac1, could arise from two reasons: the differences in image processing algorithms, and the use or not of a classification system for the modes of cell migration. With respect to the first reason, Machacek et al. analyzed the local area of the cell periphery, i.e. only the protrusive area, with image sets acquired for a shorter time interval of 10 seconds than ours of 1 minute. We, instead, focused on the global membrane dynamics of protrusion and retraction in the entire cell periphery of migrating HT-1080 cells. This could cause larger time delay values than those obtained in Machacek’s study. With respect to the second reason, we classified the modes of cell migration into four categories – wave-like, oscillatory, directional and unclassifiable – and analyzed only the cells that were categorized into the wave-like migration. By contrast, Tsukada et al. did not use any categorization. Time-delay values obtained from directionally migrating cells became much greater than those of the cells migrating in the other modes. Therefore, the use of classification resulted in shorter delay timing in our study than Tsukada’s studies (Tsukada et al., 2008). Notably, we assumed that the proposed feedback regulations between Rac1, actin cytoskeleton and MLCK took place in both protrusions and retractions in migrating HT-1080 cells for the following reasons.

(1) Data obtained from cross-correlation analysis at the front of migrating cells contributed to the total correlation coefficient value to a similar extent as that obtained at the back of migrating cells.

(2) We did not recognize any difference in the correlative relationship of Rac1 or Cdc42 activities and edge velocity between the front and back of cell movements; namely, at the front, increase in edge velocity preceded increase in Rac1 activity, whereas, at the back, decrease in edge velocity preceded decrease in Rac1 activity. It was also noted that a correlation-based multiplexing approach should be carefully applied to a system with feedback interactions (Sabouri-Ghomi et al., 2008). In our study the delay timing obtained by correlation analysis was within 2.2 minutes for Rac1 (Fig. 3); whereas, the effect of feedback regulation appeared at 4–6 minutes later (Fig. 6). Thus, it is unlikely that the feedback regulation interferes with the correlation analysis.

By employing image processing, statistical analysis and pharmacological perturbations, this study has demonstrated the molecular mechanisms underlying the ordered membrane dynamics in randomly migrating eukaryotic cells. A pivotal question to be answered is what factors initiate the increase in edge velocity prior to Rac1 activation. Our data showed that PtdIns(3,4,5)P3 accumulated concomitantly with the increase in edge velocity (Fig. 3). Additionally, it has been recently reported that membrane curvature induces actin polymerization through EFC and/or F-BAR proteins and the N-WASP–WIP complex (Takano et al., 2008). In line with this finding, maximal F-actin assembly was observed ~20 seconds after maximal edge advancement (Ij et al., 2008). These results suggest the involvement of lipid regulation, which serves as a trigger for membrane protrusion. Further studies will be needed to uncover the molecular mechanism of the dynamics in migratory cells.

Materials and Methods

FRET biosensors

The plasmids encoding FRET biosensors have been described as follows: Raichu-Rac1/1011x, pRaiuho-Cdc42/1054x (Itoh et al., 2002), pRhA-RhoA/1294x (Nakamura et al., 2005; Yoshizaki et al., 2003), pPippp-PtdIns(3,4,5)P3/1747x, pPippp-PtdIns(3,4)P2/1759x (Aoki et al., 2005; Sato et al., 2003), pPippp-PtdIns(4,5)P2/1758x (Nishikawa et al., 2008) and pRaiuho-Pak-RhoA/1110x, a negative control (Kurokawa and Matsuda, 2005).

Cells, plasmids and reagents

HT-1080 cells were purchased from the American Type Culture Collection or the Japan Cell Resource Bank, and maintained in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS). The cells were plated on 35-mm glass-bottomed dishes (Asahi Techno Glass, Tokyo, Japan), which were pre-coated with collagen type 1 (Nitta Gelatin Inc., Osaka, Japan). Plasmids were transfected into HT-1080 cells with Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen, San Diego, CA). CDNA of Lifeact, a 17- amino-acid peptide (Riedl et al., 2008), were subcloned into the pCAGGS-EGFP vector. EGFP-Long-MLCK was a kind gift from Anne R. Bresnick (Popechcnaya et al., 2000). Expression plasmids for ERED–NES, LDL (Lyn11-targeted FRB), FKBP and FKBP–FLI1 have been described previously (Aoki et al., 2007; Inoue et al., 2005). CDNA for FKBP–SH2 was subcloned into pCAGGS-GST vector to obtain pCAGGS-GST-FKBP–SH2 (Inoue and Meyer, 2008). LY294002 was obtained from Sigma-Aldrich (St. Louis, MO). Latrunculin B, cytochalasin D, ML-7 and Y27632 were purchased from Calbiochem (La Jolla, CA). PIK-93, which is a PI3K inhibitor, was obtained from Synmanis NZ (Timaru, New Zealand). Rapamycin was purchased from LC Laboratories (Woburn, MA).

