



Multiplexed GTPase and GEF biosensor imaging enables network connectivity analysis

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Here we generate fluorescence resonance energy transfer biosensors for guanine exchange factors (GEFs) by inserting a fluorescent protein pair in a structural ‘hinge’ common to many GEFs. Fluorescent biosensors can map the activation of signaling molecules in space and time, but it has not been possible to quantify how different activation events affect one another or contribute to a specific cell behavior. By imaging the GEF biosensors in the same cells as red-shifted biosensors of Rho GTPases, we can apply partial correlation analysis to parse out the extent to which each GEF contributes to the activation of a specific GTPase in regulating cell movement. Through analysis of spontaneous cell protrusion events, we identify when and where the GEF Asef regulates the GTPases Cdc42 and Rac1 to control cell edge dynamics. This approach exemplifies a powerful means to elucidate the real-time connectivity of signal transduction networks.

The cytoskeleton is regulated by dynamic, multi-layered signaling networks that interconnect Rho family small GTPases with exquisite spatiotemporal precision¹. Understanding the organization of these networks is challenging, as protein activation and interaction occur transiently and with precise subcellular localization. We and others have used fluorescent biosensors in living cells to map the activation patterns of the Rho family small GTPases relative to the changes in cell edge dynamics that they control^{2–4}.

In past studies we could determine the relative timing and location of Rac1, Cdc42, RhoA and RhoC activation, first by using fluorescent biosensors to correlate the activation of each GTPase with cell edge movements, and then using cell edge movement as a common fiducial to relate the different GTPases to one another^{3,5,6}. This allowed us to predict the relative spatiotemporal dynamics of the four signaling activities, but we could not predict whether any of the signals were directly or indirectly coupled, how specific source signals contributed to the total modulation of each target signal, and the effects of specific couplings on downstream events.

The Rho GTPases are controlled by the localized activity of numerous Rho guanine nucleotide exchange factors (RhoGEFs) that have overlapping GTPase specificity^{7,8}. This produces complex relationships between RhoGEFs and Rho GTPases, where a single GTPase may be activated by multiple GEFs, even in the same location, or one GEF may activate multiple GTPases. Here we show that statistical approaches, coupled with multiplexed biosensor imaging, can be used to ascertain the component of a GTPase signal that results from a particular GEF, or the relative influence a multi-specific GEF has on its different downstream GTPases. Furthermore, to appreciate the functional diversity of a GEF in controlling for example cell motility versus other downstream functions, it is possible to statistically determine the extent to which cell edge dynamics results from a particular GEF–GTPase interaction. We describe an approach to generate fluorescent biosensors for GEFs, multiplexed imaging of these biosensors together with red-shifted GTPase biosensors, and correlation analysis to dissect out GEF–GTPase relationships. Applying this approach to the GEFs

Asef and Vav2, and the GTPases Rac1 and Cdc42, we find that Vav2 activity is weakly coupled to cell protrusion dynamics, but Asef strongly activates Cdc42 signals that relate to cell edge movement. In contrast, the interaction between Asef and Rac1 is weaker and probably indirect.

Results

Design and testing of new RhoGEF biosensors. To perform the statistical analyses, it was first necessary to produce biosensors that report the activity of GEFs in living cells (Fig. 1). Many Dbl family RhoGEFs are regulated through occlusion of the GTPase binding interface by an autoinhibitory domain (AID)⁷, providing a route to Dbl family biosensors. We first targeted GEFs with well understood AID interactions and published crystal structures. In the RhoGEF Asef, an SH3 domain acts as the AID; it undergoes a conformational rearrangement when adenomatous polyposis coli (APC) binds to the upstream ABR (APC binding region), leading to Asef activation⁹ (Fig. 1a). We produced an Asef analog that reports these activating conformational changes by inserting two fluorescent proteins into the flexible linker between the SH3 AID and the catalytic Dbl homology (DH) domain. The activating conformational changes affected fluorescence resonance energy transfer (FRET) between the fluorescent proteins by altering their distance or relative orientation. We used high-throughput microscopy¹⁰ to test insertion of Cerulean¹¹ and Venus¹² fluorescent proteins at a series of positions between the AID and DH domain, optimizing FRET intensity and the activation-dependent difference in the donor/FRET emission ratio (Supplementary Fig. 1a). We then screened a small library combining different fluorophore pairs (Cerulean3 (ref. 13), TagCFP¹⁴ or mTFP¹⁵; combined with YPet¹⁶ or a series of YPet circular permutations; Supplementary Fig. 1a). This led to a biosensor with 75 ± 9% difference in donor/FRET ratio for wild type versus constitutively active Asef (Fig. 1a). Similar changes were seen on coexpression of known activating proteins, a fragment of APC⁹ or constitutively active Src¹⁷ (Fig. 1b). No ratio change was observed when kinase-dead Src or a nonbinding APC mutant were used,

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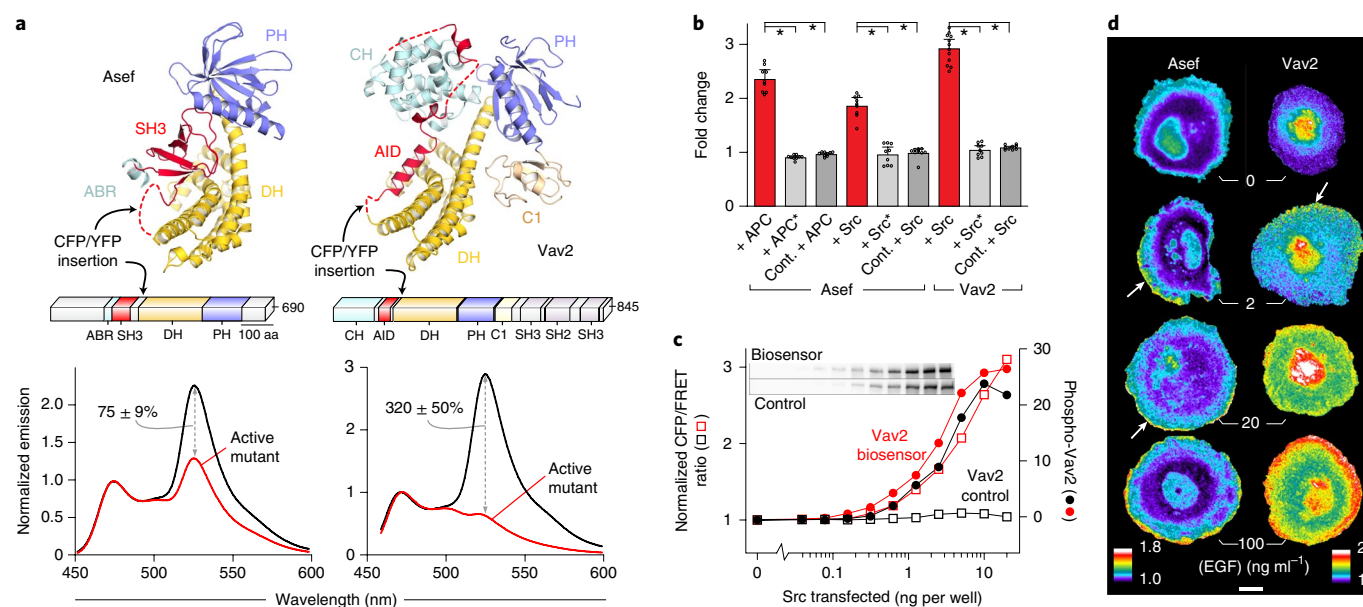


