

BIOSENSORS

A Versatile Toolkit to Produce Sensitive FRET Biosensors to Visualize Signaling in Time and Space

Rafael D. Fritz,¹ Michel Letzelter,^{1*} Andreas Reimann,¹ Katrin Martin,¹ Ludovico Fusco,¹ Laila Ritsma,² Bas Ponsioen,² Erika Fluri,¹ Stefan Schulte-Merker,^{2,3} Jacco van Rheenen,^{2,4} Olivier Pertz^{1†}

Genetically encoded, ratiometric biosensors based on fluorescence resonance energy transfer (FRET) are powerful tools to study the spatiotemporal dynamics of cell signaling. However, many biosensors lack sensitivity. We present a biosensor library that contains circularly permuted mutants for both the donor and acceptor fluorophores, which alter the orientation of the dipoles and thus better accommodate structural constraints imposed by different signaling molecules while maintaining FRET efficiency. Our strategy improved the brightness and dynamic range of preexisting RhoA and extracellular signal-regulated protein kinase (ERK) biosensors. Using the improved RhoA biosensor, we found micrometer-sized zones of RhoA activity at the tip of F-actin bundles in growth cone filopodia during neurite extension, whereas RhoA was globally activated throughout collapsing growth cones. RhoA was also activated in filopodia and protruding membranes at the leading edge of motile fibroblasts. Using the improved ERK biosensor, we simultaneously measured ERK activation dynamics in multiple cells using low-magnification microscopy and performed *in vivo* FRET imaging in zebrafish. Thus, we provide a construction toolkit consisting of a vector set, which enables facile generation of sensitive biosensors.

INTRODUCTION

To study signaling events with high spatiotemporal resolution, tools are required to measure protein activation events in single living cells, which has led to the development of fluorescence resonance energy transfer (FRET) biosensors based on fluorescent proteins. A subclass of these sensors consists of unimolecular genetically encoded fusions of a sensing module flanked with a donor fluorophore (DF) and an acceptor fluorophore (AF) that allow for simple, ratiometric measurements. With these unimolecular sensors, the spatiotemporal dynamics of second messengers (such as calcium, lipids, and cyclic nucleotides), phosphorylation events, protease activity, and the activation status of small guanosine triphosphatases (GTPases) have been visualized (1). The sensitivity of many of these biosensors, which depends on brightness and dynamic range, is limited, hampering the analysis of signaling events that are highly spatiotemporally regulated. For example, Rho family GTPases are regulated on time scales of tens of seconds and length scales of single micrometers (2, 3). Capturing these signaling events, therefore, necessitates sampling at high temporal frequency, using oil immersion, high numerical aperture (NA) objectives, which lead to bleaching of the fluorophores and phototoxicity to the cells. The efficiency of excitation and collection of emission limits the application of the currently available FRET biosensors for *in vivo* imaging in living animals. Thus, sensitive biosensors are needed, but their construction is a tedious task.

Each sensing module imposes unique structural challenges because of its dimensions and topology (compact domain or short peptide), and the

potential requirement of a free N or C terminus for lipid or other types of posttranslational modification. Therefore, the identification of a construct with a sufficient change in FRET efficiency from the ON to the OFF state and an acceptable signal-to-noise ratio typically requires generation and empiric testing of many variants. Geometrical parameters known to affect FRET, such as the distance between the fluorescent proteins and the relative orientation of their dipoles, which are modifiable through circularly permuted (cp) fluorescent protein variants, have been optimized to improve the dynamic range of various sensors (4–8). However, in these biosensors, only the AFs were modified. One further limitation is that most biosensors use a rather rigid design in which the DF and AF are placed at the N and C termini of the sensing module. In the case of biosensors that report small Rho GTPase activity, this is problematic because a native C terminus is essential for proper interaction with endogenous regulators. This was previously solved by placing fluorescent proteins internally to the sensing module (9). Thus, variation of the domain topologies of the sensing module is needed to preserve biological function and could simultaneously serve as an additional source of geometrical diversity. Therefore, we engineered a library of DF and AF fusions in which several geometrical parameters, such as fluorophore distance (using linkers of different length), dipole orientation (using cp mutants in both the AF and DF), and sensing module domain topology, were varied (Fig. 1). This approach accommodated structural constraints imposed by different signaling proteins to produce sensors with increased dynamic range.

We demonstrated the utility of these improved biosensors by analyzing the spatiotemporal dynamics of RhoA activity in neurites and filopodia in cultured neuroblastoma cells and in motile fibroblasts. RhoA is a crucial regulator of cell migration because it stimulates both membrane protrusion at the leading edge, contractility in the cell body, and retraction at the rear of the cell (2, 9, 10). We also studied the activity of extracellular signal-regulated protein kinase (ERK) in response to growth factors on a single-cell level and in zebra fish *in vivo*. ERK is a member of the Ras–Raf–MEK [mitogen-activated protein kinase (MAPK) and ERK kinase]–ERK

¹Institute of Biochemistry and Genetics, Department of Biomedicine, University of Basel, Mattenstrasse 28, 4058 Basel, Switzerland. ²Hubrecht Institute–Royal Netherlands Academy of Arts and Sciences and University Medical Center Utrecht, Uppsalalaan 8, 3584 CT Utrecht, the Netherlands. ³Experimental Zoology Group, Wageningen UR, 6708 Wageningen, the Netherlands. ⁴Cancer Genomics Netherlands, 3508 TC Utrecht, the Netherlands.

*Present address: PerkinElmer, Bahnstrasse 8, 8603 Schwerzenbach, Switzerland.

†Corresponding author. E-mail: olivier.pertz@unibas.ch

pathway that regulates cell proliferation, differentiation, survival, apoptosis, motility, and metabolism (11, 12).

RESULTS

Identification and characterization of mTFP1 cp mutants

We generated fluorescent protein fusion libraries based on the DF monomeric teal fluorescent protein (mTFP1), which we chose for its robust brightness (13), and the AF Venus (14). To manipulate the orientation between both fluorophores, we took advantage of circular permutations (cps) (6). cps are generated by connecting the native N and C termini with a linker followed by the introduction of new termini into surface-exposed loops of the fluorophore's β barrel (6, 15). Although cp sites for the *Aequorea* green fluorescent protein (GFP) are known and therefore extendable to its derivative Venus (6, 14), this is not the case for the *Clavularia*-based mTFP1. Thus, we screened all the possible mTFP1 cp variants in loop positions based on the mTFP1 crystal structure (13) (see note S1 for details). Upon expression in human embryonic kidney (HEK) 293T cells, we identified several cp sites at which mTFP1 retained its fluorescent properties (fig. S1A). We chose one mTFP1 cp variant per loop (cp105, cp159, cp175, and cp227) for detailed analysis. Although most of the cp permissive sites that we selected occurred at identical locations to those in Venus (for example, cp159, cp175, and cp227), the cp105 site was only permissive in mTFP1 and the cp49 site only occurred in Venus (fig. S1B). We expressed the four cp mTFP1 variants in *Escherichia coli* and evaluated their spectral properties (summarized in Table 1 and fig. S1, C to G). These four cp mTFP1 variants displayed absorption and emission spectra identical to the wild-type protein (fig. S1, C and D). The cp variants displayed only limited loss in brightness compared to the wild-type mTFP1, with cp175 exhibiting the largest reduction in fluorescence (26%), because of both reduced quantum yield and the smallest extinction coefficient (Table 1). However, even cp175 retained brightness that is in the same range as the cyan fluorescent proteins (CFPs) mCerulean (16) and mTurquoise (17). The cp variants retained robust pH insensitivity (fig. S1E) and maturation (protein folding and chromophore formation) speed (fig. S1F). The cp105 mutant was slightly more pH-sensitive, but not to an extent that should hamper its imaging in the cytosol (fig. S1E). All constructs retained robust photostability (fig. S1G). These cp mTFP1 variants are robust fluorophores that can be used as DFs to expand biosensor libraries.

Characterization of the RhoA biosensor library

Next, we used the preexisting biosensor for the small GTPase RhoA to evaluate our biosensor construction library. The first-generation RhoA biosensor (RhoA1G) consists of the RhoA-binding domain (RBD) of the effector rotekin that specifically binds guanosine triphosphate (GTP)–RhoA, a CFP DF, a 20–amino acid linker, the AF Citrine (18), and RhoA (9). Upon GTP loading, the RhoA portion interacts with RBD, leading to a conformational change and an increase in FRET efficiency. We constructed a RhoA biosensor library in which this architecture was varied in three distinct ways (Fig. 2A and fig. S2A). In design 1, RhoA1G DFs and AFs were exchanged for all possible combinations of the wild type or four cp variants of mTFP1 and Venus, respectively, yielding 25 constructs. From this, design 2 exchanges the 20–amino acid linker for a 3–amino acid linker in each biosensor, with the aim of enhancing the impact of fluorophore orientation by introducing increased steric hindrance of the fluorophores to limit the possible orientations that the fluorophores can adopt. In design 3, we shuffled the position of the RBD and mTFP1 DF relative to design 1 to alter sensing module domain topology. To construct these vectors, we used universal cloning strategies in which we transferred the

sensing module to backbones consisting of different fluorophore combinations (fig. S2A).

