

Spatio-temporal Rho GTPase signaling – where are we now?

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Summary

Rho-family GTPases are molecular switches that transmit extracellular cues to intracellular signaling pathways. Their regulation is likely to be highly regulated in space and in time, but most of what is known about Rho-family GTPase signaling has been derived from techniques that do not resolve these dimensions. New imaging technologies now allow the visualization of Rho GTPase signaling with high spatio-temporal resolution. This has led to insights that significantly extend classic models and call for a novel conceptual framework. These approaches clearly show three things. First, Rho GTPase signaling dynamics occur on micrometer length scales and subminute timescales. Second, multiple subcellular pools of one given Rho GTPase can operate simultaneously in time and space to regulate a wide variety of morphogenetic events (e.g. leading-edge membrane protrusion, tail retraction, membrane ruffling). These different Rho GTPase subcellular pools might be described as ‘spatio-temporal signaling modules’ and might involve the specific interaction of one GTPase with different guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and effectors. Third, complex spatio-temporal signaling programs that involve precise crosstalk between multiple Rho GTPase signaling modules regulate specific morphogenetic events. The next challenge is to decipher the molecular circuitry underlying this complex spatio-temporal modularity to produce integrated models of Rho GTPase signaling.

Key words: Rho GTPase, Fluorescence resonance energy transfer, Live-cell imaging, Cell motility, Systematic approaches

Introduction

Rho GTPases are key regulators of cytoskeletal dynamics in a wide variety of morphogenetic events, such as cell migration, axonal guidance, vesicle trafficking, cytokinesis and endocytosis (Etienne-Manneville and Hall, 2002). They can also control many other functions, such as cell-cycle progression and gene expression. Rho GTPases cycle between an active GTP-loaded and an inactive GDP-loaded state; this cycle is controlled by their interaction with guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). In the activated form, they are competent in binding to numerous effector proteins, which leads to the activation of myriad downstream signals. Their ability to regulate so many different functions, in highly dynamic cellular contexts, implies that they are tightly regulated at the spatio-temporal level.

However, most of what we know about Rho GTPase signaling has been based on a set of ‘classic’ Rho GTPase tools that do not faithfully reflect this level of regulation. These include the use of dominant-positive (DP) or -negative (DN) mutants of the three canonical Rho GTPases: Rac1, Cdc42 and RhoA. These mutants have been used to interfere with many different cellular processes. Another tool is biochemical pulldown assays, which enable the measurement of the average activation status of a given GTPase in a population of cells. These approaches have clearly established that Rho GTPases are master regulators of the cytoskeleton in many different cellular contexts. They have also shown that each GTPase can regulate a plethora of additional processes, such as microbe killing through regulation of NADPH oxidase by Rac1 (Bokoch and Zhao, 2006) and gene transcription through the serum-factor pathway by RhoA (Posern and Treisman, 2006).

In the context of the regulation of the cytoskeleton during cell migration, the use of these different tools has led to the conceptual framework that Rac1 regulates membrane protrusion at the leading

edge, Cdc42 controls filopodia formation and cell polarization, and RhoA controls contractility at the back of the cell (BurrIDGE and Wennerberg, 2004). However, many lines of evidence support an intrinsically more complicated picture of Rho GTPase signaling during this process. For example, in contrast to the clear effects of the overexpression of DN mutants, cells in which Rac1 (Wheeler et al., 2006) or Cdc42 (Czuchra et al., 2005) have been genetically ablated can still protrude lamellipodia and produce filopodia, and only display very mild defects in cell migration. This suggests that the contributions of Rac1 and Cdc42 to the process of cell migration are much more subtle than we initially thought. Another example is the finding that RhoA is linked not only to the generation of contractility, but also to the regulation of microtubule stabilization at the leading edge during directional cell migration (Bartolini and Gundersen, 2009; Palazzo et al., 2001). Rather than involving Rho kinase, this specific RhoA function requires stabilization of microtubules through the effector mDia. RhoA therefore most probably performs different functions at the front and at the back of migrating cells, undoubtedly through selective interactions with different downstream effectors. Such controversial results have led to considerable confusion in the Rho GTPase field and illustrate some of the limitations of the classic Rho GTPase toolkit.

The development of technologies such as fluorescent probes that report Rho GTPase signaling in time and space has yielded important new insights that call for a novel conceptual framework for Rho GTPase signaling. I will first briefly comment on the design of different Rho GTPase biosensors and some experimental considerations when imaging them. I will then concentrate on a series of examples that illustrate the newly revealed complexity of spatio-temporal Rho GTPase signaling compared with the classic conceptual framework. Having this in mind, I will discuss the

problems that are inherent to the classic Rho GTPase toolkit and the need for new approaches to the dissection of the molecular basis of these complex spatio-temporal signaling events.

Rho GTPase activation biosensors: experimental considerations and design

The first problems to consider when imaging the spatio-temporal dynamics of Rho GTPase activation are the low dynamic range of activation of these proteins and their subcellular localization. Unlike Ras GTPases, for which up to 40% of the cellular pool can be activated by a physiological stimulus (Sato and Kaziro, 1992), only a very small fraction of the total Rho GTPase pool is activated at one time in the cell. Even with a potent stimulus that leads to robust phenotypical changes, biochemical pulldown assays show that, at most, 5% of the total pool of RhoA is activated at any one time in a cell (Ren et al., 1999). Similar values are found for Rac1 and Cdc42 (Benard et al., 1999). Furthermore, in contrast to Ras GTPases, which are constitutively membrane anchored, most Rho GTPases partition between the cytosol and membranes because of their interaction with the Rho guanine dissociation inhibitor (RhoGDI) (Michaelson et al., 2001). This is problematic for imaging because the fluorescence signal of the large cytosolic pool of inactive GTPases will mask any of the subtle events of membrane recruitment and activation of the GTPase. This explains why simple subcellular localization of a GTPase using immunostaining or green fluorescent protein (GFP) fusions cannot reveal its activation. This illustrates the need for sensors that specifically detect the location at which Rho GTPases are activated. A large variety of such sensors have been developed over the past ten years for that purpose. Importantly, different biosensor designs might necessitate different microscopy and image-analysis techniques, and exhibit specific advantages and disadvantages. Thus, depending on the specific aspect of Rho GTPase signaling under analysis or the limitations imposed by the model cell system to be imaged, one might have to choose between the different biosensors that are available.