Fig. 1 | Biosensors for Asef and Vav2. **a**, Autoinhibited structures of Asef and Vav (PDB entries 2PZ1 and 3KY9, respectively, top) and domain architecture of full-length Asef and Vav2 (ABR, APC binding region; C1, C1 domain; CH, calponin homology; PH, pleckstrin homology; SH2, Src homology 2; SH3, Src homology 3, middle) with insertion sites of FRET pair cassettes indicated (CFP, cerulean fluorescent protein; YFP, yellow fluorescent protein). Emission spectra ($\lambda_{ex}=430$ nm) of wild type and active forms (Asef, V252E; Vav2, Y142E:Y159E:Y172E: Δ 860) expressed in HEK293T cells (bottom). Change in donor/FRET ratio on activation is indicated ($n=9$ independent transfections, $\pm 95\%$ confidence interval (CI)). **b**, Stimulation of Vav2 and Asef biosensors using Src or APC in HEK293T cells. Src* is kinase dead, APC* does not bind to Asef. In biosensor control (Cont.) the FRET pair is moved to the C terminus. Ratio normalized to empty vector control. Bars represent mean of nine independent transfections across multiple experiments. Error bars are 95% CI. * $P < 0.0001$ using a two-tailed Welch's t -test. **c**, Effect of increasing Src levels on donor/FRET emission ratio (open squares) and phospho-Tyr¹⁷² levels (solid circles) of Vav2 biosensor (red) and control (black). Normalized to empty vector transfection control. Inset, phospho-Vav blots (for full blots, see Supplementary Fig. 2e). Representative of three independent experiments. **d**, A431 cells expressing Asef (left) and Vav2 (right) biosensors after stimulation with increasing amounts of EGF. Arrows point to high ratio values at the edge of cell protrusions. Ratios in pseudocolor normalized so lowest value is 1. Scale bar, 10 μ m. Images representative of three independent experiments.

or when the FRET pair was moved to a site in the RhoGEF that does not undergo a conformational change (Fig. 1b).

We proposed that this approach could be applied to other RhoGEFs that undergo a conformational change on release of autoinhibition, so we tested Vav2, where the DH domain is blocked by an upstream helical AID¹⁸ (Fig. 1a). Autoinhibition is relieved when Src and other kinases phosphorylate tyrosines in the autoinhibitory regions^{19–21}. As with Asef, we optimized the site of fluorophore insertion between the AID and DH domains, and screened fluorophore combinations (Supplementary Fig. 1a). In addition, we tested fluorophore–fluorophore connectors of varying length and rigidity to impose constraints on the conformation of the inserted segment (Supplementary Fig. 1b). This led to three biosensors, whose fluorescence ratio changed $330 \pm 70\%$ (TagCFP donor), $320 \pm 50\%$ (mCerulean3 donor) and $130 \pm 20\%$ (mTFP donor) on activation (Fig. 1a and Supplementary Fig. 1c). The detectability of GEF activity was a function of both the extent of fluorescence change and the brightness of the fluorophores. In the imaging studies that follow, we used the brightest donor (mTFP) even though it produced less change (Supplementary Fig. 1c).

To test whether the Vav2 biosensor could report activating conformational changes in living cells, we compared the mCerulean3 biosensor with a nonresponsive control biosensor in human embryonic kidney 293 (HEK293) cells, examining response to increasing amounts of constitutively active Src. Dose-dependent phosphorylation of the biosensor and the control biosensor were equivalent, as shown by blotting with a phospho-Vav antibody, but only the real biosensor showed an increase in fluorescence emission ratio (Fig. 1c). As with Asef, this ratio change was not seen

with inactive Src (Fig. 1b). Using the optimized Vav2 biosensor as a template we also produced biosensors for other Vav family members simply through limited screening of the insertion site (Supplementary Fig. 1d).

For Asef and Vav2, high-resolution crystal structures were available to identify AID interactions. We next attempted to make biosensors for RhoGEFs proposed to have autoinhibitory regulation, but where structural information was limited. The RhoGEF Tim contains a putative helical region that is thought to directly interact with the DH domain²², equivalent to the Vav2 AID, and autoinhibition is maintained by polyproline and SH3 domains that flank the DH domain²³. For the GEFs T cell lymphoma invasion and metastasis 1 (Tiam1) and leukemia-associated RhoGEF (LARG), small-angle X-ray scattering suggests that an N-terminal domain folds over the DH domain^{24,25}, and binding sites for known regulators lie upstream of the DH domain (Ras²⁶/tyrosine kinases^{27–29} for Tiam1, and $G\alpha_{13}$ (ref. ³⁰) for LARG). The RhoGEF β -Pix has a polyproline-SH3 domain pair flanking the DH domain and multiple sites downstream that are proposed to regulate activity³¹. For each of these RhoGEFs we screened donor/acceptor insertion sites directly upstream of the DH domain, and optimized the response as before (Supplementary Fig. 1a). The final set of biosensors had dynamic ranges of 32% for LARG (Fig. 2a), 50% for β -Pix (Fig. 2b), 125% for Tiam1 (Fig. 2c) and 78% for Tim (Fig. 2d). For Tim, mutations within the autoinhibitory helix that mimic Src phosphorylation, and mutations within the SH3 domain that prevent polyproline binding, caused changes in the donor/FRET ratio as large as those produced by Src coexpression. The combined mutations had an additive effect (Fig. 2d).