To evaluate the library, we overexpressed the biosensors in HEK293T cells and compared the FRET efficiency in the ON (RhoA sensor) or OFF [RhoA sensor + Rho guanine nucleotide dissociation inhibitor (RhoGDI)] states. As previously described (9), RhoA biosensor overexpression leads to its activation. For RhoA biosensors that bind RhoGDI, this is because excess biosensor leads to titration of its cytosolic interaction partner RhoGDI, subsequently leading to accumulation of the biosensor at membranes where activation by guanine nucleotide exchange factors (GEFs) occurs. Coexpression of the sensor with excess RhoGDI maintains the biosensor in the inactive conformation and prevents its activation (9). We acquired the emission spectra of the biosensors in both the ON and OFF states in live-cell suspensions using a plate reader fluorometer and derived the percentage change in emission ratio (Fig. 2A). All emission spectra and emission ratio values are shown in fig. S2, B to D. The parental RhoA1G displayed a 28% emission ratio change (Fig. 2B). In design series 1 and 2, the most robust constructs displayed emission ratio changes of 62 and 59%, respectively. In design 3, varying sensing module domain topology enabled a further increase in dynamic range, leading to the best RhoA

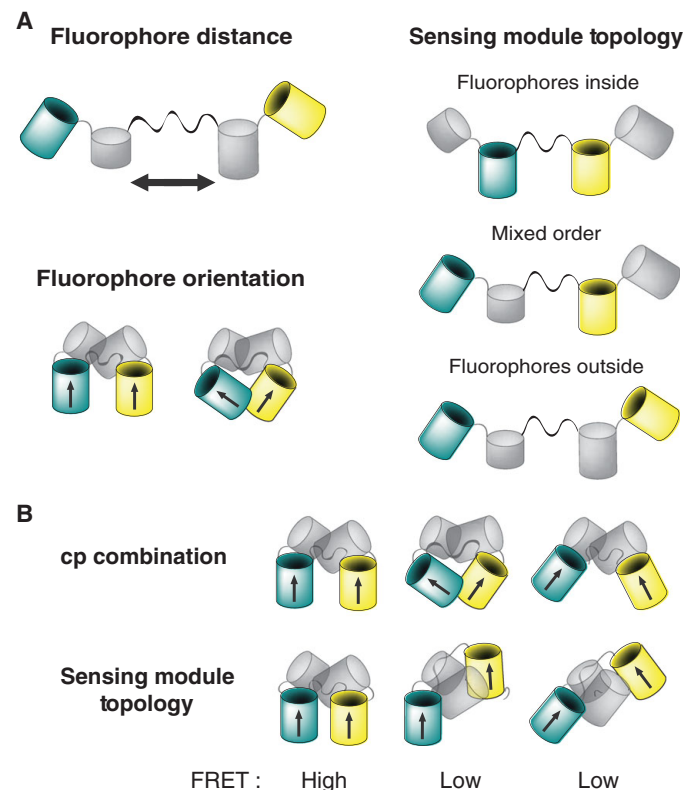


Fig. 1. Geometrical parameters that affect FRET. (A) Three sources of geometrical diversity include fluorophore distance, which can be varied with linkers of different lengths, fluorophore orientation, which can be varied using cp variants, and sensing module topology, which can be altered by changing the order of the fluorophores and sensing module components. (B) In the ON state in which the two moieties interact, a specific cp combination (top) or sensing module domain topology (bottom) might favor a spatial arrangement of DFs and AFs that produces high FRET efficiency. Blue and yellow shapes represent the fluorophores. Arrows in the fluorophores represent the orientation of the dipoles.

biosensor from this set. This biosensor (design 3 mTFP/cp175-Venus/wt) was then reevaluated using a more sensitive cuvette-based fluorometer, which revealed a 99% emission ratio change, mostly due to a higher dequenching between the ON and OFF states (Fig. 2C). Because long linkers efficiently decrease FRET efficiency in the OFF state (5, 19), we engineered a 60 (instead of 20)-amino acid linker in the best construct from design 3 (fig. S2A). This led to further dequenching in the OFF state, resulting in an emission ratio change of 147% (Fig. 2, D and E). We named this optimized RhoA biosensor RhoA2G (RhoA second generation). These experiments showed that empirically exploring different combinations of fluorophore orientation, linker length, and sensing module domain topology produced an optimized dynamic range of FRET-based ratiometric biosensors.

Using the fluorometry assay, we showed that RhoA2G responded appropriately to RhoA-specific GTPase-activating proteins (GAPs) and GEFs (fig. S2E). To avoid activation of overexpressed RhoA2G by saturating the RhoGDI binding capacity, we produced a stable HEK293T cell line expressing RhoA2G at low levels and evaluated the response of RhoA2G to GEFs, GAPs, or GDI coexpression by ratiometric imaging (Fig. 2, F and G). Expression of GEFs (Dbl or Ect2) increased FRET efficiency compared to that in untransfected cells. In contrast, we observed decreased FRET efficiency upon overexpression of p50RhoGAP or RhoGDI. Thus, RhoA2G appropriately responded to upstream regulators.

As a control, we also engineered a nonresponsive RhoA2G biosensor containing a RhoA F39A effector mutation (20), which abrogates the interaction between the rhotekin RBD and RhoA in the sensing module. We selected RhoA F39A instead of the dominant-negative RhoAT19N mutant, because RhoA F39A retains identical subcellular localization as wild-type RhoA and does not modify cell geometry by acting as a dominant-negative (9, 20). Using fluorometry, we found that RhoA2G F39A displayed FRET efficiency comparable to RhoA2G that was inactivated by coexpression of p50RhoGAP or RhoGDI (fig. S2F). A detailed map of the RhoA2G DNA and protein sequence is shown in fig. S3.

Characterization of the ERK biosensor library

As a second biosensor model system, we used the previously published ERK sensor (19), EKAR_{cyto}, which we refer to as EKAR1G (ERK activity reporter first generation). EKAR1G consists of mCerulean (16), a proline-directed WW phosphoserine- and phosphothreonine-binding domain, a linker of 72 Gly residues, a 10-amino acid ERK phosphorylation substrate peptide from Cdc25C, a 4-amino acid ERK docking motif, mVenus, and a nuclear export site. EKAR1G is the result of a large optimization process that involved generation of about 20 constructs in which the sensing module, cp variants in the AF, and linker length were varied (19). Notably, for EKAR1G, engineering cp variants in the AF did not increase the dynamic range.

We used our biosensor library to design 100 constructs in which orientation, distance, and domain topology were varied (Fig. 3A). In design 1, we substituted mCerulean at the N terminus and mVenus at the C terminus of EKAR1G with wild type or any of the four cp mTFP1 or Venus variants, respectively (fig. S4A). In design 2, we used the same, fluorophore-inside architecture (20-amino acid linker) as for the RhoA1G design 1, and placed the WW domain at the N terminus and the Cdc25C substrate peptide at the C terminus. In designs 3 and 4, we mixed fragments from designs 1 and 2 to yield biosensors with additional domain topologies in which sensing module domains were alternated with fluorophores. We evaluated this biosensor library by expression of the biosensors alone (OFF state), with a truncated constitutively active form of Raf (CA-Raf, ON state), or with dominant-negative form of MEK (MEK-DN, OFF state). CA-Raf promotes ERK activity (21), and MEK-DN inhibits ERK activity (22), which we confirmed in transfected HEK293T cells (fig. S4B).

We evaluated this ERK biosensor library by transfecting the constructs into adherent HEK293T cells in 96-well plates and then monitoring fluorescence with a plate reader fluorometer (Fig. 3A, spectra shown in fig. S4, C to G) and with an automated microscopy-based approach (Fig. 3B). For the microscopic analysis, we identified a filter set combination that enabled enhanced DF brightness for mTFP1- and Venus-based EKAR2G (EKAR second generation) biosensors compared to that of Cerulean- and Venus-based EKAR1G (fig. S5A). Using a 10× objective, we found increasing EKAR1G emission ratio values when coexpressed with increasing CA-Raf amounts, and emission ratio values were homogeneous across multiple fields of view (fig. S5B). For adequate comparison between both assays, we normalized emission ratio values of the different EKAR2G constructs to those of EKAR1G. The best biosensors were observed in the design 1 series, whereas designs 2 to 4 did not yield new variants that exhibited increased dynamic range compared with the parental construct (Fig. 3, A and B). Spectral analysis of the biosensors revealed that design 2 series displayed overall higher FRET efficiency in the ON state but could not efficiently dequench in the OFF state (fig. S4E). Design 3 and 4 series exhibited lower FRET efficiency in the ON state, again without efficient dequenching in the OFF state (fig. S4, F and G). Within the design 1 series, we observed some different results using the fluorometry and the microscopy approach (Fig. 3, A and B). We selected two biosensors for detailed characterization: EKAR2G1 (mTFP1/cp227-Venus/cp173) and EKAR2G2 (mTFP1/cp105-Venus/cp195). These two were among the best in both screens and consist of different cpDF-AF combinations. The CA-Raf titration experiment confirmed that both EKAR2G biosensors required less CA-Raf to produce detectable signal, and the dynamic range of the response was greater than for EKAR1G, indicating that these second-generation ERK biosensors were more sensitive than EKAR1G (Fig. 3C).

Table 1. Biophysical characterization of mTFP1 cp variants. wt, wild type.

mTFP1	Extinction coefficient ε (M ⁻¹ cm ⁻¹)(× 10 ⁻³)*	Quantum yield Φ	Brightness†	Photostability t _{0.5} (s)‡	Maturation t _{0.5} (min)§
wt	67	0.85	57	63	13
cp105	64	0.81	52	50	34
cp159	65	0.81	53	53	15
cp175	58	0.73	42	63	21
cp227	69	0.72	50	60	14

*Measured at 462 nm. †Product of Φ and ε × 10⁻³. Φ of the wt protein was reported previously (13). Φ of cps was calculated according to Topell and Glockshuber (15). ‡Time to bleach by 50%. Bleaching was measured by live-cell imaging in HEK293T cells exposed to a 440-nm LED light power of 100%. For details, see note S1 and Materials and Methods. §Maturation was measured at 25°C and pH 7.9.

