Synthetic spatially graded Rac activation drives cell polarization and movement

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Migrating cells possess intracellular gradients of active Rho GTPases, which serve as central hubs in transducing signals from extracellular receptors to cytoskeletal and adhesive machinery. However, it is unknown whether shallow exogenously induced intracellular gradients of Rho GTPases are sufficient to drive cell polarity and motility. Here, we use microfluidic control to generate gradients of a small molecule and thereby directly induce linear gradients of active, endogenous Rac without activation of chemoattractant receptors. Gradients as low as 15% were sufficient not only to trigger cell migration up the chemical gradient but to induce both cell polarization and repolarization. Cellular response times were inversely proportional to the steepness of Rac inducer gradient in agreement with a mathematical model, suggesting a function for chemoattractant gradient amplification upstream of Rac. Increases in activated Rac levels beyond a well-defined threshold augmented polarization and decreased sensitivity to the imposed gradient. The threshold was governed by initial cell polarity and P3K activity, supporting a role for both in defining responsiveness to Rac activation. Our results reveal that Rac can serve as a starting point in defining cell polarity. Furthermore, our methodology may serve as a template to investigate processes regulated by intracellular signaling gradients.

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Directional motility is an intrinsic ability of many eukaryotic cells to migrate to predetermined locations in an efficient manner. Migration bias is tuned by the detection of various guidance cues that often form spatial gradients. The extracellular gradients of diffusing or surface-bound ligands can lead to spatially graded occupancy of extracellular receptor (1). The spatial asymmetry in receptor occupancy is subsequently translated into an intracellular gradient of polarity effectors that can modify cytoskeleton and lead to the development of an asymmetrical cell morphology with functionally distinct front and rear “compartments.” Remarkably, many cell types can accurately detect less than a 5% difference between ligand concentration at the cell front and back (1–3). This exquisite sensitivity suggests that intracellular amplification of extracellular cues may be necessary. Indeed, various groups have demonstrated the existence of local positive feedback loops (4–7) and mutual inhibition between different regulators (8, 9) as likely candidates for response amplification. However, recent studies have also shown that directional motility can still be achieved, albeit less efficiently, when molecules, once thought to be indispensable, involved in putative amplification mechanisms are removed (10, 11). Thus, the various functions of signaling components associated with directed migration still need to be resolved.

An attractive method for resolving the roles of signaling network components in both spatial cue sensing and directed cell motility is direct activation of these components in a spatially constrained and rapid manner, independent of initiation of upstream, receptor-level signaling. Using this principle, a variety of studies have used optical activation to identify the small Rho GTPase Rac (9, 12–16), collagen (17), thymosin B4 (18), and calcium (19) as key components that are sufficient to direct cellular motility. However, an important caveat to these studies has been the reliance on highly localized activation that can create artificial regional amplification of target protein activity. In contrast, in more physiological settings, a cell processes a shallow gradient of an external cue into a graded intracellular response, as reflected in polarized effectors (20), including those of the small Rho GTPase family (21–23). Thus, it remains unclear if an induced shallow gradient of an active motility signaling component is capable of reconstituting cell polarization and motility. In particular, it is unknown if such perturbations are sufficient to override or enhance endogenous intracellular signaling of the same component. Finally, localized activation of signaling processes presents considerable challenges to quantitative analysis and coupling to detailed computational models developed to describe more natural, spatially distributed signaling events.

To address these questions, we created microfluidic devices permitting generation of precise gradients of extracellular cues (24) and interfaced them with a rapamycin-induced dimerization system (25). In this system, the addition of rapamycin leads to dimerization of two intracellularly transduced molecular components, FK506 binding protein (FKBP) and the rapamycin binding domain of FKBP-rapamycin binding protein (FRB) (26). Localization and signaling motifs can be linked to either domain, allowing spatial-temporal control of protein function. We used our combined system to study the effects of a rapidly induced intracellular gradient of activated Rac, which is an important regulator of cell polarity (27) and has been shown previously to induce migration when locally activated (9, 12–16).

Results

System Design. To activate endogenous Rac directly, we introduced two constructs into HeLa cells: a cytoplasm-localized effector unit consisting of YFP-tagged TIAM1, a Rac guanine nucleotide exchange factor (GEF) conjugated to FKBP (YF-TIAM1) and an anchor unit at the cell membrane, Lyn11–FRB (LDR). The introduction of rapamycin dimerizes these modified molecular components, thereby bringing TIAM1 in close proximity to the cell membrane, where it activates endogenous Rac (25) (Fig. L4). Due to the chemical nature of the activation, this system is amenable to generation of a gradient of Rac activity through microfluidic production of rapamycin gradients. Microfluidic tools have recently been used for the control of complex gradients of extracellular cues

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Fig. 1. Graded activation of Rac directs cellular polarity. (A) Schematic of the mechanism of Rac activation by rapamycin-induced heterodimerization. (B) Microfluidic device used to generate linear gradients of rapamycin with a sample image of the microchannels seeded with individual HeLa cells and the corresponding gradient visualized with Alexa 594 dye. Ports are labeled according to function. The red layer of the device is the fluid flow layer, and the green layer is the control valve layer. Alexa 594 dye is used to visualize the gradient (red) in all subsequent images. A sample image of a cell transfected with the Rac activator, YF-TIAM1, experiencing a gradient of rapamycin is shown below. (C–F) Four polarity states observed after the attachment period with respect to the direction of the imposed rapamycin gradient and associated polarization responses to the gradient of rapamycin. Images are rotated by 90° to aid in visualization. Cartoons illustrate the polarity of the associated state and the direction of the gradient. The green color indicates expression of YF-TIAM1. Yellow arrowheads denote the initial direction of polarity. The rapamycin gradient is shown in the 5-min image and removed in subsequent images for clarity. Red dotted lines highlight evolving changes in cell morphology. Times are given in minutes. (Scale bars, 10 μm) (G–J) Kymographs taken across cell centers (specified in accompanying image) illustrate the morphological changes of the corresponding cells in C–F over the experimental period. Blue lines trace initially polarized faces, and red lines trace initially unpolarized faces. Yellow arrowheads denote the initial response time, and their location indicates which cell face was the first to change in the gradient. White arrowheads indicate the late polarization time. Times are given in minutes.

We applied a previously developed strategy for imposing diffusion-based linear gradients onto cells housed within narrow channels (24, 28, 29). Specifically, the devices contained a series of 6-μm tall microchannels for cell experimentation, flanked by 130-μm tall main flow-through channels (device operation is described in SI Appendix, Fig. S1A and Experimental Setup). Activation of flow led to the development of a linear gradient across the shallow channels due to uneven stimulus concentration in the flow-through channels (Fig. 1B). The microchannels were designed to be on the order of a cell diameter to relegate cells to a uniaxial phenotype (Fig. 1B). HeLa cells introduced into the microchannels settled into random locations, and after a 3- to 4-h attachment period, they were categorized into different polarity states according to the existence of a lamellipodium (state I, no lamellipodium) and its direction (state II, lamellipodium toward the high side of the gradient; state III, lamellipodium toward the low side of the gradient; and state IV, lamellipodia toward both sides of the gradient) (Fig. 1 C–F). The overall distribution of phenotypes skewed toward cells with a single leading edge lamellipodium, indicating an intrinsic preference for directed motility (SI Appendix, Fig. S1B).

Direct Generation of Active Rac Gradients. To evaluate HeLa cell responses to synthetic generation of an intracellular gradient of active Rac, we exposed cells to a linear gradient of rapamycin (0–2 nM across the channel or 0.01 nM/μm, yielding front/back concentration differences ranging from ∼15–92% across varied cell lengths) visualized by Alexa 594, a fluorescent dye with a similar molecular weight (Fig. 1B and SI Appendix, Fig. S1C). We chose this concentration range to avoid saturating the FKBP-FRB system with rapamycin [K_D = 12 nM (34)]. One consequence of using these concentration values was a slow-down of the response times compared with previously reported values for saturating uniform rapamycin inputs (25). However, the slower response times allowed a better resolution of the effects of gradually accumulating Rac activity and were far below those reported to affect the function of the mammalian target of rapamycin (35). As a control, we compared basal cell motility with and without rapamycin and found that the addition of rapamycin had negligible effects (SI Appendix, Fig. S1D and E). We tracked both the initial state and subsequent cell responses by imaging over a 4-h time period.

