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Title:

Patterning of the cell cortex by self-organized Rho GTPase Dynamics

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Abstract

The Rho GTPases—Rho, Rac and CDC42—are small GTP-binding proteins that regulate basic biological processes such as cell locomotion, cell division and morphogenesis by promoting cytoskeleton-based changes in the cell cortex. This regulation results from active (GTP-bound) Rho GTPases stimulating target proteins that in turn promote actin assembly and myosin-2-based contraction to pattern the organization of the cortex. This basic regulatory scheme, well-supported by *in vitro* studies, led to the natural assumption that Rho GTPases function *in vivo* in an essentially linear matter, with a given process being initiated by GTPase activation and terminated by GTPase inactivation. However, a growing body of evidence based on live cell imaging, modelling, and experimental manipulation indicates that Rho GTPase activation and inactivation are often tightly coupled in space and time via signalling circuits and networks based on positive and negative feedback. In this Review, we present and discuss this evidence, and we address one of the fundamental consequences of coupled activation and inactivation: the ability of the Rho GTPases to self-organize. We discuss how Rho GTPase self-organization results in the formation of diverse spatio-temporal cortical patterns such as static clusters, oscillatory pulses, traveling wave trains, and ring-like waves. Finally, we discuss the advantages of Rho GTPase self-organization and pattern formation for cell function.

[H1] Introduction

The Rho GTPases—RHOA, RAC1, and CDC42 and their close relatives—are small GTP binding proteins of the Ras superfamily that are best known for their regulation of actin filaments (F-actin) and the motor protein myosin-2¹. Like other members of the superfamily, Rho GTPases undergo cycles of GTP binding and hydrolysis (Figure 1a), and these cycles are linked to their ability to signal to their targets: Rho GTPases are active when bound to GTP and can bind to and stimulate so-called effector proteins which, in turn, signal to F-actin and myosin-2. Following hydrolysis of GTP to GDP, the Rho GTPases can no longer bind to their effectors and become inactive, until they exchange GDP for GTP, completing the cycle. *In vitro*, GTP hydrolysis and GDP-GTP exchange are extremely slow; *in vivo* these steps are accelerated by GAPs (GTPase activating proteins) and GEFs (guanine nucleotide exchange factors) respectively. The GTPase cycle has a distinct spatial context within the cell, with active (GTP-bound) Rho GTPases associating with the plasma membrane via their carboxyterminal prenyl groups. Following inactivation, the GTPase can be extracted from the plasma membrane by RhoGDI (Rho guanine nucleotide dissociation inhibitor), which ensheathes the prenyl group and maintains the inactive GTPase in a soluble, cytosolic form (Figure 1a)²⁻⁴.

Targeting of the Rho GTPases to the plasma membrane is of particular significance because it gives them access to the cell cortex. The cell cortex is the outermost layer of the cell and includes both the plasma membrane and the layer of cytoplasm just beneath it⁵. The cortex is typically rich in F-actin (“cortical F-actin”) and myosin-2 (“cortical myosin-2”) and is of great importance to the cell because it drives cell shape changes needed for an enormous variety of processes including cell migration, phagocytosis, polarity establishment, cytokinesis, and morphogenesis. It is the Rho GTPases that enable the cell shape changes required for these processes, by virtue of their ability to rapidly remodel the cortical F-actin and myosin-2 via activation of their effectors at the plasma membrane⁶⁻¹⁰. Consequently, the subcellular patterning of Rho GTPases in the cortex, and how it is controlled, have long been the subject of intense interest.

The dominant model for Rho GTPase patterning has been one in which pattern control is exercised in an essentially linear manner, with a given upstream signal such as a growth factor stimulating a GEF in a particular region of the plasma membrane, which then stimulates the Rho GTPase in the same region. The high Rho GTPase activity then triggers a particular response such as cytokinesis (Figure 1b). In this activation-centric view, GEFs are the primary drivers of the response, while GAPs are considered to simply restrain or terminate the response. However, recent studies in a variety of model systems indicate that upstream signals result in the engagement of both Rho GTPase GEFs and GAPs at the same time, and show that this results in self-organization of the Rho GTPases into cortical patterns such as stable clusters, traveling waves, and oscillatory pulses¹¹⁻²¹. Thus, it is not simply activation of the Rho GTPases that matters, but the GTPase cycle itself and the resultant pattern (Figure 1c).

The objective of this Review is to present and discuss the evidence that self-organization plays a major role in the regulation of Rho GTPases. Toward this end, we first discuss Rho GTPase patterns **and** provide a brief overview of self-organizing patterns. We consider the role of positive and negative feedback in such patterns,

both in generic terms and then in terms of the Rho GTPase GEFs and GAPs known to engage in feedback in cells. We then present a series of examples of self-organizing Rho GTPase patterns, drawing on recent studies of diverse processes and model systems. Finally, we discuss the advantages that arise from the use of self-organization for signalling at the cell cortex.

[H1] Rho GTPase zones and Rho GTPase flux

Early imaging studies of Rho GTPase dynamics using different approaches (Box 1) revealed that cellular processes including yeast budding²², cytokinesis²³, plasma membrane repair²⁴, exocytosis²⁵, and cell locomotion^{26,27}, are accompanied by formation of Rho and CDC42 “zones”, regions of the cell cortex where Rho GTPase activity is highly elevated.

These zones represent local patterns such as stripes, patches, and rings that are highly enriched in GTPase activity relative to the immediately surrounding areas. They can emerge and disappear within seconds to minutes, even when occupying thousands of square micrometers. Superficially, it might seem reasonable that such patterns could be generated by Rho GTPase activation alone simply by localization of a GEF at the site of the zone **in the absence of GAP activity or other mechanisms for removal of active GTPases from the plasma membrane.**

Indeed, Rho GEFs are often targeted to distinct subcellular locations⁴³. However, any accumulation of active Rho GTPase at the plasma membrane will be counteracted by diffusion of the GTPase away from the site of activation, thereby degrading the pattern. The very low intrinsic rate of GTP hydrolysis by Rho GTPases will exacerbate this problem as the active GTPases can potentially diffuse very far away from the site of activation, essentially raising the background level of GTPase activity and thereby further degrading the pattern.

These observations led to the “GTPase Flux Hypothesis”, which posits that Rho GTPase activation and inactivation are tightly coupled within zones to counteract the effects of GTPase diffusion⁴⁴. An independent modelling study demonstrated that the high activity and fast turnover of small GTPases characteristic of activity zones requires simultaneous action of GEFs and GAPs⁴⁵. Confirmation of these concepts was provided by demonstrations that suppression of GAP expression doesn't simply increase GTPase activity but rather disrupts GTPase patterns or patterns of their targets^{40,46}, and by studies showing that Rho GTPases have very short (several seconds or less) half-lives at the plasma membrane^{30,31,47}. Put simply, steady state maintenance of the GTPase activity zones requires continuous delivery of inactive GTPase, its activation, and compensatory GTPase inactivation and removal. Consequently, the concept of GTPase flux was further extended from local GTPase cycling to GTPase transport and nucleotide cycling flux on a cellular scale⁴⁸.

Thus, activation and inactivation must be temporally coupled to account for the existence of Rho GTPase zones (Figure 1c). In one common implementation of this requirement, Rho GTPase GEFs and GAPs can be targeted to complementary compartments (e.g., an apical GEF and basolateral GAP in epithelial cells)⁴⁹⁻⁵². However, recent studies based on improved live imaging approaches, combined with theoretical modelling and experimental manipulations, have revealed another, less-intuitive mechanism cells use to generate dynamic Rho GTPase patterns: spatial coupling of the GEFs and GAPs. Specifically, studies in many systems including budding and fission yeast^{11,12}, worms¹³, flies¹⁴, echinoderms^{15,16}, frogs^{15,17} cultured mammalian cells¹⁸⁻²⁰, and cell-free extracts²¹ reveal that Rho GTPases exhibit periodic (cyclic) activity patterns including single traveling waves, traveling wave trains, and oscillatory pulses; (Figure 2). Behaviours like these are hallmarks of

signalling networks that couple positive and negative feedback to drive self-organized pattern formation^{53,54}.