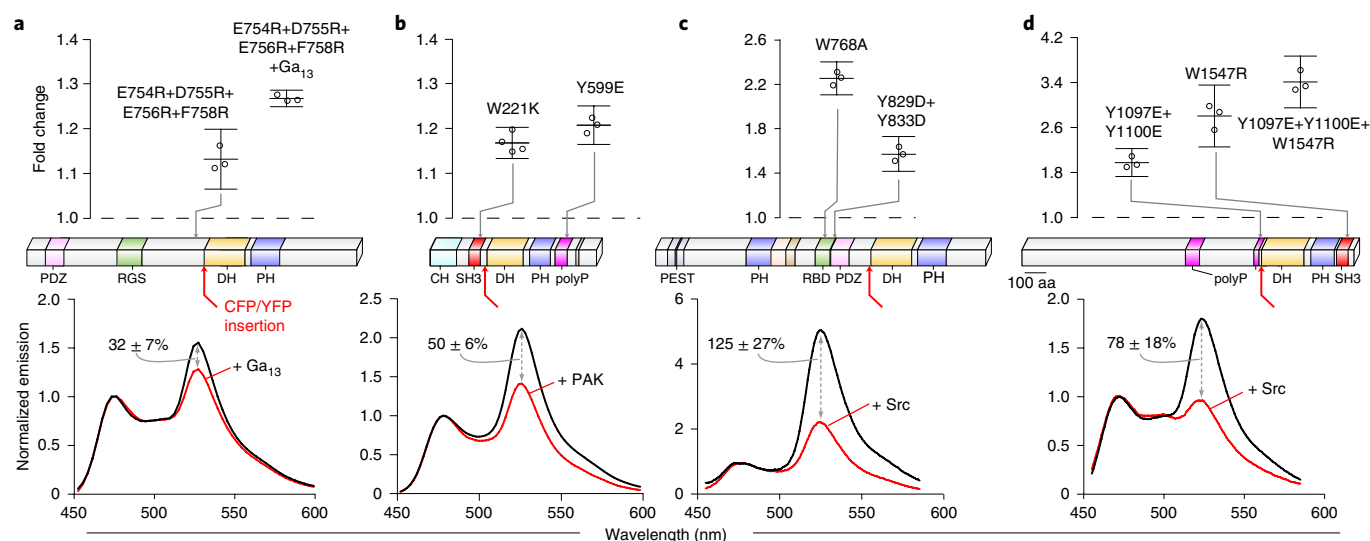


Fig. 2 | Biosensors for RhoGEFs without high-resolution structures. **a–d**, Domain structures (middle) show site of FRET pair insertion in full-length RhoGEFs LARG (**a**), β-Pix (**b**), Tiam1 (**c**) and Tim (**d**). Lower graphs show emission spectra of wild type biosensors ± indicated activators (λ_{ex} = 430 nm), change in donor/FRET ratio on activation is indicated (**a**, n = 6; **b**, n = 8; **c**, n = 9; **d**, n = 6; ±95% CI). Upper graphs show ratio change caused by indicated mutations as compared to wild type (n = 3). Bars represent mean of n independent transfections across multiple experiments. Error bars are 95% CI. PDZ, PSD95/Dlg1/zo-1; RGS, regulator of G protein signaling; polyP, polyproline; PEST, sequence rich in amino acids P, E, S and T; RBD, Ras-binding domain; other domains are as listed above.

The new LARG, Tiam1 and β-Pix biosensors provided insight into potential regulatory mechanisms. Specific residues inside the Ras-binding domain of Tiam1 caused large FRET changes, suggesting that this domain has an autoinhibitory role (Fig. 2c). Mutations of negatively charged residues in LARG³² and phosphorylation sites in β-Pix that were thought to be involved in RhoGEF regulation induced modest FRET changes, and mutation of the SH3 domain in β-Pix also affected FRET, suggesting that there may be an autoinhibitory role for the polyproline-SH3 domain pair flanking the DH domain, as there is for Tim (Fig. 2a,b).

We validated the new biosensors by examining their response to known stimuli in living cells. In response to epidermal growth factor (EGF), Vav2 and Tiam1 showed widespread activation (Fig. 1d and Supplementary Fig. 2a) while Asef was activated only within 2 μm of the cell edge (Fig. 1d). Ratio imaging was used to minimize effects of nonuniform biosensor distribution. Comparison of biosensor and activity distribution indicated that activity patterns were not simply the result of variations in cell thickness (Supplementary Fig. 3a). More detailed studies of Vav2 showed that its response was dose dependent and reversible, with activation within 30 s (Supplementary Fig. 2b). LARG and β-Pix showed activation concentrated at the leading edge of randomly migrating cells, with LARG concentrated right at the edge and β-Pix 1–2 μm away from the edge (Supplementary Fig. 2c).

GEF activity relative to cell edge dynamics. For studies of GEF–GTPase circuitry, we focused on GEFs that were stimulated by EGF, to examine protrusion–retraction cycles during EGF-induced chemokinesis. Biosensors for Asef, Vav2 and improved versions of our previously published biosensors for Rac1 (ref. ³) and Cdc42 (refs. ^{33,34}; Methods and Supplementary Fig. 4a) were stably expressed in MDA-MB-231 cells and studied in EGF-containing medium. Biosensors were kept below expression levels that perturbed motility behaviors (Supplementary Fig. 5a,b). As previously described, Rac1 and Cdc42 (Fig. 3a,b and Supplementary Video 1) showed broad gradients of activity dropping from the cell edge to 5–8 μm behind the edge, consistent with activation in protruding lamella (Supplementary Fig. 6a,b). The new biosensor showed that Asef activation was

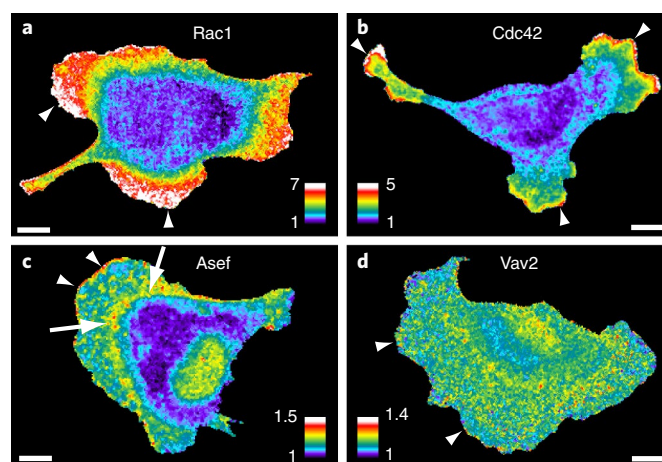


Fig. 3 | RhoGEF and GTPase activity in living cells. **a–d**, Rac1 (**a**), Cdc42 (**b**), Asef (**c**) and Vav2 (**d**) activation reported by biosensors in MDA-MB-231 cells undergoing random edge motion. Pseudocolor as in Fig. 1. Scale bars, 10 μm. Images are representative of at least three independent experiments.

generally restricted to a narrow band at the leading edge of protrusions (resembling that produced by acute EGF stimulation of A431 cells, Fig. 1d), together with a broader region 5–10 μm back from the edge, at the base of lamella, (Fig. 3c, Supplementary Fig. 6a, b and Supplementary Video 2). The latter activation occurred in the same cellular region as Rab7–mScarlet, indicating activated Asef interacts with a sorting compartment (Supplementary Fig. 6c). When we moved the FRET pair in the Asef biosensor to a region that should not undergo conformational change, activation at both cellular locations was eliminated (Supplementary Fig. 6d). In contrast, Vav2 showed diffuse activation throughout the cell, with only a few ‘hot spots’ adjacent to the edge (Fig. 3d and Supplementary Video 2). Comparison of biosensor localization with activity showed that this activity pattern was not caused by variations in cell thickness (Supplementary Fig. 7a).

