Chapter 5

Biosensors of Small GTPase Proteins for Use in Living Cells and Animals

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CONTENTS

5.1 SMALL GTPase BIOSENSOR DESIGN

Low-molecular-weight guanosine triphosphatase proteins (small GTPases, a form of G-protein distinct from heterotrimeric G-proteins) were among the first

molecules targeted for study with biosensors. Their long and interesting history illustrates the evolution of biosensor design, principles, and approaches. Although there are some exceptions, GTPase proteins almost always exist in one of two conformations—an inactive conformation bound to GDP and an active conformation bound to GTP. It is only in this active conformation that GTPases can interact productively with their downstream effector proteins (Figure 5.1). In general, the activation of GTPases is regulated by three classes of upstream proteins: guanine nucleotide exchange factors (GEFs), which mediate the binding of GTP; GTPase activating proteins (GAPs), which accelerate the hydrolysis of GTP to GDP; and guanine nucleotide dissociation inhibitors (GDI), which bind the GDP-bound form of the proteins (Bar-Sagi and Hall 2000; Reuther and Der 2000; Takai et al. 2001; Jaffe and Hall 2005). The goal of most biosensors is to track the transient localization and formation of the GTP-bound, "activated" conformation of the GTPase in living cells and animals. For example, the cycling between active and inactive nucleotide states is tightly coupled to changes in membrane localization for many GTPases. In other cases, regulatory mechanisms such as phosphorylation and degradation can influence the activity and localization of the protein. In designing a biosensor, one strives for maximum sensitivity by producing the brightest possible sensor, and for some designs, the greatest possible difference between the fluorescence

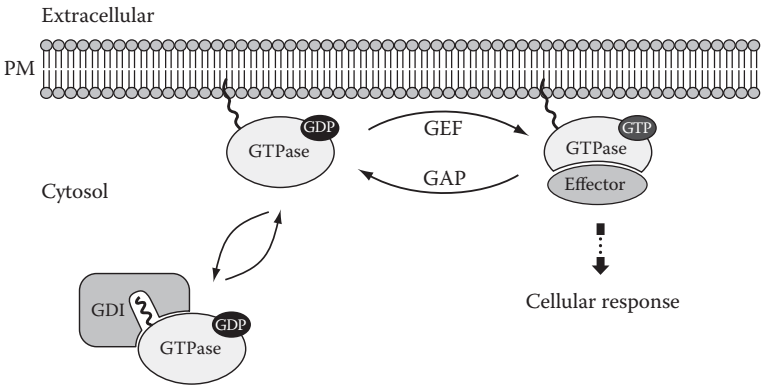

Figure 5.1 The Rho GTPase regulatory cycle. Rho GTPases are molecular switches that cycle between GDP-bound (inactive) and GTP-bound (active) states. Guanine dissociation inhibitors (GDIs) sequester GDP-bound GTPases in the cytoplasm. At the membrane (potentially still associated with GDIs), GTPases are activated by guanine nucleotide exchange factors (GEFs), which promote the exchange of GDP for GTP. Active GTPases interact with multiple effector proteins that govern a wide range of cell responses and behaviors. GEF activity is opposed by GTPase-activating proteins (GAPs), which increase the rate of GTP hydrolysis, thereby returning GTPases to their GDP-bound state.

of the active and inactive conformations. The goal is to perturb the GTPase of interest as little as possible, but it is difficult to modify a GTPase or sense its activity without in some way interfering with biologically important interactions. Most importantly, one must not alter the upstream regulatory interactions that control activation, or the downstream interactions that generate subcellular localization. As choices between different perturbations are often the only viable course, a biosensor may report only a subset of signaling events in a cell, but valuable biological information can be obtained if the limitations of the biosensor are understood. An enduring misconception in the field has been the view that biosensors act simply as "activity stains" akin to antibodies, without regard to the limitations of each design. In this chapter, we discuss the development of small GTPase biosensors, highlighting the utility and limitations of existing sensors.

The design of the first GTPase biosensor, Rac1 FLARE (Kraynov et al. 2000), was based on previously described biochemical GTPase activation assays developed to quantify GTPase activation in cell lysates. In these studies, a fragment of a downstream effector protein that binds only the active form of the GTPase (an "affinity reagent") was used to pull down the GTPases Rac1 or Cdc42 (Benard et al. 1999). Western blotting was then used to gauge the quantity of pulled-down, active GTPase. This affinity-domain paradigm was adapted to produce a biosensor by using Förster/fluorescence resonance energy transfer (FRET) to visualize the binding of the affinity reagent to the GTPase (Kraynov et al. 2000). An effector fragment from p21-activated kinase (Pak) covalently labeled with a fluorescent dye produced FRET when it bound to the active (GTP-loaded) conformation of green fluorescent protein (GFP)-tagged Rac1.

The development of fluorescent proteins (FPs) with different excitation/emission wavelengths capable of undergoing FRET made it possible to generate GTPase biosensors that were genetically encoded, an approach first applied to Ras and Rap1 (Mochizuki et al. 2001). Importantly, the use of genetically encoded components made it possible to link the affinity reagent and the GTPase in a single-chain, simplifying expression and ratio imaging. Genetically encoded versions of both single- and dual-chain designs have since been developed for multiple different GTPases (Figure 5.2). These two design types have important differences that impact the optimization and design of new biosensors, as well as the tailoring of biosensors to specific biological applications. The single-chain designs have predominated because they are simpler to apply using widefield ratio imaging, but the advent of new modes of microscopy (e.g., fluorescence lifetime imaging) for the quantitation of biosensor activity and the greater sensitivity of the dual-chain biosensors are leading to their reemergence (Hinde et al. 2011, 2013). In brief, single-chain biosensors are easier to use for ratiometric imaging, because the GTPase and affinity reagents are