The most minimalist design is a simple fusion of an effector domain with GFP. This construct will specifically translocate to regions where a given GTPase is activated (Fig. 1A). These protein fusions can be transfected or recombinantly expressed, and used as affinity reagents for staining. This strategy has been successfully used to show dynamic Cdc42 and RhoA GTPase activation patterns in a *Xenopus laevis* oocyte wound-healing model system (Benink and Bement, 2005), to monitor the pattern of RhoA inactivation during basement-membrane breakdown in an avian model of gastrulation (Nakaya et al., 2008) and to localize RhoA activation during the genesis of the ciliated epithelium of *Xenopus* embryos (Park et al., 2008). However, one caveat of such approaches is that effector domains often bind to and will report activation of multiple GTPases. For example, the effector domain of p21-activated kinase (PAK) binds to GTP-Rac1 and GTP-Cdc42 (Benard et al., 1999). The effector domain of WASP binds with high affinity to GTP-Cdc42, but can also bind with lower affinity to GTP-Tc10 (a small Rho family GTPase) (Hemsath et al., 2005). In this case, the effector domain is most probably Cdc42 specific in vivo. Finally, the effector domain of rhotekin binds to GTP-RhoA, GTP-RhoB and GTP-RhoC (Ren et al., 1999).

To solve this specificity problem, a variety of more sophisticated fluorescent probes have been engineered. A bimolecular fluorescence resonance energy transfer (FRET) sensor (Fig. 1B) has enabled the successful imaging of the location of Rac1

activation (Kraynov et al., 2000). In this case, the FRET signal occurs when a microinjected bacterially expressed domain of PAK that is labeled with an Alexa-Fluor-546 dye locally interacts with active, exogenously expressed GFP-labeled Rac1. Because the acceptor and donor fluorophores are bound to separate proteins that might have different subcellular distributions, contributions to the FRET image from donor-emission 'bleed through' and direct excitation of the acceptor make the quantification of FRET complicated. A fraction of these contributions must therefore be subtracted from the raw FRET image; this will depend on the specific filters used and must be quantified for each imaging system. This strategy has nonetheless been successful multiple times and can also be performed with genetically encoded fluorophores such as CFP and YFP (Hoppe and Swanson, 2004).

To facilitate FRET quantification, a set of unimolecular probes (Fig. 1C,D,E) has been designed in which the donor and acceptor fluorophores are fused within a single chain. As both fluorophores are then identically distributed throughout the cell, the contributions of donor bleed through and direct acceptor excitation are the same wherever the probe is analyzed. Thus, the corrections that are necessary with bimolecular probes are not needed and the FRET signal can be quantified as a simple ratio of FRET:donor emission.

Different unimolecular probe designs also exist. In the 'Raichu' probes (Fig. 1C), a Rho-binding domain (RBD) and RhoA are connected by a linker, and sandwiched between donor and acceptor fluorophores (Yoshizaki et al., 2003). A structural constraint therefore imposed by this design is that the C-terminal polybasic CAAX sequence of RhoA has to be fused to one of the GFP fluorophores, making the biosensor unable to interact with RhoGDI and targeting it constitutively to the plasma membrane. These probes can therefore monitor the effects of GEFs and GAPs at the membrane, but will miss any regulation exerted by RhoGDI at the level of cytosol-to-membrane shuttling. To resolve this issue, another Raichu probe was designed, in which the RBD effector domain is flanked with donor and acceptor fluorophores (Fig. 1D) (Yoshizaki et al., 2003). In this case, binding of endogenous active RhoA will trigger a conformational change in the RBD and loss of FRET. Raichu probes for Rac1 and Cdc42 with a design similar to the two discussed above also exist (Itoh et al., 2002). Another unimolecular probe design sandwiches the donor and acceptor fluorophores between the RBD and RhoA, leaving the RhoA C terminus free for correct geranyl-geranylation and reversible interaction with membranes (Fig. 1E) (Pertz et al., 2006).

Finally, an alternative to the FRET approach is to derive a recombinantly expressed effector domain with a solvatochromic dye that will undergo a change in fluorescence intensity in response to solvent polarity (Fig. 1F) (Nalbant et al., 2004). When the derivatized Cdc42-binding domain (CBD) interacts with endogenous GTP-loaded Cdc42, the solvatochromic dye will display an increase in fluorescence. The ratio of GFP to merocyanine dye fluorescence will therefore highlight regions where Cdc42 is active. Note that, as alluded to above, for all single-effector-domain probes (Fig. 1A,D,F), one might not be able to discern the active conformations of multiple GTPase isoforms. Further reviews about the different strategies used to build such biosensors are discussed in detail elsewhere (Hodgson et al., 2008; Pertz and Hahn, 2004), as are the different experimental considerations for imaging them (Hodgson et al., 2006).

