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Rho GTPase isoforms in cell motility: Don't fret, we have FRET

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The Rho-family of p21 small GTPases are directly linked to the regulation of actin-based motile machinery and play a key role in the control of cell migration. Aside from the original and most well-characterized canonical Rho GTPases RhoA, Rac1, and Cdc42, numerous isoforms of these key proteins have been identified and shown to have specific roles in regulating various cellular motility processes. The major difficulty in addressing these isoform-specific effects is that isoforms typically contain highly similar primary amino acid sequences and thus are able to interact with the same upstream regulators and the downstream effector targets. Here, we will introduce the major members of each GTPase subfamily and discuss recent advances in the design and application of fluorescent resonance energy transfer-based probes, which are at the forefront of the technologies available to directly probe the differential, spatiotemporal activation dynamics of these proteins in live single cells. Currently, it is possible to specifically detect the activation status of RhoA vs. RhoC isoforms, as well as Cdc42 vs. TC-10 isoforms in living cells. Clearly, additional efforts are still required to produce biosensor systems capable of detecting other isoforms of Rho GTPases including RhoB, Rac2/3, RhoG, etc. Through such efforts, we will uncover the isoform-specific roles of these near-identical proteins in living cells, clearly an important area of the Rho GTPase biology that is not yet fully appreciated.

Introduction

Cell motility is a complex process that is essential for many aspects of life. The correct functioning of the immune system, the development of the embryo and the process of wound healing all rely on cell migration.^{1–6} Furthermore, aberrant cell motility is a hallmark of many pathological conditions, including cancer metastasis and atherosclerosis.^{7,8} In order to move, cells initiate a

program of events termed the “Cell Motility Cycle.” Once an external signal is detected, the cell extends an actin-rich protrusion in the direction of motion, which adheres to the substrate in order to pull the cell forward. The rear of the cell then contracts, drawing the rest of the cell forward. The activity of Rho GTPases is essential to coordinate the stages of this cycle by virtue of their central role in regulating actin polymerization.^{9–13}

Rho GTPases are a Ras-related family of proteins that play a role in many physiological processes such as cell migration, cell division and gene expression.^{14–17} The Rho GTPases act as molecular switches that cycle between an inactive GDP-bound conformation and an active GTP-bound conformation.^{18,19} When active, the GTPase can interact with a variety of downstream signaling molecules or “effectors” to propagate different signaling outcomes. Rho GTPases are also prenylated at their C-terminus and this allows for their insertion into cellular membranes, which is essential for their correct localization and function.²⁰ Regulation of the activity of Rho GTPases is controlled by three types of proteins – GEFs, GAPs and GDI.²¹ GEFs (Guanine nucleotide exchange factors) promote the exchange of GDP for GTP, thereby activating the Rho GTPase, while GAPs (GTPase activating proteins) enhance the intrinsic GTP hydrolysis activity of the GTPase resulting in its inactivation.^{22,23} The third class of regulator, the RhoGDI, binds to the GDP-bound GTPase masking the prenylated C-terminus and sequestering the inactive Rho GTPase in the cytosol.²⁴

Due to the importance of both the localization and activity of Rho GTPases in their functioning, traditional biochemical techniques and imaging studies are not sufficient to gain detailed insight into their roles and regulation. In the last decade the introduction of the technique of FRET (Fluorescence Resonance Energy Transfer) has gained increasing popularity as it allows the simultaneous measurement of both the localization of the GTPase and its activation status. FRET is a non-radiative energy transfer process between two compatible fluorophores that require certain characteristics, including: 1) sufficient quantum efficiency of the donor fluorophore; 2) sufficiently long fluorescence lifetime of the donor fluorophore to allow for non-radiative transfer; 3) sufficient overlap in the emission spectra of the donor fluorophore and the excitation spectra of the acceptor fluorophore; and 4) correct coupling angles of the dipoles of the donor and the acceptor fluorophores.²⁵ While the theoretical aspects of FRET is beyond the scope of the current review, it is sufficient to say that the reversibility and the sensitivity of FRET is ideally