Time-lapse FRET imaging

FRET imaging was done essentially as previously described (Aoki and Matsuda, 2009). Briefly, HT-1080 cells expressing biosensors were suspended in transpyrin, plated on collagen-coated 35-mm glass-bottomed dishes, and left for approximately 1 hour. At the time that cells were being plated, the culture medium was replaced with Phenol-Red-free DMEM/F12 containing 10% FBS, and overlaid with mineral oil to prevent evaporation. Cells were imaged with an inverted microscope (IX81; Olympus, Tokyo, Japan) equipped with a 60× objective lens (Olympus), a cooled CCD camera (Cool SNAP-K4; Roper Scientific), an LED illumination system (CoolLED precisExcite; Molecular Devices), an IX2-ZDC laser-based autofocus system (Olympus) and an MD-XY30100T-Meta automatically programmable XY stage (Sigma Koki, Tokyo, Japan). The following filters were used for the dual emission imaging studies: excitation filters, 435/20 for CFP and FRET (Olympus), S492/18x for GFP and 350/26 for RFP (Olympus Optical, Brattleboro, VT); dichroic mirrors, XF2034 for CFP and FRET (Omega) and 86000hs for GFP and RFP.
Mapping of velocity and molecular activity in the leading edge

The velocity of the leading edge in migrating HT-1080 cells was calculated using a mechanical model with some modifications (Machacek and Danuser, 2006).

First, non-classifiable disordered pattern of the auto-correlation function was discriminated from the other migration patterns. Then, directional migration was analyzed by calculating the distance from the point at time $T_{2}$ to the point at time $T_{1}$, and divided by the interval time of imaging to obtain the edge velocity (supplementary material Fig. S1G). We denoted the matrices of edge velocity and molecular activity/localization as $M$ and $F$, respectively. Numerical simulation was performed using Matlab software (version R2010b; The Mathworks Inc., Natick, MA).

Velocity calculating. First, the data of the XY coordinates and FRET/CFP ratio values, which were obtained by the image processing, were normalized to 200 sampling windows. Second, to quantify the edge velocity, the window that was located at an angle of 0$^\circ$ from the centroid was set as an initial point (Fig. 2A). Third, normal vectors were defined along the cell boundary (supplementary material Fig. S1E).

Normal vector angles were affected especially in the region with strong boundary deformation (Machacek and Danuser, 2006). Therefore, fourth, the normal vector angles were corrected by averaging angles of five backward and five forward vectors (supplementary material Fig. S1F). Lastly, by using the averaged vectors, the boundary displacement at the time $T$ was obtained by calculating the maximum distance from the point at time $T$ to the point at time $T+1$, and divided by the interval time of imaging to obtain the edge velocity (supplementary material Fig. S1G). We denoted the matrices of edge velocity and molecular activity/localization as $M(x,t)|_{0}$ and $F(x,t)|_{0}$, respectively, where $M_{x}$ is spatio-temporal data of morphological changes and $F$ is spatio-temporal data of molecular activity or molecular localization. These processes were implemented using Matlab software (version R2010b; The Mathworks Inc., Natick, MA).

Correlation analysis

The auto- and cross-correlation analysis was performed essentially as described previously (Maeda et al., 2008). The auto-correlation functions of $M(x,t)$ and $F(x,t)$, and the cross-correlation function between $M(x,t)$ and $F(x,t)$ are defined as:

\[ ACF(M(x,t)) = \frac{\langle(M(x,t) + M_{ave} - M_{max})\rangle}{\langle(M_{ave} - M_{min})\rangle} \]

\[ CCF(M(x,t)) = \frac{\langle(M_{ave} - M_{max})F(x,t) - F_{ave}\rangle}{\langle(M_{ave} - M_{max})\rangle^\frac{3}{2}\langle(F_{ave} - F_{max})\rangle^\frac{3}{2}} \]

where the numbers $M_{max}$ and $F_{max}$ indicate the average value of $M(x,t)$ and $F(x,t)$, respectively. The operator $\langle \cdot \rangle$ denotes the averages over time and over space. The range of calculation is the entire period of measurement in time and space from 0 to 200. Correlation analyses were implemented using Matlab software.