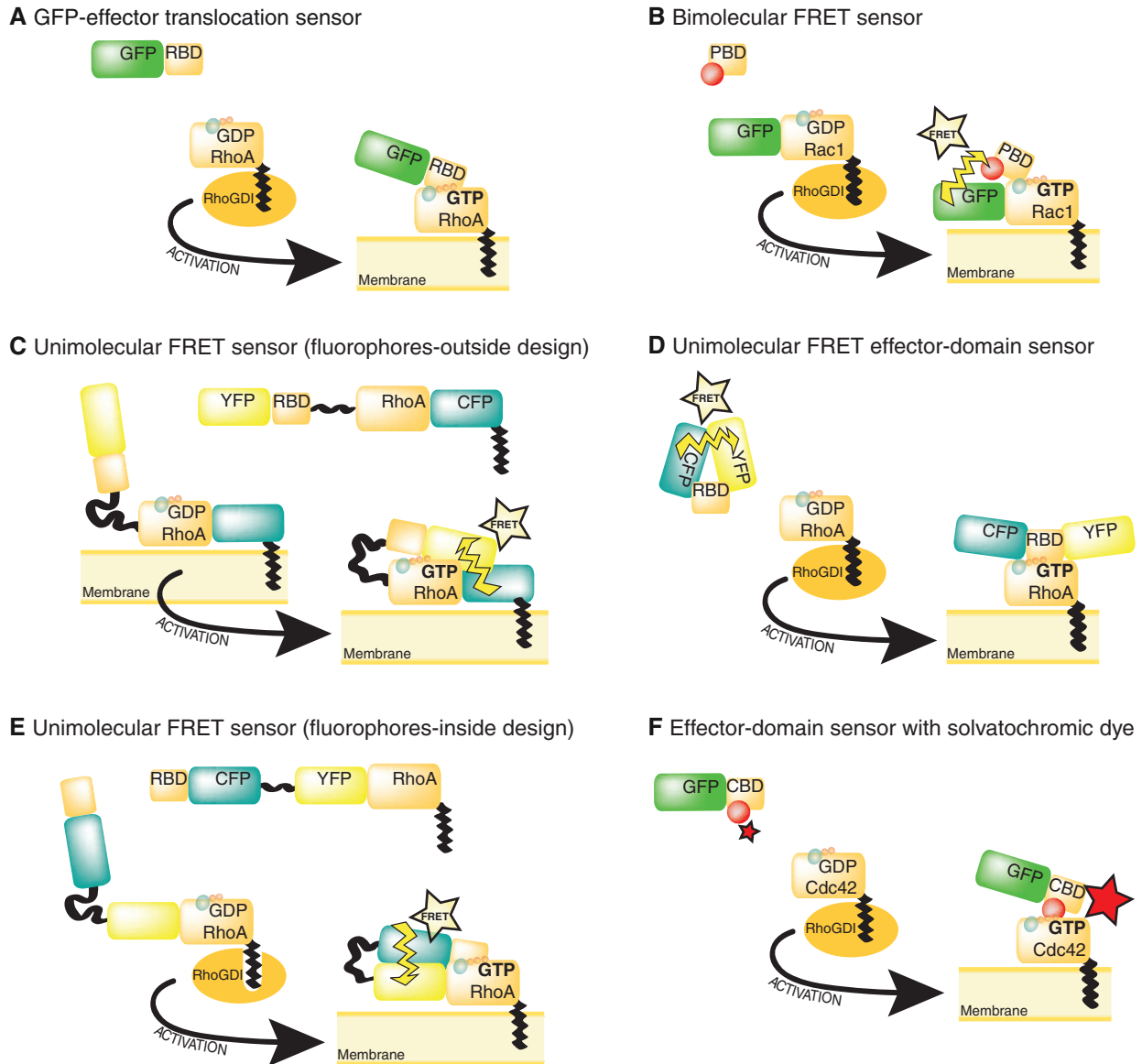
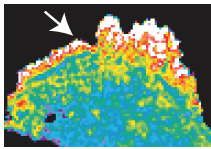


Fig. 1. Design of different Rho GTPase activation biosensors. (A) GFP-effector translocation biosensor. (B) Bimolecular FRET Rac sensor. The red circle represents a dye that was covalently coupled to the PAK-binding domain (PBD). (C,D) Raichu unimolecular FRET RhoA sensors. (E) Unimolecular FRET RhoA sensor with a fluorophores-inside design. (F) Solvatochromic-dye-based sensor. The red circle represents a solvatochromic dye that was coupled to the Cdc42-binding domain (CBD) of WASP. The red star represents the increased fluorescence emission upon binding of the probe to active Cdc42. RBD, Rho-binding domain of rhotekin.

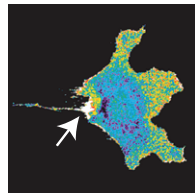
Spatio-temporal modularity of Rho GTPase signaling

The first report of a Rho GTPase FRET-based probe was published in 2000 (Kraynov et al., 2000). This probe allowed the spatio-temporal dynamics of Rac1 activation to be measured during cell migration. Not surprisingly, the probe revealed that Rac activation was confined to the leading lamellae and peripheral ruffles of migrating fibroblasts. Similar results were observed with Rac biosensors based on other designs (Itoh et al., 2002). However, when the same probe was imaged in chemotactic neutrophils, Rac activation was observed in the retracting uropod, in addition to the leading edge, suggesting that Rac can also function at the back of the cell in this context (Gardiner et al., 2002). Probes that revealed

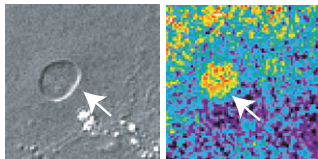
the subcellular location of active Cdc42 again reported activity at the leading edge (Itoh et al., 2002; Nalbant et al., 2004); however, depending on the cellular context, Cdc42 activity was also clearly observed simultaneously in the Golgi compartment (Nalbant et al., 2004). Surprisingly, despite the prediction that RhoA is activated in the cell body and at the back of the cell to control acto-myosin contractility (Burrige and Wennerberg, 2004), and consistent with a role for RhoA at the leading edge (Cook et al., 1998), FRET probes that reported RhoA activation (Kurokawa et al., 2005; Pertz et al., 2006) revealed that the bulk of RhoA activation occurs directly at the leading edge of protruding lamellae in fibroblasts (Fig. 2A). However, transient bursts of RhoA activation were also simultaneously observed at the back of the cell during robust