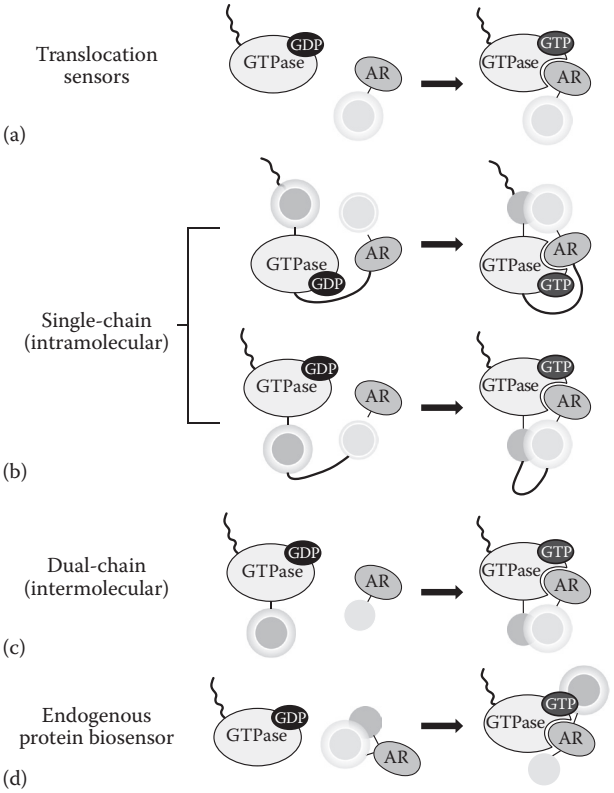

Figure 5.2 Genetically encoded biosensor designs. (a) A protein fragment that selectively binds to the active form of the GTPase (the affinity reagent, AR) is attached to a fluorescent protein. It translocates to the site of GTPase activation, revealing the localization and kinetics of activation. (b) Single-chain intramolecular biosensors. The GTPase, an AR, and fluorescent proteins capable of undergoing FRET are combined in a single chain. When the GTPase is activated, the AR binds the GTPase, affecting the separation and orientation of the fluorescent proteins and thereby affecting FRET. In some cases, the fluorescent protein is attached to the C-terminus (top). Because this destroys the motifs needed for membrane localization, a lipid is attached to the C-terminal fluorescent protein, leading to constitutive membrane localization. In other cases, the fluorescent proteins are inserted in the middle of the chain, leaving the C-terminus of the GTPase unaltered and free for interaction with the membrane and with proteins that regulate reversible membrane localization. (c) Dual-chain intermolecular biosensors. Here, the AR and GTPase are each tagged with a fluorescent protein and are expressed as separate chains. Activation of the GTPase leads to intermolecular AR binding and FRET. (d) Single-chain biosensors directed against endogenous targets. Here, a pair of fluorescent proteins that can undergo FRET is attached to the AR. Their relative positions are affected on AR-GTPase binding, leading to a change in FRET.

distributed identically and therefore do not require correction for spectral bleed-through. However, the dual-chain designs can be more sensitive because there is no residual FRET in the "off state," and bleaching does not affect quantitation in fluorescence lifetime imaging microscopy (FLIM) as it does for single-chain designs. Furthermore, dual-chain designs can be easier to make because they do not require the difficult optimization of multiple linkers and protein orientations (Baird et al. 1999). Unfortunately, dual-chain biosensors have a tendency to produce more heterogeneous data because of variations in the relative expression levels of the two chains. Dual- and single-chain biosensors can introduce distinct biological perturbations, and they differ in their propensity to produce either false negatives or false positives. Single-chain designs have a greater tendency to act as dominant negative protein analogs because, on activation, the intramolecular interaction of the affinity reagent outcompetes interactions with native effector molecules. In dual-chain designs, the affinity reagent can be competed away by endogenous effectors, leading to "false negatives." It is essential to carry out appropriate controls, including titrating the intracellular expression of the biosensor against the perturbation of cellular behavior. Past studies have shown that it is possible to use "tracer amounts" of biosensor that report activation through reversible interactions with upstream regulators while not unduly perturbing the cell behavior under study (Kraynov et al. 2000; Nalbant et al. 2004). For tissue culture cells, cell brightness per unit cell area or volume has proven to be a simple measure that is proportional to biosensor concentration. Fluorescence-activated cell sorting (FACS) can be used to isolate cell populations of the appropriate brightness. One must be careful of the fact that different fluorescent proteins show different levels of misfolding and/or degradation.

It is also important to realize that GTPase biosensor designs can affect the membrane interactions of the GTPase, an important aspect of biological regulation. In some designs, the GTPase is constitutively anchored to the membrane (Mochizuki et al. 2001; Itoh et al. 2002; Yoshizaki 2003), while in other designs the sequences required for regulation of reversible membrane interaction remain intact (Figure 5.2) (Jiang and Sorkin 2002; Pertz et al. 2006; Machacek et al. 2009). Biosensors that are constitutively anchored to the membrane have been said to indicate GEF activity rather than overall GTPase activity. Some biosensors simply eliminate membrane localization altogether, a design that is difficult to interpret biologically (Kardash et al. 2010). Clearly, more systematic studies of these effects would be valuable, as it is important to identify precisely which specific subsets of GTPase interactions are being reported by a given design.

Many GTPase biosensors are not based on FRET. So-called "translocation biosensors" monitor the change in the localization of a fluorescently tagged GTPase, or a fluorescent effector domain, on GTPase activation. GTPases often

become concentrated in specific subcellular regions upon activation, such as the cell rim during wound healing (Benink and Bement 2005), or at the bottom membrane of the cell where they can be quantified by total internal reflection fluorescence (TIRF) microscopy (Navarro-Lerita et al. 2011; Sato et al. 2012). The signal from translocation biosensors is typically much brighter than that from FRET biosensors, but changes in localization must be pronounced enough to discern over a background of diffusely localized biosensors that is fluorescing at the same wavelengths. In contrast, the sensitivity of FRET is enhanced by the unique spectral signature produced on activation. Translocation biosensors are easy to use and amenable to high-content screening, but it is difficult to use them to discern or quantify subtle activation events such as gradients of activity.

To minimize cell perturbation, it is advantageous to use designs that report the activity of endogenous proteins. The translocation of fluorescently tagged affinity reagents has been used (Table 5.1), and in other cases, sensitivity has been enhanced by covalently modifying the affinity reagent with bright, environmentally sensitive dyes that change their fluorescence intensity or wavelengths when they bind the activated conformation of an endogenous GTPase (Figure 5.3). This technique has been used successfully to study the activation of endogenous Cdc42 and other non-GTPase proteins (Nalbant et al. 2004; Loving et al. 2010; Gulyani et al. 2011). The amount of biosensor needed for visualization is substantially lower than that needed for FRET, because the dyes can be intrinsically brighter than FPs and are directly excited. However, the dye-labeled proteins must be microinjected, electroporated, and so forth, rather than simply being expressed. A final approach to report the activity of endogenous GTPases has been to fuse two FPs to the affinity reagent. The fluorophores are positioned such that FRET is altered on binding of the affinity reagent to the activated GTPase target.

Although the focus of this chapter is on live-cell imaging, we briefly note that several GTPase biosensors have been developed for *in vitro* applications. Environment-sensing dyes attached directly to GTPases report conformational changes, and fluorescently labeled nucleotides have been used *in vitro* to study the kinetics of GTPase activation/deactivation, as well as interactions with various effectors (Nomanbhoy et al. 1996; Nomanbhoy and Cerione 1999; Goguen et al. 2011).

In the following sections, we have organized the small GTPase biosensors by target molecule, hoping that this will be useful to those interested in finding available biosensors for the target(s) they are studying. We discuss the different design strategies employed and provide what we hope is a comprehensive list of published GTPase biosensors in Table 5.1. We sincerely apologize to our colleagues for the inevitable and unintentional omission of some biosensors.