[H1] Self-organized patterns of Rho GTPase activity

Self-organization is the spontaneous acquisition of order by a previously disordered system arising from local interactions of system constituents⁵⁵. Self-organization requires continuous energy investment⁵⁶, and thus represents a state far from equilibrium, even when an apparently stationary organization is achieved. This contrasts with self-assembly, wherein the process usually proceeds to equilibrium⁵⁶. At the subcellular level, self-organization is most famously associated with formation of the mitotic spindle^{57,58} but it is becoming increasingly apparent that self-organization is ubiquitous⁵⁹ with the potential to contribute to many aspects of cell behaviour, including patterning of plasma membrane⁶⁰.

To understand how Rho GTPases form self-organized patterns requires familiarity with patterning by the so-called “activator-inhibitor” systems^{61,62}. Activator-inhibitor systems are generic, idealized models of pattern formation, which can be used to explain how Rho GTPases self-organize, with the active GTPase serving as the activator and GTP hydrolysis providing the requisite energy investment (Box 2).

It is important to realize that models such as the activator-inhibitor systems are idealizations and patterning in living cells, by Rho GTPases or other self-organizing systems, is typically subject to many influences. Such influences include positional cues that can direct pattern formation to certain areas of the cell and often act by increasing the local concentration of the pattern-forming elements in particular areas of the cell. For example, as described in more detail below, formation of the CDC42-GTP cluster during yeast budding is normally confined to the site of previous budding by so-called landmark proteins. In another example, the mitotic spindle directs the concentration of high amplitude, complementary Rho-GTP and F-actin waves at the equator during cytokinesis. Molecular noise is another influence in self-organized patterns. Molecular noise is the natural (spontaneous) fluctuation of local protein copy numbers and it has the potential to impact all aspects of cell biology⁶³. In the context of self-organized GTPase pattern formation, the role of noise is expected to be particularly prominent when and where the parameters of the pattern-forming network are close to the onset of pattern formation and in which normal positional cues for pattern formation are lacking. Thus, in the budding yeast in which landmark proteins have been experimentally removed, molecular noise determines where the bud forms⁶⁴. Similarly, in the absence of external guidance cues, noise determines which ends are chosen to be the front and the back of cultured cells plated on narrow adhesive stripes^{65,66}.

The patterns presented in Box 2 are only a small sample of the patterns that the mathematical models of nonequilibrium systems can produce *in silico*^{67,68}, prompting the question of how self-organizing patterns are identified *in vivo*. While there are no absolute rules, two or more of the following criteria are typically applied. The first is identification of pattern dynamics that are characteristic of activator-inhibitor systems such as waves or oscillatory pulses simply because these are often most easily explained by self-organization. Conversely, underlying self-organization can be revealed by the onset of periodic behaviours following some manipulation. For example, the appearance of oscillatory behaviour following the removal of a pattern

regulator is often taken as a sign of a missing feedback⁶⁹. Given the importance of feedback, a second common criterion is the evidence that system component interactions can generate feedback loops. A third criterion is the evidence that the pattern can form spontaneously, even when upstream cues are compromised. For example, during the budding process, budding yeast normally form a cap of CDC42-GTP close to the site of previous cell division due to the influence of landmark proteins; when these proteins are genetically deleted, the cap still forms, but it is mislocalized⁷⁰ (see also below). A fourth criterion is the ability of a theoretical model based on the principles of self-organization to capture the features of the experimental pattern or, better still, to make testable predictions about the pattern that are confirmed by experiment. A fifth (and as yet extremely challenging) criterion is the successful reconstitution of the pattern in vitro from an initially homogenous mixture of the components⁷¹⁻⁷³.

[H1] Rho GTPase feedback loops

Activator-inhibitor models for patterning typically presuppose the existence of both positive and negative feedbacks in Rho GTPase regulation, a supposition that is fulfilled by the observation that Rho GTPases can both positively and negatively regulate their own GEFs and GAPs⁷⁴⁻⁷⁷. Indeed, a wealth of such feedbacks have been described (Figure 3; Table 1). They can be grouped based on the number of steps between the GTPase and the GEF or GAP (Figure 3): 1) Direct feedback, where the active GTPase itself binds to a GEF and modifies its activity⁷⁴. For example, at least seven Rho GEFs⁷⁸⁻⁸⁰, and at least one CDC42 GEF⁸¹ interact allosterically with Rho-GTP or CDC42-GTP, respectively, an interaction which directs the GEF to the plasma membrane and increases its activity. **Such interactions drive positive feedback (the GTPase activates its GEF, generating more active GTPase); to date there are no reported examples of active Rho GTPases binding their GAPs at sites other than active site and stimulating them allosterically (see also below).** 2) Effector-based feedback, where the GTPase effector modifies the activity or localization of a GEF or GAP. For example, active GTPases often bind scaffold proteins that also bind and stimulate their upstream GEF to drive positive feedback⁸²⁻⁸⁵; 3) Effector target-based feedback, where the downstream target of a given effector modifies the activity or localization of a GEF or a GAP. For example, F-actin, a downstream target of actin regulatory proteins such as formins (Rho and CDC42 effectors) and N-WASP (a CDC42 effector) can modulate the activity or localization of both GEFs^{84,86} and GAPs⁸⁷⁻⁸⁹.

These examples are by no means exhaustive, and the network depicted in Figure 3 and the examples presented in Table 1 omit other potential feedback mechanisms such as those that work through RhoGDI¹¹⁴. Nonetheless, a striking observation emerges: based on the number of participants in a feedback loop and the number of levels at which feedback acts, *feedback itself is a fundamental outcome of the Rho GTPase signalling*. That is, not only do the GEFs alluded to above in fact act as effectors in direct feedback, all of the major classes of effectors also participate in feedback including formins⁹⁵, N-WASP⁸⁴, p21-activated kinases (Paks)^{97,109}, Rho kinase (ROCK)¹⁰⁸, and Protein Kinase N (PKN)¹⁰⁷. With respect to downstream targets, in addition to F-actin, monomeric actin (G-actin) participates in feedback¹¹⁰, as does myosin-2¹⁰⁶, and Phosphatidylinositol (3,4,5)-trisphosphate (PIP3)^{106,110,115} is a downstream target of Rac and CDC42^{115,116}. Further, depending on the context, a given effector or target can participate in diverse feedbacks. For example, myosin-2 can both engage in both positive and negative feedback with Rho^{18,108} while F-

actin engages in negative feedback with Rho via binding to at least two different Rho GAPs^{13,18}. It is also apparent that the potential for nonlinear positive feedback is high, which is important because theoretical studies¹¹⁷ show that, for the formation of GTPase activity zones in the presence of linear GTPase inactivation by GAPs, positive feedback is required to be nonlinear. Both theory⁴⁸ and experiment⁷⁹ indicate that the mechanisms which result in recruitment of a GEF to the plasma membrane or the cortical cytoskeleton, either via allosteric interaction with a GTPase, or by other means, result in nonlinear positive feedback.

A further point implicit in the different categories of feedback are differences in feedback onset: feedbacks with more and slower steps will take longer than those with fewer, faster steps. Thus, the potential for generating different lengths of delay and therefore variation in the period of oscillatory patterns is also high. Notably, the only examples of direct feedback are positive, with the active GTPases stimulating GEFs, meaning that when paired with any negative feedback, a delay between the feedbacks is possible. But this is not the only way to achieve a delay: in principle, as long as the negative feedback has more steps, or slower steps than the positive feedback, a delay is expected. Further, differences in the extent of the delay are expected to produce differences in the pattern. Thus, pairing a direct positive feedback mechanism with an effector-based negative feedback would be more likely to produce higher frequency patterns (i.e., shorter period waves or pulses) than pairing the same direct feedback mechanism with a downstream target-based negative feedback simply because the target-based mechanism has more steps than the effector-based mechanism. This consideration leads to a final, related point: perhaps one of the virtues of Rho GTPase crosstalk—the communication between different GTPases—is that it makes longer cycles possible since feedbacks going through two or more GTPases would be inevitably slower than ones routed through a single GTPase.