Classification of migration pattern

Random migration patterns of HT-1080 cells, i.e. wave-like, oscillatory, directional, and non-classifiable migration, were categorized on the basis of the ordered pattern of auto-correlation function in edge velocity, as shown in Fig. 2F. First, non-classifiable disordered pattern of the auto-correlation function was discriminated from the other migration patterns. Then, directional migration was classified as a value of $\Delta t_{ave} > 30$ minutes defined as a threshold criteria in the temporal auto-correlation function ($\Delta t_{ave} = 0$, dashed line in Fig. 2D). Finally, wave-like migration was distinguished from oscillatory migration by the typical wavy shapes in the auto-correlation function.

Modeling and numerical simulation

All kinetic reactions in Models A–D (Fig. S5) were described by ordinary differential equations based on Michaelis–Menten kinetics. Equations and parameters in Models A to D are listed in supplementary material Tables S2–S5, respectively. Numerical simulation was performed using Matlab software. To achieve the adaptation, we assumed one constraint: the reaction rates and Michaelis constants of the buffering node ($k_{buf}$ and $k_{buf}$) were set to smaller values than those of their substrate in order to saturate their reaction rates (Ma et al., 2009).

Acknowledgements

We thank A. R. Brennick for the plasmids and Y. Sakumura for the helpful discussion. Y. Inaoka, K. Hirano, R. Sakai, A. Katsumata, N. Nonaka, and A. Kawagishi are also to be thanked for their technical assistance. We are grateful to the members of the Matsuda Laboratory for their helpful input.

Funding

This work was supported by a grant-in-aid from the JSPS [grant number 10J06338] to K.K.; the JST PRESTO program to K.A.; and the Research Program of Innovative Cell Biology by Innovative Technology (Cell Innovation) from the Ministry of Education, Culture, Sports and Science, Japan to M.M.

Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.096859/-/DC1

References


Aoki, K., Nakamura, T., Fujikawa, K. and Matsuda, M. (2005). Local phosphati-
dolinolynostosis 3,4,5-trisphosphate accumulation recruits Vav2 and Vav3 to activate Rac/Cdc42 and initiate neurite outgrowth in nerve growth factor-stimulated PC12 cells. Mol. Biol. Cell 16, 2207-2217.