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suited to the live-cell imaging experiments in which the dynamics of the protein activation states can reversibly modulate in the order of seconds. This is due primarily to the fact that FRET is exquisitely sensitive to the relative distance and the dipole-orientations of the FRET donor and acceptor fluorophores.²⁶ Unlike approaches including bifluorescence complementation,^{27,28} proximity ligation assays,^{29,30} and simple monitoring of fluorescent protein-labeled binding domain accumulations, FRET-based measurements offer live-cell, rapid, dynamic, reversible and sensitive measurements, even when bulk-accumulations and bulk-changes of the signaling molecules do not likely take place, such as in the case of the majority of Rho GTPase signaling.^{31,32} FRET studies involving Rho GTPases primarily utilize biosensors which are typically single or dual chain constructs encompassing the GTPase, an effector binding domain and two fluorescent moieties, for example CFP and YFP, that are capable of FRET transfer.³³ Activation of the GTPase results in an interaction with the effector binding domain and a consequent alteration in the relative orientations of the two fluorescent molecules. This leads to a measurable change in the efficiency of FRET in the biosensor and provides a spatial and temporal readout for the activation of the GTPase.

The Rho family of GTPases consists of 22 members of which the best characterized are RhoA, Rac1 and Cdc42. These proteins have all been classically associated with the regulation of actin cytoskeleton and cell motility and it is well established that each of these GTPases plays a specific role in regulating the signaling pathways that control actin reorganization. Classically, Cdc42 is thought to promote filopodia formation, while Rac1 regulates the protrusion of lamellipodia and RhoA is associated with cell contraction.^{9–11} However, that is not the whole story, each of these major GTPases also has different isoforms, which besides sharing much of their sequence, also share the same effectors and upstream regulators—GEFs, GAPs and GDIs. This immediately raises a number of questions. Why are these other isoforms required? Do they have different functions and if so how are these specific roles achieved given the similarity of the isoforms? We are starting to understand the role of the Rac isoforms Rac1, 2, 3 and Rho isoforms RhoA and RhoC within the context of cell migration. Recent results from a number of different laboratories, including ours, are uncovering novel functions of these isoforms that could explain their expression in different cell types and also their roles in pathological processes such as metastasis.

In this review we will focus on the recent discoveries regarding Rac, Rho and Cdc42 isoforms in cell motility and how the use of FRET-biosensor imaging technologies are revealing unexpected roles for these small GTPases.

The ABCs of Rho GTPases

Since the identification in 1985 of the genes for the Rho proteins³⁴ a vast amount of work has been done in order to understand the biology of RhoGTPases. The Rho family of GTPases encompasses three genes named RhoA, RhoB and RhoC. These different isoforms share 85% sequence homology.³⁵ The original studies from Allan Hall³⁶ and Paul Monaghan³⁷ focused on

understanding the cellular localizations of these proteins as well as on gathering the first insights into their biological function. These studies demonstrated that RhoB is localized in endosomes while RhoA and RhoC are found at the plasma membrane and associated with actin-rich cytoskeletal structures. Since then, a substantial amount of work has been done to understand the role of RhoB in intracellular trafficking and adhesion^{35,38–42} and the functions of RhoA and RhoC in regulating the actin cytoskeleton during migratory processes (reviewed in^{40,43}). These works emphasized that while highly related in sequence, the final output of the intracellular signaling by these isoforms is different. This is presumably due to the intricate way each RhoGTPase network is wired, whereby a different subset of the key GTPase regulators, GEFs, GAPs and GDIs, cooperate to trigger a unique physiological response. Given the similarities between these GTPases, how can we distinguish between the activities of these Rho isoforms within cells during migration and invasion?