RHO GTPases have what seems to be an excessively large repertoire of regulators: at least 80 different GEFs and 70 different GAPs¹¹⁸ regulating the three most abundant GTPases—RhoA, RAC1, and CDC42. By way of comparison, the three Ras GTPases, HRas, KRas, and NRas, are regulated by 3 GEFs and 6 GAPs¹¹⁹. We suggest that the explanation for the abundance and diversity of Rho GTPase GEFs and GAPs is that they enable a diversity of feedbacks which, in turn, enable a potentially limitless repertoire of self-organizing Rho GTPase patterns.

[H1] Self-organized Rho GTPase patterns in cells

In this section, we present recent examples of self-organizing Rho GTPase patterns in more detail with the goal of revealing how such patterning works in different contexts. The examples were chosen from studies that used high temporal resolution (<10s sampling intervals) imaging of Rho GTPase activity, which is needed to reveal many of the patterns considered here and based on their satisfaction of two or more of the criteria for self-organizing patterns alluded to above.

[H2] Polarized growth in yeasts

Polarized growth in fungi provides a paradigmatic example of a morphogenetic process pre-patterned by a circular cluster (“cap”) of Rho GTPase activity¹²⁰⁻¹²³. Budding yeast has two mutually exclusive morphogenetic programs that strictly require

polarized growth: budding and shmooing, i.e., formation of the mating protrusion. Fission yeast can also exhibit bipolar growth at the two opposite cell tips that can grow simultaneously. Early studies demonstrated that CDC42 and its GEF Cdc24 are both strictly required for and localize to the zones of polarized growth^{28,124-126}. The field was revolutionized by the introduction of CDC42 activity reporters^{22,26,127-129} that demonstrated that CDC42 is highly active at a disc-shaped cluster with a diameter of 1-3 μm that marks the nascent protrusion site. CDC42-GTP via its numerous effectors directly drives all morphogenetic processes including formation of polarized actin cables, vesicle secretion and, in case of budding, establishment of the septin ring^{130,131}. From the initiation of bud protrusion, the CDC42-GTP cluster translocates into the growing daughter cell and disassembles at mitosis onset when the bud growth ceases^{122,130}. Observations of the CDC42 and RAC1 clusters at the tips of cellular protrusions in other fungi suggest that they ubiquitously drive morphogenesis of polarized growth zones across the entire fungal kingdom^{120,129,132-134}. Similarly, in plants, the ROP ("Rho of plants") GTPases are active and enriched at the tips of growing pollen tubes and root hairs¹³⁵⁻¹³⁸.

The mechanism of CDC42-GTP cluster formation attracted much attention over several decades^{122,123,139,140}. Early work employing cytoskeletal poisons showed that neither microtubules nor actin are necessary for the cluster emergence^{122,141}. Rather, the CDC42-GTP cluster location on the membrane is influenced by a system of landmark proteins converging on the Ras-like small GTPase Rsr1/Bud1, which directly recruits Cdc24 as its effector⁷⁰. In the context of shmoo formation, the CDC42 cluster position is biased by the G-protein signalling activated by the mating pheromone receptor¹⁴². However, deletion of Rsr1 results in random bud positioning, but not failure of bud formation, while mutation of pheromone sensing abrogates chemotropic growth to a partner but not shmoo formation¹⁴². These results argued that the upstream signals serve only as spatial cues but are otherwise not required for the CDC42 cluster formation. In the following years the CDC42-GTP cluster emerged as a manifestation of self-organized cellular polarization and, thus, symmetry breaking^{117,143}. A pivotal point was the discovery of the positive feedback loop mediated by the scaffold-effector Bem1 that simultaneously binds CDC42-GTP and its activator, the GEF Cdc24 (refs. ^{83,144}) (Figure 4a). The key role of Bem1 in CDC42 polarization had been extensively confirmed by genetic perturbations^{11,145,146} and more recently by direct optogenetic recruitment¹⁴⁷. This feedback is also conserved in fission yeast, where the CDC42 effector Scd2 recruits the GEF Scd1¹⁴⁸. Several other non-mutually exclusive feedback loops have been suggested in the literature^{31,149} (for detailed review see¹¹⁷).

Modeling has provided essential insight into which mechanisms could, in principle, account for CDC42-GTP cluster formation. First, a complete model of a CDC42 cluster formation needs to describe spontaneous symmetry breaking¹¹⁷. Second, since polarizing yeast cells need only a single bud or a shmoo, such a model also needs to explain this uniqueness. Early models provided several physically plausible mechanisms of symmetry breaking but they required actin-cable-mediated delivery of CDC42 and did not address either nucleotide cycling of CDC42 or the uniqueness of the CDC42 cluster^{22,150,151}. The first fully mechanistic model of spontaneous CDC42-GTP cluster formation⁴⁸ was derived from the reaction network consisting of nucleotide cycling and membrane-cytoplasmic shuttling of CDC42. In agreement with experiment, this model did not require F-actin as part of positive feedback. Instead, it introduced the notion of spatiotemporal GTP-hydrolysis-driven CDC42 flux that

continuously renews the membrane-bound CDC42-GTP cluster. The flux concept also explained the uniqueness of the bud since two or more GTPase clusters cannot grow simultaneously in the same cell if they compete for the limited cellular pool of the GTPase and its GEF. The competition between several CDC42 clusters was confirmed experimentally^{11,146,152,153} and actively studied theoretically¹⁵⁴⁻¹⁵⁸. Multiple variations and extensions of the original models based on CDC42 nucleotide cycling^{48,159} have been proposed in the following years^{117,160-169}.

Addition of negative feedback turns stationary GTPase caps into moving or oscillating clusters^{22,130,170-173}. CDC42 GAPs bound to septin polymers recruited by CDC42-GTP via its effectors Gic1/2 were shown to form a negative feedback loop in the context of budding¹³⁰. Vesicle insertion into the plasma membrane has also been extensively studied as a potential negative feedback diluting CDC42-GTP on the membrane^{152,174-176}. However, in patterning of the septin ring exocytosis plays a positive feedback role by diluting septin polymers that physically block membrane secretion¹³⁰. Similarly, in fission yeast, vesicle insertion into the plasma membrane plays a positive role in CDC42 cluster formation by pushing the GAP Rga4 away from the center of the CDC42 cluster¹⁷⁷. Interestingly, in the presence of negative feedback, the competition of two CDC42 clusters can change from antagonistic winner-takes-all to oscillatory, out-of-phase coexistence--the mechanism which was proposed to explain the discovery of the tip-to-tip CDC42-GTP oscillations in fission yeast^{12,178,179}.

[H2] Pulsed contractions

Pulsed contractions driven by focal activation and accumulation of myosin-2 and F-actin are a common feature of developing animal embryos, where they drive cell and tissue shape changes¹⁸⁰⁻¹⁸⁴ and contribute to polarization via advection¹⁸⁵. The contractions are generally ascribed to transient, localized bursts of Rho activity which result in F-actin polymerization via formins¹⁸⁶ and myosin-2 filament assembly via ROCK (ref. ¹⁸⁷).

Pulsed contractions are particularly striking in early *C. elegans* embryos, where they engage in a complex interplay with Par proteins to help specifying the developmental fate of the blastomeres^{185,188}. In a recent study¹³, the mechanism of pulsed contractions was analyzed in the *C. elegans* embryos via a combination of TIRF microscopy and single molecule tracking. These approaches made it possible to distinguish between the contributions of actin and myosin filament assembly, disassembly, and contraction to the formation of individual pulses (Figure 2). Pulses have a periodicity of ~30s and are initiated by focal Rho activation. Rho activation was dependent on positive feedback (Figure 4b), based on both pulse kinetics and Rho depletion which showed that pulsing behaviour requires the active Rho concentration to exceed a certain threshold. Rho-dependent myosin-2 and F-actin filaments accumulate in pulses ~5-6 s after Rho activation. Rho activity begins to fall coincident with the recruitment of F-actin and myosin-2 and before the onset of contraction, suggesting that the loss of Rho activity is contraction-independent. This point was confirmed by myosin-2 depletion, which failed to arrest Rho pulsing although it prevented contraction. Rather, loss of Rho activity was driven by a delayed negative feedback loop based on F-actin and RGA-3/4, two functionally redundant Rho GAPs¹⁸⁹: RGA-3 was recruited to pulses coincident with loss of Rho activity and depletion of RGA-3/4 resulted in an arrest of pulsing and uniformly high cortical levels of Rho activity¹³. Further, RGA-3 colocalized with F-actin within

pulses, and pharmacological disruption of F-actin resulted in loss of cortical RGA-3 as well as cessation of pulsing. An activator-inhibitor model based on Rho positive feedback and delayed negative feedback through F-actin and RGA-3/4 captured all of the features of pulse dynamics, leading the authors to conclude that pulsed contractions are governed by a self-organizing cortical network.