TABLE 5.1 PUBLISHED GTPASE BIOSENSORS

GTPase	Design	References
Rho-Family		
RhoA	Single-chain	Yoshizaki (2003); Pertz et al. (2006)
	Single-chain, endogenous target	Yoshizaki (2003)
	Dual-chain	Murakoshi et al. (2011)
	Translocation sensor	Benink and Bement (2005)
Rac1	Dual-chain, dye-based	Kraynov et al. (2000); Del Pozo et al. (2002)
	Dual-chain, genetically encoded	Machacek et al. (2009)
	Single-chain	Itoh et al. (2002)
	Single-chain, endogenous target	Graham et al. (2001)
Cdc42	Single-chain	Itoh et al. (2002); Seth et al. (2003); Kamiyama and Chiba (2009)
	Dual-chain	Kamiyama and Chiba (2009); Murakoshi et al. (2011)
	Dye-conjugated affinity reagent	Nalbant et al. (2004); Goguen et al. (2011)
	Single-chain, endogenous target	Seth et al. (2003); Lorenz et al. (2004); Graham et al. (2001)
	Translocation sensor	Benink and Bement (2005); Kim et al. (2000); Kumfer et al. (2010)
	Direct dye labeling	Nomanbhoy et al. (1996); Nomanbhoy and Cerione (1999); Goguen et al. (2011)
RhoC	Single-chain	Bravo-Cordero et al. (2011)
	Dual-chain	Zhong et al. (2007)
TC10	Single-chain	Kawase et al. (2006)
	Dual-chain	Pommereit and Wouters (2007)
Ras-Family		
Ras (H, K, N)	Single-chain	Mochizuki et al. (2001)
	Dual-chain	Jiang and Sorkin (2002); Hibino et al. (2003); Yasuda et al. (2006)
	Translocation sensor	Bondeva et al. (2002); Chiu et al. (2002) Caloca et al. (2003); Augsten et al. (2006)
	Dye-conjugated affinity reagent	Murakoshi et al. (2004)

(*Continued*)

TABLE 5.1 (CONTINUED) PUBLISHED GTPASE BIOSENSORS		
GTPase	**Design**	**References**
Ras-Family		
R-Ras	Single-chain	Takaya et al. (2007)
Rap1	Single-chain	Mochizuki et al. (2001)
	Translocation sensor	Bivona et al. (2004)
RalA/B	Single-chain	Takaya et al. (2004)
Rab/Ran-Family		
Ran	Single-chain, endogenous target	Kaláb et al. (2002, 2006)
	Single-chain	Kaláb et al. (2002)
	Dual-chain	Plafker (2002)
Rab5	Single-chain	Kitano et al. (2008)
	Dual-chain	Galperin (2003)
Rab6	Dual-chain	Thyrock et al. (2010)
Rab35	Single-chain	Ishido et al. (2011)
Rab10	Dual-chain	Chen et al. (2009)
Sar1/Arf and Rheb-Families		
Arf6	Dual-chain	Hall et al. (2008)
Rheb	Dual-chain	Li et al. (2007)

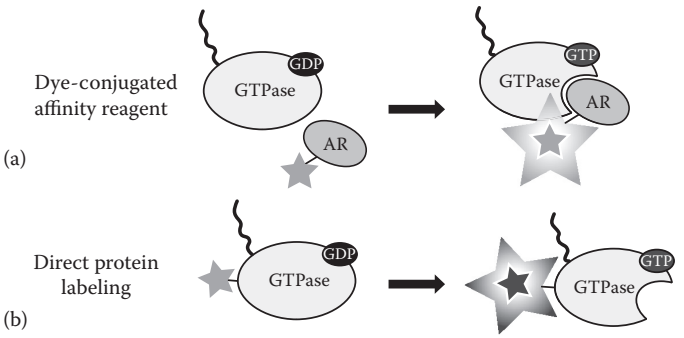

Figure 5.3 GTPase biosensors based on solvatochromic dyes. (a) The AR is covalently labeled with a dye whose fluorescence is affected by the environment. When the AR binds the GTPase, the dye encounters a more hydrophobic environment and/or undergoes interactions with specific amino acids, leading to a change in fluorescence. (b) The GTPase can be directly labeled with an environmentally sensitive fluorescent dye. Changes in conformation affect residues around the dye, altering fluorescence.

5.2 Rho-FAMILY GTPases

The Rho family of GTPases comprises 20 homologous proteins expressed ubiquitously in mammals. They are essential signaling components in a broad range of dynamic cellular events that require precise spatial and temporal control. These processes include cytoskeletal rearrangements, vesicular trafficking, cell migration, and polarization. In this section, we discuss biosensors for the three best characterized members of Rho-family proteins: RhoA, Rac1, and Cdc42.

5.2.1 Rac1

Based on sequence homology, Rac1, Rac2, Rac3, and RhoG form a subgroup within the Rho family of GTPases (Boureux et al. 2006). Rac proteins initiate lamellipodia and membrane ruffling and are also involved in membrane formation during phagocytosis. Like the Rho isoforms, each Rac subfamily member appears to have nonredundant functions. For example, whereas Rac1 and RhoG are widely expressed (Vincent et al. 1992), Rac2 expression is restricted to hematopoietic cells (Didsbury et al. 1989; Shirsat et al. 1990), and Rac3 is abundantly expressed in the brain (Haataja et al. 1997; Bolis et al. 2003; Corbetta et al. 2005). Moreover, whereas Rac1-null mice are embryonic lethal (Sugihara et al. 1998), Rac2-, Rac3-, and RhoG-null mice have no overt developmental abnormalities but present subtle cell-type–specific defects (Vincent et al. 1992; Roberts et al. 1999; Vigorito et al. 2004; Cho et al. 2005; Corbetta et al. 2005). Despite the pleiotropic effects of Rac homologues, all biosensors published in the literature have thus far centered on Rac1, but these designs may well be readily extended to the other homologues.

The first GTPase biosensor for living cells, Rac–FLARE (fluorescence activation indicator for Rho proteins), was based on two components: Rac1 fused to a GFP molecule and the p21-binding domain (PBD) of Pak1, which bound selectively to the activated conformation of the GTPase (Kraynov et al. 2000). The Pak1-binding domain (the affinity reagent) was labeled on a cysteine near its N-terminus with the FRET-acceptor dye Alexa 546. The design preserved normal binding to GDI and regulation of membrane translocation and showed a dynamic range of greater than 400%. Imaging studies with Rac–FLAIR provided our first glimpse of the highly dynamic nature of Rho GTPase signaling inside living cells. Local increases in Rac activity were shown to correlate in time and space with cell protrusion and the production of membrane ruffles, and a broad gradient of Rac activity was demonstrated at the leading edge of motile cells. These previously unattainable observations supported specific models of Rac's role in polarization and were consistent with biochemical and genetic studies indicating a role in protrusion. Genetically encoded versions of

this biosensor have since been generated and coupled with novel image analysis approaches for the precise quantitation of Rac dynamics during protrusion and retraction (Machacek et al. 2009).

Rac biosensors took advantage of FPs engineered to undergo FRET, including enhanced blue fluorescent protein (EBFP) and enhanced green fluorescent protein (EGFP). In 2001, a group led by Peter Chalk explored the latitude for preserving FRET in two fused FPs by inserting flexible linkers of increasing length between EGFP and EBFP molecules. Surprisingly, not only were linkers as long as 50 amino acids tolerated, but also the FRET between EBFP and EGFP increased in proportion to linker length (Graham et al. 2001). This suggested that biologically relevant protein sequences could be inserted between two fluorescent molecules to change FRET efficiencies on target protein binding. Such a simple arrangement could be applied as a general design to construct additional biosensors. They tested this hypothesis by inserting the PBD of Pak1 between EGFP and EBFP and employed various *in vitro* assays to assess FRET changes on binding to active Rac1 and Cdc42 (Graham et al. 2001). In experiments with purified proteins, high levels of constitutive FRET in the absence of activated GTPase were reduced by approximately 60% when the PBD bound to active Rac1. The use of the Chalk et al. biosensor in living cells was not reported, likely because of the relatively small amount of FRET produced and because EBFP is relatively dim, photobleaches rapidly, and requires "cytotoxic" excitation wavelengths. Subsequent improvements in FPs greatly aided FRET-based biosensor design by expanding the color palette of FPs and enhancing their spectral properties. Almost all Rho-family biosensors have been based on derivatives of enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP), including the ECFP variants mCerulean (Rizzo et al. 2004), CyPet (Nguyen and Daugherty 2005), Azurite (Mena et al. 2006), EBFP2 (Pédelacq et al. 2006), and TFP1 (Ai et al. 2006) and the EYFP variants mVenus (Nagai et al. 2002), YPet (Nguyen and Daugherty 2005), and Citrine (Griesbeck et al. 2001).