Strikingly, we found that the shallow linear gradient of rapamycin could direct motility of cells in all initial polarity states in the direction up the gradient (Fig. 1 C–F and Movie S1). Unpolarized cells (state I) and bipolar cells (state IV) demonstrated symmetry, breaking with either the establishment of a leading
edge or the enhancement of one lamellipodium and retraction of the other, respectively (Fig. 1 C and F and Movie S1). Cells already polarized in the direction of the gradient (state II) exhibited widening and extension of the leading lamellipodium and movement up the gradient (Fig. 1D and Movie S1). Interestingly, cells initially oriented in the direction opposite of the gradient (state III) repro- larized (Fig. 1E and Movie S1). In all states, the initiation of migra- tion was followed by a pronounced enhancement of cell polarity (Fig. 1 G–J). The directed cell polarization and migration responses were not observed in untransfected cells (SI Appendix, Fig. S1F) or if the rapamycin gradient was not imposed (SI Appendix, Fig. S1G).

For validation of our system, we applied linear gradients of rapa- mycin to similarly transfected MTLn3 cells, a rat mammary ade- nocarcinoma line used to assay chemotaxis to EGF in vitro (36) and in vivo (37) (SI Appendix, Fig. S2). MTLn3 cells exhibited initial polarity states similar to those seen in HeLa cells and polarized toward gradients of rapamycin (SI Appendix, Fig. S2). Our data suggest that our system is applicable across multiple cell types and can be used to study signaling pathways regulating chemotaxis.

To verify that the externally imposed rapamycin gradient was translating into a graded change in active Rac across cells, we car- ried out two control experiments. First, we sought to confirm that the Rac activator, YF-TIAM1, translocated to the membrane in a graded fashion over time. Our results above indicated that translocation of YF-TIAM1 caused reeling and substantial changes in cell morphology that obscure translocation; therefore, we used a YFP-tagged FKBP (YF) without TIAM1 to assess translocation. YF was previously shown to translocate with similar kinetics to YF- TIAM1 (25), and therefore could serve as a suitable proxy. We tracked the membrane-to-cytosol ratio of fluorescence intensities across the length of cells in a gradient of rapamycin over time and found that membrane translocation of YF was increasing significantly in a graded manner, with higher translocation toward the high side of the gradient (SI Appendix, Fig. S3 A–F and Movie S2). The membrane-to-cytosol ratio curve increased over time for all states but retained similar slopes (SI Appendix, Fig. S3 D–F). We did not observe a similar response when DMSO was substituted for rapamycin (SI Appendix, Fig. S3 G and H). In both conditions, we did not observe any significant morphological changes, in-dicating that translocation of FKBP constructs without effectors does not perturb morphology. Having verified that the rapamycin gradient induced graded membrane translocation of FKBP con- structs, we used a Raichu Rac FRET sensor (38) to monitor the resulting changes in Rac activity. In these experiments, we used a mCherry-tagged FKBP-TIAM1 (MCHF-TIAM1) to activate Rac. At a basal level before stimulation, state I cells did not show po- larized Rac activity (SI Appendix, Fig. S4A), whereas state II and state III cells exhibited higher levels of active Rac at lamellipodia (SI Appendix, Fig. S4 B and C). On introduction of the rapamycin gradient, we observed increases in Rac activity in the direction of the gradient in all cells, whereas state III cells exhibited an additional decrease in Rac activity in the original lamellipodium (SI Appendix, Fig. S4 A–C and Movie S3). We quantified the average Rac activity across cells over time and found that there was a gradual and spatially graded increase in Rac activity in all cell states (SI Appendix, Fig. S4 D–F). In particular, state III cells exhibited a sharp increase in Rac activity in the newly formed protrusion and a decrease in the initial opposite-facing protrusion (SI Appendix, Fig. S4F). As a control, we quantified Rac activity across cells that did not express the Rac activator, MCHF-TIAM1, but had expression of the Rac FRET sensor. Under these conditions, we did not observe any sign-ificant changes in Rac activity over time in gradients of rapamycin (SI Appendix, Fig. S4 G and H). Overall, our results suggested that an exogenously applied linear gradient of rapamycin could result in a graded increase in Rac activity, which was sufficient to direct motility and polarization of cells from a variety of preexisting polarity phenotypes.

For further validation of our system, we compared cellular responses to graded rapamycin with responses to uniform rapa- mycin stimulation. Cells given a uniform stimulus for the entire experimental period displayed extensive uniform flattening with little net motility (SI Appendix, Fig. S5A). This result was in agreement with previous experiments showing that differentiated HL-60 cells exposed to spatially uniform Rac stimulation dis- played membrane ruffling around the entire cell periphery (6). For a more detailed comparison of the effects of graded and uniform Rac activation, we exposed state II cells to a rapamycin gradient (0.01 nM/μm) for 2 h, subsequently followed by uniform stimulation (2 nM rapamycin) for 3 h thereafter. As expected, cells polarized and moved in a biased fashion during gradient stimulation; however, on the switch to uniform stimulation, cells started forming protrusions at the rear and showed a decrease in the length of the front (SI Appendix, Fig. S5 B–D and Movie S4). This effect indicated the importance of persistent gradient input but might have also reflected a gradual saturation of rapamycin binding sites after prolonged treatment. We explored the latter possibility by running the converse experiment, exposing cells to spatially uniform rapamycin (2 nM) for 2 h followed by a rapamycin gradient (0.01 nM/μm) for 3 h (SI Appendix, Fig. S5E). Protrusions developed on both sides of cells during uniform stimulation, and subsequently could not be biased by the gradient (SI Appendix, Fig. S5E). However, cells exposed to a lower uniform stimulation (1 nM) were able to polarize toward the ensuing rapamycin gradient input (0.01 nM/μm) (SI Appendix, Fig. S5F). In combination, these results indicate that cell polarity can be acutely sensitive to changes in rapamycin gradients as long as the rapamycin exposure does not exceed a threshold level. Below, we explore the effects of the rapamycin dose and exposure time on cell responses in detail. These results suggest that the spatial restriction of Rac activity within a cell is important for maintaining polarization.

Mathematical Modeling of Graded Rac Inputs. To better understand cell responses to the exogenously imposed graded Rac activation, we developed a mathematical model of cell polarity. The model is based on a simple scheme of Rac-RhoA-Cdc42 small GTPase interactions, expanding on earlier modeling studies (39, 40) (modeling details are provided in SI Appendix, Model Supplement). The model made three important qualitative predictions: A spatially graded Rac activation can polarize Rac activity across the cell (Fig. 2 A and B); the timing of that initial Rac polarization is strongly dependent on the input gradient and weakly dependent on the average input (Fig. 2 B and C); and an-tagonism between the activities of Rac and Rho small GTPases can trigger a phase transition-like change to a substantially more asymmetrical polarization, which can be stably maintained as long as the activity of Rac remains high enough (Fig. 2D). This transition is addressed in more detail below. Model details are provided in SI Appendix, Model Supplement. Our experimental setup allowed di- rect examination of these predictions.

The Effect of Rac Gradient Steepness on Timing of Celluar Responses. To validate the model predictions, we next examined the correlates of the initiation of directed cell migration and their dependencies on the local gradient and average value of rapamycin input. HeLa cell responses were characterized by either the retraction of a leading lamellipodium in cells polarized in the direction opposite of that of the gradient (state III) or the extension of a preexisting (state II) or new (state I) lamellipodium in the direction of the gradient (Fig. 2 C–F). To evaluate these effects quantitatively, we examined the width and length of the front and rear sides of cells in all states through the entire stimulation period, along with the respective concentrations experienced at each side (SI Appendix, Fig. S6). Cells that exhibited bipolar phenotypes (state IV) were relatively rare (Fig. 1F and SI Appendix, Fig. S1B), and therefore were excluded from subsequent analysis. Our analysis revealed that
these initial directed migration response times, in agreement with the model predictions, were indeed inversely dependent on the steepness of the rapamycin gradient across each cell (Fig. 2F). This trend was seen across all polarity states (Spearman correlation coefficients: −0.679, −0.655, and −0.583 for states I, II, and III, respectively). Similar dependencies were also observed in MTLn3 cells (SI Appendix, Fig. S7A–C). The initial response times of state I cells also showed a discernible but much weaker dependency on the average rapamycin concentration, consistent with the model predictions, whereas cells in states II and III had relatively weaker dependencies (SI Appendix, Fig. S8A–C) (Spearman correlation coefficients: −0.582, −0.478, and −0.377 for states I, II, and III, respectively). State II cells had consistently shorter initial response times for any given gradient steepness compared with those in states I and III (Fig. 2F and SI Appendix, Fig. S9) (F test: state II vs. state I, P < 0.0001; state II vs. state III, P < 0.0001), whereas state I and III cells behaved similarly (state I vs. state III, P = 0.54). For example, when comparing the average initial response times at a gradient of 0.01 nM/μm ± 0.001, state II cells had an average initial response time of 51 min, whereas state I cells had an average initial response time of 98 min and state III cells had an average initial response time of 71 min. These results suggest that cells are able to make migration decisions rapidly in the presence of Rac activity gradients overlapping with their initial polarization state. Together, our data demonstrate that the magnitude of active Rac gradients can influence the timing of the onset of directed cell motility.