Pulsed contractions based on focal myosin-2 activation are not restricted to embryos, but have also been observed in a variety of cultured cell types¹⁹⁰, where they have been linked to processes such as focal adhesion, stress fibre formation^{191,192} and endocytosis¹⁹³. In a recent study of U2OS cells, it was found that myosin-2 pulses which developed spontaneously were associated with and dependent on a self-organizing network that controls Rho activity¹⁸ (Figure 2): myosin-2 and Rho pulses were dependent on the Rho GEF GEF-H1 and direct positive feedback from active Rho to GEF-H1 was demonstrated via expression of GEF-H1 with a nonfunctional Rho-GTP binding site (Figure 4b). Delayed negative feedback occurred via two motor proteins: the unconventional myosin-9, which has a C-terminal Rho GAP domain, and by myosin-2 itself. The period of the Rho-GTP waves was ~80s, and dual label imaging and cross-correlational analysis revealed that the formin FHOD1 was recruited ~6 sec after Rho-GTP. F-actin (the presumptive recruiter of myosin-9) recruitment occurred ~11 sec after Rho-GTP, and myosin-2 recruitment occurred ~40 s after Rho-GTP¹⁸. Strikingly, the amplitude of the oscillatory pulses was dependent on the elasticity of the extracellular matrix, demonstrating that the signalling network was capable of responding to external signals. In a follow-up study¹⁹⁴, chemo-optogenetic targeting¹⁹⁵ and modelling were employed to further probe the relationship between GEF-H1 and Rho-GTP. Experimental recruitment of Rho-GTP to the plasma membrane was sufficient to recruit GEF-H1, directly confirming that this GEF and Rho-GTP engage in positive feedback. Graded release of GEF-H1 from mitochondrial sequestration via optogenetics demonstrated that, while a minimum level of GEF-H1 was necessary for periodic pulses, an excess of GEF-H1 reduced wave amplitude, a result that modelling revealed to be dependent on myosin-2-based noise.

[H2] Traveling waves

Traveling waves of cortical F-actin are associated with a variety of dynamic cellular phenomena^{53,196}. Such waves propagate by new actin assembly at their leading edge (i.e., the front of the wave) and disassembly of actin at their trailing edge (i.e., the back of the wave). While in many cases their upstream control mechanisms are unclear, traveling actin waves are often generated by complementary waves of Rho GTPase activation and inactivation^{15,17}.

Traveling actin waves can assume a variety of forms, one of which is the actin “coat”. Actin coating refers to the process wherein secretory granules become enveloped by F-actin after fusing with the plasma membrane¹⁹⁷. Coating is associated with exocytosis of secretory granules that contain bulky, insoluble content¹⁹⁷ and has been observed in exocytosis of cortical granules in frog eggs¹⁹⁸, pancreatic¹⁹⁹ and salivary²⁰⁰ acinar cells, pancreatic beta-cells²⁰¹, alveoli²⁰², and endothelial cells²⁰³. Coating is triggered by rapid, fusion-dependent²⁰⁴ activation of Rho GTPases on the membrane of exocytic granules^{197,200,205}. Following fusion, the active GTPases recruit a variety of effectors^{197,200,206-208} that direct accumulation of F-actin, myosin-1 (ref. ²⁰⁹) and myosin-2^{203,205,206}. Once formed, the coat contracts and compresses

the granule, which powers expulsion of the granule contents and initiates retrieval of the granule membrane to maintain plasma membrane homeostasis^{200,201,210,211}.

How is coat contraction coupled to Rho-GTP dynamics? This question was addressed using the *Drosophila* salivary gland, where exocytosis of so-called glue proteins is accompanied by and dependent on actin coating²⁰⁰. As expected, Rho activation is essential for coat formation and contraction. Accordingly, Rho suppression impairs recruitment of F-actin, ROCK and myosin-2²⁰⁰. More interestingly, however, inhibition of myosin-2 or ROCK (refs.^{88,200}) doesn't simply stall coat contraction. Instead, it results in oscillatory cycles of Rho activation and inactivation and consequent F-actin accumulation on and loss from the fused vesicles, with the cycles of the same length as normally required to attain full coat contraction⁸⁸. This observation suggested delayed negative feedback, prompting a screen for coat-localized Rho GAPs. The screen identified C-GAP, which was recruited to exocytic granules ~5 seconds after F-actin. Further, C-GAP recruitment was F-actin dependent, as was Rho inactivation (Figure 4c). Strikingly, suppression of C-GAP expression resulted in the arrest of coat contraction, although active Rho and the actin coat persisted, demonstrating that Rho *inactivation* as well as Rho activation are required for coat contraction. Based on these results, a model was proposed in which granule compression results from a traveling wave of Rho-GTP activity that is chased by a wave of negative feedback in the form of F-actin-dependent recruitment of C-GAP⁸⁸.

Single traveling GTPase waves are also evident during plasma membrane repair. In this process, damage to the plasma membrane triggers local activation of Rho, CDC42, Rac, or all three in model systems including yeast²¹², worms²¹³, flies¹⁴, frogs²⁴, and cultured human muscle cells²¹⁴. Rho GTPase signalling during plasma membrane repair has been most intensely studied in frog oocytes²¹⁵ and syncytial fly embryos²¹⁶. In these large cells, live cell imaging using Rho GTPase GBD activity reporters²⁴ (Box 1), or directly labeled Rho GTPases^{14,35} (Box 1) has shown that the GTPases are activated within ~20-60 s of plasma membrane damage and then organize into concentric zones of activity. The zone of RhoA borders the wound edge, and CDC42 and RAC1 activity form broader zones that circumscribe the Rho zone (Figure 2). Not surprisingly, activation of the GTPases is dependent on wound-recruited GEFs^{92,217}, although Rho may also be activated via wound-induced production of reactive oxygen species²¹⁴. The complementary GTPase zones direct the formation of a contractile actomyosin-based ring that closes over the wound site in concert with the GTPase zones. This results in repair of the cortical cytoskeleton and expulsion of material damaged by the wound^{24,218-220}.

Studies in frog oocytes show that movement (closure) of the Rho GTPase zones around wounds arises from the fact that the CDC42 and Rho zones are actually circular traveling waves that move at ~80 nm/sec with preferential GTPase activation at their leading edges¹⁷. The leading-edge activation of the GTPase drives the zones forward, even under conditions where actomyosin-based contraction is completely suppressed. The trailing edge of the Rho zone is defined by a ~3-fold higher rate of inactivation where it abuts the CDC42 zone¹⁷. At the same time, the CDC42 zone is apparently limited by direct extraction of active CDC42 by RhoGDI³⁵. Modelling and imaging results^{221,222} show that the zones self-organize near the wound edge due to spatially restricted bistability, such that within the area around the wound, Rho or

CDC42 activity is stable in one of only two states—high activity within the zones or low activity outside the zones. The bistability results from positive feedback: positive feedback within the Rho zone arises from Rho-GTP-dependent recruitment of the dual GEF-GAP, ABR (Figure 4c)⁹². ABR has an N-terminal GEF domain that, *in vitro*, targets RhoA, CDC42 and RAC1, and a C-terminal GAP domain that targets Rac and CDC42, but not Rho (ref.⁹³). The basis for the positive feedback within the CDC42 zone is unclear. The spatial restriction of the feedback to the wound-proximal region is thought to be due to the formation of a wound-induced “playing field”—a region within which bistability is enabled. This is likely based on elevated intracellular free calcium and protein kinase C-beta^{24,35,93,220,223}. Segregation of the Rho and Cdc42 zones results from reciprocal negative crosstalk between Rho and CDC42: the GAP activity of ABR suppresses CDC42 activity in the Rho zone. Accordingly, such expression of a GAP-dead ABR results in CDC42 invading the Rho zone while ABR overexpression expands the Rho zone at the expense of the CDC42 zone⁹². Conversely, the CDC42 zone suppresses Rho activity, apparently by recruiting a GAP. This was inferred by the higher inactivation rate of Rho in the CDC42 zone¹⁷, and by the demonstration that CDC42 suppression spreads and intensifies the Rho activity zone²²⁴. Thus, the Rho zone (wave) is chased by a wave of CDC42-dependent negative feedback.