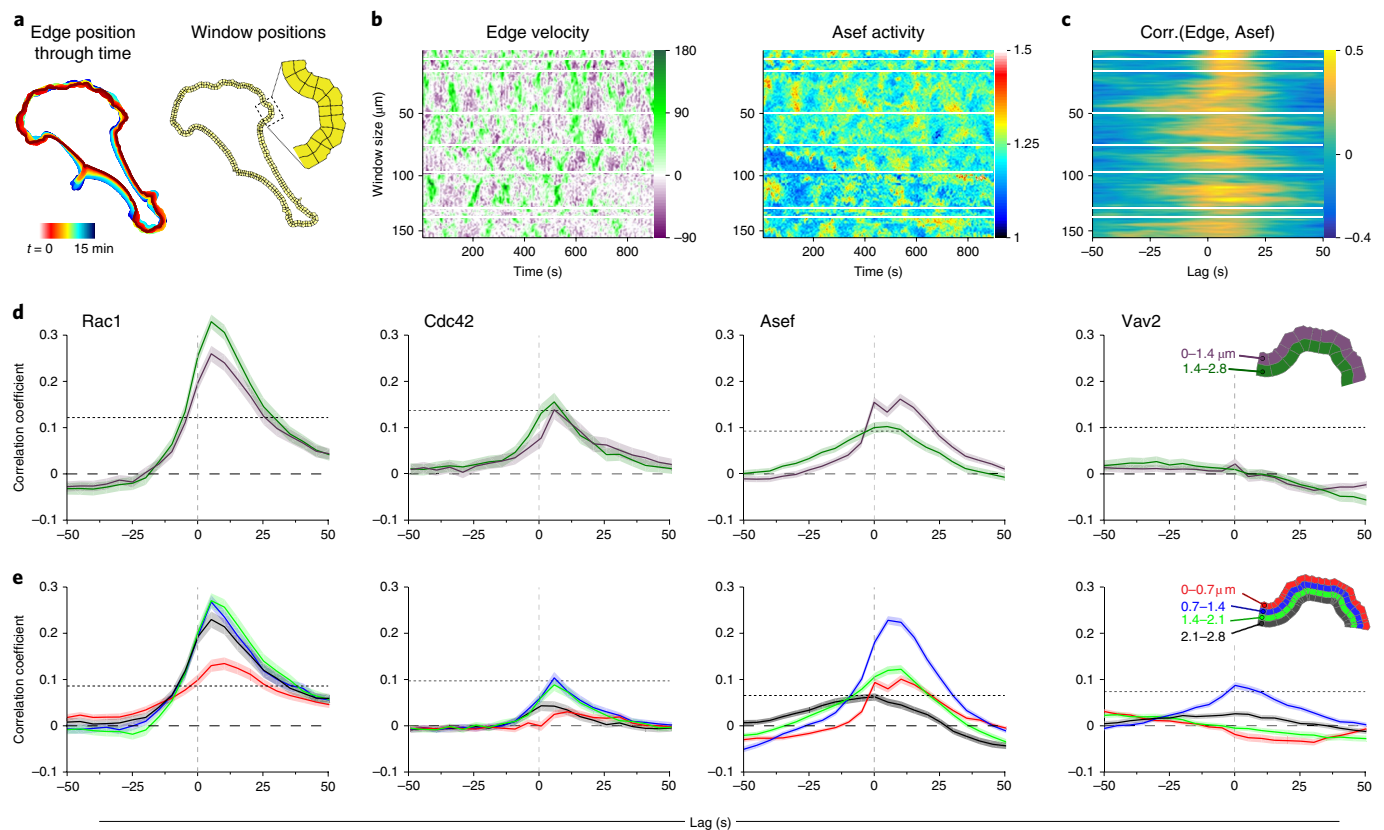


Fig. 4 | Correlation of protein activities with edge motion. **a**, Evolution of cell edge positions (color-encoded from red (early) to blue (late) time points, left). Two rows of sampling windows, each 1.4-μm deep placed at the cell edge. **b**, Maps of edge velocity (nm/s, left) and Asef biosensor activities (right) along the edge. Green regions are protruding, purple regions retracting. Red/yellow regions have high activity, blue regions have low activity. Each column is a single time point. White horizontal bars show quiescent regions that are excluded. Scale bars are shown to right of each map. **c**, Cross-correlation coefficients between edge velocity and Asef activity. Gold shows high correlation and blue negative correlation. Each row is a single position along the edge corresponding to the activity maps. **d**, Average cross-correlation functions for each biosensor. Analysis using a 1.4-μm window size (*n*, cells; *m*, windows); Rac1 (*n*=9, *m*=259); Cdc42 (*n*=6, *m*=204); Asef (*n*=8, *m*=448) and Vav2 (*n*=6, *m*=360). **e**, Average cross-correlation functions for each biosensor. Analysis using a 0.7-μm window size (*n*, cells; *m*, windows); Rac1 (*n*=9, *m*=518); Cdc42 (*n*=6, *m*=408); Asef (*n*=8, *m*=896) and Vav2 (*n*=6, *m*=720). Inset shows window size and color key. For **d** and **e**, dotted lines show the correlation coefficient above which the coupling between two variables is considered significant with 95% confidence. This depends on the number of windows (see Methods). Shading represents 95% CI about the mean correlation computed from *m* windows. The width of this interval depends on the consistency of the correlations across windows and cells (see Methods).

To quantify the relationship between biosensor activity and edge displacement, we used previously described local cross-correlation analysis³, which integrates data from multiple cells to assess the coupling between the two cues despite heterogeneous signaling and motion along the cell periphery. Specifically, we tracked the cell edge over time and then divided the perimeter of the cell into small edge sectors and associated sampling windows (Fig. 4a). Sampling windows were placed in layers of increasing but constant distance from the edge. As the cell boundary moved, the windows were rearranged such that they maintained a stable one-to-one relationship with the associated sector. Thus, we could sample for each edge sector the local, instantaneous velocity and the corresponding biosensor activity at any layer (Fig. 4b). These cell shape- and motion-invariant data representations then permitted a straightforward analysis of the relationship between edge motion and signaling activity using Pearson's correlation (Fig. 4c).

Improvements in instrumentation since our first published correlation analyses of biosensor fluctuations enabled us to enhance spatial resolution by sampling 0.7 × 0.7 μm windows rather than the 1.4 × 1.4 μm windows used previously. Consistent with the earlier work in fibroblasts, Rac1 correlated best with edge motion in the two layers between 0.7–2.1 μm (Fig. 4d,e). Also consistent with the earlier studies, correlation was strongest when we incorporated a

lag between edge movement and Rac1 activity, but the lag seen in MDA-MB-231 cells was considerably shorter than in fibroblasts (5 s instead of ~40 s, Fig. 4d,e). The shortest time lag to protrusion–retraction cycles was in these two middle layers, suggesting that Rac1 molecules are activated in this zone and then diffuse or are transported to the cell front³. The correlation of Cdc42 with edge motion also peaked at a distance 1–2 μm from the cell edge, and with similar time lag, but with overall lower magnitude than Rac1 (Fig. 4d,e), suggesting that Rac1 is the more dominant GTPase in the regulation of MDA-MB-231 protrusion and retraction. We suggest that the delays of both Rac1 and Cdc42 relate to the upregulation of Arp2/3-mediated dendritic network growth after protrusion onset, which is required for persistent edge protrusion against increasing membrane tension^{35–37}. Arp2/3 is the primary nucleator of the branched actin filament network and is an effector of the Rac1-WAVE and Cdc42-N-Wasp pathways.