RhoA activation dynamics during fibroblast cell migration and neuronal differentiation

To demonstrate the advantage of the optimized sensor in a biological system, we performed wound healing experiments and monitored RhoA activation dynamics in motile mouse embryonic fibroblasts (MEFs) expressing low levels of the RhoA biosensor, which is the same system we had previously studied for RhoA activation dynamics with RhoA1G (9). When using RhoA1G, one limitation is that its fluorescence signals are relatively dim, which necessitates excitation with high fluorescence light intensity that can lead to substantial phototoxicity. In time-lapse experiments, to avoid phototoxicity, we used either oxyrase or medium perfusion to remove reactive oxygen radicals generated during the imaging process (9). Because RhoA2G has enhanced spectral properties, we imaged with

low light intensity [using light-emitting diodes (LEDs) at low power rather than classic mercury or xenon arc lamps] with high frequency and for long time periods. As previously shown with RhoA1G (9), with RhoA2G, we observed RhoA activity at the leading edges (Fig. 4A, white arrows, and movie S1). The distribution of RhoA2G was mostly cytosolic and excluded from the nucleus. RhoA activity was also evident at the retracting tail of the cell (Fig. 4A, red arrow, and movie S1). As previously shown, the leading edge protrudes and retracts on a time scale of minutes, and RhoA activity correlates with membrane protrusion but switches off during membrane retraction (2). This variation with protrusion and retraction was also evident with RhoA2G (Fig. 4, B to D). Because RhoA2G is brighter than RhoA1G, we could image leading-edge filopodia, which are very thin structures in which the RhoA activity

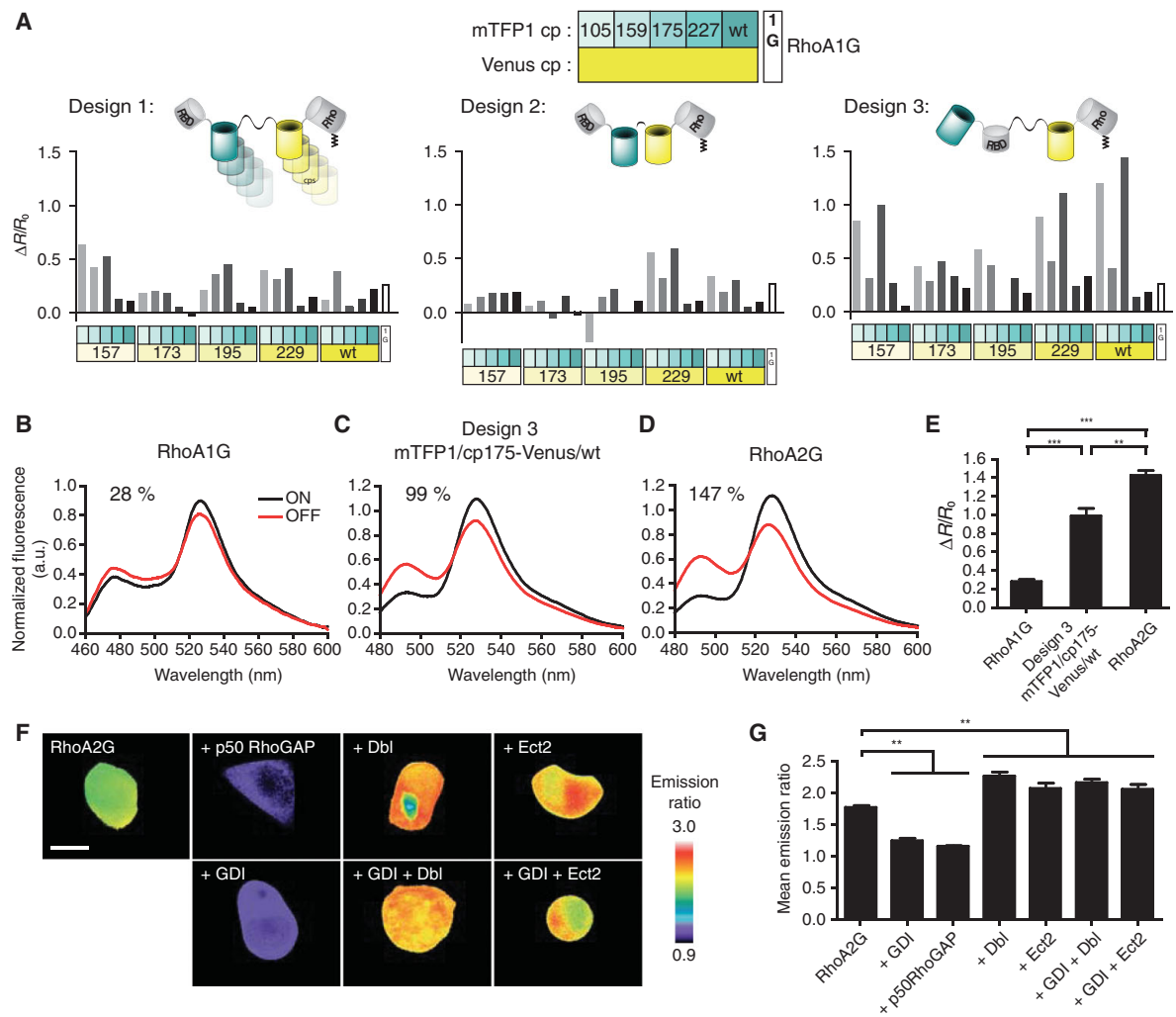


Fig. 2. Characterization of the RhoA biosensor library. (A) Evaluation of the dynamic range of the RhoA biosensor library using a plate reader fluorometer. $\Delta R/R_0$ values represent changes in FRET efficiency between the ON and OFF states and were derived from emission spectra displayed in fig. S2, B to D. A key indicates the specific mTFP1 and Venus cps. wt, wild type. (B to D) Normalized emission profiles of the indicated RhoA biosensors measured with a cuvette-based fluorometer. The percent emission ratio change was calculated at the peak wavelengths. a.u., arbitrary units. (E) $\Delta R/R_0$ values of the indicated biosensors. Bar graph: mean $\pm$ SEM; $n \geq 3$ measurements; $**P < 0.01$, $***P <$

0.001, Student's *t* test. (F) Evaluation of stably expressed RhoA2G in HEK293T cells using fluorescence microscopy. Cells were transiently transfected with GEFs (Dbl1, Ect2), GAP (p50RhoGAP), and GDI. Emission ratio pictures are color-coded according to activation intensity. Scale bar, 10 μ m. (G) Mean emission ratio of cells expressing the RhoA2G biosensor with the indicated proteins. Bar graph: mean $\pm$ SEM; $n \geq 10$ cells per experiment; $**P < 0.01$, one-way analysis of variance (ANOVA). The brackets and lines above the bars show that RhoA is compared to RhoGDI and p50RhoGAP collectively. RhoA is also compared to Dbl, Ect2, RhoGDI + Dbl, and RhoGDI + Ect2 collectively.

signal is so dim that it cannot be detected with RhoA1G. We observed RhoA activity during filopodia protrusion, and these structures were also positive for F-actin (Fig. 4, E and F, and movie S2), which we visualized with the Lifeact-mCherry reporter (23).

We also tested if RhoA2G can detect the platelet-derived growth factor (PDGF)-induced reduction in RhoA activity at the leading edge, which has been previously shown using RhoA1G (9). PDGF stimulation increased leading-edge displacement and decreased RhoA activity (Fig. 4, G and H, and movie S3). These results showed that the RhoA2G sensor can be used to detect different modes of RhoA signaling that depend on the stimulus that triggers membrane protrusion.

Growth cones are specialized cellular extensions through which neuronal cells form neurites. We evaluated the usefulness of RhoA2G in monitoring RhoA activity dynamics during differentiation of neuron-like N1E-115 neuroblastoma cells. When grown on laminin, nondifferentiated N1E-115 cells are highly motile and display flat lamellipodia containing prominent filopodia, whereas, in response to serum starvation, N1E-115 cells adopt a neuronal phenotype, becoming round and producing dynamically extending and retracting neurites (24). We observed RhoA activity at the tip of protrusive structures, lamellipodia in nondifferentiated N1E-115 cells, and growth cones and extending neurites of differentiated N1E-115 cells (Fig. 5, A and B, and movie S4). RhoA activity was low in

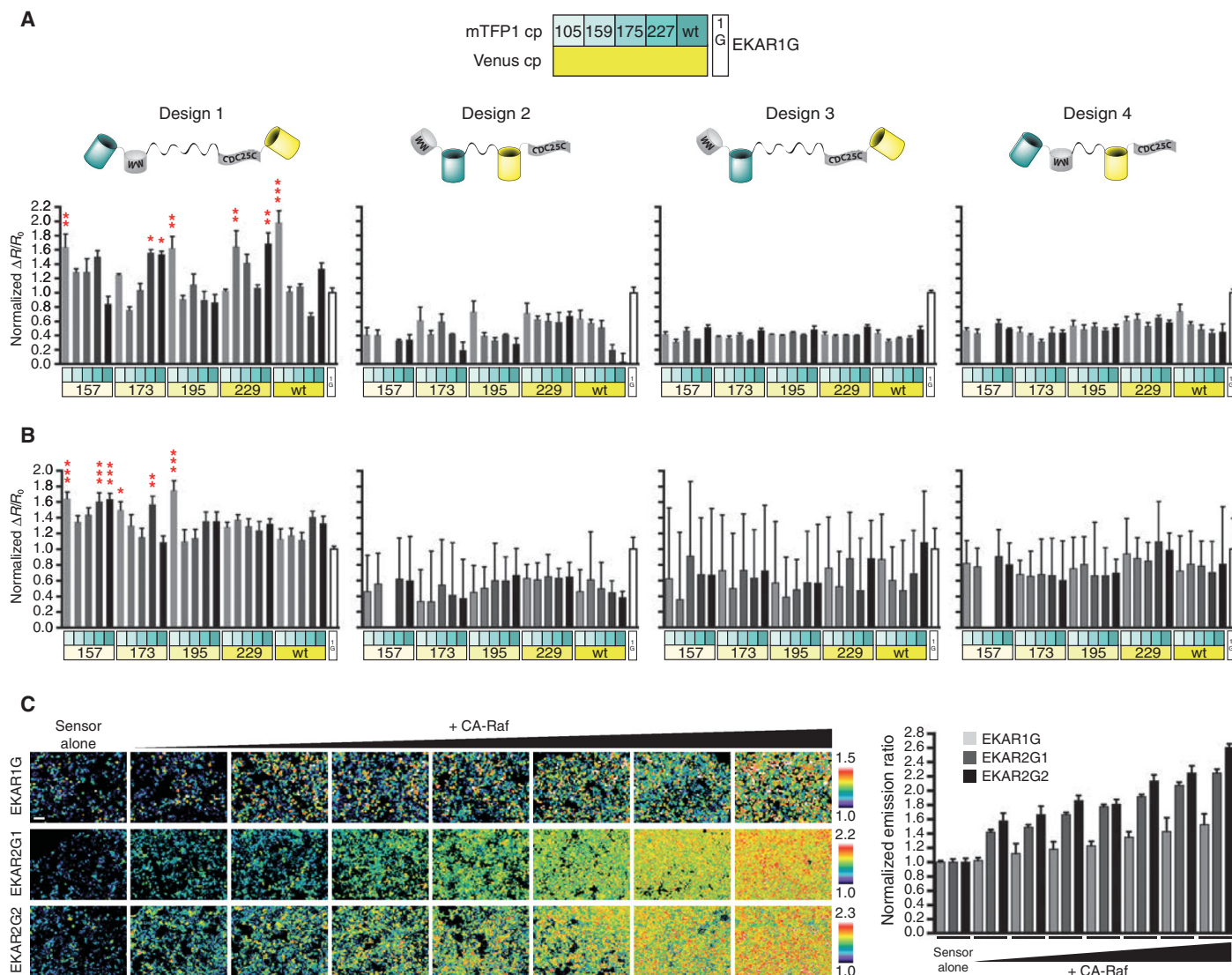


Fig. 3. Characterization of the ERK biosensor library. (A) Evaluation of the dynamic range of the ERK biosensor library using a plate reader fluorometer. FRET efficiency values were derived from emission spectra displayed in fig. S4, C to G. $\Delta R/R_0$ values were normalized to EKAR1G to allow for comparison between both methods in (A) and (B). Bar graph: mean $\pm$ SEM ($n = 3$). (B) Evaluation of the dynamic range of the ERK biosensor library by microscopy. Bar graph: mean $\pm$ SEM of nine fields of view per experiment for