In addition to the initiation of biased cell migration, spatially graded Rac activation eventually triggered a striking enhancement in cell polarization, with a substantial enlargement of the directed leading lamellipodium (Fig. 3A). This unexpected morphological change was consistent with the model
prediction that crossing a threshold of Rac activity triggers a rapid, strong, and stable polarization, akin to a phase transition. According to the model, a gradual variation of Rac GEF levels causes an abrupt transition from a moderately polarized to highly polarized state, expressed mathematically as a bifurcation in the model response (Fig. 3B). We hypothesized that the use of low rapamycin concentrations allowed us to observe this effect by gradually titrating intracellular Rac GEF and Rac activity levels, ultimately leading to this strong polarization. The gradual stimulus-induced Rac GEF build-up was expected to follow a simple mathematical representation of accumulation of Rac GEF concentration over the duration, $t$, of exposure to rapamycin:

$$\Delta \text{[Rac GEF]} = k \cdot \text{[Rapamycin]} t$$  \hspace{1cm} [1]$$

where $k$ is the constant defining tripartite rapamycin–FKBP–FRB complex formation. This expression allowed us to test the hypothesized existence of a Rac activity threshold mediating enhanced polarization.
One of the immediate consequences of the expansion of the lamellipodium in the direction of the rapamycin gradient was an apparent dimming of the YFP fluorescence signal from the cell body when observed with a wide-field epifluorescence microscope (Fig. 3 A and C). The dimming of the fluorescence intensity was likely due to a redistribution of cytoplasmic volume from the cell body to the expanding lamellipodium and an increase in the translocation of YF-TIAM1 complexes to the membrane (Fig. 3 D and E). We thus used the fluorescence intensity of the cell body as a metric for the timing of late polarization. Our analysis indicated that the late polarization time exhibited a linear dependency on the average local rapamycin concentration (Fig. 3F) (Pearson correlation coefficients: −0.608, −0.783, and −0.698 for states I, II, and III, respectively), in agreement with model predictions. MTLn3 cells also showed similar dependencies on mean rapamycin concentrations (SI Appendix, Fig. S7 D–F). A weaker dependence on the sharpness of the rapamycin gradient, consistent with the model, was also detected in these experiments (SI Appendix, Fig. S8 D–F) (Pearson correlation coefficients: 0.103, −0.511, and −0.228 for states I, II, and III, respectively). As with observations of early cell responses, state II cells reached late polarization significantly faster than cells in other states for a given rapamycin concentration (Fig. 3F) and SI Appendix, Fig. S10) [analysis of covariance (ANCOVA) test of intercept: state II vs. state I, P < 0.0001; state II vs. state III, P < 0.0001], whereas the difference between the other two states was negligible (state I vs. state III, P = 0.13). We also found that a subpopulation of state III cells exposed to lower concentrations of rapamycin completely failed to reach late polarization (Fig. 3F). In combination, these results suggested the existence of a threshold for late polarization, which is variable across individual cells and dependent on the initial polarity state.

Transient Graded Rac Activation. Eq. 1 suggests that the duration of rapamycin exposure is as critical as the local rapamycin concentration in exceeding the polarization threshold. Based on in vitro estimates of rapamycin–FKBP–FRB complex formation, the characteristic binding time estimated for 1 nM rapamycin is in the range of tens of minutes (34). To test this prediction, we varied the time interval of rapamycin stimulation, taking into account the earliest polarization time observed for the rapamycin concentrations tested (i.e., 30 min) and the estimated equilibration time above. We exposed cells to transient rapamycin gradients of 0.01 nM/μm for 30 min or 1 h, followed by perfusion of the devices with rapamycin-free media for the rest of the experiment. We found no cells undergoing late polarization after a 30 min rapamycin gradient exposure (Fig. 4A–C and Movie S5); however, after a 1-h stimulation, we found subsets of cells in all polarity states able to undergo late polarization provided that they were exposed to sufficiently high concentrations of rapamycin (Fig. 4 D–F and Movie S6). The polarization responses were morphologically similar to those seen earlier during continuous stimulation (Fig. 4 D and E and Movie S6), with the timing to late polarization indistinguishable from that observed for continuous stimulation (Fig. 4F). Responding cells continued to polarize even after the stimulus was withdrawn, suggesting fixation of the induced polarity and migration states (Fig. 4D–E and Movie S6) in contrast to their loss during the transition to spatially homogeneous Rac activation (SI Appendix, Fig. S5A). This behavior was in agreement with model predictions of a stable polarization beyond a Rac activity threshold, which enables cells to maintain a strongly polarized state whose direction is based on but not continuously informed by the input gradient. Using a classification algorithm to separate responding and nonresponding cells, we found that the minimum concentrations needed to elicit a response varied across the polarity states (Fig. 4F). In agreement with the earlier observation of distinct responses in state II cells, cells in this state possessed the lowest response threshold (Fig. 4F) (state I = 1.5 nM, state II = 1.1 nM, and state III = 1.4 nM). These data further support the existence of a well-defined initial polarity-dependent Rac activation threshold essential for the rapid and profound induced changes in polarized cell morphology.

Inhibition of Upstream Activators. Direct activation of Rac allows bypassing of many signaling species commonly thought to be either upstream of Rac or involved in a regulatory feedback with this molecule. A well-studied example of such a molecule is phosphatidylinositol 3,4,5-triphosphate (PIP₃) (6, 41, 42). In chemotactic gradients, PI3K is recruited to the plasma membrane and phosphorylates the abundant phosphatidylinositol 4,5-bisphosphate to yield PIP₃ (2, 43). Due to spatial regulation of PI3K recruitment, PIP₃ is often enriched at the front areas of migrating cells (43), displaying an intracellular gradient that is sharper than the gradient of the extracellular chemoattractant (2, 44). It is thought that PI3K can influence cell guidance through its interaction with small GTPases and actin, but the mechanism of these interactions and the resultant role of PI3K in regulating chemotaxis are still under investigation (10, 11). If, as sometimes assumed, PI3K is upstream of Rac activation in chemotactic signaling systems, its perturbations are not expected to lead to alteration of cell responses to rapamycin-based Rac GEF stimulation. If, on the other hand, PI3K forms a feedback loop with Rac (4, 6, 13) or otherwise enables Rac-mediated outputs, cell responses might be affected by its inhibition, with the change in cell behavior potentially suggesting the mechanism of PI3K regulatory involvement. We used a pharmacological inhibitor of PI3K, LY294002, to inhibit PI3K activity during stimulation with a gradient of rapamycin. As a control, we first observed the effects of LY294002 with and without rapamycin on basal cell motility and found no significant effect (SI Appendix, Fig. S1 D and E). After induction of the graded stimulus, in contrast to the responses of cells in which PI3K was not perturbed, we observed large subsets of cells exhibiting no response, both in terms of the initial and late polarization, for all three initial polarity states across various gradients and concentrations (Fig. 5 A–F, SI Appendix, Fig. S11, and Movie S7). Interestingly, the cells that did undergo the initial migration and late polarization responses did so with the same kinetics as observed in the absence of PI3K perturbation (Fig. 5A). This was consistent with the model prediction that a decrease in simulated strength of the PI3K-mediated feedback to Rac could lead to an increased threshold for cell responsiveness, requiring a sharper effective internal Rac activity gradient for cells to respond (Fig. 5B and C). As a consequence, the stochastic differences in internal states of the cells, defining cell sensitivity to the graded signaling input, can lead to a greater degree of cell population separation into responding and nonresponding cells, without affecting the timing of responses in responding cells.