A major breakthrough in the understanding of RhoGTPase biology came with the development of biosensors for Rho family GTPases in the early 2000s.^{44,45} For the first time, these tools allowed the direct observation of the localization of RhoGTPase activation in real-time in living cells. These studies revealed the localized patterns of GTPase activation within subcellular compartments and during physiological processes, such as in the actin-rich protrusions that are critical for cell migration.^{31,46,47} The observed activity patterns opened the window to a new field of GTPase biology that focuses on understanding the spatiotemporal regulation of GTPase activation during biological processes at subcellular compartments. More importantly, these tools provided new approaches for gaining insight into how upstream molecules, including GEFs, GAPs and GDI, regulate GTPase activity. The intricate activation dynamics of GTPases suggested that the regulation of upstream GEFs and GAPs are likely to confer specificity to the pattern and dynamics of Rho GTPase activation that we see in cellular processes.²² Specifically, the RhoA biosensor revealed important aspects of its spatiotemporal regulation. Traditional biochemical and imaging approaches had shown a key role for RhoA in mediating cellular contraction during motility. Unexpectedly, biosensor imaging showed that RhoA activity localized to the leading edge of migrating cells,^{44,48} suggesting a novel role in leading edge protrusion. By combining biosensor imaging with a computational approach, Machacek et al.⁴⁶ demonstrated that RhoA GTPase activation dynamics during leading edge protrusion are directly coordinated with edge movement immediately at the cell periphery. In tumor cells it was also shown that in the absence of a lamellipodium, RhoA displayed high activation levels in mDia1-dependent finger like protrusions that formed after EGF stimulation.⁴⁹ Furthermore, biosensor technology has shown how RhoA activity is regulated in other cellular processes such as cytokinesis⁵⁰ and T-cell trans-endothelial migration.⁵¹ The RhoA biosensor design has evolved since the first version^{44,45} (Fig. 1A) to more recent forms,^{44,52} which maintain an intact C-terminus and allowed the adaptation of the design to other isoforms, including RhoC⁵³ (Fig. 1B). This new biosensor has revealed some important aspects of the spatiotemporal regulation of RhoC in tumor cells and put in

context the distinct roles of RhoA and RhoC proposed during tumor cell invasion.^{54,55}

Depletion of RhoC in tumor cells inhibits invasion⁵⁶ and metastasis.⁵⁷ The RhoC biosensor was used to reveal the mechanism by which RhoC contributes to the invasion of tumor cells. Spatial activation of RhoC surrounding the core of invasive

protrusions named invadopodia⁵⁶ or within regions located behind the leading edge⁴⁹ triggers a signaling pathway that phosphorylates cofilin⁵⁸ and confines cofilin activity and actin polymerization at the core of invadopodia or to the tip of the leading edge, a localization which is essential for efficient invasion. These activation zones are regulated by the spatial and geometric