Asef had a maximum correlation in the first layer, immediately adjacent to the edge (0–1.4 μm, Fig. 4d). In contrast to the GTPases, we observed a near twofold increase in the correlation peak magnitude when we switched to analyzing correlation with the new, smaller windows, despite the decreased signal to noise ratio that they produced (compare Fig. 4d,e). This indicated that the portion of the Asef signal related to cell motion was activated in a highly

confined zone 0.7–1.4 μm behind the cell edge, which corresponds to the zone of cell adhesion formation (Supplementary Fig. 6b). Like Rac1 and Cdc42, the Asef activation was slightly delayed relative to protrusion–retraction cycles. Together, these results indicate that spatially precise RhoGEF activity produces more diffuse effector activation, probably due to diffusion of the effector from the point of activation. Unlike Asef, Vav2 showed no significant correlation with edge motion at the coarser analysis resolution (Fig. 4d), and only a weak correlation in finer analyses using the 0.7–1.4 μm layer (Fig. 4e). This indicates that Vav2 does not play a main role in coordinating signals that specifically regulate cell protrusion and retraction events in unstimulated migration. Given the strong response of Vav2 to acute EGF stimulation (Fig. 1d), we conjecture that Vav2 could be important in translating directional cues to the cell protrusion machinery, or may be disconnected from the pathways that promote constitutive protrusion–retraction cycles.

Simultaneous imaging of RhoGEF and Rho GTPase activities.

In view of the significant correlation between Asef activity and cell edge movement, we decided to focus on the interactions of Asef with the GTPases Rac1 and Cdc42. To complete our statistical analysis, we needed to correlate the activation of Asef and each GTPase, so we produced red-shifted GTPase biosensors that could be imaged in the same cell as the Asef biosensor. We modified our published GTPase biosensors³ by changing the fluorescent proteins to LSSmOrange³⁸ and mCherry³⁹ (Fig. 5a,b). Because of LSSmOrange's long Stokes shift, we could capture emission from both biosensors at once using a single excitation, for rapid imaging and reduced photo-toxicity (Fig. 5a,b). The new biosensors responded correctly to RhoGEFs and RhoGAPs (Supplementary Fig. 4), and their correlation analysis showed GTPase–edge coordination consistent with previous findings (Supplementary Fig. 8). The GTPase biosensors were transfected into cells stably expressing Asef biosensor, at levels that minimized perturbation of cell motility (Supplementary Fig. 5c,d). Cells with two biosensors showed activation patterns such as those of single biosensor cells (by correlation analysis and by visual inspection; see Fig. 5b, Supplementary Video 3 and Supplementary Fig. 7) but did show some increase in edge velocity (Supplementary Fig. 5). With two biosensors in the same cell we could employ the dynamic grid of sampling windows to extract the local correlation between Asef and each GTPase (Fig. 5c,d). Asef activity correlated with both Rac1 and Cdc42, in both the 0–1.4 and 1.4–2.8 μm layers. Overall, both correlation peaks were substantially higher for Cdc42 than for Rac1. This is consistent with biochemical data that Asef directly interacts with Cdc42, but can activate Rac1 only indirectly^{9,40}, although our imaging data now shows that all three signals are active in the same zone. Any delay between activation of Asef and Cdc42 was too short to be captured by the 5 s sampling intervals we needed when imaging both biosensors together. This means that the communication between GEF and GTPase as resolved by these sensors and the affordable image sampling is quasi-instantaneous. We noted that the overall positive correlation lobes in the second layer dip at $t=0$. Thorough investigation of the causes unveiled a systematic amplification of subpixel errors in cell edge segmentation and sampling window positioning that depresses the correlation values specifically at zero time lags (Supplementary Fig. 9). Taken together, this analysis revealed specific regions and times when activation of Asef is correlated with Cdc42 and Rac1 activation during cell edge movement. During protrusion, the GTPases Rac1 and Cdc42 are coregulated by multiple RhoGEFs, which our data show includes Asef but not Vav2. Using a previously published Cdc42 biosensor⁴¹ combined with our new red-shifted Rac1 biosensor, we tested whether Cdc42 and Rac1 correlate directly. Indeed, this was the case, again with a time lag of zero (Fig. 5b,e and Supplementary Video 4), suggesting that at the time scale of our sampling, the two GTPases are modulated concurrently.

Partial correlation analysis of RhoGEF and Rho GTPase activities at the edge. A critical question is why a RhoGEF activates multiple GTPases, and how much interaction with each one contributes to the downstream effector responses that actually produce edge motion. In concrete terms here, how much of the Cdc42 activation that is produced specifically by Asef contributes to the modulation of edge motion and how much is contributed by Asef's indirect activation of a Rac1 signal? By imaging all possible combinations of three variables (GEF–GTPase, GEF–edge and GTPase–edge), we could address this question using partial correlation analysis.

Given three confluctuating variables (X , Y and Z), partial correlation analysis can quantify how much the fluctuations in Y and Z remain coupled after removing the effects of a third variable X , which controls both Y and Z (Fig. 6a). Using this approach, we first computed the correlation between Cdc42 activation and edge motion after eliminating the influence of Asef on Cdc42 and edge motion (a 'numerical knockout' of Asef). We focused on the layer 1.4–2.8 μm from the edge, where Asef displayed significant confluctuation with edge motion and Cdc42 (Figs. 4e and 5d). Compared to the direct correlation between Cdc42 and edge motion, the peak value was about a third smaller (0.2 versus 0.13; Fig. 6b), indicating that 36% of the Cdc42 signal that influences edge motion is triggered by Asef. Using short-hairpin RNA to reduce Asef levels experimentally also produced a reduction in the correlation between Cdc42 and edge motion (Supplementary Fig. 8b). The difference between the 36% reduction seen with partial correlation analysis and the 25% reduction in the shAsef experiment may be marginally significant, but it should be noted that reducing the concentration of one GEF can be compensated by other GEFs. The numerical analysis by partial correlation is not subject to compensation by other GEFs. Thus, it is reasonable for the partial correlation analysis to report stronger differences. In stark contrast, for Rac1 the correlation with edge motion increased after eliminating the contribution of Asef (Fig. 6c). This implies that the Asef to Rac1 signaling link desynchronizes the coupling of Rac1 and edge velocity. Such a conclusion can only be reached using numerical analysis. An actual perturbation of Asef would have effects on cell edge velocity that preclude analysis of the changes in the relationship between Rac1 and velocity; for example, inducing shifts in Cdc42 signaling.

This finding is consistent with an indirect activation of Rac1 by Asef, potentially via cross-talk between Cdc42 and Rac1 (ref. ⁴⁰). Alternatively, interactions between Asef and Rac1 mediated without Cdc42 may be elicited for cell functions other than protrusion, again with an adversarial effect on the interactions between Rac1 and edge motion. Distinguishing these two explanations will require concurrent imaging of Asef, Cdc42 and Rac1, which is impossible at this point. Nonetheless, these findings reveal the limitations of multi-functional signaling networks, where signaling for one purpose may impair the precision in signaling for another purpose.

Previous studies suggest that expansion of the plasma membrane during protrusion produces mechanical feedback on GEF–GTPase interactions, especially in the adhesion-rich zone near the cell edge^{36,42}. We therefore applied partial correlation analysis to assess the influence of cell edge motion on Asef–Cdc42 and Asef–Rac1 relationships. The correlations between GEF and GTPase fluctuations remained unchanged on controlling for common fluctuations associated with edge motion. This shows that both interactions are nearly independent of edge motion (Fig. 6d,e). Hence, Asef is unlikely to be a mediator of mechanical feedback to Rac1 and Cdc42.