The results in Fig. 3F suggested the existence of a relatively high (re)polarization threshold for state III cells. Thus, we explored whether there would be synergy between this threshold and the increase in polarization threshold caused by inhibition of PI3K. We found that this threshold was indeed shifted in the presence of PI3K inhibition to a higher rapamycin concentration level (Fig. 5D) (1.0 nM for untreated state III vs. 1.4 nM for LY294002-treated state III). These results thus support the notion that PI3K can serve to sensitize cells to spatially graded Rac activation, allowing them to exceed polarization and repolarization thresholds more readily.

Discussion The results presented in this report argue that directly induced, spatially graded membrane translocation of a Rac activator, TIAM1, can trigger unambiguous polarization and directed movement of cells aligned with the direction of the stimulation gradient. TIAM1 is a specific Rac GEF (45), and another key regulator of polarity, CDC42, is considered upstream of Rac (46); therefore, we attribute our observed phenotypes to be originating from di-
Rect Rac activation. The gradients of the inducer of Rac activation, the exogenously added rapamycin, can be effective with values as low as 15% across the cell length, with the rates of cellular responses to the stimulation being defined by the gradient steepness. The results suggest that even mild initial Rac GEF gradients can trigger strongly polarized cell responses, potentially providing insights into the levels at which graded inputs can be amplified in the signaling network. However, the response kinetics can be enhanced with sharper input gradients (Fig. 2F) or if these gradients are amplified upstream of Rac activation.

We propose that rapamycin-induced graded Rac activation can induce qualitatively similar polarization responses to those seen in chemoattractant gradients. For example, state I cells exhibit initially low, mostly homogeneous Rac activity, followed by the induction of high Rac activity at a newly formed front when a gradient of rapamycin is applied. This pattern of Rac activity is similar to that seen in neutrophils polarizing to a gradient of f-Met-Leu-Phe (47). Additionally, state III cells repolarize when given sufficiently high gradients of rapamycin by forming a new front at the rear and retracting the previous front. This behavior is seen when chemoattractant gradients are presented at the rear of polarized cells through microfluidics or a micropipette. Chemoattractant-induced repolarization can be seen in neutrophils (48), social amoebae (49), and breast cancer cells (36). Given that rapa-
mycin-induced graded Rac activation can mimic polarization behaviors seen with chemotactic gradients, we believe that polarization can be defined at the level of Rac, or at least starting from the level of Rac.

The research platform described here enabled the screening of the effects of a slow variation in the total cellular Rac activity. Both a simple model describing a feedback-based interplay between small GTPases in a cell and the corresponding experimental observations support the unique finding that a rapid and pronounced transition to a much stronger degree of polarization can occur if Rac activity exceeds a threshold level. This threshold was found to be strongly affected by the initial polarization status of the responding cell. Cells initially polarized in the direction of the applied gradient have lower response thresholds, on average, than cells that are unpolarized or polarized in the opposite direction. Moreover, only a fraction of cells initially polarized away from the gradient responded to the gradient of Rac activator. These results are consistent with the following view supported by the model: An existing endogenous gradient of Rac activity in state II cells would lead to a smaller difference between the maximum local initial Rac activity within a cell and the polarization threshold value.

Our results further suggest that signal processing upstream of Rac activation in the context of chemotactant stimulation may limit the degree of total Rac activation, and thus the ability of the cell to reach the threshold controlling the transition into the strongly polarized state. Thus, a single ligand may not induce such a transition. However, the threshold might potentially be reached if multiple inputs converging on Rac activation, which may be affected by cell type-specific peculiarities of the signaling apparatus, such as basal levels of Rac activation and the expression of the signaling proteins.

The rapamycin stimulation system described here also allows a more detailed study of the interplay between Rac activation and activity of other signaling species, including those that might be involved in various feedback interactions. This analysis is akin to the more common epistasis assays, but with subtler phenotypes related more closely to gradient sensing responses. In particular, our analysis suggested that PI3K interplay with Rac activation, although consistent with the recently proposed formation of an AND gate in terms of the response (6), in which both inputs are necessary to induce directed migration, acts more specifically by controlling the threshold of cell responsiveness to Rac activity gradients. Whereas PI3K inhibition does not prevent the ability of the cells to undergo directed cell polarization or migration responses, it can strongly reduce the fraction of cells capable of these responses within the same set of experimental conditions.

The analysis here represents a more general framework extendable to other rapamycin-activatable signaling molecules (25, 50, 51), as well as to other cell types and multicellular systems. Furthermore, the effects of gradients of other proteins engineered to be sensitive to small membrane-permeable molecules, such as ATP analogs (52) and imidazole (53), could also be analyzed to refine our understanding of the mechanisms of cell responses to graded intracellular signaling activity. As also demonstrated in this report, such efforts could help develop qualitatively and quantitatively improved mathematical and computational models of gradient sensing and chemotaxis phenomena, extending common approaches to these processes. We suggest that as the repertoire of methods for direct control of cellular events increases, microfluidics-based tools will play an important role in exploitation of these methods in cell navigation research.
Cell Culture and Transfection. HeLa cells were maintained in DMEM with 10% (vol/vol) FBS and 1% penicillin streptomycin (Gibco). MTLn3 cells were cultured in α-minimum essential medium supplemented with 5% (vol/vol) FBS and 1% penicillin streptomycin (Gibco). Both cell lines were kept in a 37 °C and 5% CO2 environment during culture and in experiments. MTLn3 cells were kindly provided by the laboratory of Jeffrey Segall (New York City, NY). The constructs, YF-TIAM1, YF, and LDR, were transfected into cells using Fugene HD (Roche) per the manufacturer’s recommendations. The Raichu Rac FRET probe was kindly provided by the laboratory of Michiyuki Matsuda (Kyoto, Japan) and was transfected in a similar manner. During FRET experiments, MF-TIAM1 was used in place of YF-TIAM1. MF-Tiam1 has Rac FRET probe was kindly provided by the laboratory of Michiyuki Matsuda (Kyoto, Japan) and was transfected in a similar manner. During FRET experiments, MF-TIAM1 was used in place of YF-TIAM1. MF-Tiam1 has mCherry fluorescence in place of YFP and was used to avoid spectral overlap with the FRET probe.

Imaging. Microfluidic experimental imaging was performed using an inverted Zeiss Axiovert 200M epifluorescence microscope at 37 °C and 5% CO2, coupled to a Cascade II:1024 EMCCD camera (Photometrics) using a 40x, 1.3-N.A. oil immersion objective (Zeiss). The microscope was driven by Slidebook software (Intelligent Imaging Innovations). Images were taken every 5 min in the YFP channel using a 494-nm excitation filter and 530-emission filter (Semrock), and Alexa 594 dye was imaged using a 572-nm excitation filter and 628-emission filter (Semrock) over a 4-h period. A spectral 2D template autofocus algorithm was used between images to account for any focus fluctuations. To correct for uneven illumination, all images were normalized with the following correction C = (I − I0)/(F − D) − M, where C is the corrected image, I is the original image, D is the darkfield image, F is the flatfield image, and M is the mean of difference between flatfield and darkfield images. The flatfield and darkfield images were taken as averages of multiple images. FRET images were taken using a cyan fluorescent protein (CFP) excitation filter (Semrock), an appropriate dichroic filter (Semrock), and YFP/CFP emission filters (Semrock). Volume analysis was performed on an inverted Zeiss Axiovert 200 spinning disk confocal microscope, coupled to a CCD camera (Hamamatsu) using a 40x objective (Zeiss). The microscope was driven by Metamorph 7.5 imaging software (Molecular Devices). YFP excitation was trigged with an argon laser (CVI Melles Griot) which was fiber-coupled (OZ Optics) to the spinning disk confocal unit (CSU10; Yokogawa) mounted with a YFP dichroic mirror (Semrock) and an appropriate YFP filter (Chroma Technology).