Traveling Rho GTPase waves are also evident during cell division, but as wave trains (multiple traveling waves in the same cell) rather than single waves. One example is provided by cytokinesis which, in animal cells, is initiated by the activation of Rho in a narrow zone at the equatorial cortex^{23,225,226} and, at least in some systems, suppression of Rac activity in the same region²²⁷. Rho activation results from spindle-mediated concentration of the Rho GEF ECT2 on the equatorial plasma membrane^{5,43,228}. ECT2 localization is controlled at least in part by its interaction with the centralspindlin complex, which comprises the microtubule motor MKLP1 and MgcRacGAP (aka Cyk4)^{225,229}. The role played by MgcRacGAP is complex and controversial²³⁰ but, at a minimum, it contributes to cytokinesis by localizing ECT2 (refs.^{225,229}), and suppressing activation of Rac in the equatorial cortex^{227,231,232} while also somehow promoting ECT2 activation^{233,234}, and maintaining Rho activity within a focused, narrow zone⁶⁹.

High spatiotemporal resolution imaging in starfish embryos revealed that the Rho zone is constituted from traveling Rho activity waves that first appear in anaphase and are rapidly amplified and concentrated at the equatorial cortex by the mitotic spindle¹⁵ (Figure 2). The Rho-GTP waves have a period of ~60-80s and give rise to and are chased by waves of F-actin that likewise concentrate at the equatorial cortex. The waves persist as the formation of the cytokinetic furrow is initiated, and then eventually transition into a state in which Rho activity is more uniformly high as the furrow deepens. Modelling and experiments indicated that the waves reflect a self-organizing, activator-inhibitor system wherein ECT2 and Rho-GTP are responsible for positive feedback¹⁵. This conclusion is supported by the observation that ECT2 is allosterically activated by binding to Rho-GTP via its PH domain⁸⁰. In what was at the time a major surprise, the negative feedback was found to be F-actin dependent¹⁵. Similar results were obtained in *Xenopus* embryos, with the added feature of high amplitude waves of F-actin that persist throughout the cell cycle but which are excluded from the equatorial cortex as the cytokinetic Rho-GTP waves

develop^{15,235}. In both starfish and frog, the waves are regulated not only by the mitotic spindle per se, but also by Cdk1 activity independently of the spindle^{15,235}.

Recently, the basis of the F-actin-dependent negative feedback in cytokinetic Rho waves was shown to rely on RGA-3/4 (ref. ¹¹¹) (Figure 4c). RGA-3/4 was previously implicated in negative Rho regulation during cytokinesis in human cells and *C. elegans*^{236,237}. In both starfish and frog, RGA-3/4 was shown to form waves that chase Rho-GTP waves in an F-actin dependent manner; like the Rho-GTP waves, the RGA-3/4 waves are focused and amplified at the equatorial cortex during anaphase. Moreover, ectopic expression of ECT2 and RGA-3/4 in immature frog oocytes, which do not naturally display excitable cortical waves^{15,111}, was sufficient to induce high amplitude, traveling waves of Rho-GTP that are chased by waves of F-actin and RGA-3/4. A model based on activation-inhibition captured not only the basic features of the cytokinetic waves, but also the more complex wave dynamics seen in immature oocytes. Reconstitution of cortical excitability in vitro using frog egg extract on supported lipid bilayers produced stationary Rho activity pulses and solitary propagating waves, further supporting the self-organized nature of cortical excitability²¹.

Wave trains are not restricted to embryos but have also been observed in adherent mammalian cells. Studies of Rho GTPase dynamics in a cultured mammalian mast cell model (RBL cells) revealed that this cell type generates self-organized oscillatory pulses (standing waves) and traveling waves of CDC42 activity in response to antigen exposure in interphase¹⁹. In metaphase, traveling CDC42 waves are also observed (Figure 2). These are accompanied by low amplitude traveling Rho activity waves or pulses²⁰. The mitotic CDC42 waves are regulated by cell adhesion and have been linked to spindle position control in these cells²⁰ while the mitotic Rho waves may be related to those involved in cytokinesis in other cell types (see above).

Recently, mitotic Rho dynamics were analyzed in detail in nocodazole-treated RBL cells, a manipulation that both increases Rho wave amplitude due to activation of GEF-H1 (ref. ²³⁸) and arrests the cells in M-phase, permitting a detailed analysis of Rho wave control mechanisms²³⁹. Mitotic Rho waves varied dramatically in terms of period and amplitude in different cells in the population, with long period (~3 min), high amplitude waves in some cells, and short period (~30s), low amplitude waves in others. In a limited number of cells, mixed mode waves were observed, in which the fast and slow wave cycles were superimposed. By manipulating the levels of specific phosphoinositides, it was found that different wave types could be interconverted, allowing the contribution of different feedback relationships to wave dynamics to be deduced: the fast waves are regulated by PI3K and PIP(3,4,5)3 via an activator-inhibitor mechanism (see Box 2), while the slow waves are regulated by PI(4)P via an activator-depleted substrate mechanism (see Box 2) in which PI(4,5)P2 is the rate-limiting consumed substrate. Exactly how these phospholipids regulate Rho remains to be determined, but GEF-H1 and ECT2, the two Rho GEFs likely to be involved have been previously linked to PI3K (ref. ²⁴⁰) and PI(4,5)P2 (ref. ²⁴¹), respectively. More importantly, the results show that different classes of feedback (activator-inhibitor and activator-depleted substrate) can produce quantitatively different GTPase patterns in the same cell and, based on the presence of mixed mode oscillations, can coexist, resulting in different oscillation patterns occurring at the same time.

[H2] Cell-cell junction self-organization and epithelial homeostasis.

Vertebrate epithelial cells are linked by tight junctions, which provide barrier function, and adherens junctions, which mechanically integrate the cells. These junctions are linked to a contractile apical actomyosin network that supports epithelial tissue integrity and drives cell shape changes. In order to maintain epithelial homeostasis, cell-cell junctions must dynamically respond to changes in tissue tension, cell density, and other insults such as tissue damage or disease that threaten homeostasis.

Live cell imaging using Rho GBD activity reporters^{108,242} or a FRET sensor²⁴³ (Box 1) revealed that a zone of Rho activity encircles the apical surface of each epithelial cell, regulating the contractility of the apical actomyosin bundle. Both adherens junctions and tight junctions are sites of complex and dynamic Rho GTPase signalling^{223,244,245}. The normal balance of junctional Rho GTPase activity is maintained by a long list of GEFs, GAPs, and scaffolding proteins that act in different epithelial contexts^{245,246}. In the adherens junctions of both mammalian and frog epithelial cells, the key regulators include several proteins originally identified as conserved cytokinesis regulators: the Rho GEF Ect2 (ref. ²⁴⁷), the centralspindlin component MgcRacGAP (ref. ²⁴⁸), and the scaffolding protein anillin^{242,249}. Despite the outwardly static appearance of the junctional Rho zone, recent work³⁰ indicates that the turnover of Rho within zone is quite rapid ($t_{1/2}$ of ~ 1 s) and revealed that one factor that contributes to junctional Rho dynamics is anillin, the knockdown of which reduced the half-life of junctional Rho even further³⁰. Anillin locally concentrates PI(4,5)P₂ in the plasma membrane, which increases the membrane retention of active Rho, allowing for extended downstream signalling from active Rho to its effectors and their targets³⁰. Another mechanism that regulates junctional Rho dynamics is a complex but fascinating feedback mechanism in which junctional myosin-2 maintains a balance of Rho activation and inactivation via ROCK-dependent phosphorylation of the noncanonical Rho GTPase, Rnd3 which, in turn, modulates the recruitment of p190BRhoGAP to the junctions¹⁰⁸. A model based on diffusion counteracted by myosin-2 powered advection explains how the tight localization of Rho-GTP to the junction is maintained in the face of rapid turnover²⁵⁰.