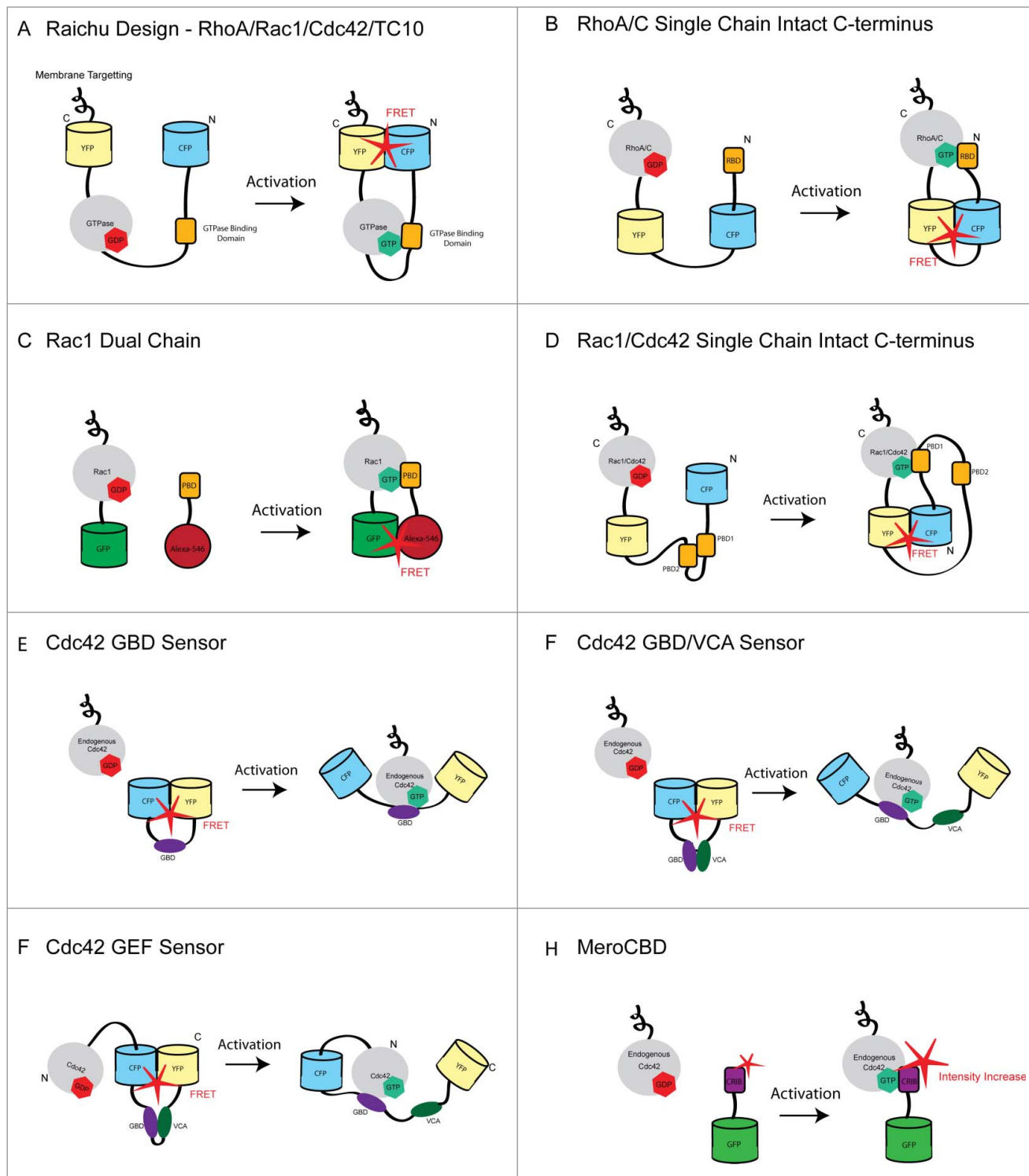


Figure 1. For figure legend, see page 529.

arrangements of the direct upstream regulators p190RhoGEF and p190RhoGAP.

This biosensor also allowed the direct comparison of the activation patterns of RhoA and RhoC at these subcellular structures.^{49,53,56} At invadopodia, RhoC has a geometrically confined activation pattern, while RhoA displays a more random activation pattern.⁵⁶ At leading edge protrusions, RhoC activation is confined spatially behind RhoA activation.⁴⁹ Also, using computational approaches in fibroblasts, the activation of RhoC was quantitatively compared with that of RhoA.⁵³ This analysis showed that areas of RhoC activation appeared to be more diffuse and evenly distributed at the edge of the cell.⁵³ Furthermore, the authors showed that RhoA and RhoC activities have different kinetics during lamellipodial protrusions. These different activation dynamics suggest that the key determining factors that control subcellular GTPase activation dynamics is likely the spatial distribution of their upstream regulators.^{49,56} Recent studies have also extended the use of Rho biosensors to *in vivo* systems. For example, Timpson et al.,⁵⁹ have shown how RhoA activity is modulated in an animal model of pancreatic cancer using the fluorescence lifetime imaging (FLIM)-FRET measurements of the Raichu-RhoA biosensor.

The development of new imaging tools capable of measuring the real-time spatial activation patterns of RhoA and RhoC has significantly extended our understanding of the differential regulation of these isoforms in the context of tumor cell invasion and migration. However, there is more to be done, for example, how is RhoB spatiotemporally regulated? The door is open for the development of biosensors for this particular isoform in order to answer this question.

Rac GTPases: Lighting Up the Lamellipodia

The Rac family of GTPases consists of three closely related isoforms Rac1, Rac2, Rac3, which share more than 90%

homology. Differences in these isoforms are found in the C-terminal 15 amino acids, which is the region important for localization of the molecules.⁶⁰⁻⁶³ In addition, this family of proteins includes the more distantly related RhoG, which has about 70% homology to Rac1.⁶⁴ Rac1 is the prototypical member of this family and its functions were first elucidated in the classical studies of Ridley and Hall. Microinjection of Rac1 into fibroblasts resulted in the formation of lamellipodia and membrane ruffles and also promotes the formation of focal contacts and E-cadherin dependent cell-cell contacts.^{9,10,65} The importance of Rac1 was further highlighted by the generation of a knockout mouse that revealed that Rac1 is essential for embryonic development, as in its absence gastrulation fails to proceed, due to defects in lamellipodia formation and cell adhesion that compromise the ability of the embryonic cells to migrate.⁶⁶ Since these original studies, Rac1 has been established as a key regulator of cell motility (reviewed in^{67,68}).