Analysis of Gradient and Mean Values of Rapamycin. All analyses were performed using custom-written codes in MATLAB 2007b (MathWorks). Cell-based data (e.g., cell length, centroid) were obtained from the YFP images, whereas gradient-based data were obtained from the Alexa 594 dye images. Cells were segmented from the YFP channel based on intensity. The signal-to-noise ratio was sufficiently high to preclude the use of more sophisticated segmentation techniques. Once cell boundaries were determined from the segmentation, concentration lines spanning the width of the channel were generated from the front and back of the cell to obtain local concentration data. The concentration lines were restricted to the width and length of an individual channel by manually selecting the boundaries of the channel from the Alexa 594 dye image. For any cell that extended out of the channel, the concentration lines were restricted to the rostrum ends of the channel to avoid spurious measurements associated with the height difference between the arms of the flow channels and the cross-channels. Dye intensities were extrapolated to the intensities at the respective ends of the channel to determine concentration.

Cell Tracking. Cell velocity was tracked by obtaining the coordinates of cell nuclei using a semiautomated script written in MATLAB.

Quantification of the Membrane Distribution of YFP-FKBP. The translocation of YFP-FKBP to the membrane was quantified by taking the ratio of intensities at the cell periphery compared with the intensity in the cytoplasm. First, the cytoplasm and membrane were segmented by intensity thresholding from cell images, and a subsequent morphological erosion was used to obtain each component separately. To obtain the membrane intensity across the cell length, the maximum value of each row of pixels in the membrane segmentation was taken. The cytoplasm intensity was obtained as the mean value of each row of pixels from the cytoplasm segmentation. Ratio values across the length of the cell were normalized between 0 and 1 and smoothed with a 10-point moving average. To aggregate data from multiple cells, time 0 was chosen as 30 min before visible translocation and subsequent profiles were normalized to values at time 0. For control cells, time 0 was chosen at times comparable to cells positioned in similar locations during rapamycin experiments.

FRET Analysis. FRET analysis was done according to a previously described protocol (56). Briefly, FRET images were analyzed by first subtracting background from each individual CFP and YFP FRET image (CFP excitation, YFP emission). Images were thresholded and subsequently aligned with a discrete Fourier transform (DFT) registration algorithm (57). The FRET ratio was calculated by dividing the YFP FRET image by the registered CFP image pixel by pixel. Final images were Gaussian-filtered to reduce noise. To obtain the distribution of FRET activity across the cell length, the mean value of each row of pixels composing the ratio image was taken and final values were normalized between 0 and 1 for the cell length and smoothed with a 10-point moving average. To aggregate data from multiple cells, time 0 was chosen as 30 min before the appearance of morphological changes in accompanying MCHF-TIAM1 images. FRET profile data at later time points were normalized to time 0. For control cells, time 0 was chosen based on MCHF-TIAM1 images from comparably located stimulated cells.

Volume Analysis. Cell heights were determined by first taking confocal z slices of cells before and after rapamycin treatment. Afterward, the cell body was segmented and superimposed to obtain the final image. The cell volume was determined by taking a region of interest (ROI) in the cell body from 3D reconstructions carried out using MATLAB. The number of pixels in the ROI was then converted to micrometers based on precalibration of the slice height.

Measurement of Initial Response Time and Late Polarization Time. All measurements were performed using MATLAB. To determine the length of a cell’s front and back, the cell nucleus was tracked by manually fitting an ellipse to the nucleus image and taking the centroid of the fitted ellipse as the position of the nucleus. Nuclei were clearly distinguishable in all YFP images and used to exclude nonresponder cells. Cell front and rear locations were determined by taking the coordinates of the front and rear of cells from segmented images and calculating the distance to the nucleus position (SI Appendix, Fig. S6). To assay the width of the front and rear of each cell, the distance between the rightmost and leftmost coordinates of the cell front (top 10% of pixels) and rear (bottom 10% of pixels) was calculated (SI Appendix, Fig. S6). The initial response time was taken as the time to reach 20% of the total magnitude of the first morphological change toward the gradient (SI Appendix, Fig. S9). The initial response time was taken at the 50% level for MTLn3 cells due to the faster kinetics associated with their responses. To find the late polarization time, the decrease in fluorescence of the cell body was measured as a function of time. The fluorescence intensity was determined in ROIs, chosen automatically based on the end coordinates of the cell (front and back) (SI Appendix, Fig. S10). The ROIs were then further eroded by several pixels to avoid any effects from the cell membrane. The late polarization time was taken as the time to reach 50% of the full fluorescence intensity drop in the ROI from the peak intensity value.

Population Separation. Thresholds between nonresponding cells and responding cells were determined using quadratic discriminant analysis (“classify” function) in MATLAB. The two populations, along with their corresponding concentrations, were input into the function as training data, and a separation point was generated from a given vector of concentrations.

Statistical Analysis. Statistical analysis was carried out with SigmaPlot (Systat) and Prism (GraphPad) software. Experimental results were expressed as means with error bars equal to SEM. Comparisons between two groups were linearly fit with a 95% confidence interval.
carried out using an ANCOVA test. First, the difference between slopes was compared. The difference was insignificant, a comparison between the y-axis intercepts was performed. To compare fitted curves, an F test was conducted using the standard procedure.

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**Fig. S1 Characterization of the microfluidic device and motility controls.**