design 1 ($n = 4$ experiments) and mean $\pm$ range for designs 2 to 4 ($n = 2$ experiments). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA for design 1. (C) Comparison of EKAR1G, EKAR2G1, and EKAR2G2 sensors by low-magnification, low-NA air objective microscopy. Biosensors were co-expressed with increasing amounts of CA-Raf. Normalized emission ratios are shown. Scale bar, 100 μm . Bar graph shows quantification of multiple (9 to 18) fields of view. Presented as mean $\pm$ SD ($n = 2$).

the cell soma. In both lamellipodia and growth cones, RhoA2G revealed highly focused RhoA activity patterns capping the distal part of F-actin bundles that form the filopodia in both growth cones and lamellipodia (Fig. 5, C and D, and movie S5). Conversely, during spontaneous neurite collapse, filopodia disappeared and RhoA was activated globally in the growth cone (Fig. 5, B and E, and movie S5). In nondifferentiated N1E-115 cells, the control RhoA2G F39A probe displayed lower FRET efficiency than did RhoA2G, and the RhoA2G F39A signal remained homogeneous across the cell (Fig. 5, F and G), showing that the FRET

activation patterns detected with RhoA2G at the cell periphery were not artifacts because of cell geometry or low signal-to-noise ratio in these structures. Finally, we compared growth cone RhoA activation patterns using RhoA1G (a cytosolic and RhoGDI-binding biosensor) (9), Raichu-RhoA (a membrane-localized biosensor that does not bind RhoGDI) (25, 26), and RhoA2G (a cytosolic and RhoGDI-binding biosensor) (fig. S6). More filopodia on the growth cones were detected with RhoA2G than with RhoA1G, indicating that RhoA2G had enhanced sensitivity. The RhoA activity patterns detected with RhoA2G and Raichu-RhoA were

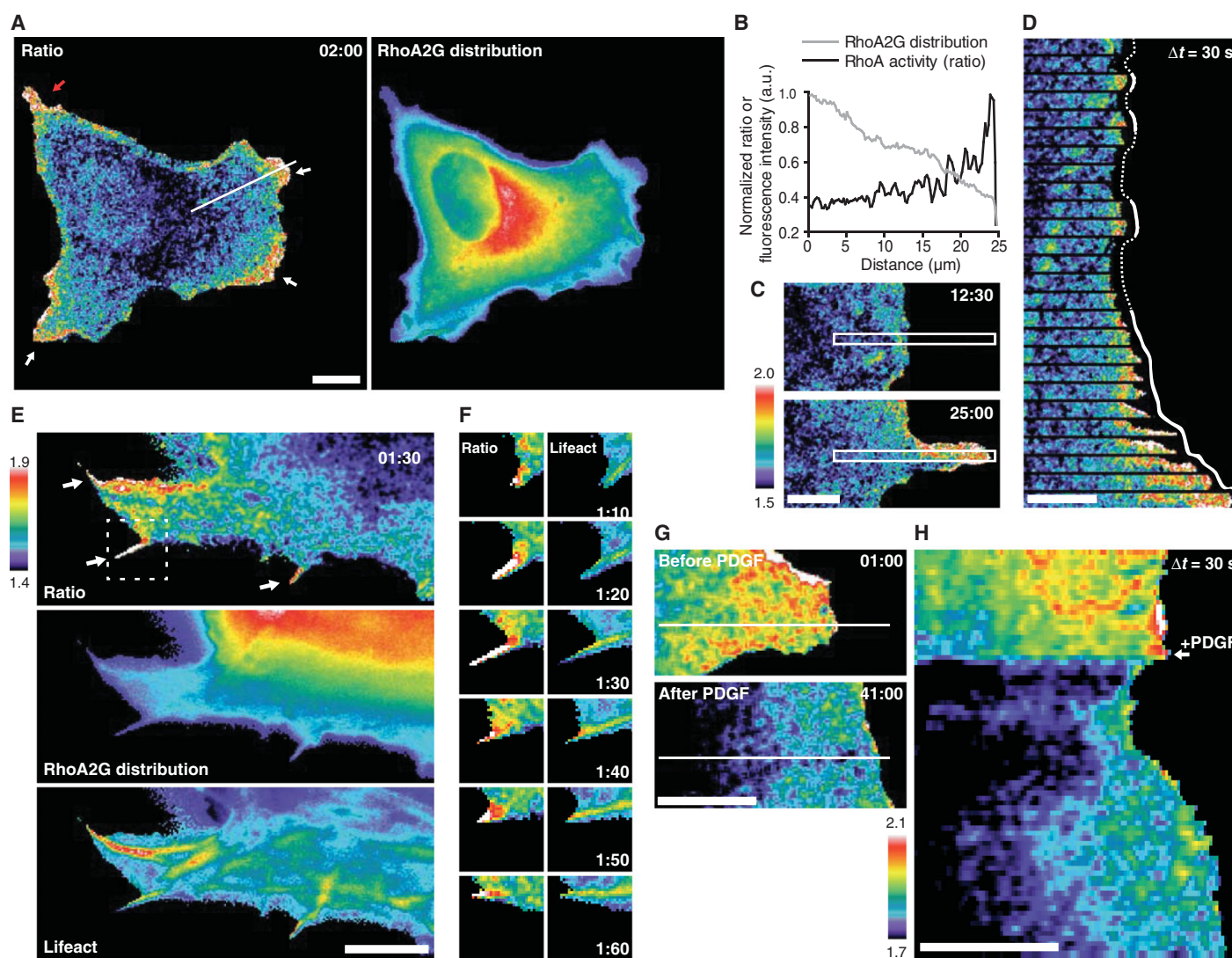


Fig. 4. RhoA activation dynamics in migrating MEFs. RhoA activation (ratio), RhoA2G distribution, and F-actin (Lifeact) are color-coded for signal intensity. (A) RhoA activation in migrating fibroblasts. White arrows, leading-edge membrane protrusions; red arrow, retracting back of the cell. (B) Linescans of ratio and RhoA2G distribution (normalized to maximum intensity) in a protruding part of the leading edge indicated by the white line in (A). (C and D) Kymograph analysis of the dynamics of RhoA activation during leading-edge membrane protrusion and retraction cycles. (C) Images show start and end time points of leading edge, and white box shows region used for kymograph analysis. (D) Images taken at 30-s intervals. Dotted and solid

lines represent periods of retraction and protrusion, respectively. Intensity scale to the left of (C) applies to both (C) and (D). (E) Dynamics of RhoA activity in filopodia (white arrows). (F) Time series of filopodium RhoA and F-actin dynamics from the box in (E). Intensity scale to the left of (E) applies to both (E) and (F). (G and H) Kymograph analysis of the dynamics of RhoA activation in response to PDGF (40 ng/ml). A representative cell is shown. (G) Images show start and end time points of leading edge used for kymograph analysis along the white line. (H) Kymograph analysis of RhoA activation before and after PDGF stimulation. Intensity scale below (G) applies to both (G) and (H). All scale bars, 10 μ m. All time scales: minutes:seconds.