A Signaling modules: RhoA**RhoA module 1:**
GEF 1, GAP 1, effector 1

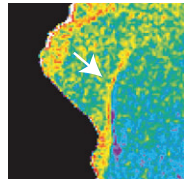
Membrane protrusion

RhoA module 2:
GEF 2, GAP 2, effector 2

Tail retraction

RhoA module 3:
GEF 3, GAP 3, effector 3

Macropinocytosis

RhoA module 4:
GEF 4, GAP 4, effector 4

Membrane ruffling

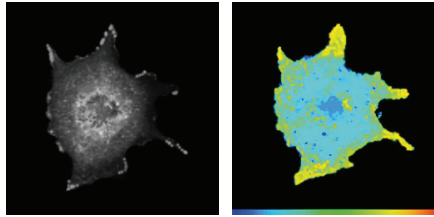
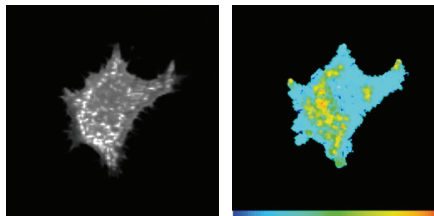
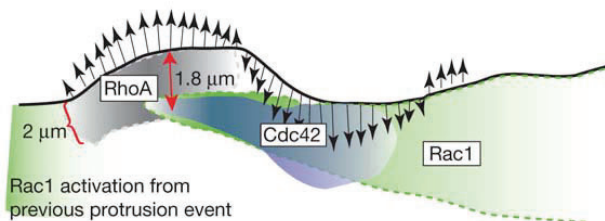
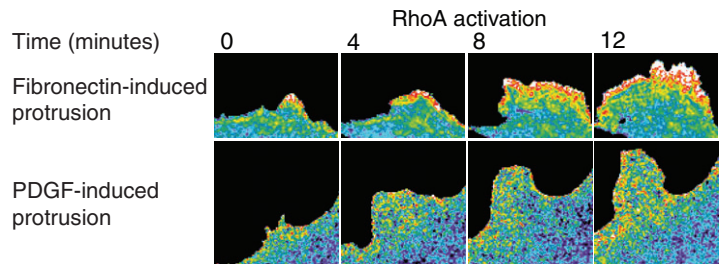
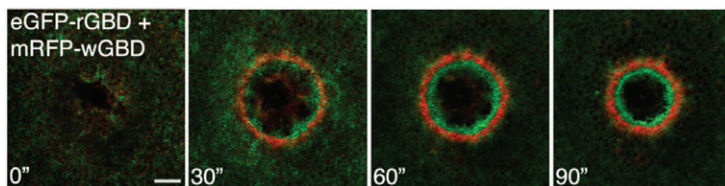
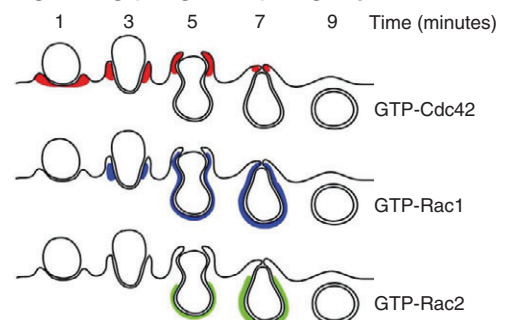
B Signaling modules: Cdc42GFP-PAK FLIM signal:
GFP-PAK / Cy3-Cdc42**Cdc42 module 1:**
GEF 1, GAP 1, **PAK1**
Membrane protrusionGFP-N-WASP FLIM signal:
GFP-N-WASP / Cy3-Cdc42**Cdc42 module 2:**
GEF 2, GAP 2, **N-WASP**
Endosomal traffic**C Signaling program: membrane protrusion****D Different signaling programs in membrane protrusion****E Signaling program: oocyte wound healing**Active Rho
Active Cdc42**F Signaling program: phagocytosis**

Fig. 2. Visualizing spatio-temporal Rho GTPase signaling. (A) RhoA activation patterns during cell migration, as visualized with a FRET RhoA sensor (Pertz et al., 2006). Different morphogenetic behaviors are shown. The RhoA activation signal is coded so that 'warm' and 'cold' colors represent regions of high and low activation, respectively. White arrows point to the specific subcellular zones at which RhoA is activated. Image reproduced from Pertz et al. (Pertz et al., 2006) with permission. (B) Differential coupling of Cdc42 with different effectors at different subcellular locations. The interaction of a GFP-tagged effector and Cy3-labeled Cdc42 is measured using FLIM (Parsons et al., 2005). The images are color coded so that warm colors represent regions where each effector interacts with Cdc42 (red and yellow, interaction with effector; blue, no interaction). Image reproduced from Parsons et al. (Parsons et al., 2005) with permission. (C) Model of the dynamic spatio-temporal activation profiles of Rac1, Cdc42 and RhoA during leading-edge membrane protrusion (Machacek et al., 2009). The black line represents the leading-edge boundary, and arrows indicate membrane protrusion and retraction behavior. Different-colored zones represent RhoA (grey), Cdc42 (blue) and Rac1 (green) activation. Image reproduced from Machacek et al. (Machacek et al., 2009) with permission. (D) Different RhoA GTPase signaling patterns during membrane protrusion induced by fibronectin or PDGF stimulation (Pertz et al., 2006). Images are color coded as in A. Image reproduced from Pertz et al. (Pertz et al., 2006) with permission. (E) Cdc42 and RhoA activation zones during wound healing of a *Xenopus* oocyte. eGFP-rRBD, GFP fusion to the effector domain of rothekin; mRFP-wGBD, mRFP fusion to the effector domain of WASP. Image reproduced from Benink and Bement (Benink and Bement, 2005) with permission. (F) Model of Cdc42, Rac1 and Rac2 activation during phagocytic uptake of an IgG-opsonized erythrocyte (Hoppe and Swanson, 2004). Each different-colored zone represents the activation pattern of one specific GTPase. Image reproduced from Hoppe and Swanson (Hoppe and Swanson, 2004) with permission.

tail-retraction events. Further zones of RhoA activation were observed on peripheral ruffles and on actively moving macropinosomes (Fig. 2A).

In addition to the surprising finding that direct measurement of the subcellular location of Rho GTPase activation sometimes contradicts the conceptual framework derived from the classic Rho GTPase toolkit, many other novel insights are evident. One important lesson is that one Rho GTPase can be simultaneously activated at multiple, discrete subcellular locations, where it seems to regulate distinct cellular functions that are important for cell morphogenesis (Fig. 2A). Most likely, each of these individual pools of active Rho GTPase represents the specific interaction of a GTPase with different upstream regulators, such as GEFs and GAPs, and distinct downstream effectors to regulate precise morphogenetic cell behavior. This becomes evident when the interaction of one GTPase with two different downstream effectors is visualized using FRET and fluorescence lifetime imaging (FLIM) (Parsons et al., 2005). In breast cancer cells, the interaction of active Cdc42 with its PAK effector occurs at the cell periphery, especially in cell protrusions. However, the interaction of active Cdc42 with its N-WASP effector occurs within an endosomal compartment that localizes throughout the cell (Fig. 2B). This high degree of specificity probably occurs through the formation of precise macromolecular complexes in time and space.