(a) The schematic of the microfluidic device used in experiments. The layer labeled in blue is the control layer containing fluidically actuated valves used to control the separate fluidic layer below, labeled in red. The green layer indicates the region where the microchannels are housed and where cells are seeded. (b) Quantification of the fractions of HeLa cell polarity phenotypes observed after a four hour attachment period. Data are presented as the mean from n = 8 experiments, with error bars representing the standard error of the mean (SEM). (c) Quantification of the gradients of Alexa 594 dye in the microchannels generated to visualize the distribution of rapamycin, with colored lines quantifying fluorescence intensity to demonstrate the linearity of the gradient profiles and consistency of the gradients in multiple adjacent channels. (d) Images of HeLa cells on fibronectin coated glass before and after the addition of indicated chemicals. Times are in minutes. Scales bars, 50 µm. (e) Average velocities of HeLa cells in various treatment conditions. There is no significant difference between conditions. Number of samples are indicated. (f-g) Representative examples of untransfected HeLa cells experiencing a gradient of the dye as in (c), with added rapamycin (e) and without added rapamycin (f). The cells show limited morphological changes through a four hour period. Time values are in minutes. Scale bars, 10 µm.
**Fig. S2 MTLn3 cell responses to rapamycin gradients.** (a-d) MTLn3 cells in various initial polarity states, similar to those seen in HeLa cells, become polarized towards gradients of rapamycin. The green color indicates expression of YF-TIAM1, while the red color indicates the rapamycin gradient. Yellow arrows indicate initial protrusions. Times are in minutes. Scale bars, 10 µm.
**Fig. S3** Quantification of graded membrane translocation of FKBP constructs. (a-c) HeLa cells in states I-III transfected with YFP-FKBP exposed to a gradient of rapamycin from top to bottom. Time 0 is chosen as 30 minutes before translocation. Cell length plot indicates axis of quantification in plots (d-f). Yellow arrows indicate initial protrusions. Times are in minutes. Scale bars, 10 µm. (d-f) Quantification of the average membrane to cytoplasm intensity ratio across state I-III cells over time. Ratios after time 0 are normalized to the membrane to cytoplasm fluorescence intensity ratio at time = 0 (shown in black). Thinner colored lines indicate S.E.M of corresponding ratio profiles. Numbers of samples are indicted. (g) Control cells exposed to a gradient of DMSO instead of rapamycin do not show translocation. Cell length plot indicates axis of quantification in (h). Times are in minutes. Scales bars, 10 µm. (h) Quantification of the membrane to cytoplasm intensity ratio across control cells over time. The ratio fluctuates around the basal level over time. Numbers of samples are indicated.
Fig. S4 Quantification of graded Rac activity. (a-c) HeLa cells in state I-III transfected with a Raichu-Rac FRET probe and an mCherry tagged FKBP-TIAM1 (MCHF-TIAM1) experiencing a gradient of rapamycin from top to bottom over time. Time 0 is chosen as 30 minutes before morphological changes in MCHF-TIAM1 images. Cell length plot indicates axis of quantification in plots (d-f). Yellow arrows indicate initial protrusions. Times are in minutes. Scales bars, 10 µm. (d-f) Quantification of average FRET activity across state I-III cells over time in gradients of rapamycin. FRET profiles after time 0 are normalized to the initial profile at time 0 (shown in black). Thinner colored lines indicate S.E.M of corresponding average profile. Numbers of samples are indicated. (g) Control cells that do not express the Rac activator (MCHF-TIAM1) exposed to gradients of rapamycin. Only slight fluctuations in Rac activity were detected. Cell length plot indicates axis of quantification in (h). Times are in minutes. Scale bars, 10 µm. (h) Quantification of the average FRET profile across control cells over time. FRET activity fluctuates around the basal level at time 0. Numbers of samples are indicated.
**Fig. S5 Comparison between graded and uniform rapamycin stimulation.** (a) Cells exposed to a uniform concentration of rapamycin (2 nM) flatten and do not show any directed polarity. (b) Cells observed for one hour without stimulation and subsequently exposed to a gradient of rapamycin for a two hour period, followed by a switch to a uniform stimulation (2 nM) for three hours. The gradient directs cell motility and amplifies the existing lamellipodium. The switch to a uniform stimulation leads to a protrusion formation in the rear of the cell and retraction of the previously amplified lamellipodium. Times in minutes. Scale bars, 10 µm. (c) A kymograph taken from the cell center (specified in accompanying image) depicting the morphological changes of the cell shown in panel (b) over various treatment periods. The blue line traces the front of the polarized cell, whereas the red line traces the unpolarized face. (d) Quantification of the length changes in cell front and back during the rapamycin gradient exposure and after the switch to uniform rapamycin stimulation. Numbers indicate total number of cells. Error bars are SEM. (e-f) Cells observed for one hour, exposed to uniform 2 nM rapamycin (e) or uniform 1 nM rapamycin (f) for two hours, and a gradient for three hours. Cells given a 2 nM uniform dose exhibit protrusions on both sides, while cells given a 1 nM uniform dose can still be guided by the gradient. Yellow arrows indicate initial and final enhanced protrusions. Times are in minutes. Scale bars, 10µm.
Fig. S6 Tracking changes in cell morphology to assay the initial response time. The plots correspond to the cells in different states, as seen in Figure 2. Various morphology metrics are illustrated in the schematic accompanying the graphs. Fractional values of the metrics are shown, with values normalized to those at the beginning of the analysis. (a-c) Tracking of changes in the lengths of the cell front and back over time during gradient stimulation. (d-f) Tracking of changes in widths of the cell front and back. The time to reach 20% of the maximum magnitude of the first morphological response to gradient exposure was taken as the initial response time. The drop line indicates the initial response time, while the circle indicates the intercept between the dropline and the corresponding metric value.
Fig. S7 Initial response and late polarization times of MTLn3 cells. (a-c) Initial response times as a function of gradient values for cells in state I, II, and III respectively. (d-f) Late polarization times for cells in state I, II and III, respectively as a function of mean concentration. State I (a,d) n = 8, state II (b,e) n = 10, state III (c,f) n = 11.
Fig. S8 Additional information on dependence of the initial response time and late polarization time on rapamycin gradient and local concentration values. (a-c) The relationship between initial response time and mean rapamycin concentration for all states. Spearmann correlation coefficient values for each curve were determined as follows, state I: -0.582 (a), state II: -0.478 (b), state III: -0.377 (c). (d-f) Late polarization times determined for different gradient values for cells in all states. Pearson correlation coefficient values for each curve were determined as follows, state I: 0.103 (d), state II: -0.511 (e), state III: -0.228 (f). Within each plot, the data is binned into three mean concentration levels. State I (green) n = 27, state II (blue) n = 37, and state III (red) n = 29.
Fig. S9 State II cells undergo the initial response faster than cells in other states. (a-c) Examples of the morphological changes in cells in different states experiencing the same gradient of rapamycin (0.01 nM/µm +/- 0.001). Cells were exposed to the gradient for the entire experimental period but only the 5 minute image showing both cells and the gradient visualization is shown for each state to add clarity. Yellow arrows indicate initial protrusions. Times are in minutes. Scale bars, 10 µm. (d-f) Quantification of the morphological changes seen in the examples shown in (a-c). The dropline indicates the initial response time. The circle indicates the metric used to determine it, with the color of the circle corresponding to the metric.
**Fig. S10 State II cells undergo the late polarization response faster than cells in other states.** (a-c) The late polarization response of cells in all three states experiencing the same concentration gradient and the same local concentration of rapamycin. Yellow arrowheads indicate the direction of initial cell polarity. (d-f) Quantification of the average fluorescence intensity in cell bodies of the cells shown in (a-c) (see the schematic for the cell body definition). Drop lines indicate the late polarization times for each example. Note that the cell in state II reaches the late polarization phase faster than the cells in the other two states. Times are in minutes. Scale bars, 10 µm.
**Fig. S11 Suppression of polarization responses with LY294002 treatment.** (a-c) Time lapse imaging of sample cells selected from subpopulations in different states during LY294002 addition. LY294002 was included at a concentration of 10 µM and was included in both cell medium solutions used to generate the rapamycin gradient. In all examples shown, cells fail to respond within the experimental time frame across all states. Yellow arrows indicate the direction of the initial cell polarity. Times are in minutes. Scale bars, 10 µm. (d) Initial response time vs. rapamycin gradient values in cells responding in the presence of LY294002. State I (green) n = 21, state II (blue) n = 31, and state III (red) n = 27. Diamonds represent non-responder cells while circles represent responding cells. Grey dots on each plot illustrate the initial response times seen for cells not exposed to LY294002 (Fig. 2f).
Synthetic spatially graded Rac activation drives cell polarization and movement

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Supporting Notes

1. Model Supplement

Introduction

As described in our previous computational article\textsuperscript{(1)}, our model (shown in Model Figure 1) is related to previous work\textsuperscript{(2, 3)}. It was assembled in several stages, with modifications and geometric considerations relevant to the specific experimental system described in the main text of this paper. Here, we provide a brief description of the ultimate model and how it was analyzed.

We consider the three GTPases implicated in polarized cell morphology, control, and chemotaxis, Cdc42, Rac and Rho (While in principle similar results are obtained without Cdc42, this master signaling component was included for completeness). For each GTPase, we track the levels of active and inactive forms bound to the membrane, \( G, G^{\text{mi}} \), as well as an inactive (GDI-bound) cytosolic form \( G^c \), (where \( G = C, R, \rho \) represent Cdc42, Rac and Rho concentrations). Each variable is a function of time \( t \) and position \( x \) in a given cell. See below for details of the cell geometry. We assume that an inactive GTPase can (un)bind from the membrane, cycling between \( G^c \) and \( G^{\text{mi}} \) and that GEF/GAP activity interconverts the membrane bound species \( G^{\text{mi}} \), \( G \). We integrate over the depth direction (see below). Every point in the resulting domain is considered to have both cytosolic and membrane components. Crosstalk shown in the figure is directed at GEFs, with enhanced/reduced GEF activity depicted by arrows/inhibitory connections. Linear GAP activity is used in all cases. For phosphoinositides, we track PIP, PIP\(_2\), and PIP\(_3\) (whose levels are denoted \( P_1, P_2, P_3 \)). Their interconversions, mediated by kinases and phosphatases) are assumed to be enhanced by Rac or Rho as indicated in the schematic (Model Fig. 1). The feedback from the PIP layer to the GTPase layer is governed by a tunable parameter \( f_1 \).

Model Figure 1- Diagram of the Rho-GTPase/Phosphoinositide signaling network used in the one dimensional spatial cell model; activation is denoted by (\( \rightarrow \)) and inhibition by (\( \perp \)). The signal is \( S(x,t) = s_0 + s_1 x / L \), where \( s_0 \) and \( s_1 \) are the basal and gradient input components, and \( L \) is cell length.
1.2. **Small GTPase module.** Equations for each of the active (membrane bound) and inactive (cytosolic) GTPases are as follows, for a total of 9 partial differential equations (PDEs).

\[
\frac{\partial G}{\partial t} = I_G - \delta_G G + D_m \Delta G,
\]

\[
\frac{\partial G_c}{\partial t} = k_{off} G_{mi} - k_{on} G_c + D_c \Delta G_c,
\]

\[
\frac{\partial G_{mi}}{\partial t} = -I_G + \delta_G G - k_{off} G_{mi} + k_{on} G_c + D_m \Delta G_{mi},
\]

where \(D_m, D_c\) are membrane and cytosolic rates of diffusion, \(\delta_G\) is GAP-mediated inactivation rate, \(k_{off}\) is the membrane disassociation rate constant, and \(k_{on}\) the membrane association rate constant. The term \(I_G\) is GEF-mediated rate of activation that depends on the availability of inactive GTPase, and on crosstalk from other active species. Rates of diffusion in membrane and cytosol are estimated as \(D_m = 0.1 \, \mu m/s^2\) and \(D_c = 100 \, \mu m/s^2\) as used previously(3, 4). These equations, supplemented with no-flux boundary conditions conserve the total amount of each GTPase.