By harnessing these genetically encoded FRET pairs and incorporating innovations such as a single-chain design, the Matsuda group generated Rho-family biosensors that were substantially more practical than their predecessors. These included iterations of their "Raichu" design scheme, as well as an improved version of the Chalk et al. biosensor. Their first sensor, named Raichu–Rac, included an N-terminal EYFP followed by the PBD of Pak1, a flexible linker, Rac1, CFP, and the C-terminal farnesylation moiety from K-Ras (Itoh et al. 2002). In this arrangement, the biosensor changes conformation when the PBD binds to active Rac1, bringing the two terminal FPs together to produce FRET. This design resulted in an *in vitro* dynamic range of approximately 80%. Their second biosensor, named Raichu–CRIB, employed the design of Chalk et al. but substituted EGFP and EBFP with EYFP and ECFP. Raichu–CRIB could report the activity of both endogenous Rac1 and Cdc42 (Itoh et

al. 2002). This biosensor responded to active Cdc42 with a dynamic range of approximately 40% and to Rac1 with approximately 10%. In the Raichu sensor designs, the C-terminus of the GTPase is replaced by ECFP. Because this displaces the C-terminal lipid modifications required for membrane localization, a C-terminal fusion of the farnesyl moiety from K-Ras was added, leading to constitutive membrane localization. The authors reported that these modifications abrogated binding to RhoGDI, eliminating this pathway for downregulating GTPase activity (Itoh et al. 2002). Newer derivatives of these biosensors, in which the terminal FP has been shifted to an internal site, enable native regulation of GTPase localization (Benard et al. 1999; Pertz et al. 2006). Raichu–Rac1 that is constitutively membrane bound likely reports GEF activity at the membrane rather than the GTPase cycle itself. Others have modified the initial Raichu–Rac1 design by removing the K-Ras localization sequence altogether, thereby generating a cytosolic Rac1 sensor (Kardash et al. 2010). Such designs must be approached with caution, as membrane localization is an important determinant of GTPase specificity.

5.2.2 RhoA

RhoA, along with its highly homologous isoforms RhoB and RhoC (85% amino acid sequence identity), is well known for its ability to regulate cell contractility and stress fiber formation when overexpressed in fibroblasts (Wheeler and Ridley 2004). The cellular functions of Rho proteins have been studied extensively with dominant-negative strategies or with clostridial enzyme C3 transferase, which modifies and inhibits all three Rho isoforms. However, recent evidence suggests that individual Rho isoforms have distinct roles in cellular events. For example, dominant-negative RhoA, RhoB, and RhoC each have distinguishable effects on cells (Rondanino et al. 2007), and genetic studies suggest that RhoB-null and RhoC-null mice have distinct functions *in vivo* (Liu et al. 2001; Hakem et al. 2005).

RhoA biosensors have been designed by the Matsuda group based on the Raichu framework (Raichu–RhoA) (Yoshizaki 2003). Several effector domains of RhoA were tested as affinity reagents, along with different modular orientations of RhoA and the affinity reagent to develop a single-chain activity reporter with optimized dynamic range. The best response was achieved using the RhoA-binding domain (RBD) of protein kinase N (PKN), with other components remaining in the order described for Raichu–Rac1 (EYFP–PKN–linker–RhoA–ECFP–farnesyl moiety). This resulted in a constitutively membrane-bound Raichu–RhoA sensor with an *in vitro* dynamic range of 33%. The second sensor was based on the design of Raichu–CRIB, with two modifications: the RBD of Rhotekin was used instead of the CRIB domain to generate a biosensor specific for RhoA (Raichu–RBD), and the K-Ras farnesyl moiety was

removed from the C-terminus (Yoshizaki 2003). The resulting Raichu–RBD biosensor produced an *in vitro* dynamic range of approximately 20%. Intriguingly, the response of Raichu–RBD to overexpression of wild-type RhoA was indistinguishable from its response to constitutively active RhoA (RhoA–Q63L).

Our group also created a RhoA biosensor, named RhoA–FLARE (Pertz et al. 2006). This single-chain design consisted of the RBD of Rhotekin, CFP, an unstructured linker of optimized length, YFP, and full-length RhoA (RBD–CFP–linker–YFP–RhoA, with C-terminus intact for interaction with GDI and membrane localization). On RhoA activation, the RBD binds RhoA, bringing the two fluorescent proteins into closer proximity to enhance FRET. The biosensor has a dynamic range of approximately 40%, as assessed in intact HEK293 cells. Similar to Raichu–RBD, wild-type RhoA–FLARE and constitutively active RhoA–FLARE mutants initially yielded similar results. However, the elevated response of wild-type sensors was greatly reduced when the biosensor was coexpressed with RhoGDI, suggesting that overexpression of RhoA biosensors can saturate the ability of endogenous GDI to inactivate the biosensor. This response to GDI concentration has been seen with biosensors that have an intact C-terminus, but is not seen when the C-terminus is altered. These observations illustrate the profound influence that molecular design can impart on biosensor behavior and interpretation. By titrating down the intracellular concentration of the GDI-responsive sensor to a level where reversible membrane localization was observed, we were able to visualize activation events that had previously been obscured, and activity at the membrane was greatly reduced.

A notable difference between biosensors is the composition of their linkers. Detailed studies examining linker length and composition have demonstrated that both factors can have profound effects on the fluorescence properties of FRET-based systems (Graham et al. 2001; Ohashi et al. 2007), but a neglected aspect has been the effect on degradation, which can generate species that affect biology yet produce no fluorescence response. Most designs have used extended polypeptides rich in glycine and serine or threonine residues because they confer enhanced solubility and unrestricted movement of the protein domains. Detailed protein engineering studies examining extended Gly-Ser/Thr linkers in single-chain Fv antibody fragments have found that such linkers are subject to aggregation and proteolytic cleavage (Whitlow et al. 1993). The RhoA–FLARE biosensor employs an optimized linker that has been demonstrated to remain flexible when fused to proteins, resist aggregation, and resist proteolytic cleavage (Whitlow et al. 1993), and may therefore be useful in other biosensors.

5.2.3 Cdc42

Cdc42 is an ancient molecule with a conserved role in regulating cell polarity and the actin cytoskeleton. It is involved in a wide range of eukaryotic

cell processes including yeast budding, epithelial polarity, migratory polarity, and cell-fate specification. It was first identified as a cell cycle mutant in *Saccharomyces cerevisiae*, as the loss of Cdc42 prevents budding and mating projection (Adams et al. 1990). It is the most heavily studied Rho-family GTPase in terms of the diversity of biosensors that have been developed to investigate its regulation *in vitro*, in live-cell imaging, and in whole-animal studies.