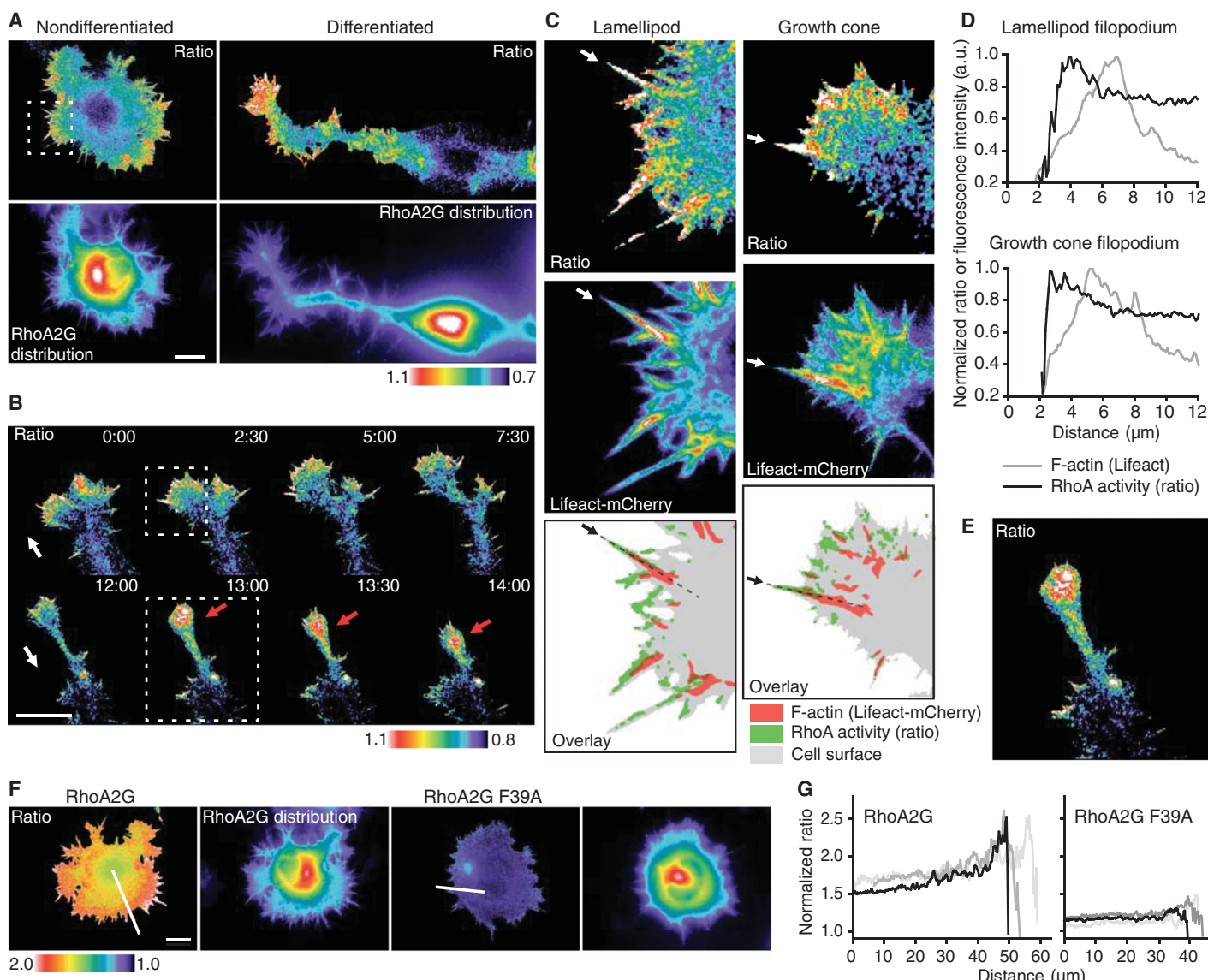


Fig. 5. Dynamics of RhoA activation in growth cone filopodia and lamellipodia. (A) RhoA activation (ratio) and RhoA2G distribution in nondifferentiated and differentiated N1E-115 cells. (B) Time series showing RhoA activation in an extending and collapsing growth cone from a differentiated N1E-115 cell. White arrows indicate direction of growth cone movement; red arrows point to global RhoA activation during collapse. Time scale: minutes:seconds. (C) High-magnification images of a lamellipod of the outlined region in (A) and a

protruding growth cone outlined in the top row of (B). Arrows indicate filopodia. (D) Linescans of RhoA activation and F-actin signals (normalized to maximum intensity) from images in (C). (E) High-magnification image of RhoA activation in a collapsing growth cone from the lower row of (B). (F and G) Cell geometry control experiments with RhoA2G and RhoA2G F39A mutant. (F) Ratio and distribution images are scaled identically. (G) Ratio linescans from three cells, including the cell in (F). All scale bars, 20 μm .

different, which likely reflects the different cellular localizations and abilities in binding RhoGDI of these two biosensors (note S2).

In vitro and in vivo measurements of endogenous ERK activation

To evaluate the usefulness of the EKAR2G biosensors, we produced HEK293T stable cell lines expressing EKAR2G1 and EKAR2G2, and measured ERK activity dynamics in response to epidermal growth factor (EGF). As demonstrated by Western blot analysis, in cells stimulated with EGF (100 ng/ml), the abundance of phosphorylated and activated ERK transiently increased,

peaking at 5 to 10 min and subsequently returning to baseline (fig. S7A). Because of the enhanced brightness and dynamic range of EKAR2G1, as well as high expression level in the stable cell lines, signals could be detected with a high-NA oil immersion objective, which allowed excitation with very low light intensity (1 to 5% intensity of the LED lamp; for details, see Materials and Methods) and thus measurement of ERK activity without substantial bleaching or phototoxicity. We observed similar ERK activity kinetics with EKAR2G1 (Fig. 6, A and C, and movie S6) as those detected by Western blot analysis (fig. S7A). We obtained data of comparable quality with a low-NA 20 $\times$ air objective, enabling the simultaneous analysis of large numbers

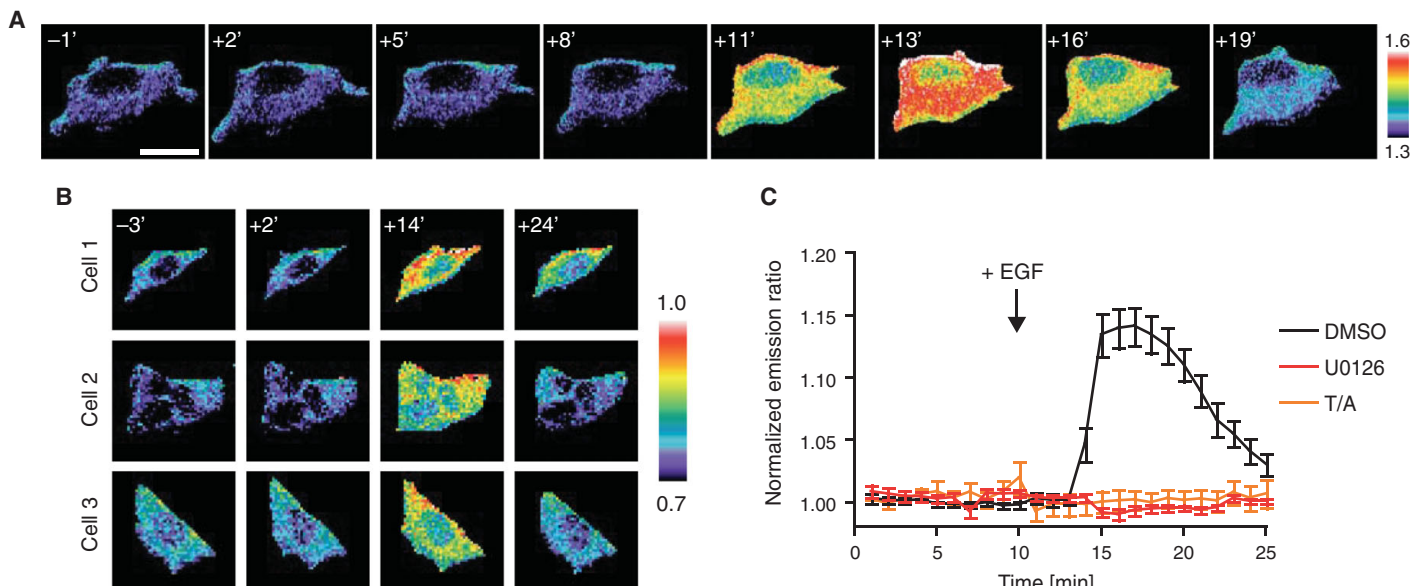


Fig. 6. Dynamics of EGF-induced ERK activity in HEK293T cells measured by FRET with EKAR2G biosensors. (A) Time series imaged using a 60 $\times$ oil immersion objective. (B) Three representative time series of cells from one field of view imaged with a 20 $\times$ air objective. In (A) and (B), time (minutes) is indicated with

of cells (Fig. 6, B and C, and movie S7). Pretreatment of cells with the MEK inhibitor U0126 or when the EKAR2G1 phospho-acceptor Thr residue in the Cdc25C peptide was mutated to an Ala eliminated the transient ERK activity produced by EGF stimulation (Fig. 6C). Using the 20 $\times$ air objective and EKAR2G1, we monitored EGF-induced ERK activity with a 10-s time resolution for more than 1 hour (fig. S7B), demonstrating its robustness in terms of photostability and brightness. EKAR2G2 behaved identically as EKAR2G1 (fig. S7C), showing that different cpDF-AF combinations did not affect the sensing module and were equally viable for generation of sensitive biosensors.

To obtain quantitative data with our EKAR2G biosensors, we performed multiphoton fluorescence lifetime imaging microscopy (FLIM) experiments, from which we calculated the fraction of phosphorylated sensor molecules. Similar to multiphoton ratiometric FRET measurements (Fig. 7A), FLIM showed an EGF-induced increase in EKAR2G1 FRET efficiency, as reflected by decreased donor lifetime (Fig. 7B). From the lifetime of the mTFP1 donor alone, we calculated EKAR2G1 FRET efficiency in conditions in which ERK activity would be different (Fig. 7C, top; corresponding lifetime histograms are shown in fig. S7, D to F). To calculate the percentage of phosphorylated sensor molecules in a given cell, we measured the sensor lifetimes in the completely nonphosphorylated state (cells treated with U0126 to inhibit basal ERK activity) and phosphorylated state (cells overexpressing CA-Raf to stimulate ERK activity). Assuming completely dephosphorylated and phosphorylated EKAR2G1 states, we found that, relative to the amount induced by CA-Raf, ~20% of EKAR2G1 was phosphorylated in serum-starved, unstimulated cells (Fig. 7C, bottom), which was qualitatively consistent with results obtained by Western blot (fig. S7A). At the peak of EGF-induced activation, EKAR2G1 phosphorylation was ~90% of that induced by CA-Raf (Fig. 7C, bottom). Thus, even when abundant due to high expression, most of EKAR2G1 responded to EGF stimulation.

To test the functionality of our ERK biosensors *in vivo*, we performed intravital imaging with zebrafish. Injection of sensor-containing DNA in-

respect to the addition of EGF (100 ng/ml). (C) Whole-cell, normalized EKAR2G1 wt [dimethyl sulfoxide (DMSO)] and Thr-to-Ala (T/A) mutant ratio profiles of EGF-stimulated cells (100 ng/ml) in the presence or absence of 10 μ M U0126. Error bars: SEM; $n \geq 10$ cells per experiment, three experiments. Scale bar, 10 μ m.

duced broad mosaic expression of EKAR2G2 without causing visible harm to the developing embryos. After coinjection of a constitutively active Ras mutant, RasV12 and EKAR2G2, we performed intravital multiphoton FLIM measurements and detected increased ERK activities in individual cells coexpressing RasV12 compared to EKAR2G2-expressing cells without RasV12 (Fig. 7, D to F, and fig. S7, G and H). These experiments demonstrate some of the applications and robustness of our second-generation ERK biosensors.