As with RhoA and RhoC, over the last 10–15 years, much progress has been made on the development of FRET-based biosensors that show the localized activation status of Rac1 in live cells. Despite many studies highlighting the important function of Rac1 in actin polymerization and cell migration, it was only with the advent of fluorescent biosensors that specific insights into the high-resolution spatiotemporal regulation of Rac1 activity were elucidated.^{31,46,47} Initial studies utilized a dual chain FRET biosensor (Fig. 1C) to demonstrate high levels of Rac1 activity in membrane ruffles in response to PDGF stimulation.³¹ In keeping with the role of Rac1 in lamellipodia formation and cell migration, this study also showed high levels of Rac1 activity at the leading edge, which declined in a broad gradient. A subsequent study used an improved Rac1 biosensor combined with a computational multiplexing approach to compare the activity patterns of Rac1, RhoA and Cdc42 in migrating fibroblasts.⁴⁶ This study unexpectedly revealed that Rac1 activity is highest not at the protruding front of the cell but in fact peaks at a distance of approximately 2 μ m from the plasma membrane, directly

Figure 1 (See previous page). Schematic showing the various designs used in GTPase Biosensors. (A) The single-chain Raichu design was used to generate genetically encoded biosensors for RhoA, Rac1, Cdc42 and TC10. In this design, the FRET pair (CFP/YFP) is placed on the N- and C-terminus of the molecule. Thus, the C-terminus of the GTPase is not free and these biosensors are insensitive to GDI. The CAAX box (membrane targeting) from K-Ras is encoded at the C-terminus of the YFP in order to place the probe constitutively in the membrane. Upon activation of the GTPase, it interacts with the GTPase binding domain (specific to each GTPases), resulting in a conformational change that brings the fluorescent proteins into close proximity leading to increased FRET signal. (B) The RhoA single chain biosensor places the donor and acceptor fluorescent proteins internally allowing the native C-terminus of RhoA to be maintained. High FRET occurs when the active GTPases interacts with the Rho binding domain (RBD) of Rhotekin. (C) The first bimolecular Rac1 biosensor in which Rac1 is linked to EGFP and the Pak binding domain (PBD) of PAK1 is coupled to the dye Alexa-546. FRET occurs when the interaction of Rac1 and the PBD bring EGFP and Alexa-546 into close proximity. This biosensor was later modified to be fully genetically encoded by replacing GFP and Alexa-546 with YFP and CFP respectively. (D) The newest version of the Rac1 biosensor is genetically encoded and uses mCerulean1 and mVenus as the FRET pair. This biosensor maintains the C-terminus of Rac1 in its native state. In addition, a tandem PBD module is utilized to regulate the interaction with Rac1 and further decrease the chances of spurious FRET. This design was recently adapted to generate a single chain Cdc42 biosensor (E) The Cdc42 GBD sensor detects active endogenous Cdc42. Upon binding of active endogenous Cdc42 to the GBD of WASP, a conformational change occurs that causes CFP and YFP to be forced apart. This results in low FRET signal where active Cdc42 is present in the cell. (F) In a modification of (E) the VCA domain of WASP is incorporated into the biosensor. This domain competes with Cdc42 for binding to the GBD and this may result in larger differences in the on and off states of the biosensor. (F) This biosensor was generated by adding Cdc42 to the N-terminus of the GBD/VCA sensor. Upon activation of Cdc42 by a GEF, Cdc42 binds to the GDB and results in decreased FRET. A caveat of this biosensor is that the rate of GTP hydrolysis is significantly slower than for wild type Cdc42 and in addition, the C-terminus of Cdc42 is occluded. (G) The MeroCBD biosensor allows measurements of the activation of endogenous Cdc42 by using a dye that changes fluorescence emission intensity as a function of the local solvent polarity. The dye is coupled to the GTPase effector and changes in fluorescence emission occur upon interaction with the active GTPase. The dye/effector moiety is also labeled with GFP, which is used as reference for ratiometric intensity measurements.

behind a focused region of RhoA activation.⁴⁶ The activation of Rac1 was also delayed with respect to the protrusion of the membrane. This study shed new light on the function of Rac1 in cell migration and challenged the hypothesis that Rac1 initiates the protrusion of the leading edge in migrating cells.^{9,69-71} Furthermore this study gave support to the theory that Rac1 antagonizes RhoA activity^{72,73} and may serve to restrict RhoA signaling to a narrow region at the leading edge.⁴⁶ Preliminary studies with the single chain Raichu biosensor (Fig. 1A), which eliminates the issues of equal expression of both biosensor components that is inherent in dual chain designs, also showed that Rac1 activity is highest directly behind the leading edge and further demonstrated that Rac1 activity decreases sharply with the plasma membrane retraction that occurs when the cell changes direction.⁴⁷