Consistently, there are several cases in which multiple components of the Rho GTPase signaling machinery are found in large macromolecular complexes. For example, the Rac1- and Cdc42-specific GEF Pix has been found to bind efficiently to the effector PAK (Obermeier et al., 1998). During neutrophil chemotaxis, G protein $\beta\gamma$ subunits that have been liberated by chemoattractants acting on the G_i heterotrimeric G protein can recruit a complex containing Pix, PAK and Cdc42 to the leading edge; this is essential for directional sensing and persistence of polarized cell migration (Li et al., 2003). Here, PAK functions both as a scaffold for the recruitment of Pix and as a target of Cdc42, with both interactions enabling the formation of an autoactivation loop. This suggests that this protein complex can work as a discrete signaling unit. Similar complexes have been described between the Rac-specific GEF Tiam1 and the effectors IRSp53 and Wave2 (Connolly et al., 2005), and between Tiam1, the effectors Par3, Par6 and aPKC (atypical protein kinase C), and the GTPase Cdc42 (Mertens et al., 2005; Pegtel et al., 2007). Thus, an attractive hypothesis is that preformed macromolecular complexes allow the coupling of GEFs with specific effectors upon interaction with an activated GTPase, enabling the spatio-temporal modularity of Rho GTPase signaling that is obvious from imaging methods. Such spatio-temporal signaling complexes that operate in time and space to regulate specific morphogenetic events (leading-edge protrusion, tail retraction, etc.) might be referred to as 'spatio-temporal signaling modules'.

In the context of single-cell biology, this suggests that we can no longer have a 'Rho-GTPase-centric' vision of Rho GTPase signaling, in which one GTPase performs one specific function. Rather, we now have to discern different Rho-GTPase-dependent functions that operate in time and space. The challenge that lies ahead is thus to define the molecular composition of these spatio-temporal signaling modules, which consist of specific Rho GTPases, GEFs, GAPs, effectors and additional interacting proteins that operate upstream or downstream of the given GTPase. At the same time, we must also gain insight into the mechanistic function of each of these modules. Importantly, this provides a conceptual

framework that can explain the finding that GEFs, GAPs and effectors outnumber their GTPase targets (Bishop and Hall, 2000; Moon and Zheng, 2003; Rossman et al., 2005).

Spatio-temporal Rho GTPase signaling programs

Another insight provided by spatio-temporal Rho GTPase measurements is that complex spatio-temporal signaling programs that involve precise crosstalk between multiple GTPases are put in place by the cell to fine-tune specific morphogenetic events. For example, in the context of cell migration, FRET probes and biochemical experiments clearly indicate that all three canonical Rho GTPases are activated at the leading edge of migrating fibroblasts or other cells (Cho and Klemke, 2002; Itoh et al., 2002; Kravynov et al., 2000; Kurokawa et al., 2005; Nalbant et al., 2004; Pertz et al., 2006). This then begs the question of how crosstalk occurs between all three GTPases in time and space to regulate the leading-edge extension process. Using computer vision approaches to merge spatio-temporal measurements of RhoA, Rac1 and Cdc42 activation performed in different cells, and using different biosensors, it was possible to identify the time and location of the activation of the three GTPases during fibroblast leading-edge membrane protrusion. This revealed a characteristic pattern of GTPase activation in highly confined subcellular domains, on the order of micrometers, and occurring with precise time shifts, on the order of tens of seconds (Machacek et al., 2009) (Fig. 2C). In these fibroblasts, leading-edge advancement occurs in cycles of protrusion and retraction, with one cycle lasting 90 seconds. RhoA is activated directly at the leading edge at the onset of membrane protrusion, but is switched off during and uncoupled from membrane retraction. Rac1 and Cdc42 are switched on later during the protrusion phase, and persist longer during retraction. Antagonistic crosstalk between the GTPases is obvious, as zones of Rac1 and Cdc42 activation lag behind and are mutually exclusive with the zone of RhoA activation that occurs directly at the leading edge. This illustrates the complexity of Rho GTPase signaling during membrane protrusion and challenges the notion that Rac1 and Cdc42 are the initiators of membrane protrusion in this precise cellular context.

An additional level of complexity came from the discovery that there are different modes of Rho GTPase signaling at the leading edge induced by different extracellular cues (Pertz et al., 2006). The complex spatio-temporal Rho GTPase signaling program discussed above, which involves precise activation of all three GTPases, was observed in fibroblasts randomly migrating on a fibronectin matrix (Machacek et al., 2009) and therefore probably depends on integrin signaling. However, when the same fibroblasts are induced to extend protrusions through stimulation by platelet-derived growth factor (PDGF), virtually no RhoA activation is observed in membrane protrusions (Kurokawa et al., 2005; Pertz et al., 2006) (Fig. 2D). Thus, additional signaling through a receptor tyrosine kinase seems to induce a different Rho GTPase signaling program that might lead to cell migration with distinct morphodynamics. This remains to be tested, but will require the establishment of an assay that challenges fibroblasts with gradients of PDGF to induce robust chemotactic behavior. A similar signaling program is also evident in specialized chemotactic cells, such as neutrophils, in which RhoA activation is absent from the leading edge and is observed solely in the retracting uropod and on peripheral ruffles (Wong et al., 2006). Most likely, Rac1 and Cdc42 are the sole players in the process of leading-edge extension in these two cell-migration systems.

A complex Rho GTPase signaling program was also observed during wound healing in single *Xenopus* oocytes (Benink and Bement, 2005). Rapid accumulation of F-actin filaments is triggered around the wound to allow its closure. Multiplexed activation measurements revealed dynamic, mutually exclusive RhoA and Cdc42 activation zones that move inward as the actin array closes (Fig. 2E). This process depends on calcium and is controlled by input from the cortical actin cytoskeleton and microtubules, and crosstalk between the GTPases themselves. Another important example is the phagocytic uptake of IgG-opsonized erythrocytes (Hoppe and Swanson, 2004). Highly defined zones of Rho GTPase activation occur and correlate with different stages of the formation and maturation of the phagocytic cup. In the first phase of particle binding, Cdc42 is activated within a subcellular domain that localizes to a band of actin that triggers pseudopod extension (Hoppe and Swanson, 2004) (Fig. 2F). Shortly delayed Rac1 activation then occurs at the same location. During the subsequent phase of phagosome contraction and closure, Rac1 is activated a second time on the inner phagosomal membrane; Rac2 is concurrently activated at the base of the phagosome.