5.2.3.1 Dye-Based Cdc42 Biosensors

The first biosensors to probe the regulation of Cdc42 and its interactions with effectors *in vitro* were described in a series of papers by Cerione and colleagues in the 1990s. Nomanbhoy et al. (1996) reported that a specific native lysine could be selectively labeled with a fluorescent succinimidyl ester (sNBD) and that the attached dye underwent a change in fluorescence intensity upon GTP loading. A nucleotide-dependent conformational change in Cdc42 likely altered the quenching of dye fluorescence by surrounding residues. The kinetics of GDP/GTP exchange and GTP hydrolysis were not affected by this covalent modification. Although the authors did not pursue live-cell studies, their work paved the way to derivatize Cdc42 with environmentally sensitive dyes designed for *in vivo* imaging. Direct labeling of endogenous proteins with reporter dyes has proven effective for tracing protein activity in vivo (Hahn et al. 1992; Toutchkine et al. 2003; Garrett et al. 2008; Macnevin et al. 2013) and has the advantage of reduced biological perturbation.

In contrast to the direct labeling of Cdc42, our laboratory took an alternate approach to visualize the activation of endogenous, untagged Cdc42. A solvent-sensitive dye with properties optimized for live-cell applications was attached to a fragment of the Cdc42 effector Wiskott Aldrich syndrome protein (WASP) (Nalbant et al. 2004). This fragment bound selectively to the active conformation of Cdc42, leading to a change in the fluorescence of the attached dye that revealed the localization and kinetics of Cdc42 activation. This approach was potentially less perturbing than some others because bright dyes were directly excited, and because there was no need to express exogenous modified Cdc42. This biosensor, termed MeroCBD (merocyanine dye–Cdc42 binding domain) did not interact with homologous Rho-family GTPases such as RhoA and Rac1 but did bind to the very closely related GTPase TC10 (Figure 5.4). The MeroCBD sensor revealed the dynamics of Cdc42 activation at broad protrusions, filopodia, and the Golgi apparatus of motile fibroblasts. Cdc42 was shown to be activated during protrusion in a microtubule-dependent manner, and activity was correlated in time and space with the extension/retraction of the cell edge (Machacek et al. 2009). Although this design enabled the detection of endogenous protein activity with high sensitivity, it had to be introduced into the cell using microinjection.

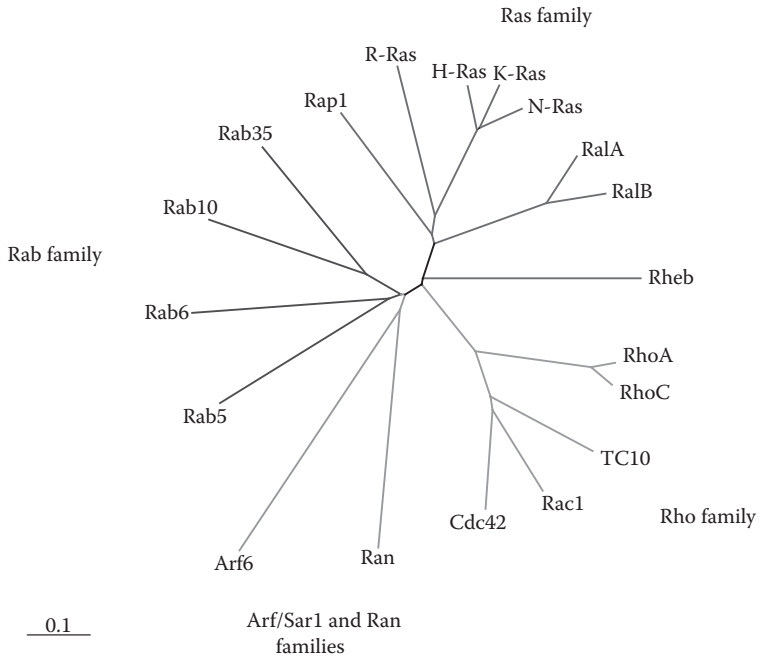

Figure 5.4 Phylogenetic tree of the small monomeric G-protein family. The amino acid sequences of human monomeric GTPases were aligned using the ClustalW program, and an unrooted phylogenetic tree was generated using the TreeView program. The 19 representative members of the family can be classified into four subfamilies. These include the Rab, Ras, Rho, and Arf/Sar1 and Ran subfamilies. Scale bar represents 0.1 amino acid substitutions per site.

5.2.3.2 Cdc42 Translocation Biosensors

In addition to dye-based biosensors, several translocation biosensors have been used to study Cdc42. Kim et al. (2000) studied E-cadherin–dependent Cdc42 activation in MCF-7 epithelial cells by fusing the Cdc42-binding domain of WASP to EGFP and imaging its accumulation at cell-cell junctions. Benink and Bement (2005) used a similar design to co-image activated Cdc42 and RhoA simultaneously in a *Xenopus* wound-healing assay. They fused the RhoA binding domain of Rhotekin to EGFP and the Cdc42 binding domain of N-WASP to monomeric red fluorescent protein (mRFP), thereby demonstrating the formation of spatially discrete rings of RhoA and Cdc42 activity around the wound. These distinct zones of GTPase activity formed rapidly and moved inward as the wounds healed. Through the use of these sensors, they demonstrated

that the formation, segregation, and/or movement of these discrete rings were affected by microtubules, the actin cytoskeleton, and crosstalk between RhoA and Cdc42. In a series of elegant experiments, this group observed changes in the formation and segregation of both the Cdc42 and RhoA rings, leading them to hypothesize that RhoA negatively regulates Cdc42. Moreover, RhoA requires a small amount of Cdc42 to become active, but RhoA is inhibited by specific localized regions of Cdc42 activity.

Although highly useful, sensing activation by observing the accumulation of effector-binding domains presents difficulties in quantitation and in observing some activation events. High contrast is required to resolve changing activation in small subcellular structures such as filopodia, and ratio imaging against volume indicators must be used to determine whether changes in fluorophore intensities are due to GTPase dynamics or alterations in cell thickness and/or illumination. Despite these challenges, translocation sensors have been very valuable because of their ease of design and implementation and have provided unambiguous readouts of Cdc42 activation where translocation clearly occurs.

The straightforward readout provided by translocation sensors can simplify studies in complex environments. Kumfer et al. (2010) adapted the Cdc42 translocation sensor to *Caenorhabditis elegans* to study the spatiotemporal dynamics of Cdc42 activation in establishing and maintaining cell polarity in the single-cell worm embryo. They fused the Cdc42-binding domain of the *C. elegans* WASP homolog (WSP-1) to EGPF and imaged GTPase activity throughout the developing embryo. Using this probe, they demonstrated the coordinated movement of Cdc42 activity from the posterior to the anterior embryo at specific stages in development and then used an RNAi library to identify regulators of this movement. Based on sequence analysis, they selected 18 putative GEFs and 22 putative GAPs and determined how knockdown of each protein affects the posterior–anterior migration of Cdc42 activity. They identified a novel GEF and GAP for Cdc42 in *C. elegans* and demonstrated that precise spatiotemporal coordination of these regulators is required to achieve proper cell polarity.