Finally, we asked how eliminating the influence of the GTPases would affect the correlation between Asef and edge motion. For both Cdc42 (Fig. 6f) and Rac1 (Fig. 6g) the correlation curves substantially decreased, confirming that the interaction between Asef and motion is indeed mediated by Rac1 and Cdc42. With Cdc42 effects removed, the Asef/motion correlation was markedly reduced

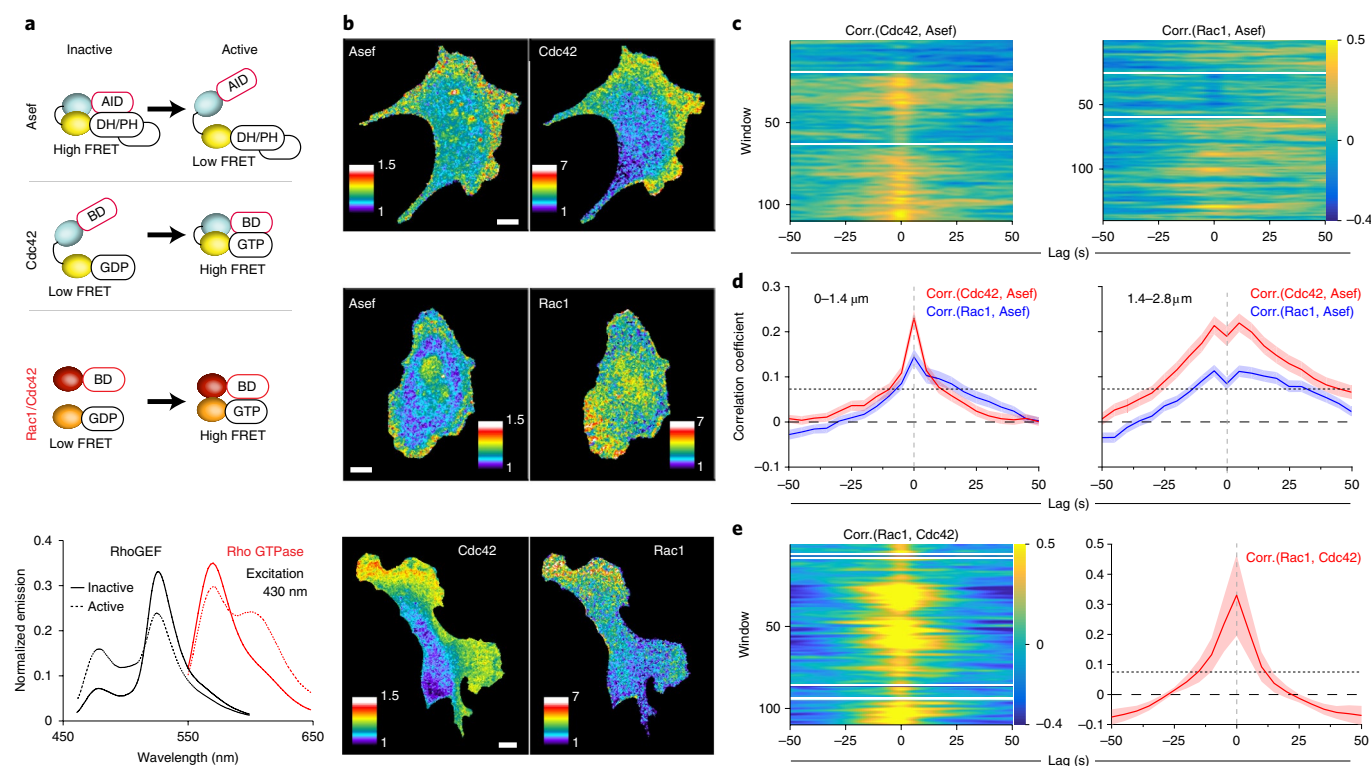


Fig. 5 | Multiplexed imaging of two protein activities. **a**, Biosensor designs and fluorescent proteins used for multiplexing (upper). Emission spectra of representative RhoGEF and Rho GTPase biosensors (lower). Inactive biosensors in solid lines, activated forms in dotted line. The two spectra were obtained using the same excitation wavelength (430 nm). **b**, RhoGEF and Rho GTPase biosensors imaged simultaneously in MDA-MB-231 cells undergoing constitutive edge motion. Pseudocolor as in Fig. 1. Scale bars, 10 μ m. Images are representative of at least three independent experiments. **c**, Cross-correlation coefficients between Rho GTPase activity and Asef activity in the 1.4–2.8 μ m layer. Gold shows high correlation, blue negative correlation. **d**, Average cross-correlation functions for each biosensor combination: 0–1.4 μ m layer, left; 1.4–2.8 μ m layer, right (n , cells; m , windows); Cdc42 ($n = 5$, $m = 729$); Rac1 ($n = 6$, $m = 719$). Dotted lines show the correlation coefficient above which the coupling between two variables is considered significant with 95% confidence. This depends on the number of windows (see Methods). Shading represents 95% CI about the mean correlation computed from m windows. The width of this interval depends on the consistency of the correlations across windows and cells (see Methods). **e**, Single cell cross-correlation coefficients (left) and average cross-correlation functions (right) between Rac1 activity and Cdc42 activity in the 1.4–2.8 μ m layer (left) ($n = 7$, $m = 684$).

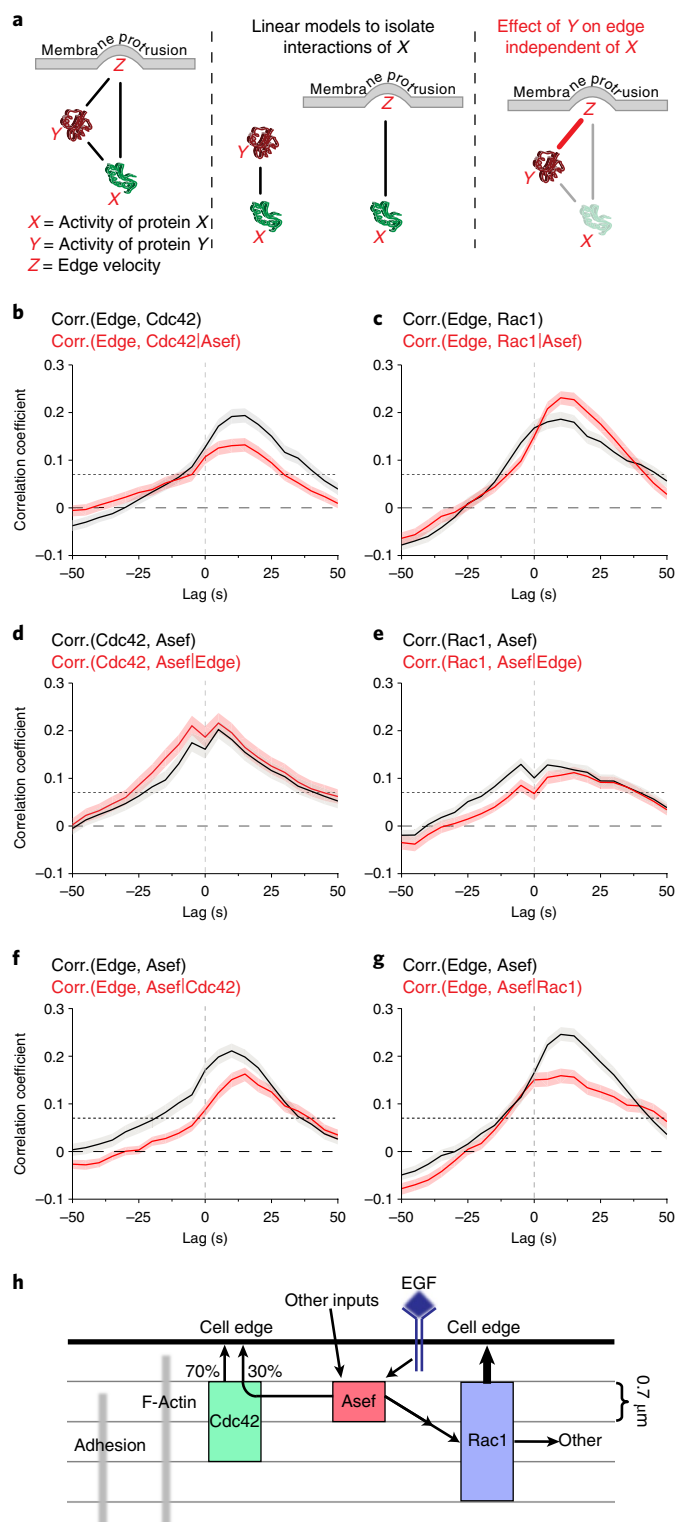
at negative time lags and between 0 and 25 s (Fig. 6f), whereas removal of Rac1 inputs reduced the Asef/motion correlation at lags between 0 and 50 s (Fig. 6g). This suggests that coupling of Asef to edge motion early in protrusion is dependent on Cdc42, while later coupling depends more on Rac1. Again, this finding is consistent with the interpretation that Asef activates Cdc42 directly, but Rac1 is coupled to Asef only via an indirect connection. Together, these analyses and conclusions indicate how direct multiplexed imaging of an activator and receiver signal in the context of a dynamic cell function allows statistical dissection of very complex signaling relations with unprecedented precision.