These different studies clearly define the scales of time and length at which the signaling events regulating different morphogenetic processes occur, and illustrate the need to analyze these signaling events with adequate spatio-temporal resolution. Importantly, the common practice of analyzing single snapshots of the signaling state of a given Rho GTPase, even with high spatial resolution, cannot provide a clear understanding of Rho GTPase signaling. Rather, the analysis of the whole continuum of spatio-temporal Rho GTPase activation is needed. For each morphogenetic event, it will be important in the future to identify the players (GTPase, GEF, GAP and effector) that operate within each spatio-temporal signaling module and examine how crosstalk occurs between these modules within the relevant signaling program. Understanding which effectors are operating downstream of each GTPase will explain the specific contribution of each signaling module to a specific morphogenetic process. Identification of the GEFs, GAPs and their upstream signaling networks relevant to each signaling module will allow us to understand the complex crosstalk between each GTPase. In that respect, the observation of antagonistic RhoA and Cdc42 activation zones during both fibroblast leading-edge membrane protrusion (Machacek et al., 2009) and oocyte wound healing (Benink and Bement, 2005) suggests that there are conserved mechanisms to set up such spatio-temporal Rho GTPase signaling programs. This might be the real significance of the classic antagonistic relationship between Rac1, Cdc42 and RhoA that has been documented using global Rho GTPase signaling manipulation (Nimmual et al., 2003; Sander et al., 1999).

The limits of global manipulations and measurements

Although the classic Rho GTPase toolkit has enabled us to understand the foundations of Rho GTPase signaling, the fine modularity that is evident from spatio-temporal Rho GTPase measurements is not necessarily compatible with most of the insights provided by the global measurements and manipulations that have been performed until now. Given the spatial and time scales on which different Rho GTPase signaling modules and programs operate, it becomes clear that the global manipulations imposed by the classic Rho GTPase toolkit might also induce artifacts.

Overexpression of a DP Rho GTPase mutant should, in principle, lead to substantial signaling by all the effectors expressed in a cell that usually operate within the different spatio-temporal signaling modules that depend on this GTPase (Fig. 1A). One can therefore ask why global manipulation of the signaling activity of a given Rho GTPase does not give information about all the functions of the different effectors activated by this GTPase, rather than inducing the highly specific cytoskeletal phenotypes that are typically observed (e.g. overexpression of DP RhoA leads to a robust contractile phenotype, but does not reveal a role for leading-edge extension). The most obvious explanation is that, in contrast to the *in vivo* situation (in which the levels of active Rho GTPases are tightly regulated and occur in highly defined subcellular regions), when a Rho GTPase is globally activated, its effectors might aberrantly signal outside of the spatio-temporal context of their specific signaling modules. In this situation, the contributions of some effectors might then simply be dominant over others and there will thus be bias towards a specific phenotype. Therefore, the robust contractile phenotype induced by RhoA hyperactivation, which occurs through its effector Rho kinase, might simply be stronger than and mask any contributions from other RhoA-dependent effectors that are linked to membrane protrusion or other functions. Importantly, when cells are induced to have high RhoA, but low Rho kinase activity, the contributions of RhoA to membrane protrusion are obvious (Tsuji et al., 2002). Therefore, the classic phenotype induced by DP RhoA might not be very informative about all the functions that depend on this GTPase.

Because they most probably act by titrating active GEFs, overexpression of DN Rho GTPase mutants has the potential to affect several pathways and thus will globally interfere with multiple spatio-temporal signaling modules. Furthermore, because many GEFs can activate multiple GTPases (Rossman et al., 2005), a DN GTPase mutant might target more GTPases than the one it intends to. This provides an explanation for the clear differences in cell-migration phenotype observed when fibroblasts devoid of Cdc42 or expressing DN Cdc42 are compared (Czuchra et al., 2005). In fact, any global manipulation, such as overexpression of GEFs, GAPs and effectors in wild-type or mutant form, is likely to switch Rho GTPase regulation from local to global to some extent, leading the overexpressed signaling component to function outside of the proper context of its precise spatio-temporal signaling module. In the case of upstream regulators (GEFs, GAPs), this might lead to the non-discriminant activation of multiple signaling modules, rather than only one. In the case of effector pathways, the downstream signaling events will no longer be restricted to the precise subcellular zones where they should, in principle, occur.

Finally, biochemical pulldown assays, which measure an average of the signaling states of thousands of cells, will also not be able to capture the underlying spatio-temporal complexity of Rho GTPase signaling. These experiments often require that the signaling state of the cell population be homogenized by overly gross stimuli that are unlikely to be physiologically relevant. Again, this might switch Rho GTPase signaling from a local to a global mode, leading to large-scale effects that mask any of the more subtle spatio-temporal patterns that are important for the regulation of cell morphogenesis.

Spatio-temporal signaling modularity and the need for systematic approaches

If Rho GTPase signaling involves the precise, coordinated action of multiple signaling modules in space and time, then the correct

description of a given cell behavior will require insight into all the players operating in each of the signaling modules. This will require systematic approaches to identify and characterize all the components of the multiple signaling modules that cooperate to regulate a given cellular process (cell migration, neurite outgrowth, phagocytosis, etc.). As suggested above, this will not be accessible with global measurements and manipulations using most of the commonly available tools, but will require more subtle perturbations. This can, in principle, be achieved by individually

targeting the different players in the multiple signaling modules that regulate a specific morphogenetic process using loss-of-function approaches (i.e. gene-knockout or RNA-interference approaches).

A panel of recent studies illustrates such systematic approaches to studying Rho GTPase signaling modularity. In 3D environments, tumor cells can switch between mesenchymal or amoeboid modes of invasion, which depend on high levels of Rac1 or RhoA activation, respectively. To identify the specific players that achieve