5.2.3.3 FRET-Based Cdc42 Biosensors

Matsuda and co-workers produced a single-chain, intramolecular FRET biosensor for Cdc42 based on the Raichu design, which was made and tested in conjunction with Raichu–Rac1 (Itoh et al. 2002). Both biosensors use a fragment of Pak as the affinity reagent and are based on the following design: CFP–GTPase–PAK fragment–YFP–K-Ras membrane-targeting sequence. In this study, both Rac1 and Cdc42 were shown to localize to the leading edge in motile HT1080 cells. However, Cdc42 was very tightly localized to the edge, whereas Rac1 was more broadly activated.

Seth et al. (2003) developed two biosensors based on different designs, each described and characterized as reporters of Cdc42 GEF activity. The GTPase-binding domain of WASP was inserted between terminal ECFP and EYFP fluorophores, either alone or with a C-terminal VCA domain. The biosensor was characterized carefully both *in vitro* and in live-cell assays, but they found that the *in vivo* sensitivity of these biosensors was too low to detect endogenous protein. They therefore performed experiments in cells by coexpressing the biosensor along with ectopic GTPase. Interestingly, they found that the rate of GEF exchange for this sensor was comparable to that of endogenous Cdc42, but the rate of GAP-mediated hydrolysis was 16-fold slower, leading the authors to classify this as a GEF biosensor. With important implications for GTPase biosensors in general, the authors suggested that single-chain sensors may have greater difficulty in reporting deactivation kinetics, as GAPs and effectors typically bind to overlapping domains on the GTPase. Affinity reagents derived from effector proteins would have similar competitive binding interactions.

Finally, Kamiyama and Chiba (2009) describe a FRET-based biosensor designed for whole-animal studies. They developed both single- and dual-chain activation probes termed A-probe.1 and .2, respectively. A-probe.1 is fully genetically encoded and uses a p21-binding domain from *Drosophila* WASP in the configuration CFP–Cdc42–PBD–YFP. In contrast to A-probe.1, the dual-chain version is not fully genetically encoded but instead consists of EGFP–Cdc42 and PBD conjugated to the dye Alexa546. To use A-probe.2, the embryos were dissected, fixed prior to incubation in a bath of Alexa546-PBD, and washed before imaging. The authors report qualitatively similar results with both probes and performed the majority of studies with A-probe.1. Using a constitutive promoter to drive the expression of A-probe.1 in the whole embryo, Kamiyama and Chiba demonstrated that, although Cdc42 is ubiquitously expressed in the developing embryo in all stages, the GTPase is not active until more than two-thirds of development is complete. This was also observed when the sensor was restricted to a specific tissue such as the trachea or CNS. Interestingly, the deletion of Cdc42 is 100% fatal, but the majority of observable development proceeds normally. Their biosensor data provide a potential explanation for these results. The authors also investigated the spatiotemporal dynamics of Cdc42 activation in specific cells such as aCC motor neurons. Typically, neuronal polarization initiates with the specification and selection of the axon, and dendrogenesis occurs after axon specification. In aCC motor neurons, the authors found no detectable FRET in the axons until 4 h after axon initiation. Active Cdc42 was restricted primarily to the proximal axon just before dendrogenesis, suggesting a spatially restricted concentration of Cdc42 during neuronal morphogenesis.

5.3 Ras-FAMILY GTPases

The Ras subfamily consists of 36 members with three main branches: Ras, Rap, and Ral (Reuther and Der 2000; Takai et al. 2001; Malumbres and Barbacid 2003). Ras is the founding member of a GTPase superfamily that now comprises more than 150 proteins. Ras-family GTPases are responsible for mediating mitogenic processes including cell growth, proliferation, and differentiation. Their role in these essential functions, as well as their contribution to many cancers, has led to more than 30 years of very active research into Ras-family proteins.

5.3.1 Ras

H-Ras, N-Ras, K-Ras-4A, and K-Ras-4B, collectively referred to as Ras, are highly homologous proteins (approximately 80% homology). They were initially identified as viral oncogenes, and mutations in Ras have been implicated in approximately 20–30% of all cancers. As with many homologous GTPases, the various Ras proteins were initially thought to mediate functionally redundant processes, but this idea has been challenged on a number of fronts. Although all Ras isoforms are ubiquitously expressed, the relative amounts of each variant differ across cell types and, typically, mutation of a specific isoform is enriched in a given type of tumor (Almoguera et al. 1988; Grady and Markowitz 2002; Mitsuuchi and Testa 2002). The greatest variability among the isoforms is in the C-terminal hypervariable region (HVR), and this region is the primary determinant of localization and membrane association (Reuther and Der 2000; Castellano and Santos 2011). Ras proteins are regulated in part by differential membrane association mediated by posttranslational lipid modification of the HVR. H-Ras, N-Ras, and K-Ras-4A undergo palmitoylation and are brought to the plasma membrane via the secretory pathway. In contrast, K-Ras-4B does not undergo palmitoylation and is shuttled directly from the endoplasmic reticulum to the plasma membrane, bypassing the Golgi and vesicular trafficking (Reuther and Der 2000). In addition to differences in lipid modification and localization, evidence is mounting that regulators of Ras activity (e.g., GEFs and GAPs) have distinct specificity toward various isoforms, and individual isoforms show preferential specificity toward different effectors (Castellano and Santos 2011). It is well accepted that Ras signals from the plasma membrane, but whether or not signaling occurs from the endoplasmic reticulum, Golgi, and/or mitochondrial membranes has been the subject of vigorous debate. Several researchers have attempted to address questions regarding both the spatial and temporal regulation of Ras signaling through the use of live-cell biosensor imaging.

5.3.1.1 Ras Translocation Biosensors

The majority of Ras biosensors are translocation sensors, typically utilizing various domains of the effector protein Raf1 fused to GFP. The first such sensors, reported in 2002, led to new insight into Ras signaling but also gave rise to apparently conflicting conclusions. Using a GFP-tagged Raf1 RBD in conjunction with ectopic H-Ras expression, Chiu and colleagues observed the accumulation of active Ras in the endoplasmic reticulum and Golgi and in the plasma membrane in response to serum stimulation (Chiu et al. 2002). They also found that when H-Ras was mutated to prohibit palmitoylation, thereby blocking secretory pathway trafficking, the GFP–RBD sensor no longer accumulated in internal membranes. Strikingly, they showed that the kinetics of translocation were different at the endoplasmic reticulum and Golgi versus at the plasma membrane, leading the authors to suggest that Ras participates in distinct signaling pathways at these different membranes. At the same time, Bondeva and colleagues used a similar GFP–RBD probe to assay endogenous Ras activity in normal and virally H-Ras-transformed NIH3T3 cells (Bondeva et al. 2002). These authors found that both the RBD and an additional Raf1 domain, the cysteine-rich domain (CRD), were required to monitor translocation of the probe to detect endogenous Ras. They found significant translocation (and therefore activation) at the plasma membrane but not at the Golgi. Similarly, Augsten et al. (2006) developed a translocation sensor designed to image endogenous Ras. They used the oligomerization of up to three Raf1 RBD domains fused to GFP to increase the affinity of the sensor for Ras. This increase in affinity enabled the characterization of Ras activation in the absence of exogenous expression. They found that in response to growth factor stimulation, endogenous Ras was active on the plasma membrane but not in the Golgi. It has been suggested that the discrepancies between these studies can be explained by ectopic expression of Ras. However, differences in experimental conditions, cell types, growth factors, and coexpressed molecules, and so forth, preclude the drawing of firm conclusions. As discussed in Section 5.1, these translocation probes require a high contrast between probe accumulation and background. Unfortunately, GFP–RBDs are broadly localized and concentrated in the thickest part of the cell, near the nucleus and Golgi, making it difficult to identify activity in this important region (Bondeva et al. 2002; Bivona et al. 2004). Ras-family GTPases such as Rap1 have effector-binding domains identical to Ras and bind many of the same effector proteins. Therefore, Ras translocation probes that have been engineered to have a high affinity for Ras may also interact with endogenous Rap.