Recently, a new single chain Rac1 biosensor, which maintains the C-terminus of the protein in its native state, has been used to explore the role of Rac1 in tumor cell invasion (Fig. 1D).⁷⁴ This study found that Rac1 activity is suppressed during invadopodia formation but is subsequently activated to promote the disassembly of the invadopod structure. Unlike earlier versions of Rac1 biosensors, a key strength of this biosensor is that it is responsive to the full complement of endogenous GTPase regulatory mechanisms, including RhoGDI. Therefore this biosensor is likely to provide fresh insight into the roles and regulation of Rac1 in myriad physiological and pathological processes. Together the data from these biosensor studies demonstrated the clear importance of high spatial and temporal regulation of Rac1 activity in the control of cell migration and invasion. However, many questions still remain as to how this spatiotemporal activation is mediated. Rac1 biosensors have also been used to shed light on these questions. For example, the pathways regulating activation of Rac1 in the control of *Drosophila* border cell migration were elucidated in a number of studies using the Rac1 biosensor.^{75,76} Furthermore, the importance of the Rac1 GEFs, PLC ϵ , Tiam1 and Trio in fibroblast chemotaxis, T-cell migration and invasion and tumor cell invasion, respectively were determined in studies that employed Rac1 FRET biosensors.^{74,77,78}

In addition to probes that monitor the activation status of Rac1 in cells, optical tools have also been developed that allow the direct, localized manipulation of Rac1 activity. A genetically encoded photoactivatable Rac1 was engineered in which Rac1 is fused to the LOV domain, which sterically hinders the ability of Rac1 to interact with downstream effectors.⁷⁹ Upon irradiation with 458 or 473nm light, a conformational change occurs that removes this hindrance and allows Rac1 to signal. This tool has been effectively employed both in cellular and multicellular models to demonstrate that activation of Rac1 is required for directional cell migration.^{75,79-82} Recently a number of studies have combined the use of biosensors to monitor the activity of Rac1 with the manipulation of this protein by specific photoactivation have been used to determine the functions of Rac1 in *Drosophila* border cell migration and tumor cell invasion.^{74,83} Studies such as these which combine different microscopy and optical approaches to dissect the mechanism of GTPase signaling in different contexts will pave the way for a complete understanding of the biology of these proteins.

Since the original cell-based studies using Rac1 FRET biosensors, much progress has been made in imaging Rac1 activity in multicellular organisms. The first *in vivo* analysis of Rac1 activity was performed in migrating neural crest cells in xenopus embryos.⁸⁴ More detailed *in vivo* analysis of Rac1 activity was performed in migrating border cells in *Drosophila*. In this study, a combination of PA-Rac1 and Raichu-Rac1 biosensor showed that Rac1 activation regulates the directionality of border cell migration in *Drosophila* embryos.⁸⁰ It was found that the directionality of the cells depends on the asymmetric localization of Rac and that the establishment of asymmetry is dependent on the guidance receptors, PVR and EGFR. Furthermore, Rac1 activity has been observed in migrating germ cells in developing zebrafish embryos.⁸⁵ A recent study also showed that the activity of Rac1 is high in invasive glioblastoma cells that are engrafted into rat brains.⁸⁶ In addition, a transgenic mouse that ubiquitously expresses the Raichu-Rac1 biosensor has recently been generated.⁸⁷ In this study Johnsson et al., imaged Rac1 activity in primary MEFs and chemotaxing neutrophils, as well as in intestinal crypt cultures, where it was found that Rac1 activity is higher in the cells at the base of intestinal crypts compared with distally located cells.⁸⁷ Furthermore, these mice were crossed with two cancer mouse models to demonstrate that Rac1 activity is higher both in pancreatic and mammary tumors compared with non-cancerous tissues.⁸⁷ These studies have given great insights into the importance of Rac1 signaling *in vivo*, however much remains to be done to fully understand the patterns and regulation of Rac1 signaling and its physiological significance in mammals.