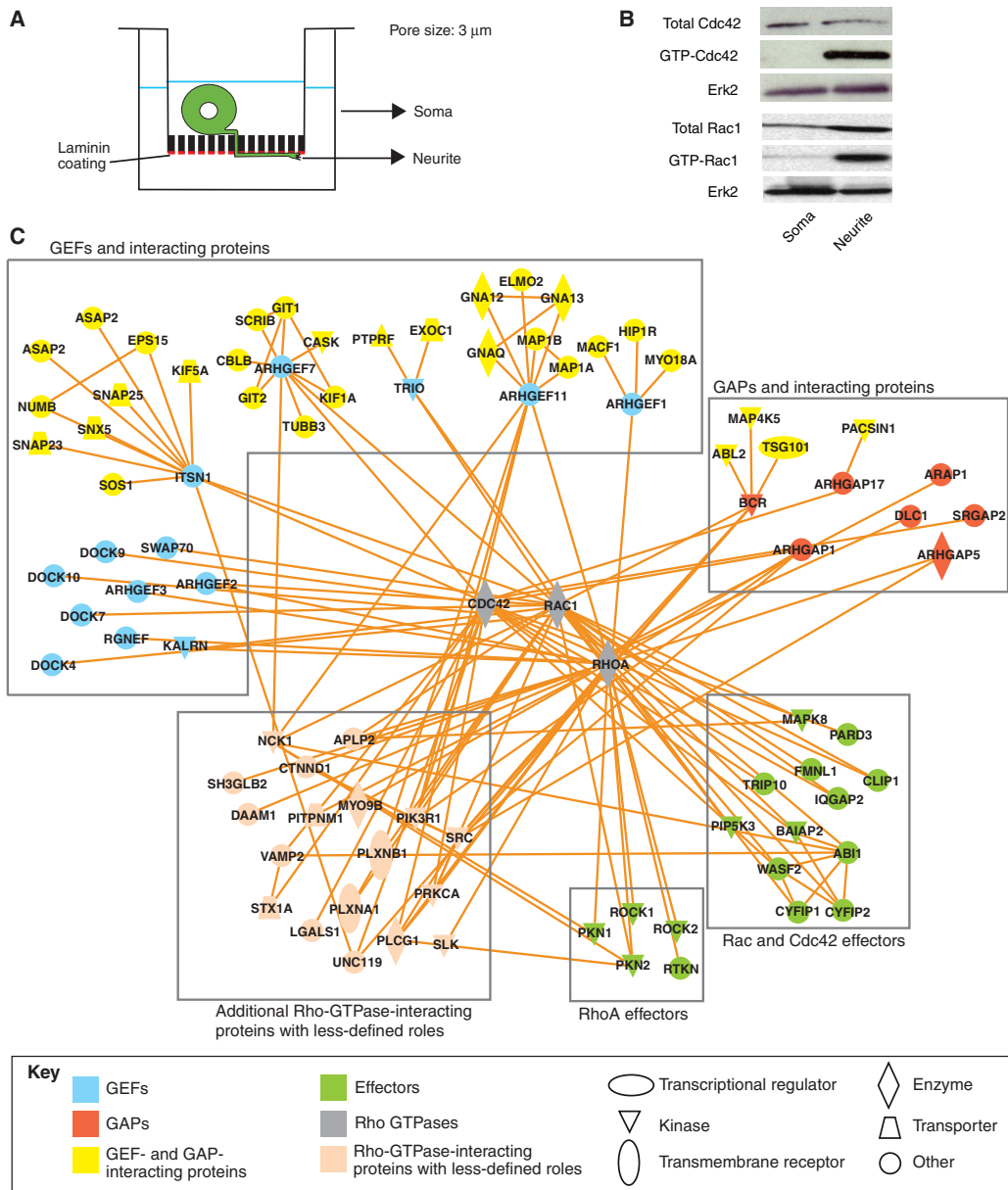


Fig. 3. Rho GTPase signaling network regulating neurite outgrowth. (A) Strategy for the purification of extending neurites. Neuroblastoma cells are plated on 3 µm microporous filters that are coated with laminin on the bottom to allow directional neurite outgrowth as described by Pertz et al. (Pertz et al., 2008). The small pore size ensures that only neurites squeeze through and enables selective purification of neurite and soma fractions. (B) Rac1 and Cdc42 activation status in extending neurites. Pull-down was performed on equivalent amounts of neurite and soma lysate. Erk2 serves as a loading control. (C) Rac1, Cdc42 and RhoA interaction network in the neurite. The neurite proteome was mined for proteins that are known to interact directly with the three GTPases or with their GEFs and GAPs using Ingenuity Pathways (<http://www.ingenuity.com>). Each line represents a protein-protein interaction documented in the literature. Proteins are named according to their official gene symbol in Entrez gene (<http://www.ncbi.nlm.nih.gov>). Further information about these proteins is found in supplementary material Table S1. Images in A and B reproduced from Pertz et al. (Pertz et al., 2008) with permission.

the high activation levels of Rac1 or RhoA, a small interfering RNA (siRNA) screen that targets all Rho-GTPase-specific GEFs and GAPs was performed using the characteristic morphology of each cell migration mode as read-out (Sanz-Moreno et al., 2008). A Rac-specific GEF, DOCK3, regulates Rac and its WAVE2 effector to drive mesenchymal movement and suppress amoeboid movement. Conversely, in amoeboid movement, RhoA signaling through ROCK and the Rac-specific GAP ARHGAP22 leads to Rac1 inactivation and suppression of mesenchymal movement. An important insight provided by this study is that the effectors that are important for the regulation of the specific cytoskeletal behaviors downstream of each GTPase also feed into the negative regulation of the specific Rho GTPase that is repressed in each respective program. Given the highly penetrant phenotypes observed, the players that have been identified here are certainly the master switches in regulating the interconversion between cell-invasion modes. However, the complexity of the spatio-temporal activation patterns described in the preceding sections suggests that there will be additional layers of Rho GTPase signaling that operate on top of these signaling pathways to allow the fine-tuning of the precise morphodynamics of these cell behaviors. These might have been missed in the screen because of their more subtle nature and might need much more sensitive read-outs.