1.3. **Geometry and simplification.** Cells are narrowly confined in microfluidic channels, so that their width is constrained and time independent. In view of this fact, it is reasonable to approximate cell shape as a 3D box of length \(L\), width \(w\), and depth \(d\) satisfying \(d < w << L\). Due to controlled signal and the physical constraints of the experimental apparatus, it is reasonable to neglect gradients in all but the length direction. Define a 1D projection of the variable \(G_c\) as

\[
G_{pc}(x) = \int_0^w \int_0^d G_c(x, y, z) dy dz = dw G_c(x).
\]

Here we have approximated \(G_c\) as nearly uniform across the width and depth directions. It follows directly that

\[
\frac{\partial G_{pc}}{\partial t} = wd k_{off} G_{mi} - k_{on} G_{pc} + D_m \Delta G_{pc}.
\]

Over the timescale considered, the volume of the cell \(V \approx w \cdot d \cdot L\) is roughly constant. Channel diameter determines width \(w\), so the observed lengthening of the cell must be accompanied by depth change. We take the initial values of \(d_0 = 0.2 \, \mu m, L_0 = 20 \, \mu m\) for a pre-stimulated cell. As \(L\) (but not \(d\)) is directly observable experimentally, we use \(wd = V/L\) to eliminate the less easily measurable cell depth.
1.4. A composite inactive form. We consider the cycling of the inactive GTPase between membrane and cytosol to be in quasi steady state, as before(2). We find the fraction of the inactive forms on the membrane and in the cytosol to be be \( \chi(L) := k_{on} / (k_{on} + [V/L] k_{off}) \) and \( (1-\chi(L)) \), respectively. A composite inactive form, \( G^i \), is defined as

\[
G^i = G^{mi} + G^{pc}, \quad G^{mi} = \gamma(L)G^i, \quad G^{pc} = (1-\gamma(L))G^i.
\]

This accounts for the fact that only the membrane bound fraction of this composite is available for GEF activation. We also define an “effective diffusion constant”

\[
D_{mc} (L) = \gamma(L)D_m + (1-\gamma(L))D_c.
\]

This weights the diffusion constant of the composite form according to the proportion of time spent on the membrane and the cytosol. A full parameter set is determined by assuming \( D_{mc} (L_0) = 50 \), consistent with previous work(2-4) and \( k_{on} = 1s^{-1} \). \( k_{off} \) is then determined by

\[
k_{off} = \frac{L_0k_{on} (D_m - D_{mc} (L_0))}{V (D_{mc} (L_0) - D_c)},
\]

completing the parameter set associated with membrane cycling.

1.5. Reduced GTPase model. The system is now reduced to a set of three GTPases, each described by a single composite inactive form \( G^i (x) \) and an active form \( G(x) \), the total of which are conserved over the (1D) domain on the experimental timescale. We use the cross-talk depicted in Model Figure 1 to formulate a system of 6 PDEs. Linear inactivation by GAP’s is assumed for each GTPase and up/down regulation of GEF activation pathways are assumed to take generic functional forms leading to

\[
\frac{\partial G}{\partial t} = I_G \frac{\gamma(L)}{\gamma(L_0)} G^i - \delta_G G + D_m \Delta G,
\]

\[
\frac{\partial G^i}{\partial t} = -I_G \frac{\gamma(L)}{\gamma(L_0)} G^i + \delta_G G + D_{mc} \Delta G^i,
\]

with \( G = C, R, \rho \) and GEF activation rate functions

\[
I_c = \frac{\tilde{l}_c}{1 + (\rho/a_f)^n} \frac{\gamma(L)}{\gamma(L_0)} C^i,
\]

\[
I_R = \frac{\tilde{l}_R}{1 + f_1 \frac{P_1}{P_{30}}} + \alpha C + S(x,t) \frac{\gamma(L)}{\gamma(L_0)} R^i,
\]

\[
I_\rho = \frac{\tilde{l}_\rho}{1 + (R/a_z)^n} \frac{\gamma(L)}{\gamma(L_0)} \rho^i.
\]
A more complete discussion of the forms of these kinetic terms was given earlier(3). Note that $n \geq 2$ is required for this system to exhibit appropriate ("wave-pinning"(5)) polarization behavior. Normalization by $\gamma(L_0)$ makes for convenient parameterization. Here $f_i$ represents PI feedback strength to the GTPase module via Rac.

1.6. Phosphoinositide module. A PI feedback module(6), modified from earlier work(2) is based on the following equations:

$$\frac{\partial P_1}{\partial t} = I_{p1} - \delta_{p1} P_1 + k_{21} P_2 - \frac{k_{PSK}}{2} \left(1 + \frac{R}{R_i}\right) P_1 + D_p P_{1xx},$$

(1.11) $$\frac{\partial P_2}{\partial t} = -k_{21} P_2 + \frac{k_{PSK}}{2} \left(1 + \frac{R}{R_i}\right) P_1 - \frac{k_{PSK}}{2} \left(1 + \frac{R}{R_i}\right) P_2 + \frac{k_{PTEN}}{2} \left(1 + \frac{\rho}{\rho_i}\right) P_3 + D_p P_{2xx},$$

$$\frac{\partial P_3}{\partial t} = \frac{k_{PSK}}{2} \left(1 + \frac{R}{R_i}\right) P_2 - \frac{k_{PTEN}}{2} \left(1 + \frac{\rho}{\rho_i}\right) P_3 + D_p P_{3xx},$$

Terms in round braces are feedback from Rac and Rho. For parameter values see Model Table 1.

1.7. Simulation Method. Simulations of the model equations (1.9), (1.10), (1.11) are performed with an implicit diffusion, explicit reaction scheme. Initial GTPase profiles are spatially uniform. The system is allowed to settle to a (parameter dependent) homogeneous steady state by integrating for 50 time units. At $t = 50$, the signal $S(x, t)$ is applied to Rac GEF as shown in Eqn (1.10) and the model is integrated to $t = 500$. We observe that a new polarized steady state emerges on a typical time scale of 100-300 time units. In all simulations, the rest state was stable to small amplitude noise. The results of a sample simulation are shown in the kymograph of Figure 2a. GTPase asymmetry/polarization strength (Figs. 3b and 5c) is measured as the absolute difference between the highest and lowest active Cdc42 ($C$) levels at the final time. ($R$ or $\rho$ can also be used to quantify polarization with similar results.) Response times, shown in Fig. 2b, are based on a generic inverse relationship between cytoskeletal reorganization rate and GTPase polarization strength, i.e., response time = 1/response strength.

1.8. Predictions. Here we provide a brief overview of the model, referring the interested reader to our companion paper(1), where a detailed development and analysis has been provided.

The model described here, has at its core a polarization mechanism based on a wave of activity that sweeps across a cell and freezes to produce a static profile with large differences between cell poles. This stalling wave behavior has been termed "wave-pinning"(5). A key feature of this mechanism is that a threshold stimulus (either localized or distributed as gradient or noise) is needed to initiate a response. This feature stems from a combination of several factors: (a) active and inactive GTPases diffuse very differently due to their membrane (cytosol) residence. (b) The GTPase circuit shown in Model Fig. 1 contains an effective positive feedback. This causes the active GTPase to promote nearby activation. (c) Cycling between these forms preserves a constant total amount, leading to depletion effects as more and more activity is turned on. This ultimately freezes the wave in its tracks. Thus, if a stimulus provokes local activity exceeding a threshold, it self-amplifies and spreads, but only so long as inactive GTPase is available to be
activated. It has been shown that this leads to a plateau of high (low) activity at the cell poles, but the details of the mathematical analysis are technical and beyond our scope.

In contrast to the above, many commonly used models for cell polarization (reviewed in (7)) have no threshold for patterning, and a response is triggered by arbitrarily small amplitude noise, here denoted by the informal term “ultrasensitive”. Such pattern formation is commonly termed Turing-type. Absence of a stable rest state, makes this type of polarization mechanism unsuitable for the HeLa cell system described in this article. Further discussion of various polarization models and their properties appears in (7).