5.3.1.2 FRET-Based Ras Biosensors

Several single- and dual-chain Ras biosensors have been developed, potentially addressing some of the ambiguities discussed in Section 5.3.1.1. The first FRET-based Ras biosensor was the founding member of the Raichu sensor family

developed by Matsuda and colleagues (Mochizuki et al. 2001), named for the Ras and interacting protein chimeric unit (and/or for the fire-loving Pokémon character). Raichu–Ras consists of H-Ras and the Raf1-binding domain flanked by CFP and YFP and includes a C-terminal K-Ras4B tag to constitutively target the construct to the plasma membrane (YFP–Ras–Raf1 RBD–CFP–KRas-4B tag). This sensor revealed that Ras is activated predominately at the plasma membrane in EGF-stimulated COS-1 cells and that sustained Ras activation in nerve growth factor (NGF)-stimulated PC12 cells is seen only in extended neurites. A dual-chain Ras biosensor developed by Jiang and Sorkin (2002) consists of YFP–RBD and CFP–Ras. This design enabled regulation of the CAAX-box protein domain involved in membrane targeting. Using this sensor as part of a larger study on signaling initiated by the epidermal growth factor receptor (EGFR), the authors found that Ras is activated both at the plasma membrane and in endosomes. Furthermore, they showed that although both H-Ras and K-Ras are ubiquitously expressed, H-Ras is seen to a greater extent in endosomes, whereas K-Ras is preferentially localized to the plasma membrane. Therefore, as with the translocation sensors, different conclusions have been drawn from experiments utilizing these distinct designs, leading to a vigorous debate regarding the regulation of Ras activity in specific cellular compartments. The distinction between biosensors responding to different modes of regulation could inform this discussion.

The majority of Ras biosensors have been developed to determine the membrane from which Ras signals and the kinetics of its activation in distinct subcellular compartments. In contrast, Murakoshi et al. (2004) designed a novel biosensor to enable single-molecule studies of activated Ras in an effort to understand how Ras mediates protein–protein interactions and thereby orchestrates complex signaling networks. These authors stably expressed YFP-tagged Ras in KB cells and microinjected dye-labeled GTP. This design utilizes YFP as a donor and the dye BodipyTR as an acceptor for FRET. Using these two bright fluorophores in TIRF microscopy enabled the detection of single activated Ras molecules. Ras was shown to be activated in the plasma membrane in response to EGF, and activation strongly affected the diffusion rate of Ras molecules. These findings led the authors to hypothesize that Ras becomes part of a large, fixed signaling complex upon activation and does not mediate downstream signaling by random collision with effector molecules. Furthermore, K-Ras and H-Ras had different diffusion rates under certain experimental conditions, providing evidence that these highly homologous GTPases are not simply redundant. Single-molecule studies were also carried out by Hibino et al. (2003), primarily using a full-length GFP-tagged Raf1 translocation sensor. Using this technique, the authors found that activated Ras in the plasma membrane is localized specifically to ruffles in EGF-stimulated HeLa cells and that Raf1 dissociates from the plasma membrane on two different characteristic time scales. Tools developed

for quantitative microscopy, such as two-photon fluorescence lifetime imaging (2pFLIM), would facilitate quantitation of signaling on a subcellular scale. In a series of elegant studies, Yasuda and colleagues developed 2pFLIM-optimized dual-chain biosensors of Ras, Rac1, and Cdc42 to enable the study of very fine structures including individual dendrites and spines in CA1 hippocampal neurons (Yasuda et al. 2006; Murakoshi et al. 2011).

5.3.2 Rap

The small GTPases Rap1A and Rap1B are responsible for mediating both mitogenic and adhesion signaling. Rap1A and Rap1B are 95% homologous and mediate overlapping functions, though they show differences in cell-type–specific expression and localization (Wittchen et al. 2011). Rap1 was initially cloned based on homology to Ras and identified as a Ras antagonist capable of reversing Ras-mediated transformation (Kitayama et al. 1989). Two decades of work on Rap proteins have since identified Ras-independent mitogenic and adhesion-based signaling roles. Rap1 has been shown to regulate cell differentiation and proliferation as well as integrin activation and cadherin-mediated cell junction formation (Kitayama et al. 1989; Hattori 2003; Bos 2005; Frische and Zwartkruis 2010). Rap1 is important in adhesion and extravasation of immune cells, owing largely to its role in mediating integrin processes, and is also essential in maintaining the epithelium (Hogg et al. 2011; Wittchen et al. 2011). Furthermore, Rap1 has been shown to be the master regulator of polarization in both neurons and T cells, where it regulates Rho-family GTPases such as Cdc42 and Rac1, as well as the Par polarity complex, to orchestrate cellular asymmetry (Schwamborn and Püschel 2004; Gerard et al. 2007; Schwamborn et al. 2007; Iden and Collard 2008). The ability of Rap1 to regulate such diverse processes and its role in highly localized phenomena such as junction formation and polarization has led to the development of tools to try to address some of these questions in live cells.

A single-chain Raichu–Rap1 biosensor was made using the configuration CFP–Rap1–Raf1 binding domain–YFP (Mochizuki et al. 2001). As with Raichu–Ras, the membrane localization of Raichu–Rap1 was achieved using a K-Ras4B tag that induced constitutive membrane localization. Strikingly, despite the plasma membrane localization tag, the biosensor was activated predominantly in the perinuclear region of COS-1 cells stimulated with EGF. Raichu–Ras and Raichu–Rap were used in the same study to understand how these similar molecules achieve specificity. Mochizuki et al. (2001) found that whereas Rap1 was predominantly activated in perinuclear regions, Ras was activated exclusively at the plasma membrane (see above), leading the authors to suggest that these GTPases mediate distinct processes through disparate cellular localization. In a subsequent study, a different sensor design was used to probe the dynamic regulation of Rap1 localization and trafficking. Bivona et al. (2004) constructed

a translocation sensor using the Rap1 binding domain of RalGDS fused to GFP to track the location of GTP-loaded Rap1. With this biosensor, Rap1 was found to be broadly localized in the perinuclear region, endosomes, and cytosol, whereas the activated protein was detected only at the periphery in the plasma membrane. Further study is needed to resolve these discrepancies; the development of biosensors that quantitatively report on the activity of Rap1 under native regulation may prove useful.