**Fig. S1. Image processing for calculating edge velocity.** The procedures for image processing are as follows. FRET (FRET biosensor) or GFP (Lifeact-EGFP and EGFP-Long-MLCK) images (A) are acquired and processed by a binary operation with an arbitrary threshold value (B). The images are treated with an Erode operation to reduce the noise of the cell periphery (C). Then, the outline of the eroded images is captured by line scan to correct the data of XY coordinates and the FRET/CFP ratio value of the cell periphery. The XY coordinates are normalized to 200 sampling windows. Then, normal vectors are obtained along the cell periphery (E). The angles are corrected by averaging angles of 5 backward and 5 forward vectors to obtain averaged vectors (F). By using the averaged vectors, the boundary displacement at the time of T is obtained by calculating the minimum distance from the point at the time of T to the point at the time of T + 1, and divided by the interval time of imaging to obtain the edge velocity.
Fig. S2. Effect of the expression of FRET biosensors on the mode of cell migration. The acquired image data were grouped into GFP reporter-expressing cells (N = 39) and FRET biosensor-expressing cells (N = 69). The mode of migration was classified into four categories as described in Materials and Methods. The data show the effect of FRET biosensors on the cell migration is negligible.
Fig. S3. Representative data of cross-correlation analysis. Each data set consists of 4 panels: upper left, edge velocity map; upper right, molecular activity/localization map; lower left, cross-correlation function (CCF); lower right, temporal cross-correlation function (TCCF), which corresponds to the white dotted line of CCF. The biosensors are as follows: Cdc42 (A), RhoA (B), PI(3,4,5)P3 (C), PI(3,4)P2 (D), PI(4,5)P2 (E), F-actin (F), MLCK localization (G), and a negative control (H). These data are available upon request.
Fig. S4. Quantification of the dynamics between morphological change and signaling molecules by cross-correlation analysis. The temporal cross-correlations between edge velocity and Cdc42 activity (A), RhoA activity (B), PI(3,4)P2 (C), and PI(4,5)P2 (D) are shown for individual cells (gray). Red lines indicate the average temporal cross-correlation function; N = 6 (Cdc42), N = 6 (RhoA), N = 7 (PI(3,4)P2), N = 5 (PI (4,5)P2). See supplementary materials Fig. S3 and Table S1 for the details.
Fig. S5. Effect of the interval of time-lapse imaging on the cross-correlation analysis. HT-1080 cells expressing Raichu-Rac1 (A) or Lifeact-EGFP and ERed-NES (B) were imaged for CFP and FRET every 30 sec to the temporal cross-correlations between edge velocity and Rac1 activity (A), F-actin (B) are shown for individual cells (graylines). Red lines indicate the average temporal cross-correlation function; N = 5 (Rac1), N = 5 (F-actin).
Fig. S6. Perturbation analysis of PI3-K-Rac1-Actin signaling by chemical inhibitors. (A and B) HT-1080 cells expressing Pippi-PI(3,4,5)P3 (A) or Raichu-Rac1 (B) were treated with 10 µM Cytochalasin D, and imaged every 1 min. FRET/CFP ratios of Pippi-PI(3,4,5)P3 or Raichu-Rac1 for the individual cells (black) were expressed by measuring the increase over the basal activity, which was averaged over 10 min before cytochalasin D addition. Red lines indicate the average FRET/CFP ratio; N = 4 (A), N = 4 (B). (C and D) HT-1080 cells expressing Pippi-PI(3,4,5)P3 (C) or Raichu-Rac1 (D) were treated with 10 µM PIK-93, and imaged every 1 min. FRET/CFP ratios of Pippi-PI(3,4,5)P3 or Raichu-Rac1 for the individual cells (black) were expressed by measuring the increase over the basal activity, which was averaged over 10 min before PIK-93 addition. Red lines indicate the average FRET/CFP ratio; N = 7 (C), N = 5 (D).
Fig. S7. Excluded models. Four signaling models are illustrated as in Fig. 5A. These models reproduce an adaptation of Rac1 activity induced by PI3-K inhibition; however, the models cannot represent a decrease of Rac1 activity upon inhibition of Actin polymerization.
Fig. S8. Numerical simulation of Model B. Model B in Fig. 5 was described by the Michaelis-Menten scheme and numerically solved. (A) Each graph shows the time course of PI(3,4,5)P3 (orange, top left), F-actin (green, top right), Rac1 (red, bottom left), and node (blue, bottom right) as a function of time after PI3-K inhibition. PI3-K inhibition decreases to 10% of the PI3-K level at time 0. (B) Each graph shows the time course of PI(3,4,5)P3 (orange, top left), F-actin (green, top right), Rac1 (red, bottom left), and node (blue, bottom right) as a function of time after actin polymerization inhibition reduces the F-actin level to 0 at time 0. All reactions, parameters and simulation conditions are described in supplementary materials Table S3.
Fig. S9. Numerical simulation of Model C. Model C in Fig. 5 was described by the Michaelis-Menten scheme and numerically solved. (A) Each graph shows the time course of PI(3,4,5)P3 (orange, top left), F-actin (green, top right), Rac1 (red, bottom left), and node (blue, bottom right) as a function of time after PI3-K inhibition. PI3-K inhibition decreases to 10% of the PI3-K level at time 0. (B) Each graph shows the time course of PI(3,4,5)P3 (orange, top left), F-actin (green, top right), Rac1 (red, bottom left), and node (blue, bottom right) as a function of time after actin polymerization inhibition reduces the F-actin level to 0 at time 0. All reactions, parameters and simulation conditions are described in supplementary materials Table S4.
Numerical simulation in Model D