To analyze the behavior of models with slow and fast rates of diffusion, we extended a recently developed technique termed "Local Perturbation Analysis" in (1). This technique approximates the reaction-diffusion PDEs with simpler ordinary differential equations (ODEs) for the local (slow diffusing) and global (fast diffusing) components of the system. The analysis of the resulting LPA system of ODE’s allowed us to (a) find interesting parameter ranges and classify the dynamics as wave-type or Turing-type behavior, (b) easily explore how changes in model assumptions affect such behavior, and (c) understand how inhibition or upregulation influences the behavior of the system.

As described in the companion paper(1), we used LPA to identify the wave-pinining parameter regime for the model. To investigate the experimentally modulated Rac GEF activity levels, we tested the model against manipulations of terms corresponding to Rac GEF activity levels. To do so, we defined a stimulus, $S(x,t) = s_0 + s_1 \frac{x}{L}$, affecting the Rac GEF term $I_R$, with $s_0$ the background level and $s_1$ the stimulus gradient steepness. (see expression for $I_R$ in Eqs. (1.10) and $S(x,t)$ in Model Fig. 1)

We ran several tests with various values of $s_0$ and $s_1$ and studied how the model responds to increases in one of these parameters at various values of the other. We found that $s_0$ modulates the wave pinning response threshold in the PDE model, (Figure 3b). For fixed $s_1$, sufficiently large $s_0$ provokes a response, as expected in the presence of a threshold. Further, the higher the background GEF activity level $s_0$, the lower the threshold to be breached for a response. Essentially, $s_0$ sets the response threshold, and $s_1$ produces spatial heterogeneity in the system. If $s_1$ is large enough that the solution breaches that threshold, a response similar to a phase transition results. The significance of this finding is that basal GEF activity levels, which can be modulated either internally by the cell or externally by the environment, aids in controlling the sensitivity of a cell to a directed stimulus.
2. Experimental setup

First, control valves (marked blue in SI Appendix (Fig. S1a)) were primed by connecting syringes filled with deionized water and pressurized to 20 psi. Experimental solutions were made using DMEM F-12 (Gibco) as a base medium. In a typical experiment, two solutions were injected into the device, one containing rapamycin and the other without. Rapamycin concentrations in microfluidic chips were titrated to uniform stimulation experiments in open chambers by comparing temporal responses. The rapamycin solution was injected into inlet “1” (Marked red in SI Appendix (Fig. S1a)). Once the solution reached the intersection of the channel and valve “1”, the valve was pressurized to keep the solution pinned at this location. In the corresponding ‘0’ inlet, the rapamycin-free solution was injected and allowed to flush through inlets marked ‘wash’ to wash away any rapamycin which may have entered into the channel. After the wash period, plugs were placed into the “wash” inlets. For experiments utilizing LY294002, a 10 μM concentration of the drug was introduced into both experimental solutions. In all experiments, a 10 μg ml⁻¹ fibronectin solution was injected into the ‘cell’ inlet prior to cell introduction. Actuation of valve marked “5” forced the fibronectin solution through the cross channels into the right flow through chamber and out of the outlet labeled ‘out’. Fibronectin coating was performed for 50 min at 37°C. After the coating period, another rapamycin-free solution was placed into the outlet and the fibronectin solution was removed from the ‘cell’ inlet. A HeLa cell suspension, at a concentration of 1 x 10⁶ cells ml⁻¹, was injected into the ‘cell’ inlet with a loading pipette. The outlet pressure was lowered below the atmospheric one, causing flow of the cell suspension and subsequent seeding of cells into the cross channels via the same principle used above for the fibronectin coating. Excess cells in the main flow arms were washed out. In experiments utilizing LY294002, a rapamycin and LY294002 free solution was injected into one of the ‘wash” inlets to flush away excess cells to prevent prior exposure of cells to the inhibitor. HeLa cells were allowed to attach for 4 h at 37°C and 5% CO₂.

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**Model Table 1 - Parameter set used for model simulations**

<table>
<thead>
<tr>
<th>Parameter Name</th>
<th>Value</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_t, R_t, \rho_t$</td>
<td>2.4, 7.5, 3.1</td>
<td>Total levels of Cdc42, Rac, and Rho</td>
</tr>
<tr>
<td>$\dot{I}_C, \dot{I}<em>R, \dot{I}</em>\rho$</td>
<td>2.95, 0.2, 6.6</td>
<td>Cdc42, Rac, and Rho activation rates</td>
</tr>
<tr>
<td>$a_1, a_2$</td>
<td>1.25, 1.0</td>
<td>Cdc42 and Rho half-max inhibition levels</td>
</tr>
<tr>
<td>$n$</td>
<td>3</td>
<td>Hill coefficient for inhibitory connections</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>0.65</td>
<td>Cdc42-dependent Rac activation</td>
</tr>
<tr>
<td>$\delta_C, \delta_R, \delta_\rho$</td>
<td>1.0</td>
<td>GAP decay rates of activated Rho-proteins</td>
</tr>
<tr>
<td>$I_{P1}$</td>
<td>10.5</td>
<td>PIP₁ input rate</td>
</tr>
<tr>
<td>$\delta P_1$</td>
<td>0.21</td>
<td>PIP₁ decay rate</td>
</tr>
<tr>
<td>$k_{PI5K}, k_{PI3K}, k_{PTE}$</td>
<td>0.084, 0.00072, 0.432</td>
<td>Baseline conversion rates</td>
</tr>
<tr>
<td>$k_{21}$</td>
<td>0.021</td>
<td>Baseline conversion rate</td>
</tr>
<tr>
<td>$P_{3b}$</td>
<td>0.15</td>
<td>Typical level of PIP₃</td>
</tr>
<tr>
<td>$L_0$</td>
<td>20</td>
<td>Cell Length</td>
</tr>
<tr>
<td>$D_m, D_{mc}(L_0), D_P$</td>
<td>1.0, 50.0, 5.0</td>
<td>Diffusion Rates</td>
</tr>
</tbody>
</table>


Movie S1. This movie demonstrates the polarization responses of cells across different polarity states to gradients of rapamycin. The green color of each cell represents expression of YF-TIAM1, and the red color illustrates the gradient of rapamycin. Each cell polarizes toward the high side of the gradient.
Movie S2. This movie illustrates graded translocation of YFP–FKBP complexes to the cell membrane in cells in states I–III in a gradient of rapamycin arriving from the top. There are only slight morphological changes and no polarization behavior over time.

Movie S2
**Movie S3.** This movie illustrates the graded increase in Rac activity in cells in state I–III visualized with a Raichu Rac FRET probe. The pseudocolor depicts the FRET ratio values, with higher values having warmer colors and lower values having cooler colors. The scale is indicated in *SI Appendix, Fig. S4.*

[Movie S3]
Movie S4. This movie depicts a cell experiencing a gradient of rapamycin followed by a uniform stimulation of rapamycin. The green color of the cell represents expression of YF-TIAM1, and the red color illustrates the gradient of rapamycin. The cell is visualized for 60 min prestimulation, followed by a gradient for 120 min and a uniform stimulation for 180 min thereafter. During the gradient stimulation, the cell shows enhancement of the cell front and motility toward the gradient. After the switch to a uniform stimulation, the cell begins spreading at the cell rear and lose the enhancement at the front.
Movie S5. This movie illustrates a cell experiencing a gradient of rapamycin for a period of 30 min, followed by washout for the remaining time period. The green color of the cell represents expression of YF-TIAM1, and the red color illustrates the gradient of rapamycin. As seen here, the cell shows little morphological change and does not respond to the gradient within the time frame.
Movie S6. This movie shows cells in different polarity states experiencing a gradient of rapamycin for a period of 60 min, followed by washout for the remaining time period. The green color of each cell represents expression of YF-TIAM1, and the red color illustrates the gradient of rapamycin. Each cell shows polarization toward the gradient with morphologies similar to those seen in Movie S1. Responses continue even when the rapamycin is washed out.
Movie S7. This movie depicts cells in different polarity states experiencing a gradient of rapamycin with the addition of LY294002, a PI3K inhibitor. The green color of each cell represents expression of YF-TIAM1, and the red color illustrates the gradient of rapamycin. Cells did not polarize toward the gradient of rapamycin and remained quiescent throughout the experimental period.

Other Supporting Information Files

SI Appendix (PDF)