Other Rac Isoforms: Shaping the Immune System and the Brain

Insights into the roles of the other Rac GTPases have revealed both distinct and overlapping functions for these isoforms. Rac2 expression is restricted to hematopoietic cells where it plays an important role in regulating the migration and chemotaxis of neutrophils, macrophages and B- and T-lymphocytes.⁸⁸⁻⁹¹ Deletion of Rac2 in mice revealed numerous other defects in the immune system as actin polymerization, superoxide production and phagocytosis are impaired in the absence of Rac2.⁹² Combined deletion of both Rac1 and Rac2 resulted in more severe migratory defects than are observed in Rac2^{-/-} cells alone indicating that there is some synergy in the functioning of these GTPases.^{93,94} A dual chain biosensor approach was used to demonstrate that active Rac2 is localized at both the leading edge and in the following tail of chemotaxing neutrophils.⁹⁵ This study showed similar results using the dual chain Rac1 biosensor and was the first report of a role for Rac signaling in tail retraction in migrating cells.⁹⁵ Rac2 also exhibits isoform specific functions in cell migration, as in its absence neutrophils cannot chemotax in response to fMLP. In contrast, loss of Rac1 in neutrophils does not result in chemotactic deficits in response to fMLP.⁹⁶

Rac3 has also been linked to cell migration as well as the invasion of metastatic cancer cells. Gest et al. demonstrate that

depletion of Rac3 in the breast carcinoma cell line MB-MDA-231 leads to decreased migration in wound healing assays, however the ability of glioblastoma cells to migrate in a similar assay was only slightly affected by the loss of Rac3.^{97,98} Conversely, depletion of Rac1 strongly affected the ability of the glioblastoma cells to migrate in two dimensions.⁹⁷ Similarly, the loss of Rac1 but not Rac3 was found to impair the migration of non-tumor primary fibroblasts in Dunn chamber chemotaxis assays.⁹⁹ In contrast, Rac3 and Rac1 appear to perform synergistic roles in the development of the nervous system as loss of both isoforms results in defects in the migration and differentiation of a number of brain cell types.^{100–103} Loss of either isoform in the brain does not lead to obvious defects.¹⁰⁰ These results highlight that each of the Rac isoforms had been specialized for a very particular function and displays its own specific biology.

It is clear that much is known about the distinct roles that the Rac family isoforms play in cell motility, which contribute to a wide range of physiological functions including the normal action of the immune system and neuronal development as well as the pathogenesis of cancer. However many questions remain, for example, the activation pattern of Rac2 has only been characterized using a dual chain approach while that of Rac3 has not yet been studied at all. Furthermore the upstream signaling pathways that control the differential regulation of these isoforms remain unclear. Thus the challenge ahead is to design biosensors that can be used to visualize the activation dynamics of Rac2 and Rac3 in order to shed light on these pertinent questions. The generation of specific tools to observe the activation of the Rac GTPases will enable a greater understanding of the functions of these isoforms as well as the regulatory mechanisms that enable such closely related molecules to play distinct roles.

The Cdc42 Family—Not just Filopodia

As with Rho and Rac, the identification of Cdc42 as a key regulator of cytoskeletal dynamics was identified in classical microinjection experiments. Activation of Cdc42 in Swiss 3T3 fibroblasts results in the rapid induction of filopodia, which subsequently develop into lamellipodia if Rac is present.¹⁰⁴ The same study also identified a role for Cdc42 in promoting focal complex formation.¹⁰⁴ Since then, key roles for Cdc42 in chemotaxis and directed cell migration as well as in the establishment of epithelial cell polarity have been identified (reviewed in⁶⁷). The first report showing the activation dynamics of Cdc42 in live cells came from the Matsuda group, which developed the Raichu-Cdc42 biosensor based on a similar design as the Raichu-Rac1 biosensor⁴⁷ (Fig. 1A). This study showed that, like Rac1, activation of Cdc42 increases in a gradient toward the leading edge of motile cells but that in contrast to Rac1, the activation of Cdc42 is highest at the tip of the leading edge. A subsequent study resulted in the development of three sensors that can be used collectively to report on the activation status of Cdc42.¹⁰⁵ Two of these sensors involve either the GTPase binding domain (GBD) of WASP or the GBD and the VCA region of WASP sandwiched between CFP and YFP (Fig. 1E,F). Upon the specific interaction