A PI3-K inhibition

B Actin polymerization inhibition

Fig. S10. Numerical simulation of Model D. Model D in Fig. 5 was described by the Michaelis-Menten scheme and numerically solved. (A) Each graph shows the time course of PI(3,4,5)P3 (orange, top left), F-actin (green, top right), Rac1 (red, bottom left), and node (blue, bottom right) as a function of time after PI3-K inhibition. PI3-K inhibition decreases to 10% of the PI3-K level at time 0. (B) Each graph shows the time course of PI(3,4,5)P3 (orange, top left), F-actin (green, top right), Rac1 (red, bottom left), and node (blue, bottom right) as a function of time after actin polymerization inhibition reduces the F-actin level to 0 at time 0. All reactions, parameters and simulation conditions are described in supplementary materials Table S5.
Fig. S11. Effect of MLCK expression level on the cross-correlation between MLCK accumulation and morphological change. Time lag values of the maximum (left) and minimum (right) cross-correlation coefficient are plotted as a function of MLCK expression level (arbitrary unit, AU), which is a value of total EGFP-Long-MLCK fluorescence in a whole cell. The regression line through the data points shows $y = -0.0093x - 3.7621$ (left) and $y = -0.0028x + 0.5714$ (right) and coefficient of determination indicates 0.0128 (left) and 0.0006 (right).
Fig. S12. Perturbation analysis of Rac1 signal by ROCK inhibitors. HT-1080 cells expressing Raichu-Rac1 were treated with 30 µM Y27632, and imaged every 1 min. (A) FRET images at the indicated time points after Y27632 addition are shown in IMD mode. Ratio ranges in IMD mode are shown at the right of image. The scale bar is 10 µm. (B) FRET/CFP ratios of Raichu-Rac1 for the individual cells (gray) were expressed by measuring the increase over the basal activity, which was averaged over 10 min before Y27632 addition. Red lines indicate the average FRET/CFP ratio; N = 4.
Table S1: Summary of cross-correlation analyses between edge velocity and molecular activity.

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Rac1 (60 sec)</th>
<th>Rac1 (30 sec)</th>
<th>Actin (60 sec)</th>
<th>Actin (30 sec)</th>
<th>PI(3,4,5)P&lt;sub&gt;3&lt;/sub&gt; (60 sec)</th>
<th>Cdc42 (60 sec)</th>
<th>RhoA (60 sec)</th>
<th>PI(3,4)P&lt;sub&gt;2&lt;/sub&gt; (60 sec)</th>
<th>PI(4,5)P&lt;sub&gt;2&lt;/sub&gt; (60 sec)</th>
<th>MLCK (Max) (60 sec)</th>
<th>MLCK (Min) (60 sec)</th>
<th>NC (60 sec)</th>
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<tbody>
<tr>
<td><strong>Time lags</strong></td>
<td><strong>(Average and SD)</strong></td>
<td><strong>(-2.17±0.75, -2.40±0.74, -0.25±1.49, -0.90±0.42, -1.14±0.69, -1.33±0.82, -2.67±1.21, -1.57±0.79, -1.40±0.55, -4.00±0.89, 0.50±1.22, NA)</strong></td>
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<tr>
<td><strong>95% Confidence Interval</strong></td>
<td><strong>(-2.96, -1.38, -3.32, -1.48, -1.49, -0.99, -1.78, -0.50, -1.81, -0.48, -3.94, -1.40, -2.30, -0.84, -2.08, -0.72, -4.94, -3.06, -0.79, 1.79)</strong></td>
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<tr>
<td><strong>Peak of TCCF</strong></td>
<td><strong>(Average and SD)</strong></td>
<td><strong>(0.41±0.07, 0.28±0.08, 0.19±0.05, 0.20±0.03, 0.24±0.07, 0.33±0.15, 0.08±0.04, 0.24±0.09, 0.29±0.07, 0.15±0.08, -0.22±0.08, 0.04)</strong></td>
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<tr>
<td><strong>95% Confidence Interval</strong></td>
<td><strong>(0.33, 0.48, 0.19, 0.38, 0.15, 0.24, 0.16, 0.24, 0.18, 0.31, 0.018, 0.49, 0.04, 0.12, 0.16, 0.32, 0.20, 0.37, 0.07, 0.23, -0.30, -0.14)</strong></td>
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</table>

Relative time delay from edge velocity was quantified by cross-correlation analysis. The time lag and peak value were obtained from temporal cross-correlation function (Fig. 2) and represented with s.d. 95% confidence interval was also calculated and represented in bracket.
### Table S2: Equations and parameters in Model A