In addition to the zone of Rho activity that supports cell-cell junction homeostasis, the tight junctions of the frog embryonic epithelium also exhibit local, transient bursts (or “flares”) of Rho activation^{242,251}. These Rho flares occur in response to the mechanical strain imposed by developmentally controlled cell shape changes, which cause local tight junction leaks. The Rho flares direct the repair of the junctions by promoting local accumulation of actomyosin, and are initiated by stretch-induced opening of mechanosensitive calcium channels²⁵². The flares are short-lived (~ 3 min) and their kinetics strongly suggest fast positive feedback and delayed negative feedback. Consistent with this hypothesis, the GEF responsible for Rho flares, p115RhoGEF (ref. ²⁵³), was previously shown to engage in direct positive feedback with Rho-GTP via its PH domain⁷⁹. Additionally, ROCK inhibition resulted in repeating cycles of Rho activation and inactivation²⁵¹, suggesting that ROCK-dependent delayed negative feedback is involved.

[H2] Context dependency of Rho GTPase pattern formation

Two important points emerge from consideration of these examples. First, there is no “typical” way in which cells implement feedbacks during Rho GTPase signalling.

For example, during yeast budding, the positive feedback is effector-dependent and involves GEF stimulation, during cytokinesis it is direct and involves GEF stimulation, and during junctional assembly it is effector target-dependent and acts through inhibition of a GAP. And these are merely the mechanisms that have been well-characterized. Further, while several of the examples of negative feedback are F-actin dependent, this likely reflects the fact that rapid manipulation of F-actin is relatively straightforward, making it comparatively easy to test the role of F-actin in feedbacks. Certainly, there are many other mechanisms for negative feedback (Table 1). Second, simply looking at signalling network diagrams for Rho GTPase regulation such as those presented in Figure 4 does not permit one to predict what kind of pattern will form. The diagrams for *C. elegans* polarization and embryonic cytokinesis are identical but one produces pulsed contractions and the other wave trains throughout the cell cortex. Similarly, the mechanism for *C. elegans* polarization resembles that for actin coating but the latter produces single waves that wrap around exocytic secretory granules rather than cortical contractions. This ambiguity extends to finer levels of mechanistic detail, in that the impact on patterning of parameter manipulations such as raising or lowering feedback strength or altering the abundance of downstream GTPase targets is highly context dependent. For example, increasing the Rho GAP to Rho GEF ratio in immature frog oocytes promotes a transition from pulses to waves, while the same manipulation in starfish oocytes reduces wave amplitude¹¹¹.

[H1] Benefits of self-organizing Rho GTPase patterns

The examples presented above represent organisms from six different phyla, prompting the question, what are the evolutionary benefits that arise from self-organized Rho GTPase patterns? Some benefits of self-organization based on positive and negative feedback are well-known, such as the ability to respond quickly or to filter out noise, which is likely to be important for cells to interpret and filter internal and external inputs during processes such as cell migration, when cells must navigate complex environments²⁵⁴, or plasma membrane repair, where cells must be able to respond within seconds to damage²⁵⁵.

The examples suggest another benefit, namely, pattern (and thus response) variation. That is, the same core players can produce very different patterns in different systems as in pulsed contractions in *C. elegans* and cytokinesis in starfish and frog embryos which both rely on Rho, F-actin, Ect2, and RGA-3/4 (refs. ^{13,111}). Further, even within a single cell, engaging the same core players, considerable pattern diversity is possible. The manipulations in RBL cells demonstrate that modulation of different phosphoinositide lipids produce dramatic differences in wave properties²³⁹ while in U2OS cells increasing the expression of GEF H1 promotes formation of Rho-GTP waves over pulses¹⁸. Similarly, increasing expression of RGA-3/4 against a constant level of Ect2 in immature frog oocytes produces a dramatic progression from pulses, to trains of short-lived waves, to persistent spiral waves¹¹¹, while the pulsed contractions in *C. elegans* embryos can be pushed toward or away from wavelike behaviour by manipulation of a Rho effector levels²⁵⁶. And even under conditions where the same players are present in the same cell, pattern transitions can happen via redistribution of one or more of the players to a different subcellular location. For example, modelling results indicate that the transition from waves of Rho-GTP and F-actin to a stationary cytokinetic zone of

Rho-GTP and F-actin can arise simply from the spindle-dependent concentration of Ect2 on the equatorial cortex past some critical threshold⁵⁴.

There are also less intuitive advantages that can arise from self-organized patterns such as those that produce periodic behaviour. Perhaps the best example is provided by pulsed contractions (although similar arguments would apply to activity waves). As described above, pulsed contractions based on F-actin and myosin-2 are common outcomes of Rho GTPase signalling in both developing organisms and cultured cells and, in the former, are harnessed to drive tissue morphogenesis. In many cases, such pulses are associated with “ratcheting” in which each pulse results in a reduction of the apical domain of the cell or one side of the apical domain of the cell and subsequent pulses result in further reductions¹⁸⁰. The net effect is tissue bending in a manner that minimizes competition between contracting cells—a contracting cell does not have to overcome neighbor contractions to achieve a cell shape change²⁵⁷. Similarly, empirical and modelling studies indicate that spatially and temporally heterogeneous contractile events are important during collective cell movement²⁵⁸ and junctional shortening²⁵⁹ in epithelial cells. modelling work also indicates that pulsed contractions permit developing systems to maintain persistent, large scale contractions in the face of local disconnections or breakages in the contractile network²⁶⁰. Additionally, pattern transitions induced by increasing negative feedback can, paradoxically, increase the amplitude of local Rho GTPase activity¹¹¹ meaning that more local contractility could be possible in the presence of a Rho GAP than in its absence. Moreover, despite their seeming simplicity, self-organized patterns can template very complex dynamic cytoskeletal arrays that are nonetheless remarkably resilient^{218,219}.

Lastly, there is an additional, broader benefit of self-organization that may be particularly important for the Rho GTPases, given that they control the cell’s contractile machinery—actin filaments and myosin-2. This machinery can be enormously powerful, such that when it is improperly harnessed, it can literally tear the cell to pieces²⁶¹. Yet somehow this same machinery must be employed to drive processes that require considerable precision, such as splitting the cell in half between the separating chromosomes or compressing a secretory granule following exocytosis. And somehow, the contractile machinery must do these things in exactly the right place and time, generally with a minimum of delay. Self-organization makes this possible, by both poising the cell to respond to diverse signals, and by ensuring that the output of the contractile machinery is modulated in a manner appropriate to the task at hand.

[H1] Conclusions and Future Directions

The examples of self-organized Rho GTPase signalling presented above represent the tip of the iceberg, as the selection criteria applied were stringent. Nonetheless, other examples of what are likely to be self-organized Rho GTPase patterns include: frustrated phagocytosis^{262,263}, invadopodia²⁶⁴, periodic pulses of RhoB on internal membranous compartments²⁶⁵, adhesive actin waves²⁶⁶, apical constriction in *Drosophila* embryonic epithelial cells¹⁸⁷, formation of microridges on the apical surface of epithelial cells^{267,268}, plant cell patterning by ROPs²⁶⁹, and, of course, the many examples of cell migration that are associated with dynamic Rho GTPase patterns but whose control circuitry awaits characterization²⁷⁰⁻²⁷⁴.

In short, available evidence indicates that Rho GTPase self-organization is not simply a curiosity restricted to a select few cell types or situations but rather a fundamental feature of Rho GTPase regulation and function. Because the self-organization dictates the Rho GTPase patterns, and the Rho GTPase patterns dictate the outcome for the cortex and the cell, an emphasis on Rho GTPase self-organization is obviously warranted. That is, if we are to understand mechanistically how Rho GTPases contribute to complex cellular processes such as cytokinesis, cell migration, and morphogenesis, it will no longer be enough to simply suppress their function. Rather, we will need to understand their pattern forming mechanisms with sufficient level of mechanistic detail to permit the manipulation of these patterns.