DISCUSSION

RhoA activation dynamics during neurite outgrowth and cell motility

Using the optimized RhoA2G biosensor, we detected subtle filopodium activation patterns that were not detectable with RhoA1G. The reduced excitation light requirements of RhoA2G circumvented problems associated with bleaching and phototoxicity and enabled sampling at high frequency to capture RhoA signaling at tens of seconds temporal resolution. The role of RhoA activity in leading-edge protrusions, such as lamellipodia, is well established (2, 3, 9). In filopodia, the GTPase Cdc42 is thought to be the primary Rho family GTPase regulating filopodia (27). RhoA activates actomyosin contractility (28), which is not thought to occur in filopodia. Thus, the finding of RhoA activity in filopodia is intriguing. In growth cone filopodia and fibroblasts, the highly polarized RhoA activity pattern at the tip of F-actin bundles suggested that it may regulate nucleation of linear actin filaments, and this may occur through its effector mDia2 (29). In the same MEF cell system that we used, Cdc42 activity has been observed at the base of filopodia (30). Tight spatiotemporal control of RhoA and Cdc42 activity might position distinct actin regulators to fine-tune filopodium dynamics. Such coordination between multiple spatiotemporally regulated Rho GTPase pools has been reported during wound healing (closure of a hole from a needle) in single *Xenopus* oocytes (31) or fibroblast pseudopod extension (2).

During neurite retraction, diffuse RhoA activation throughout the whole collapsing growth cone most likely activates Rho kinase to promote myosin contractility that is necessary for this process (32). This distinct pool of active RhoA (in contrast to the highly focused pool observed in filopodia) suggests different modalities of activation presumably involving different GEFs, GAPs, and effectors than those regulating RhoA activity in filopodia. Our results suggested the existence of spatially distinct pools of RhoA that may regulate conserved functions that can be used interchangeably in different cell types and behaviors (for example, filopodium formation in growth cones or lamellipodia, contractility during tail retraction, or growth

cone collapse). The coming challenge is to dissect this spatiotemporal signaling complexity.

Improved EKAR2G biosensors for robust measurements of endogenous ERK activation in vitro and in vivo

The EKAR2G biosensors facilitated robust measurements of growth factor-evoked ERK activation dynamics by microscopy using low-magnification, low-NA air objectives. This was not only because of the biosensors' enhanced spectral properties but also because high amounts of EKAR2G biosensors were well tolerated by the cell and, even when highly expressed, ERK activation dynamics occurred with kinetics similar to those in cells that were not expressing the biosensors. Furthermore, FLIM experiments showed that 90% of the biosensor pool responded to growth factor stimulation, which is likely possible because ERK1 and ERK2 exhibit high cytosolic concentrations in the cell [in the range of 1 μ M (33)]. Because of these characteristics, the EKAR2G biosensors could be used to study ERK activation dynamics at the single-cell level in large populations of cells (34) or be used in high-throughput imaging assays. The experiments with zebrafish embryos showed that these second-generation ERK biosensors were sensitive enough to use for intravital imaging.

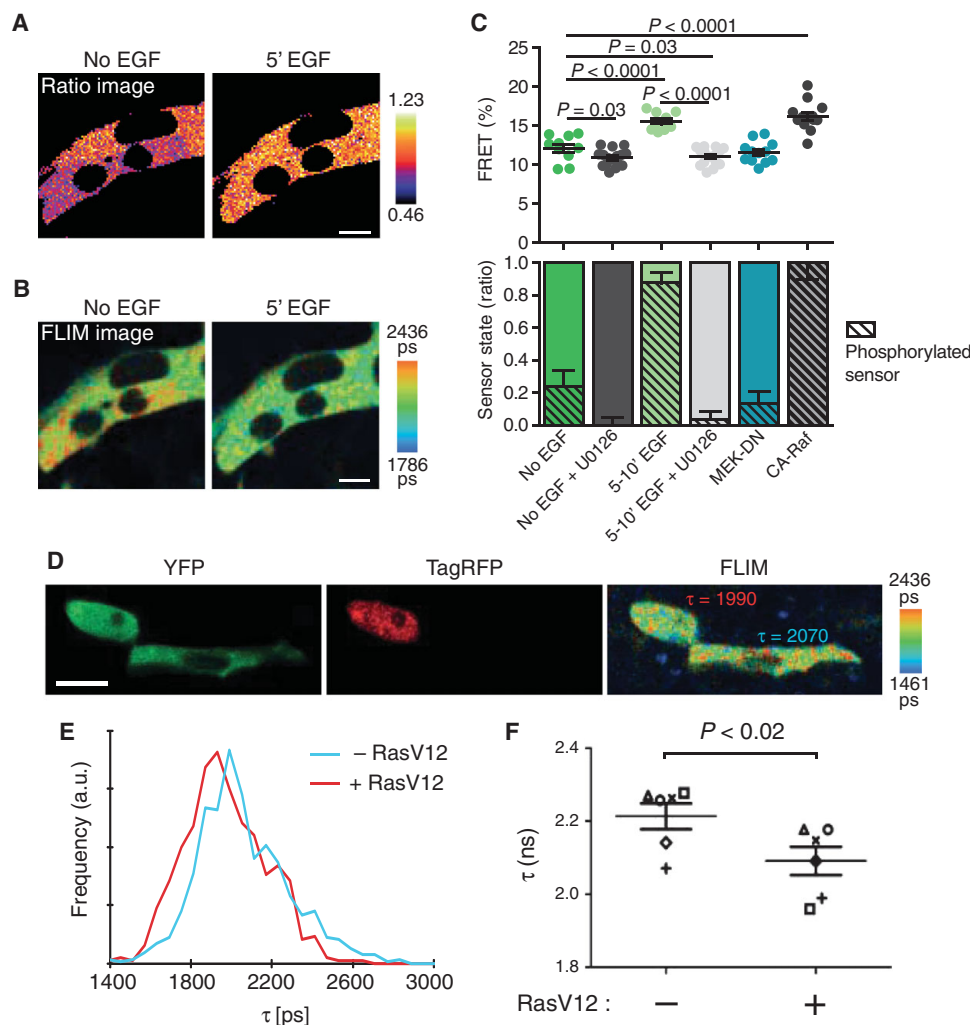


Fig. 7. Analysis of ERK activity with the activity with the EKAR2G biosensors by two-photon microscopy in cultured cells and zebrafish embryos. (A) Ratiometric analysis of representative cells expressing EKAR2G1 before and 5 min after stimulation with EGF (100 ng/ml). (B) FLIM analysis of the same cells as in (A). (C) Top: FRET efficiency of EKAR2G1 measured in cells with different amounts of ERK activity using FLIM. Bottom: Proportion of phosphorylated EKAR2G1 in the cells based on data in the top graph. Mean $\pm$ SEM of $n = 10$ cells. (D) FLIM measurements of EKAR2G2 activity in response to ectopic RasV12 expression in live zebrafish at 1 day after fertilization. Adjacent, sensor-expressing cell duplets (YFP) were selected for the presence of one cell positive and one negative for RasV12 (TagRFP). Average lifetimes per cell are indicated (ps). (E) Lifetime frequency plots of the two cells in (D). (F) Lifetimes of multiple direct comparisons of RasV12-positive and RasV12-negative, EKAR2G2-positive cell pairs ($n = 6$; mean $\pm$ SEM, one-tailed Wilcoxon test). Scale bars, 10 μ m.

ERK activation dynamics occurred with kinetics similar to those in cells that were not expressing the biosensors. Furthermore, FLIM experiments showed that 90% of the biosensor pool responded to growth factor stimulation, which is likely possible because ERK1 and ERK2 exhibit high cytosolic concentrations in the cell [in the range of 1 μ M (33)]. Because of these characteristics, the EKAR2G biosensors could be used to study ERK activation dynamics at the single-cell level in large populations of cells (34) or be used in high-throughput imaging assays. The experiments with zebrafish embryos showed that these second-generation ERK biosensors were sensitive enough to use for intravital imaging.

A flexible toolkit to produce sensitive FRET biosensors

Our DNA construct library, in which a given sensing module can be flanked with different cpDF-cpAF combinations using universal cloning strategies, should enable efficient generation of a broad range of biosensors. With the combination of (i) cp mutants in the AF and the DF, (ii) linkers of different lengths, and (iii) variations in domain topologies, empirical exploration of a large geometrical space, which would accommodate specific structural requirements imposed by a given sensing module, can be performed. Screening the library bypasses the need for empirical rounds of biosensor engineering; however, library screening depends on a robust assay to induce biosensor ON and OFF states and an appropriate detection method. Our microscopy-based approach (Fig. 3B) represents a rapid screening platform.

We showed that using our biosensor toolkit, we could improve two ratiometric FRET biosensors with already good spectral properties. Evidence that our approach allows sampling of geometric constraints imposed by a given sensing module comes from the observation that within the three different RhoA biosensor design series, distinct cpDF-cpAF combinations had improved spectral properties compared to the RhoA1G biosensor (Fig. 2A). In contrast,

we observed similar spectral responses with biosensors that contained wild-type and cp227 mTFP1 AFs or wild-type and cp229 Venus DFs (Fig. 2A, designs 1 to 3). These cp variants contain the small C-terminal part of the fluorophores fused to the N terminus. Thus, constructs with mTFP1 cp227 and Venus cp229 are likely to give similar results as their wild-type counterparts. Together, these findings exclude the possibility that optimal spectral characteristics of the best biosensor are due to intrinsic properties of specific cp DFs or AFs. The most effective RhoA biosensor required the right combination of a certain cpDF-cpAF pair, a specific linker, and a specific domain topology, validating the latter parameter as an additional source of geometric optimization for this biosensor. Thus, testing cp mutants in both the DF and AF in the context of a biosensor that has to accommodate multiple structural constraints is advantageous. The following represent the kinds of structural constraints that may be important in biosensor generation: (i) The sensing module consists of two compact, well-folded domains, and (ii) one sensing module component must be placed at the C terminus because of posttranslational modification or appropriate interaction with endogenous regulators or both. Our biosensor construction system extends the previous biosensor architecture in which the fluorescent proteins flanked the sensor domains (fluorescent protein–outside architecture) and, thus, accommodates diverse structural constraints that may be exhibited by various sensing modules. The library could be used to create biosensors that report on different GTPase families that require free N or C termini for lipid modification (35). Our comparison of RhoA2G (biosensor with a free C terminus) and Raichu-RhoA (biosensor with a fluorescent protein fused at the C terminus) in growth cones suggested that the domain topology affected the signal, because we found different activation patterns that might be due to the fact that Raichu-RhoA cannot interact with the essential regulator RhoGDI.