This higher degree of complexity became apparent in another study, in which the Rho GTPase signaling program that regulates neurite outgrowth has been analyzed. To get a biochemical handle on spatio-temporal signaling, a fractionation method that separates extending neurites from the soma of neuronal-like cells was engineered (Pertz et al., 2008) (Fig. 3A). Effector pulldown assays revealed that Rac1 and Cdc42 activities are confined to the extending neurite (Fig. 3B). Using quantitative proteomics and bioinformatic approaches, a highly complex, neurite-localized potential signaling network was unveiled involving Rac1, Cdc42, RhoA, a large number of GEFs, GAPs and effectors, and additional proteins known to interact directly with Rho GTPases (Fig. 3C). Obviously, the complexity of this network is consistent with the elaborate signaling programs that are observed using FRET biosensors, but not with the simple view that Rac1 and Cdc42 are solely responsible for neurite extension (da Silva and Dotti, 2002). To explore the significance of this complexity, a small part of this interaction network was functionally probed using a small siRNA screen targeted against a set of neurite-enriched Rac1- and Cdc42-specific GEFs and GAPs. Surprisingly, irrespectively of whether a GEF or a GAP is knocked down, increased neurite outgrowth is observed in most cases, whereas loss of neurite outgrowth is never observed. However, taking a more sensitive approach to resolving the dynamics of the neurite-outgrowth process using time-lapse microscopy revealed a large spectrum of subtle morphodynamic phenotypes that simply were not accessible with steady-state measurements. The candidate genes targeted here displayed phenotypes that consisted of 'subfunctions' of neurite outgrowth, such as establishment of polarity during neurite path finding, initiation of neurite outgrowth or regulation of the stability of filopodia on the growth cone. This approach thus again illustrates a more complex level of regulation than initially anticipated, and allows the assignment of different GEFs and GAPs to the distinct spatio-temporal signaling modules that regulate a large variety of specific functions in the cell.

These examples illustrate why it will be important in the future to take systematic approaches to deconstruct the spatio-temporal signaling modules and programs that underlie different cellular

behaviors (cell migration, phagocytosis, etc.). A roadmap for the complete elucidation of such spatio-temporal signaling networks might be as follows. In a first, exploratory, phase, siRNA screens (or loss-of-function screens using other methods) will allow us to identify and pinpoint the specific functions of a Rho GTPase interactome in a given cell behavior. Obviously, knockdown of proteins that are part of a specific signaling module will produce a spectrum of morphodynamic phenotypes affecting one specific cellular function. Thus, phenotypic clustering should allow the prediction of which components are part of a specific signaling module. Once the different spatio-temporal signaling modules have been identified, further experiments will have to be performed for their validation and further characterization. Perturbation of the signaling components upstream of the GTPase (GEFs, GAPs and upstream pathways) in a specific signaling module should lead to aberrant spatio-temporal Rho GTPase activation profiles that correlate with specific morphodynamic phenotypes. These will then be evaluated using fluorescent biosensors of Rho GTPase activation. Validation of the downstream signaling components will require imaging methods to measure the transient and highly localized interactions between a GTPase and an effector of a given signaling module. As mentioned above, this can be done using a FRET approach (Parsons et al., 2005). Simultaneous visualization of cytoskeletal and adhesion dynamics with Rho GTPase activation profiles, in the native and disturbed states, will also give essential cues about the effector functions of specific signaling modules. Because of the fine spatio-temporal crosstalk between Rho GTPases, one should also keep in mind that affecting one signaling module might also lead to collateral effects on other ones.

The full characterization of such complex signaling programs will require multidisciplinary approaches and the establishment of novel technologies to quantify the complex space-and-time-resolved datasets. One important problem of single-cell biology is noise. Whereas the phagocytosis and oocyte wound-healing examples (Fig. 1E,F) are intrinsically robust behaviors, and relatively simple image-analysis techniques allow us to quantify their dynamics, cell migration is much more prone to noise in that many morphodynamic phenotypes are observed. Here, the multiplexing of the activation profiles of multiple GTPases at the leading edge of migrating fibroblasts (Machacek et al., 2009) (Fig. 1C) was only possible using sophisticated computer vision tools that allowed the pooling of 'noisy' measurements from multiple cells into statistically relevant data sets. Only then was the determination of both the spatio-temporal crosstalk between Rho GTPases and how it relates to cell behavior possible. This gives a flavor of the methods of analysis that we will have to use in the future. Similar image-analysis methods are needed for the comprehensive quantification of complex morphological phenotypes that, in the cases of 3D cancer-cell invasion and neurite-outgrowth systems mentioned above, were only verbally described. Such an image-analysis approach has been used with neural networks to extract quantitative morphological signatures from static pictures of migrating cells in which different signaling molecules have been genetically disturbed (Bakal et al., 2007). This enabled the complex morphological phenotypes of migrating *Drosophila melanogaster* cells to be unraveled, and allowed unbiased clustering of genes associated with different cellular processes, such as cell protrusion, adhesion and tension.

Another approach might be to try to experimentally eliminate the cellular noise by devising precise microenvironments that recapitulate the extracellular cues observed in vivo to induce robust

prototypical cell behaviors. In the context of cell migration, this could be achieved by using microfluidic devices to challenge cells with highly defined chemokine gradients to induce robust and standardized directional movement (Chung et al., 2007). Finally, we will also need methods to acutely manipulate Rho GTPase signaling in time and space. Two recent examples illustrate the possibility of 'caging' the activation of Rac1 in single living cells (Levskaia et al., 2009; Wu et al., 2009). Both studies show that localized Rac photoactivation is enough to trigger membrane protrusion. Importantly, whereas constitutive membrane protrusions of fibroblasts display high Rac1 and RhoA activation, protrusions that were induced by acute, local Rac1 photoactivation show low RhoA activation (Wu et al., 2009). This suggests that, upon a certain activation threshold at the leading edge, Rac1 locally antagonizes RhoA activation and is sufficient to drive membrane protrusion. This technique will facilitate the study of crosstalk between different GTPases in different Rho GTPase signaling programs.

Concluding remarks

Although the classic Rho GTPase toolkit has laid the foundations for studying Rho GTPase signaling, it is now time to move from a global 'Rho-GTPase-centric' view to a precise understanding of the spatio-temporal modularity of Rho GTPase signaling. This will hopefully resolve many controversial results observed in the past and provide novel conceptual frameworks. As emphasized in this Commentary, the future of spatio-temporal Rho GTPase signaling is about the systematic mapping of the multiple signaling modules that regulate different morphogenetic cell behaviors and understanding how crosstalk between these signaling modules establishes complex signaling programs. This should lead to an integrated view of Rho GTPase signaling. Because this endeavor necessitates more sensitive cellular read outs, this will enhance further understanding of many Rho GTPase effectors, GEFs and GAPs that have been only marginally studied until now, and thus is likely to expand the repertoire of Rho GTPase functions. This will require interdisciplinary approaches and the establishment of novel quantitative technologies to manipulate and measure Rho GTPase signaling. Ultimately, the combination of these different quantitative approaches should provide data that are amenable to modeling, yielding an accurate understanding of these complex signaling networks.

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