With this view in mind, several research directions are likely to be especially important. First, increased attention to high temporal resolution study of Rho GTPase dynamics may reveal that apparently stationary patterns are actually periodic. Second, an increased emphasis on feedback circuitry and in particular, negative feedback, is likely to be fruitful. Third, continued imaging tool development for both the Rho GTPases themselves^{41,42} and their regulators²⁷⁵, will be essential, particularly since there is increased interest in studying Rho GTPase dynamics in more complex samples, such as tissues, which are likely to be more challenging than single cells²⁷⁶⁻²⁷⁸. Fourth, more structural and biochemical analyses of GEFs and GAPs will be needed to identify and characterize novel feedback mechanisms¹¹⁰. Finally, increased application of single molecule imaging¹³ and reconstitution approaches^{21,223} are likely to provide much-needed information about how, exactly, Rho GTPase cycles are executed.

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Table 1: Feedbacks to Rho GTPase GEFs and GAPs

Regulator	GTPase	Feedback From	Feedback Type	Refs
GEFs				
P190 RhoGEF; p115 RhoGEF; PRG; LARG; GEF-H1; LBC	Rho	Rho-GTP	Direct, Positive	79,90,91
Ect2	Rho	Rho-GTP	Direct, Positive	80
Abr	Rho	Rho-GTP	Direct, Positive	92,93
Dock180	CDC42/Rac	CDC42-GTP	Direct, Positive	81
P190 RhoGEF	Rho	Rac-GTP	Direct, positive, crosstalk	90
Beta-Pix	CDC42/Rac	CDC42-GTP	Direct, positive, crosstalk	94
ArgGEF7	CDC42/Rac	Coronin-1a	Effector, positive	87
Intersectin	CDC42/Rac	N-WASP	Effector, positive	84,86
LARG	Rho	Dia1	Effector, positive	95
Trio	Rho	Filamin	Effector, positive	96
Prex2	CDC42/Rac	Pak1	Effector, negative	97
Dock180	CDC42/Rac	PIP3	Target, positive	98
Dock2	CDC42/Rac	PIP3	Target, positive	99
Dock4	CDC42/Rac	PIP3	Target, positive	100
Fgd1	CDC42/Rac	Cortactin	Target, positive	101
Beta-Pix	CDC42/Rac	Paxillin	Target, positive	102
Tiam1	CDC42/Rac	Arp2/3	Target, positive	103
Trio8	CDC42/Rac	SESTD1	Effector, negative	104
Prex1	CDC42/Rac	Pak1	Effector, negative	105
GEF-H1	Rho	F-actin, MYOSIN-9	Target, negative	18
GEF-H1	Rho	Myosin-2	Target, negative	18
Beta-Pix, Vav, Tiam1, Dbs	CDC42/Rac	Myosin-2	Target, negative crosstalk	106
GAPs				
ArhGAP18	Rho	PKN	Effector, positive	107
P190RhoGAP	Rho	Rnd3	Effector, positive	108
ArhGAP15	CDC42/Rac	Pak1, Pak2	Effector, negative	109
ArhGAP9, 12, 15, 27, 32, 33	CDC42/Rac	G-actin	Target, negative	110
RGA-3/4	Rho	F-actin	Target, negative	13,111
Ophn1	Rho Rac	F-actin	Target, negative	112
ArhGAP12,25	CDC42/Rac	PIP3	Target, negative	113
ArhGAP15	CDC42/Rac	PIP3	Target, negative	109
Abr	CDC42/Rac	Rho-GTP	Direct, negative crosstalk	92,93

Figure Legends

Figure 1. Basic principles of Rho GTPase Regulation. **a.** Schematic diagram depicting the Rho GTPase cycle. Rho GTPase inserts into the plasma membrane via its carboxyterminal prenyl group. Activation of Rho GTPase (ie exchange of GDP for GTP) results from interaction with a GEF. Active GTPase can then bind to an effector resulting in changes in the cortical cytoskeleton. GTPase inactivation results from interaction with a GAP and is followed by extraction of the GTPase from the plasma membrane by RhoGDI, rendering the GTPase soluble in the cytoplasm. **b.** The activation-centric view of Rho GTPase signalling. In this view, the path from the stimulus to the response is essentially linear, with the stimulus activating a GEF, the GEF activating the GTPase, and the active GTPase directing the response, while the contributions of GAPs to the response by GAPs are considered to merely limit or terminate the response. **c.** The Self-Organizing view of Rho GTPase signalling. In this view, the path from the signal to the response is highly non-linear, with the stimulus activating both the GEF and the GAP resulting in continuous GTPase cycling and self-organization of the GTPases into patterns which dictate the response. PM = plasma membrane; R-GDP = Rho GTPase (Rho, Rac or CDC42) bound to GDP; R-GTP = Rho GTPase bound to GTP; GAP = GTPase activating protein; GDI = RhoGDI = guanine nucleotide displacement inhibitor; GEF = guanine nucleotide exchange factor.

Figure 2. Self-organizing Rho GTPase patterns. **a.** Pulsed contractions in *C. elegans* embryo. Top: single frame from TIRF movie showing Rho-GTP (green) and myosin-2 (red); anterior end of the embryo is on the left; posterior on the right. Bottom: kymograph derived from embryo in top panel; T = time; D = distance; total elapsed time is 200 s. Pulses are evident in the kymograph as streaks which on average move toward the anterior end of the embryo over time. Rho activity rises before myosin-2 in the contractions. **b.** Mitotic CDC42-GTP wave in RBL cell from a TIRF movie. Image shows a composite of three successive timepoints with each time point colored differently to reveal movement—red (t=0s), blue (t=4s), green (t=8s). The image captures a target pattern wave (ie one that forms from a spot and spreads outward from the spot) of CDC42 activity. **c.** Experimentally induced Rho-GTP and F-actin waves in frog oocytes. Single frame from timelapse light sheet movie showing traveling wave trains of Rho-GTP (cyan) chased by F-actin (red) from frog oocyte expressing the Rho GEF Ect2 and the Rho GAP RGA-3/4. Both target and spiral wave patterns are evident. **d.** Pulsed contractions in U2OS cell. Left: series of images from timelapse TIRF movie of nocodazole-treated U2OS cell showing Rho-GTP (green) and myosin-2 (magenta); images taken 30 s apart. Right: kymograph corresponding to white arrow on leftmost image; elapsed time is 770 s. Rho activity rises ahead of myosin-2 recruitment in the pulsed contractions **e.** Traveling waves of Rho GTPase activity in wounded frog oocyte. Top: Single frame from confocal movie of wounded frog oocyte showing CDC42-GTP wave (red) and Rho-GTP wave (cyan); the CDC42-GTP wave encircles the Rho-GTP wave. Bottom: kymograph from cell depicted in top panel; elapsed time is 240s. Single waves of Rho-GTP and CDC42-GTP converge on the wound. **f.** Cytokinetic Rho and F-actin waves in starfish blastomere. Left: Single frame from confocal movie of dividing starfish blastomere showing Rho-GTP (green) and F-actin (orange). The cell is undergoing cytokinesis and the Rho-GTP and F-actin waves are confined to

the equatorial cortex. Right: kymograph taken from area indicated by box on left. Elapsed time is 960 s. Furrow waves of Rho-GTP and chasing F-actin waves are evident as angled lines. **a** provided courtesy of John Michaux and Ed Munro, U. Chicago. **b** provided courtesy of Cheesan Tong and Min Wu, Yale university. **c**. provided courtesy of Ani Michaud, Promega Corp. **d** provided courtesy of Melanie Graessl, Perihan Nalbant, and Leif Dehmelt, University of Duisburg and Technical University of Dortmund. **e** provided courtesy of Lila Hoachlander-Hobby, University of Wisconsin-Madison. **f** provided by the authors.