Discussion

The study of RhoGEFs has been limited to deciphering biochemical interactions *in vitro*, and to molecular perturbation *in vivo*^{43,44}. However, due to the complexity of RhoGEF–GTPase interaction networks, which contain numerous feedbacks, crosstalks and redundancies, an understanding of GEF function requires new methods to determine connectivity and function in the context of space and time. To unravel the effects of RhoGEFs on multiple GTPase targets, we first developed a generalizable approach to Dbl family GEF biosensors. The method has been applied to the GEFs described here and to GEF-H1 (ref. 45). It is applicable to diverse structures comprising 19 of the 70 Dbl family RhoGEFs (Supplementary Fig. 2d), and potentially to ten GEFs that are similarly regulated. This enabled correlation of GEF activity and GTPase activity with the motions

of the cell edge. We then modified our existing GTPase biosensors for simultaneous imaging of GEF and GTPase activity, providing the ability to directly correlate their spatiotemporal dynamics in living cells. With pairwise correlation of edge motion, GEF activity and GTPase activity in hand, we could apply partial correlation analysis to locally sampled time series and thereby determine that the RhoGEF Asef has stronger interactions with Cdc42 than with Rac1, albeit both interactions are statistically significant. We could show that specific, spatially localized Asef activations contribute to specific Cdc42 activation events, and determine to what extent the Cdc42 activation promoting cell edge activity was due to Asef (Fig. 6h). For Asef activation of Rac1, several observations (timing, diffuse response and weaker coupling between Asef and Rac1) indicated that Rac1 is activated via indirect interactions, consistent with published biochemical studies. Moreover, while Asef activity clearly has an activating effect on Rac1 signaling, our partial correlation analysis indicates that the Asef-triggered Rac1 activity does not contribute to the modulation of edge protrusion—this despite the fact that the correlation between Rac1 and edge motion is substantially stronger than the correlation between Cdc42 and motion (Fig. 4d,e). Rac1 may be more involved in the regulation of constitutive protrusion–retraction cycles. This highlights that each signal can best be understood through *in situ* analysis in the context of a cell function.

The GEF biosensor design used here exemplifies a valuable alternative to current biosensor approaches. It can produce protein analogs that report conformational changes while maintaining upstream



and downstream interactions. This contrasts with biosensors whose components bind selectively to the activated state of the target protein, thereby generating dominant negative effects. Unlike biosensors based on protein substrates, the new design will be less prone to false negative readouts where substrate cannot reach the target. The GEF biosensors described here could potentially be expressed by replacing the genes of their endogenous counterparts. Like other biosensors, in the current study we had to carefully control expression level to minimize overexpression artifacts, forcing us in some cases to work at concentrations near the limit of signal/noise for robust image analysis.

Fig. 6 | Partial correlation analysis. **a**, Model for partial correlation analysis using three variables, X, Y and Z, which are all connected by pairwise relationships (solid lines, left). Correlations describing specifically the relationships between X and Y, as well as X and Z (middle). The contribution of X to both Y and Z is then subtracted from each (gray arrows), and the relationship between the residual Y and Z is calculated, giving the partial correlation (red line, right). **b-g**. Comparison between total and partial correlations for all combinations of Asef, Rho GTPase and edge velocity (total correlation, black line; partial correlation, red line ((n, cells; m, windows); Cdc42 (n=5, m=729); Rac1 (n=6, m=719))). Edge to GTPase correlations, controlled for Asef input (Cdc42 (**b**), Rac1 (**c**)). GTPase to Asef correlations, controlled for edge input (Cdc42 (**d**), Rac1 (**e**)). Edge to Asef correlations, controlled for GTPase input (Cdc42 (**f**), Rac1 (**g**)). Dotted lines show the correlation coefficient above which the coupling between two variables is considered significant with 95% confidence. This depends on the number of windows (see Methods). Shading represents 95% CI about the mean correlation computed from m windows. The width of this interval depends on the consistency of the correlations across windows and cells (see Methods). **h**. Spatiotemporal integration of Asef, Cdc42 and Rac1 signaling in the control of constitutive cell protrusion-retraction cycles. Asef directly activates Cdc42 to control ~30% of the effect of Cdc42 on edge motion. Asef also contributes indirectly to the activation of Rac1, but this contribution does not play a role in controlling the edge. Instead, other GEFs must be responsible for the motion-relevant signaling of Rac1, which is stronger than Cdc42's contribution. The relative localizations of each protein are shown. Note that activation of Asef is tightly localized compared to Rac1 and Cdc42, supporting localized GTPase activation by Asef followed by GTPase diffusion or transport to the edge.

Building on multiplexed imaging of biosensors, future correlation analysis can assign functions to the perplexing multitude of RhoGEF-GTPase interactions, in the context of diverse cellular behaviors. This can ultimately be extended to a host of other complex signaling circuits.