We achieved less spectral enhancement for EKAR2G than for RhoA2G. This may reflect that the first-generation biosensor was well optimized because the sensing module of EKAR, which relies on a compact domain and a short flexible phospho-acceptor peptide, has less stringent structural requirements than the RhoA sensing module. Only the classic fluorescent protein–outside biosensor architecture produced any biosensors with enhanced spectral properties (EKAR2G1, EKAR2G2, and mTFP1/cp105-Venus/cp157) (Fig. 3, A and B, design 1); the other cpDF-cpAF combinations performed as well as the parental construct. Thus, for biosensors with less stringent structural requirements, cp mutations are less effective. Thus, in biosensors with floppy sensing module components, the classic fluorescent protein–outside architecture might be the most efficient design. Because two EKAR2G biosensors based on different cpDF-cpAF combinations exhibited identical biological responses, the fluorescent proteins per se did not influence the sensing module in our system.

Within the framework of the fluorescent protein–outside biosensor architecture, Komatsu *et al.* also reported a second-generation EKAR construct (5). Their FRET probe construction strategy took advantage of the *Aqueora* DF CFP and AF YPet (36), which are thought to robustly dimerize with an optimal spatial arrangement, leading to high FRET efficiency when brought together in the ON state (37, 38). Low FRET efficiency is then obtained by engineering a long flexible linker to efficiently separate the fluorescent proteins in the OFF state. mTFP1 and Venus lack a propensity to dimerize and, thus, should not display high FRET efficiency in the ON state. In the absence of fluorescent protein dimerization, optimization of fluorescent protein spatial orientation is achieved by identification of a specific cpDF-cpAF combination for a particular sensing module. For the RhoA2G biosensor, we found that introduction of a long linker contributed to FRET efficiency modulation between the ON and OFF states (Fig. 2, C and D).

These different approaches represent two alternative strategies to constructing sensitive biosensors. However, the approach presented by Komatsu *et al.* might only apply to the classic fluorescent protein–outside biosensor architecture and, thus, might not accommodate the structural constraints of the sensing module, such as those imposed by RhoA.

An additional advantage of our biosensor construction approach is the ability to easily produce stable cell lines, because the fluorescent proteins are derived from two different species, which eliminates on the tendency of *Aqueora*-based CFP and YFP (yellow fluorescent protein) derivatives to recombine CFP and YFP sequences (39). The ability to generate homogeneously expressing cells or cell lines enables highly reproducible emission ratio changes across a large number of cells, as we showed with the EKAR2G1-expressing cells. Stability of the fluorescent protein–encoding sequences is also important for stable transgenesis into model organisms, such as the zebrafish or the mouse, in which homologous sequences can be recombined after genomic insertion.

Combined with an effective strategy to easily identify the biosensor with adequate spectral properties, our approach is widely applicable for the rapid construction of various biosensors. We provide a biosensor construction toolkit, called cp-based FRET (cpFRET), comprising a set of vectors that can be used for the generation of further biosensors. Information about this toolkit is provided in figs. S2 to S4, S8, and S9 and in Materials and Methods.

MATERIALS AND METHODS

DNA constructs

Starting with RhoA1G as a template (9), all building blocks of the RhoA sensor libraries were prepared by polymerase chain reaction (PCR) amplification, assembled by a series of consecutive subcloning steps, and introduced into a derivative of the eukaryotic expression vector pTriEX4 (Novagen), which bears His6 and myc tags at the N terminus. Using overlap-extension PCR, we produced the RhoA F39A effector mutant in RhoA2G. See figs. S2 and S3 for details about the library constructs and the sequence of RhoA2G.

For the ERK sensor libraries, we used custom gene synthesis to generate two sensing modules with different topologies with which we constructed the design 1 and 2 biosensor series. These were then cloned in the pTriEX4 derivative. We mixed and matched fragments from both designs to produce design 3 and 4 biosensor libraries. See fig. S4 for details about the library constructs, fig. S8 for the sequences of EKAR2G1 and EKAR2G2, and fig. S9 for the cloning strategy.

To generate stable cell lines, RhoA2G and EKAR2G biosensors were shuttled from the pTriEX4 plasmid into the lentiviral vector pRRL-SV40(puro)_CMV(mcs) (a gift from M. Kaeser) by homologous recombination with the In-Fusion PCR cloning system (Clontech). For zebrafish transgenesis, EKAR2G2 was amplified with PCR, then was cloned into a pCS2+ empty vector (Eco RI/Xho I), and from there ligated (Eco RI/Not I) into an adapted miniToI2 vector containing 14×UAS and a β -actin minimal promoter, resulting in miniToI2-14×UAS:EKAR2G2 polyA(SV40). pDCR×CMV:RasV12-IRES-TagRFP-polyA(SV40) was generated by ligating IRES-TagRFP+polyA(SV40) (Bam HI/Hin dIII) directly behind the stop codon of RasV12 in the cytomegalovirus (CMV) promoter-containing pDCR×RasV12 plasmid (a gift from K. Jalink). To generate the intermediate construct with IRES-TagRFP, an internal ribosomal entry site (IRES) sequence was amplified and ligated into the empty miniToI2 vector (Bgl II); TagRFP+polyA(SV40) was subsequently isolated from pCS2-TagRFP (Bam HI/Not I) and ligated behind the IRES of miniToI2-IRES (cut Bgl II/Not I).

Practical notes for using the FRET sensor construction kit

For future usage of the biosensor toolkit, referred to as cpFRET, we provide two sets of 25 plasmids consisting of the EKAR2G design 1 and 2 series, with all possible combinations of wild-type and cp mTFP1 and Venus fluorescent proteins. cpFRET is available at Addgene (<http://www.addgene.org/>; toolkit ID: 1000000021) and can be used to construct new biosensor libraries by exchanging the EKAR sensing module with other sensing modules using the unique restriction sites. The different constructs are provided in the pTriEx4 vector (Novagen), which enables expression of the biosensors in *E. coli*, baculovirus, or eukaryotic cells from one single vector. Thus, biosensor screening can be performed in various expression systems. The design 1 series enables construction of a library of biosensors with sensing module components internal to the DF and AF (the classic fluorophore-outside design) (fig. S8A). The design 2 series enables construction of a library of biosensors with sensing module components placed external to DF and AF fusions (fluorophore-inside design) (fig. S8B). The plasmids in designs 1 and 2 can then be combined to produce the design 3 and 4 biosensor libraries with additional domain topologies in which sensing module components, AF, and DF are alternated (fig. S9). Briefly, Bam HI/Xma I-restricted insert fragments from the design 1 biosensor series can be transferred into Bgl II/Bsp E1-restricted vector fragments from the design 2 biosensor series to produce the design 4 biosensor series. Conversely, Bgl II/Bsp E1-restricted insert fragments from the design 2 biosensor series can be transferred into Bam HI/Xma I-restricted vector fragments from the design 1 biosensor series to produce the design 3 biosensor series.

We do not recommend adapting the system for recombination cloning approaches (such as Gateway technology) because recombination approaches might involve specific sequences that have the potential to introduce deleterious amino acids in the linker regions between sensing module components and fluorescent proteins. In our library, each sensing module component, linker, and fluorescent protein can be exchanged by a unique set of restriction sites. We specifically used restriction sites that encode amino acid sequences compatible with unstructured linker features. Note that most of our restriction sites are engineered to be in frame. However, the Not I restriction site has an additional nucleotide added to its 3' end so that the protein sequence remains in frame.

Cell culture, transfections, and generation of stable cell lines

N1E-115 neuroblastoma cells, MEFs, and HEK293T cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin. N1E-115 cells were transfected with the indicated plasmids as previously described (40). For differentiation, N1E-115 cells were starved for 24 hours in serum-free Neurobasal medium (Invitrogen) supplemented with 1% L-glutamine and 1% penicillin/streptomycin. For imaging of neurite outgrowth and retraction dynamics, cells were detached with PUCK's saline and reseeded on coverslips previously coated with laminin (10 μ g/ml) (Millipore-Chemicon) and imaged 6 to 8 hours after plating.

For production of stable cell lines, we produced lentiviral vectors expressing RhoA2G, EKAR2G1, or EKAR2G2. Briefly, HEK293T cells (Invitrogen) were transfected with lentiviral and packaging constructs (pVSV, pMDL, and pREV). Supernatant was then collected and concentrated with a Lenti-X concentrator kit (Clontech). HEK293T and MEF cells were infected and selected with puromycin (1 or 0.5 μ g/ml, respectively). HEK293T cells stably transfected with EKAR2G were then sorted by flow cytometry to obtain a stable cell line that expressed homogeneous and high biosensor levels.

For wound healing experiments, MEFs stably expressing the RhoA2G sensor were starved overnight in Ham's F-12 nutrient mixture supplemented with 0.5% FBS, 0.5% bovine serum albumin, 1% L-glutamine,

and 1% penicillin/streptomycin and mixed with nontransfected MEFs at a 1:2 ratio, and 7×10^3 cells were seeded into Culture-Inserts (ibidi) stitched to glass coverslips that were precoated with fibronectin (10 μ g/ml final). MEFs were allowed to adhere for 3 hours to fibronectin-coated glass coverslips before removal of the Culture-Inserts (wound creation) and subsequent time-lapse analysis. In some experiments, cells were stimulated with PDGF (40 ng/ml) after wounding (rat; Sigma).

For growth factor stimulation experiments, HEK293T cells stably expressing the EKAR2G sensors were plated on poly-L-lysine (PLL)-coated (20 μ g/ml final) glass-bottom multiwell plates (MatTek) at single-cell confluence. Four hours after seeding, cells were starved overnight with phenol red-free DMEM supplemented with 0.2% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. Human EGF (100 ng/ml final) was then added to induce ERK activation.