Figure 3. Feedbacks via Rho GTPase GEFs and GAPs. **a.** Schematic diagram showing overview of known mechanisms of positive and negative feedback of Rho GTPases acting through their GEFs and GAPs. Feedback is considered positive if the end result is an increase in the activity of the Rho GTPase; negative if the end result is a decrease in the activity of the GTPase. Thus, stimulation of a GEF by its target GTPase is considered positive feedback while stimulation of a GAP by its target GTPase is considered negative feedback; Rho=Rho, Rac, or Cdc42; D = Direct feedback (active GTPase binds GEF); E = Effector-based feedback (effector binds or modifies GEF or GAP); T = effector target-based feedback (downstream target of effector binds to or modifies GEF or GAP). Positive interactions indicated by arrows with pointed ends; negative interactions indicated by arrows with flat ends. See table 1 for specific examples. **b.** Example mechanisms of the different classes of feedback. Direct feedback: an active Rho GTPase binds allosterically to its GEF via the PH domain in the GEF, thereby targeting it to the plasma membrane and exposing the active site (DH) which can then activate an inactive GTPase. Effector-based feedback: an active GTPase binds an effector which binds a GEF or GAP, targeting it to the plasma membrane. Effector target-based feedback: an active GTPase stimulates an effector which promotes formation of (in this case) F-actin which, in turn targets the GEF or GAP to the plasma membrane. ***Direct feedback has only been described for positive feedback; effector-based feedback and effector target-based feedback can be either positive or negative.***

Figure 4. Proposed self-organizing feedbacks for examples of self-organization presented in text. Pointed arrows indicate positive regulatory interactions; flat-headed arrows indicate negative regulatory interactions. R-GTP = active Rho; R-GDP = inactive Rho; C-GTP = active CDC42; C-GDP = inactive CDC42. ? = players assumed but not yet identified. For each example, the upstream signal is indicated at the top and the pattern produced is indicated at the bottom. Circled plus signs (cyan) indicate positive feedback loops; circled minus signs (red) indicate negative feedback loops. **a.** Formation of the polarizing CDC42 cluster in budding yeast relies on at least one positive feedback (via CDC42-GTP to Bem1 to the GEF Cdc24) and two negative feedbacks (from CDC42-GTP to septins and the GAP Bem2 and CDC42-GTP to F-actin cables and secretory vesicles). **b.** Pulsed contractions in *C. elegans* may arise from direct positive feedback from Rho-GTP to the GEF Ect2 and from negative feedback from Rho-GTP to F-actin to the GAP RGA-3/4. Pulsed contractions in U2OS cells arise from direct positive feedback from Rho-GTP to the GEF GEF-H1 and two negative feedbacks: from Rho-GTP to myosin-2 which inhibits GEF-H1 and from Rho-GTP to F-actin to the GAP myosin-9. **c.** Traveling waves during actin coating of secretory vesicles arise from negative feedback from Rho-GTP to F-actin to the GAP, C-GAP; the basis of positive feedback has yet to be identified. Traveling waves during plasma membrane repair arise from positive

feedback from Rho-GTP to the dual GEF-GAP, ABR; ABR is also responsible for negative cross talk from Rho-GTP to CDC42-GTP and participates in positive feedback for Rho. CDC42-GTP is responsible for negative cross talk to Rho-GTP by an as yet unidentified Rho GAP. Traveling waves during embryonic cytokinesis arise from direct positive feedback from Rho-GTP to the GEF, Ect2 and from negative feedback from Rho-GTP to F-actin which engages in negative feedback with the GAP, RGA-3/4. **d.** Homeostasis in adherens junctions relies on positive feedback from Rho-GTP to ROCK and myosin-2 which negatively regulate Rnd3 which positively regulates the GAP, p190RhoGAP. The basis of the negative feedback has not been identified. Homeostasis in tight junctions is restored following junction stretching via positive direct positive feedback from Rho-GTP to p115 Rho GEF. The basis of negative feedback is unknown but it may be dependent on ROCK.

Box 1: Live cell imaging approaches for Rho GTPases

Three general approaches have been used for live cell imaging of Rho GTPases. One is expression of N-terminally-tagged fusion proteins^{28,29} (C-terminal tagging prevents prenylation) (figure part a). This technique is simple and permits fluorescence recovery after photobleaching or photoactivation to monitor GTPase turnover³⁰. However, it does not distinguish between active and inactive GTPases and in some cases the fusion proteins do not properly reflect normal Rho GTPase localization or function³¹⁻³³. Additionally, expression of exogenous Rho GTPases can upset the stoichiometric balance of the Rho GTPases with RhoGDI resulting in aggregation and degradation of the GTPases³⁴. Insertion of the fluorescent protein into an exposed surface loop of the GTPase (sandwich or internal tagging) improves localization and function^{31,32,35} and the balance with RhoGDI can be maintained by either gene replacement or co-expression with RhoGDI³⁵.

Förster resonance energy transfer (FRET)-based probes^{27,36,37} are more complex, in that they typically contain the GTPase of interest, a GTPase binding domain (GBD) that binds specifically to the activated GTPase, and two fluorescent proteins, one a donor and one an acceptor (figure part b). When the GTPase within the probe is activated by a GEF, the fluorescent proteins are brought together, permitting the donor to excite the receptor thus generating FRET fluorescence. Comparing the local ratio of donor fluorescence to acceptor fluorescence reveals areas where the probe is preferentially activated. These probes can be GTPase subtype specific (distinguishing, for example, RhoA from RhoB) and do not interfere with the function of the endogenous Rho GTPase. However, they often have limited dynamic range, making it difficult to visualize the FRET signal against background and do not report on the endogenous GTPase activity per se, but rather the local GEF availability²⁷. Their dynamic range can be improved by modifications in probe design³⁸.

Fluorescent GBDs work by recruitment from the cytoplasm to areas of high GTPase activity at membranes^{22,24-26} (figure part c). They are simple to use and report on endogenous, active GTPases. However, at high levels they interfere with endogenous GTPase function and they do not distinguish between GTPase subtypes (e.g. RhoA vs. RhoB). Moreover, their utility in different cell types varies widely³⁹. Their performance can be improved by total internal reflection microscopy or confocal microscopy^{18,19,40}, by tight control of expression¹⁸, and by increasing the number of GBDs or fluorophores per probe^{39,41,42}. In two recent, and very useful studies^{41,42} mammalian cell-based assays were used to vet a variety of GBDs. The interested reader is strongly encouraged to read these studies before embarking on Rho GTPase imaging in living cells.

Box 2 Self-Organizing Rho Patterns

Self-organization is a process in which a disordered system spontaneously acquires some form of order, such as a pattern, due to interactions between system parts. Self-organized patterns commonly arise as a result of combined positive and negative feedback. One well-studied example of how positive and negative feedback give rise to self-organized patterns is the “activator-inhibitor” system (figure part a). In activator-inhibitor systems, an activator (in this case, the Rho GTPase in its active, GTP-bound state) stimulates its own activation via positive feedback (e.g. by stimulation of a GEF) while also stimulating the production of an inhibitor (I) which

antagonizes the activity of the GTPase via negative feedback (e.g. by stimulation of a GAP). A variation of this theme is the “activator-depleted substrate” system in which negative feedback arises not from an inhibitor, but from consumption of a limiting substrate needed for activation of the activator. Because the active GTPase is produced from the inactive GTPase, active and inactive forms of any GTPase always make an activator-depleted substrate pair.

To avoid having the feedbacks simply cancel each other out, they operate on different length or time scales. For example, if the inhibitor diffuses faster than the activator, stationary patterns such as stripes or spots can be produced as the activator (and thus positive feedback) becomes confined to islands surrounded by seas of fast-moving inhibitor (figure part b). A common way to analyze such patterns over time is by use of kymographs (also known as “space-time plots”). Kymographs are generated by making a very narrow slice (represented by a dotted line) on a movie file and then collecting one slice for each time point of the movie. The slices are then positioned next to each other in order, like a montage but without the border. One arrow (T) indicates which axis is time; the other (D) indicates which axis is distance (or space). Stable patterns will appear as vertical stripes in the kymograph; moving patterns will be angled in the kymograph.

If the production of the inhibitor (and negative feedback) is delayed relative to the positive feedback, a variety of dynamic patterns can be produced such as waves or oscillatory pulses (figure part c). Here, the wave of activator moves away from a wave of inhibitor into regions free of inhibitor (i.e., the inhibitor “chases” the activator). For activator-substrate depletion systems, waves can form as the activator moves away from areas of substrate depletion into areas of high substrate concentration.

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