5.4 OTHER GTPase FAMILIES

5.4.1 Ran

The small GTPase Ran has long been known to maintain and compartmentalize genomic DNA within the cell (Clarke and Zhang 2008), to regulate spindle assembly (Clarke and Zhang 2001; Kaláb et al. 2006), and to control nuclear transport (Moore 1998). The only known Ran GEF, RCC1, has a high affinity for chromatin and is localized to the nucleus, whereas RanGAP is cytosolic. Indeed, the affinity of RCC1 for chromatin allows it to be used as a global marker for chromosomal organization. This cellular polarization of positive and negative Ran regulators was thought to heighten Ran activity in the nucleus, while diminishing its activity in the cytosol, and establish a gradient of Ran activity. Recent studies have demonstrated important roles for active Ran in the cytosol as well. For example, Ran mediates retrograde flow from the tips of axons toward the cell body in injured nerves, and neurons deficient in Ran show increased axonal branching and blebbing (Yudin and Fainzilber 2009). Moreover, the known Ran binding protein RanBP10 binds to microtubules in the cytoplasm of megakaryocytes and has been shown to have nucleotide exchange activity toward Ran, challenging the assumption that nuclear RCC1 is the only RanGEF (Schulze et al. 2008). Several Ran biosensors have been developed in an effort to study these phenomena *in vivo*.

Although many researchers hypothesized that local gradients of active Ran were required to facilitate Ran's varied functions, Kaláb et al. (2002) developed biosensors that could demonstrate their existence experimentally. They designed two biosensors, each with a binding domain flanked by the fluorophores CFP and YFP. In the first design, the affinity reagent directly bound RanGTP, and the interaction between the probe and Ran resulted in decreased FRET. In the second design, the affinity reagent (IBB) bound importin-β, because the interaction between IBB and importin-β is disrupted only in the presence of activated RanGTP. This latter design reported the activation of Ran through an increase in FRET efficiency. These biosensors demonstrated the existence of a sharp RanGTP gradient during spindle formation and revealed a role for RanGTP as a chromosomal positional marker. Unfortunately, the authors

found that the expression of IBB, derived from importin-α, disrupted cell cycle progression and could be toxic to somatic cells. Therefore, they refined their design to incorporate an importin-β–binding domain from snurportin 1 that is not toxic and they used the fluorophores EYFP and Cerulean for fluorescence lifetime microscopy (Kaláb et al. 2006). This new biosensor, termed Rango, is a measure of the RanGTP-mediated binding of importin-β to an affinity reagent and is not a direct measure of RanGTP itself.

5.4.2 Arf6

Members of the Arf family of GTPases were first identified as the cellular factors required for the toxic effect of cholera toxin through ADP ribosylation of the Gs heterotrimeric G protein (Kahn and Gilman 1986; O'Neal 2005). This ADP ribosylation factor (ARF) activity is shared by several homologous proteins, which were subsequently numbered Arf1–Arf6. Like other GTPases, the Arf proteins bind to their downstream effector targets only when bound to GTP. Nonetheless, there are several unique characteristics of Arf proteins that make them divergent from other small GTPases. Foremost among these, Arf proteins are not true GTPases. Like other GTPases, Arf proteins cycle through active and inactive states that are dependent on GTP binding and hydrolysis. However, Arf proteins do not possess intrinsic hydrolase activity and require the activity of a GAP to hydrolyze GTP. Moreover, Arf proteins share a set of structural features that define a larger family that encompasses Sar1 and a set of Arf-like (Arl) proteins (Pasqualato et al. 2002; Kahn et al. 2006). These proteins have a myristoylated N-terminal amphipathic helix rather than the C-terminal lipid modification common to other GTPases. GTP binding displaces the N-terminal amphipathic helix from a hydrophobic pocket within the protein, and this promotes the insertion of the helix into the plasma membrane (Goldberg 1998; Pasqualato et al. 2002). Such structural and mechanistic divergence from classical small GTPases has provided unique challenges in designing biosensors for the Arf family.

Nearly all genetically encoded GTPase biosensors fuse an FP to either the N- or C-terminus of the protein of interest. Unfortunately, neither terminus could be altered in Arf sensors. A group led by Martin Schwartz constructed a dual-chain FRET biosensor for Arf6 using the crystal structure to identify internal insertion sites (Hall et al. 2008). First, they identified an exposed loop composed of amino acid residues 140–148. Whereas direct insertion of a circular permutant of GFP after Ile144 resulted in an unstable protein, the introduction of 6-amino acid spacers at the N- and C- termini of the insertion site and the use of CyPet resulted in a stable chimeric protein. A fragment of the clathrin adaptor protein GGA was used as an affinity reagent. Using structural information from cocrystallized Arf6 and GGA, the second chain was created by fusing YPet to residues 148–303 from GGA3 (Hall et al. 2008). This resulted in a biosensor that showed

a 200% difference in FRET between constitutively active (Q67L) and dominant-negative (T27N) Arf6 mutants. This effect was also observed when the biosensor was coexpressed with ACAP1, a GAP for Arf6, or ARNO, an Arf6 GEF, indicating that the biosensor was subject to upstream regulators of GTPase activity. Indeed, the authors found that in fibroblasts stimulated with PDGF, Arf6 activity increased in a rapid but transient manner (approximately 10 min duration), suggesting that endogenous receptors and signaling molecules could function to properly regulate the biosensor's activation/deactivation cycle. However, the two components of the biosensor showed obvious differences in localization; YPet–GGA3 was primarily at the Golgi apparatus, whereas Arf6–CyPet was predominantly cytosolic and often concentrated within vesicles. When ratiometric imaging techniques were used, this required careful bleed-through correction.

5.5 CONCLUSION

The ability to observe dynamic changes in GTPase activity in living cells has provided tremendous insights into the molecular events that govern cell behavior. Unlike classical genetic methods, which do not reveal spatiotemporal information, biosensors shed light on the dynamic nature of protein interactions and structural changes. Clearly, a rich ensemble of experimentally validated biosensor designs now exists for Rho GTPases, and understanding the design elements used to formulate these reagents will allow the creation of additional small GTPase biosensors. For example, the Rab-family GTPases, known to regulate vesicle trafficking, are just beginning to be explored (Kitano et al. 2008; Chen et al. 2009; Thyrock et al. 2010).

Many approaches have been used to construct biosensors, but as highlighted here, no design is perfect. Importantly, each biosensor reports specific subsets of interactions, and each is subject to specific restrictions. This is not necessarily a disadvantage. Carefully characterizing and understanding of the limitations and focus of each biosensor will produce a deeper understanding the spatiotemporal dynamics that only biosensors can reveal. The wealth of biosensor designs with different sensitivities presents a means to compare the roles of different regulatory pathways. For example, negative results with one biosensor and not another, examined in light of their designs, can demonstrate the localized activity of negative regulatory pathways reflected by one biosensor.

Future work will couple new biosensor designs with new modes of microscopy and new computational methods to simplify animal imaging of protein activity, enhance quantitation of signaling dynamics with minimal perturbation, and enable imaging of multiple different molecules in the same cell (Welch et al. 2011). Protein labeling within living cells and protein import will enhance the applicability of dye-labeled biosensors (Griffin et al. 1998; Meyer et al. 2006;

Slavoff et al. 2011), and biosensors based on engineered protein scaffolds will provide access to previously intractable targets (Chen et al. 1994; Brient-Litzler et al. 2010; Gulyani et al. 2011).

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