of the GBD or GBD/VCA with active Cdc42, a conformational change occurs that results in a decrease in FRET efficiency. Unlike the majority of FRET sensors, these probes can detect the activity of the endogenous GTPase. The third sensor consists of Cdc42 coupled at its C-terminus to the CFP-GBD-VCA-YFP sensor (Fig. 1G). This acts as a sensor of Cdc42 specific GEFs but again has the caveat that the C-terminus of Cdc42 is occluded which is likely to affect the correct localization of the sensor. Detailed biochemical analyses revealed that this sensor reports the timing of Cdc42 activation accurately but that there is 16-fold slower rate of GTP hydrolysis by the GEF sensor than by wild type Cdc42. Thus this sensor may not be optimal for monitoring processes in which the rapid turnover of Cdc42 activation is important. Imaging studies showed that these Cdc42 sensors can be expressed in cells and respond to the presence of GEFs and GAPs, however a detailed analysis of Cdc42 activation using these probes have not been performed.¹⁰⁵

Greater insight into the function of Cdc42 came from a study in which another novel biosensor capable of reporting the activity of endogenous Cdc42 was developed.¹⁰⁶ This biosensor, termed MeroCBD, comprises the CRIB (GBD) domain of the Cdc42-specific effector protein WASP, covalently labeled with a fluorescent dye (Fig. 1H). Upon binding to active GTP-bound Cdc42, the dye undergoes a measurable change in fluorescence.^{106,107} Microinjection of this biosensor into fibroblasts showed that after attachment, Cdc42 is activated in a thin band in areas of the plasma membrane that are extending filopodia, although no activity is observed within the filopodia themselves. At later time-points after adhesion, Cdc42 activity is localized in larger dynamic protrusions. This study also showed that the indicated fluctuations in Cdc42 activity are correlated with extension and retraction of cellular protrusions and suggested that Cdc42 is important for the initiation but not the maintenance of these protrusions.¹⁰⁶ More recently, a new genetically encoded single chain Cdc42 biosensor has been developed¹⁰⁸ (Fig. 1D). Like the new RhoA, RhoC and Rac1 biosensors (Fig. 1B,D), this biosensor maintains the native C-terminus of Cdc42 and thus undergoes correct membrane targeting and interaction with GDI. As well as confirming the previously documented activation of Cdc42 at the leading edge of motile cells, the authors of this study also extended their observations to macrophages and showed that Cdc42 is activated during phagocytosis and after cytokine stimulation. Furthermore, Cdc42 was found to be transiently activated during the formation of podosomes, which are adhesive and degradative structures involved in extracellular matrix remodeling and cell migration.¹⁰⁹ While these papers collectively reveal the patterns of Cdc42 activation in different contexts, studies utilizing Cdc42 biosensors to examine the regulation of its activation are still lacking.

Like the Rho and Rac families, different isoforms of Cdc42 have also been identified. These are termed TC10 (RhoQ) and TCL (RhoJ). This family of proteins shares 70–85% homology.^{110,111} TC10 has been shown to play a role in the regulation of glucose transport, while TCL is highly expressed in endothelial cells and is important for vascular morphogenesis.^{110,112,113} Like Cdc42, expression of these GTPases in cells results in modulation

of the morphology of the actin cytoskeleton.^{110,111} The activity of TC10 has been visualized using a modified version of the Raichu biosensors¹¹⁴ (Fig. 1A). This study showed that TC10 is highly activated on exocytosing vesicles and recycling endosomes, but not on early or late endosomes in Hela cells. Furthermore, hydrolysis of GTP by TC10 was shown to be necessary to promote the fusion of vesicles with the plasma membrane.¹¹⁴ This function of TC10 was found to be conserved in neurites, suggesting that TC10 activity is broadly important for exocytosis.¹¹⁵

Conclusion

FRET-biosensors have come in multiple flavors since their early development, but technology has vastly improved and we now have powerful tools that allow the precise analysis of GTPase activation in the time scale of seconds and at subcellular resolution. The expansion in the use of FRET-biosensor imaging to address biological question is changing the way we understand and study GTPase biology. Computational analysis combined with FRET imaging is revealing unpredicted roles for GTPases

in different subcellular compartments. The field is wide open, we must now expand our use of biosensor technology to analyze the activity of upstream GEF and GAP regulators and GDIs in order to complete the entire picture of GTPase regulation.

FRET-biosensor imaging technology has been widely used to address the functions of RhoGTPases during motility but there are many other processes in which these proteins play a major role including cell division, membrane trafficking, polarity and proliferation. The application of FRET biosensor technology to these fields will greatly improve our understanding of these critical physiological processes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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