Fluorometric analysis of the biosensors

Fluorescence emission spectra analysis of the RhoA sensor library was performed on a Synergy4 microplate reader (BioTek). HEK293T cells (4×10^5) were seeded on PLL-coated 12-well cell culture plates (20 μ g/ml final) and transfected with the adequate mixes of plasmids [typically 100-ng DNA of biosensor (first or second generation), 400 ng of regulator, and 3.5 μ l of Metafectene; Biontex]. After 48 hours, cells were detached with brief trypsin treatment and resuspended in ice-cold phosphate-buffered saline (PBS) in a 96-well plate with an opaque bottom. For parental, CFP- and YFP-based biosensors, the donor was excited at 433 nm, and emission spectra were recorded from 460 to 600 nm. For second-generation mTFP1- and Venus-based biosensors, the donor was excited at 453 nm, and emission spectra were recorded from 480 to 600 nm. Fluorescence emission spectra analysis of the ERK sensor library was acquired on an Envision microplate reader (PerkinElmer). CA-Raf and MEK-DN expression plasmids were a gift from M. Wymann. HEK293T cells (7×10^3) were seeded on 96-well optical plates coated with PLL (20 μ g/ml) and transfected (typically 20 ng of biosensor, 80 ng of regulator, and 0.5 μ l of Metafectene). After 48 hours, excitation spectra were read directly from the adherent cell monolayer. Spectra were recorded from 460 to 560 nm with a step of 2 nm. Confirmation of the RhoA screen results was obtained with a fluorometer (PerkinElmer LS50b). Cells were resuspended 48 hours after transfection in ice-cold PBS, transferred to a quartz cuvette, and recorded with a step of 0.5 nm. Spectra were background-subtracted with spectra of nontransfected samples acquired in the same conditions. Spectra were normalized according to their spectrum integral to take into account different expression levels.

Live-cell imaging, EKAR microscopy-based screen, and image analysis

All epifluorescence, ratio-imaging experiments were performed on an Eclipse Ti inverted fluorescence microscope (Nikon) with Plan Apo VC oil 60 $\times$ (NA 1.4) or Plan Apo air 20 $\times$ (NA 0.75) objectives controlled by NIS-Elements software (Nikon) or MetaMorph (Molecular Devices). Laser-based autofocus was used throughout the experiments. LED lamps [440 nm (excitation of CFP, mCerulean, and mTeal), 480 nm (excitation of Venus), and 565 nm (excitation of mCherry)] were used as light sources (CoolLED). According to the manufacturer, the total power and intensity at 440 nm are 520 mW and 210 mW/cm², respectively, based on measurement of the collimated beam at a distance of 200 mm from the light source. Acquisitions were done with a Hamamatsu Orca R2 camera at a 16-bit depth. The experiments with the RhoA2G sensor in N1E-115 cells were performed with 10 to 40% of the 440-nm LED light power at $\times 60$ magnification. The acquisition time for the donor and the FRET channels usually spanned between 200 and 500 ms at binning 2×2 . In experiments with the EKAR2G sensors expressed in HEK293T cells, we typically used 1 to 5%

of the 440-nm LED light power at $\times 60$ and 10% at $\times 20$ magnification. The acquisition time for the donor and the FRET channels usually spanned between 20 and 100 ms according to the binning (4×4 or 2×2) and the magnification ($\times 20$ or $\times 60$). Donor and FRET images were acquired sequentially using filter wheels with the following excitation, dichroic mirrors, and emission filters (Chroma): donor channel: 430/24 $\times$, Q465LP, 480/40 m; FRET channel: 430/24 $\times$, Q465LP, 535/30 m; acceptor channel: 500/20 $\times$, 89006bs, 535/30 m; mCherry channel: ET572/35, 89006bs, 632/60 m. Cells were imaged in phenol red-free DMEM or Ham's F-12 nutrient mixture supplemented with 0.5 to 2% FBS, 1% L-glutamine, and 1% penicillin/streptomycin at 37°C.

The microscopy-based screen for ERK biosensors was performed in 96-well plates (Falcon). HEK293T cells (3×10^4) were seeded and transfected with 20 ng of biosensor and 160 ng of CA-Raf. Forty-eight hours after transfection, images of nine fields of view per condition were recorded in PBS at 37°C with a 10 $\times$ Plan Apo air objective. Illumination settings were kept constant for all measurements. Filter settings for CFP-based EKAR1G were as follows: 430/24 $\times$, 89006bs, 470/24 m (donor channel) and 430/24 $\times$, 89006bs, 535/30 m (FRET channel). Filter settings for mTFP1-based EKAR2G are described above. Average ratios per field of view were calculated with MetaMorph (Molecular Devices).

FLIM analysis was performed with a Leica TCS SP5 inverted microscope with a 25 $\times$ (HCX IRAPO, NA 0.95, working distance 2.5 mm) water objective, which was adapted for TCSPC (time-correlated single-photon counting) FLIM with a Becker and Hickl SPC 830 card using 64 time channels. The samples were excited with a femtosecond titanium chameleon Ti:sapphire-pumped optical parametric oscillator (80 MHz; Coherent Inc.). Images were obtained with a linescan speed of 400 Hz. Two-photon excitation was performed with a wavelength of 860 nm, and fluorescence was detected between 450 and 500 nm. The fluorescence decays obtained were fitted using a single exponential decay model with Becker and Hickl SPCImage software v2.9.9, 29107, and the lifetimes were portrayed in false color maps. To determine the sensor state, we used the following formula:

$$\text{Amp}^{\text{on}} = 1 - \text{Amp}^{\text{off}} \\ (\tau^{\text{off}} \times \text{Amp}^{\text{off}}) + (\tau^{\text{on}} \times \text{Amp}^{\text{on}}) = \tau^{\text{weighted}}$$

Thus,

$$\text{Amp}^{\text{off}} = \frac{\tau^{\text{weighted}} - \tau^{\text{on}}}{\tau^{\text{off}} - \tau^{\text{on}}}$$

where Amp^{on} is the percentage of phosphorylated sensor, Amp^{off} is the percentage of nonphosphorylated sensor, τ^{off} is lifetime of the biosensor in the OFF state (2219.7 ps), τ^{on} is lifetime of the biosensor in the ON state (2086.8 ps), and τ^{weighted} is the measured lifetime.

Processing of epifluorescence ratio-imaging data sets was performed with the Biosensor Processing Software 2.1 (Danuser laboratory: <http://lccb.hms.harvard.edu/software.html>). Images were sequentially thresholded on each channel, shade- and background-corrected, masked, and registered before ratios were calculated. An optional photobleaching correction was applied (mainly for N1E-115 experiments with the RhoA2G sensor). Further image analyses were performed with MetaMorph (Molecular Devices). Ratio images are color-coded so that warm and cold colors represent high and low biosensor activity, respectively. Biosensor distribution was monitored through excitation of mTFP1 and collection of Venus emission light. To visualize F-actin, the Lifeact-mCherry signal was volume-corrected through division by the FRET signal (RhoA sensor distribution, which is a good volume approximation at cell periphery). This increased the contrast of F-actin structures. Mean emission ratio changes on a per cell basis after EGF stimulation during EKAR2G experiments were analyzed for each in-

dividual cell of various fields of view. These were normalized for the baseline signal before stimulation and plotted against time.

Zebrafish injections and FLIM imaging

Zebrafish adults and embryos were maintained in the Hubrecht Institute under standard husbandry conditions. DNA plasmids 14 $\times$ UAS:EKAR2G2 and CMV:RasV12-IRES-TagRFP were mixed in 1:3 concentration ratio (together 30 ng/ μ l), diluted 1:1 with Tol2-transposase mRNA (50 ng/ μ l), and injected into one cell-stage eggs from incrossed Tg(Gal4-91101) zebrafish, which exhibit broad neuronal and somitic Gal4 expression. At 1 day after fertilization, injected embryos were selected for mosaic TagRFP fluorescence and Gal4-activated expression of ERK sensor. These were anesthetized in MS-222, embedded on a glass coverslip in E3 medium containing low-melting point agarose (0.5%), and imaged at 28°C on the Leica SP5 inverted confocal microscope with a 25 $\times$ water lens (NA 1.0). Embedded embryos were subsequently scrutinized to identify cell duplets within a single field of view, showing equal expression levels of ERK sensor, but with only one cell showing TagRFP fluorescence. In this manner, all intravital FLIM measurements directly compared RasV12-positive and RasV12-negative cells. Lifetime acquisition was done as mentioned above.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/6/285/rs12/DC1

Note S1. Generation and characterization of the mTFP1 cp mutants.

Note S2. Comparison of RhoA2G with the RhoA1G and Raichu-RhoA biosensors.

Fig. S1. Identification and characterization of mTFP1 cp mutants.

Fig. S2. Schematics and further characterization of the RhoA biosensor library.

Fig. S3. Sequence of RhoA2G biosensor.

Fig. S4. Schematics and further characterization of the ERK biosensor library.

Fig. S5. Characterization of filters compatible with mTFP1- and Venus-based biosensors and imaging screening assay for EKAR2G biosensors.

Fig. S6. Comparison of RhoA1G, Raichu-RhoA, and RhoA2G biosensors in N1E-115 cells.

Fig. S7. Characterization of EKAR2G by epifluorescence ratio and two-photon FLIM.

Fig. S8. Sequences of EKAR2G design 1 and 2 biosensors.

Fig. S9. Cloning strategy to construct design 3 and 4 EKAR2G biosensors.

Movie S1. RhoA activation dynamics during membrane protrusion and retraction in MEFs.

Movie S2. RhoA activation patterns in leading-edge filopodia of MEFs.

Movie S3. RhoA activation dynamics in response to PDGF.

Movie S4. RhoA activation dynamics during N1E-115 neuroblastoma neurite growth and collapse.

Movie S5. RhoA activation dynamics in a growing and subsequently collapsing growth cone in an N1E-115 cell.

Movie S6. ERK activity dynamics in response to EGF stimulation in HEK293T cells stably expressing EKAR2G1 (high magnification).

Movie S7. ERK activity dynamics in response to EGF stimulation in HEK293T cells stably expressing EKAR2G1 (low magnification).

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