<table>
<thead>
<tr>
<th>Model A</th>
<th>Reactions</th>
<th>kf</th>
<th>Kmf</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \frac{d[PIP3]}{dt} = k_f [PI3K] \frac{1-[PIP3]}{1-[PIP3]+Kmf} - k_b [PTEN] \frac{[PIP3]}{[PIP3]+Kmb} )</td>
<td>0.1</td>
<td>1.0</td>
<td>Arbitrary</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.01</td>
<td>1.0</td>
<td>Obtained by fitting the data in Fig. 4K.</td>
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<td></td>
<td>( \frac{d[F\text{-}actin]}{dt} = k_f [PIP3] \frac{1-[F\text{-}actin]}{1-[F\text{-}actin]+Kmf} - k_b [Node] \frac{[F\text{-}actin]}{[F\text{-}actin]+Kmb} )</td>
<td>0.05</td>
<td>1.0</td>
<td>Arbitrary</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>1.0</td>
<td>Arbitrary</td>
</tr>
<tr>
<td></td>
<td>( \frac{d[Rac1]}{dt} = k_f [F\text{-}actin] \frac{1-[Rac1]}{1-[Rac1]+Kmf} - k_b [GAP] \frac{[Rac1]}{[Rac1]+Kmb} )</td>
<td>0.01</td>
<td>1.0</td>
<td>Obtained by fitting the data in Fig. 4D.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.01</td>
<td>0.001</td>
<td>These parameters were estimated to reproduce the data in Fig. 4.</td>
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</table>

PIP3 corresponds to PtdIns(3,4,5)P3. kf and kb are rate constants of forward and backward reaction, respectively. Kmf and Kmb are Michaelis constants of forward and backward reaction, respectively. Initial conditions of other parameters are as follows: \([PI3K] = 1.0 \) (constant), \([PTEN] = 1.0 \) (constant), \([GAP] = 1.0 \) (constant). PI3-K inhibition decreases 90% of the PI3-K level at time 0. Actin polymerization inhibition reduces the F-actin level to 0 at time 0.
### Table S3: Equations and parameters in Model B

<table>
<thead>
<tr>
<th>Reactions</th>
<th>kf (obtained by fitting the data in Fig. 4K)</th>
<th>Kmf</th>
<th>Kmb</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>d[PIP3]/dt = kf*[PI3K]<em>(1-[PIP3])/(1-[PIP3]+Kmf) - kb</em>[PTEN]*[PIP3]/([PIP3]+Kmb)</td>
<td>0.1</td>
<td>1.0</td>
<td>Arbitrary</td>
<td></td>
</tr>
<tr>
<td>d[F-actin]/dt = (kf1*[PIP3]+kf2*[Node])<em>(1-[F-actin])/(1-[F-actin]+Kmf) - kb</em>[F-actin]/([F-actin]+Kmb)</td>
<td>0.03</td>
<td>1.0</td>
<td>Obtained by fitting the data in Fig. 4K.</td>
<td></td>
</tr>
<tr>
<td>d[Rac1]/dt = kf*[F-actin]<em>(1-[Rac1])/(1-[Rac1]+Kmf) - kb</em>[GAP]*[Rac1]/([Rac1]+Kmb)</td>
<td>0.01</td>
<td>1.0</td>
<td>Arbitrary</td>
<td></td>
</tr>
<tr>
<td>d[Node]/dt = kf*(1-[Node])/(1-[Node]+Kmf) - kb*[Rac1]/([Node]+Kmb)</td>
<td>0.02</td>
<td>0.001</td>
<td>These parameters were estimated to reproduce the data in Fig. 4.</td>
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</tbody>
</table>

PiP3 corresponds to PtdIns(3,4,5)P3. kf and kb are rate constants of forward and backward reaction, respectively. Kmf and Kmb are Michaelis constants of forward and backward reaction, respectively. Initial conditions of other parameters are as follows: [PI3K] = 1.0 (constant), [PTEN] = 1.0 (constant), [GAP] = 1.0 (constant). PI3-K inhibition decreases 90% of the PI3-K level at time 0. Actin polymerization inhibition reduces the F-actin level to 0 at time 0.
Table S4: Equations and parameters in Model C

Model C

<table>
<thead>
<tr>
<th>Reactions</th>
<th>kf</th>
<th>Kmf</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>d[PI3K]/dt = kf*[PI3K]<em>(1-[PI3K])/(1-[PI3K]+Kmf) - kb</em>[PTEN]*[PI3K]/(PI3K+Kmb)</td>
<td>0.1</td>
<td>1.0</td>
<td>Arbitrary</td>
</tr>
<tr>
<td>d[F-actin]/dt = (kf1*[PI3K]+kf2*[Node])<em>(1-[F-actin])/(1-[F-actin]+Kmf) - kb</em>[F-actin]/(F-actin+Kmb)</td>
<td>kf1=0.03, kf2=0.01</td>
<td>1.0</td>
<td>Arbitrary</td>
</tr>
<tr>
<td>d[Rac1]/dt = kf*[F-actin]<em>(1-[Rac1])/(1-[Rac1]+Kmf) - kb</em>[GAP]*[Rac1]/(Rac1+Kmb)</td>
<td>0.01</td>
<td>1.0</td>
<td>Arbitrary</td>
</tr>
</tbody>
</table>
| d[Node]/dt = kf*[Node]/(1-[Node]+Kmf) - kb*[PI3K]*[Node]/([Node]+Kmb)      | 0.05 | 0.55| These parameters were estimated to reproduce the data in Fig. 4.  

PI3K corresponds to PtdIns(3,4,5)P3. kf and kb are rate constants of forward and backward reaction, respectively. Kmf and Kmb are Michaelis constants of forward and backward reaction, respectively. Initial conditions of other parameters are as follows: [PI3K] = 1.0 (constant), [PTEN] = 1.0 (constant), [GAP] = 1.0 (constant). PI3-K inhibition decreases 90% of the PI3-K level at time 0. Actin polymerization inhibition reduces the F-actin level to 0 at time 0.
### Table S5: Equations and parameters in Model D

<table>
<thead>
<tr>
<th>Model D</th>
<th>Reactions</th>
<th>kf</th>
<th>Kmf</th>
<th>kb</th>
<th>Kmb</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$d[\text{PIP3}] / dt = kf<a href="1-%5Ctext%7B%5BPIP3%5D%7D">\text{PI3K}</a>/(1-\text{[PIP3]}+Kmf)$</td>
<td>0.1</td>
<td>1.0</td>
<td>0.01</td>
<td>1.0</td>
<td>Arbitrary</td>
</tr>
<tr>
<td></td>
<td>\quad - kb*[\text{PTEN}][\text{PIP3}]/([\text{PIP3}]+Kmb)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Obtained by fitting the data in Fig. 4K.</td>
</tr>
<tr>
<td></td>
<td>$d[\text{F-actin}] / dt = kf<a href="1-%5Ctext%7B%5BF-actin%5D%7D">\text{PI3K}</a>/(1-\text{[F-actin]}+Kmf)$</td>
<td>0.05</td>
<td>1.0</td>
<td>0.05</td>
<td>1.0</td>
<td>Arbitrary</td>
</tr>
<tr>
<td></td>
<td>\quad - kb*[\text{GAP}][\text{F-actin}]/([\text{F-actin}]+Kmb)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Arbitrary</td>
</tr>
<tr>
<td></td>
<td>$d[\text{Rac1}] / dt = kf<a href="1-%5Ctext%7B%5BRac1%5D%7D">\text{F-actin}</a>/(1-\text{[Rac1]}+Kmf)$</td>
<td>0.01</td>
<td>1.0</td>
<td>0.01</td>
<td>1.0</td>
<td>Arbitrary</td>
</tr>
<tr>
<td></td>
<td>\quad - kb*[\text{GAP}][\text{Rac1}]/([\text{Rac1}]+Kmb)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Obtained by fitting the data in Fig. 4D.</td>
</tr>
<tr>
<td></td>
<td>$d[\text{Node}] / dt = kf<a href="1-%5Ctext%7B%5BNode%5D%7D">\text{PI3K}</a>/(1-\text{[Node]}+Kmf)$</td>
<td>0.05</td>
<td>0.003</td>
<td>0.05</td>
<td>0.53</td>
<td>These parameters were estimated to reproduce the data in Fig. 4.</td>
</tr>
<tr>
<td></td>
<td>\quad - kb*[\text{Node}]/([\text{Node}]+Kmb)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PIP3 corresponds to PtdIns(3,4,5)P3. kf and kb are rate constants of forward and backward reaction, respectively. Kmf and Kmb are Michaelis constants of forward and backward reaction, respectively. Initial conditions of other parameters are as follows: [PI3K] = 1.0 (constant), [PTEN] = 1.0 (constant), [GAP] = 1.0 (constant). PI3-K inhibition decreases 90% of the PI3-K level at time 0. Actin polymerization inhibition reduces the F-actin level to 0 at time 0.