Online content

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Methods

RhoGEF biosensor design. The biosensors were optimized and tested in a sequential fashion. Initially, a cassette comprising mCerulean or mCerulean3, a flexible linker³, and mVenus or YPet was inserted into a series of sites between the AID and DH domains of full-length RhoGEFs and emission was measured using fluorimetry for Vav2 or high-throughput microscopy for the other RhoGEFs (see detailed procedures below). For Vav2 we compared wild type to constitutively active (Y140E:Y159E:Y172E) mutants, and for the other RhoGEFs we used coexpression of an activator. For Vav2 we also tested linker variants. These comprised a flexible unit⁴⁶ and a structured helical unit⁴⁷ combined in different topologies and repeat numbers (two, three and four repeats for short, medium and long, respectively). Once we had identified the best insertion site and linker combination, we combinatorially combined donor proteins (mCerulean3, TagCFP and mTFP) with acceptor proteins (YPet, along with circular permutations of YPet) to form a library of variants of each biosensor that were screened using high-throughput microscopy for the effects of activation. The biosensor constructs were inserted into a tet-off inducible retroviral expression system and stable lines were produced in tet-off MDA-MB-231 cells (Johnson laboratory, UNC-CH). Cells were maintained in DMEM (Cellgro) with 10% FBS (Hyclone) and 0.2 $\mu\text{g ml}^{-1}$ doxycycline to repress biosensor expression. Biosensors were named FLARE.a as part of a nomenclature system based on the biosensor design (for example, Asef FLARE.a).

Rho GTPase biosensors. The Rac1 FLARE.dc1g biosensor is a modification of our previously reported dual chain biosensor design³. To improve brightness and dynamic range we used Turquoise fluorescent protein¹³ rather than CyPet. In brief, YPet¹⁶ is fused upstream of residues 60–145 of human PAK1, and Turquoise is fused to the N terminus of full-length Rac1. The two biosensor chains were expressed on one open reading frame with two consecutive 2A viral peptide sequences from porcine teschovirus-1 (P2A) and *Thossea asigna* virus (T2A) inserted between them, leading to expression of the two separate biosensor chains⁴⁸. The Cdc42 and RhoA FLARE.dc1g biosensors use an identical topology, wherein YPet is fused upstream of N-WASP (200–293) or Rhotekin (6–98), for Cdc42 and RhoA, respectively, and mCerulean3 is fused to the N terminus of the GTPase, with the two biosensor chains separated by the 2A sequences. The biosensor constructs were inserted into a tet-off inducible retroviral expression system and stable lines were produced in tet-off MDA-MB-231 cells (Johnson Laboratory, UNC-CH). Cells were maintained in DMEM (Cellgro) with 10% FBS (Hyclone) and 0.2 $\mu\text{g ml}^{-1}$ doxycycline to repress biosensor expression.

For the red-shifted versions of the GTPase biosensors, YPet was exchanged for mCherry and the donor was exchanged for LSSmOrange. Both donor and acceptor contain the R1251 mutation to increase intramolecular FRET⁴⁹. For dual biosensor experiments, these red-shifted GTPase biosensors were transfected into the RhoGEF biosensor-expressing stable cell lines. For experiments with two GTPase biosensors in the same cell, the Cdc42 biosensor⁴¹ was transfected into cells stably expressing the red-shifted Rac1 biosensor.

Spectral analysis of biosensors. Emission spectra of biosensors were obtained using a Fluorolog fluorometer (Horiba). HEK293T cells grown in six-well plates (Nunc) were transfected with biosensor DNA plus regulator if required. After 24 h, cells were detached by trypsinization (Cellgro) and resuspended in cold PBS (Sigma) + 1%FBS (Hyclone), washed and then resuspended in cold PBS. Samples were excited at 430 nm and spectra obtained from 460 to 600 nm for biosensors with Cerulean3/TagCFP/mTFP, and 550 to 650 nm for LSSmOrange biosensors. Dual chain biosensors were corrected for acceptor bleed-through.

High-throughput microscopy screening. Using a modification of our published procedure¹⁰, HEK293T cells were plated in 96-well plates with μ -clear plastic bottoms (Greiner Bio-one) coated with poly-L-lysine (Sigma). Cells were transfected in triplicate with a library of biosensor DNA plus regulator if required and imaged after 24 h. Growth media was replaced with Hank's balanced salt solution (Sigma) with 1% FBS and 10 mM HEPES (Gibco) before imaging. Cells were imaged using a $\times 10$, 0.4 numerical aperture objective on an Olympus IX-81 inverted microscope and using Metamorph screen acquisition software (Molecular Devices) and mercury arc lamp illumination. Filters used were excitation, ET436/20x; emission, donor, ET470/24M; FRET, ET535/30M and a 445/505/580 ET dichroic mirror. Images were obtained on a Flash4 sCMOS camera (Hamamatsu). Images were analyzed using MATLAB (Mathworks). Briefly, four fields were taken of each well and the intensity was summed for each channel. These were then background subtracted using values from wells that were mock transfected, and ratios obtained from these background-subtracted values. For regulator titration experiments, regulator DNA was first titrated against the biosensor DNA and then transfection complexes formed in 96-well plates before transfection. Regulators used were APC (309–798), Src (FL, Y529F), G α_{13} (FL, Q226L), PAK (FL D389R:S422D:T423E), Dbl (495–826), Vav2 (191–573), Asef (FL), Tiam1 (C1199), p115RhoGEF (FL), RhoGDI (FL), p50RhoGAP (FL) and RacGAP1 (FL).

Src stimulation of Vav2 biosensors. Cells were transfected and imaged as above. After lysis, triplicate samples were pooled and western blotted for phospho-Vav

levels using a phospho-specific Tyr-172 antibody (Abcam). Samples were normalized to biosensor expression using an anti-GFP antibody (Clontech).

Stimulation experiments. A431 cells (ATCC) were plated in six-well plates 24 h before transfection. Cells were incubated with transfection complexes for 5–6 h and then replated onto no. 1.5 coverslips (Warner Instruments) coated with Collagen IV (Gibco). Cells were allowed to spread in DMEM/10% FBS. After 2–3 h the media was replaced with DMEM/0.5% delipidated bovine serum albumin (BSA) and cells were starved overnight before imaging. Cells were then imaged in Hams/F12 (Caisson Labs) with 0.5% BSA, 10 mM HEPES (Gibco), 100 μM Trolox (Sigma) and 0.5 mM Ascorbate (Sigma). Cells were imaged using a $\times 40$ 1.3 numerical aperture Silicon oil objective on an Olympus IX-81 inverted microscope using Metamorph software and 100 W Hg arc lamp illumination. Excitation filters used were FF-434/17 and FF-510/10 combined with a FF462/523 dichroic mirror. Donor and FRET images were simultaneously captured using a TuCam system (Andor) fitted with FF-482/35 and FF-550/49 and an imaging flat FF509-FDi01 dichroic, together with two Flash4 sCMOS cameras (Hamamatsu). To visualize endogenous Vav2 in these cells, mock-transfected A431 cells were stimulated in exactly the same way, then fixed in 4% formaldehyde (EMS microscopy supplies), 0.1 M Pipes, 1 mM EGTA and 1 mM MgSO₄ (all Sigma). They were washed in PHEM buffer (60 mM Pipes, 25 mM HEPES, 2 mM MgSO₄, 10 mM EGTA, all Sigma) then blocked with BSA. The cells were incubated with anti-Vav2 antibodies (Epitomics) in BSA/PHEM buffer, washed with PHEM buffer and then visualized using Alexa-633 labeled secondary antibodies.

Constitutive migration experiments. Biosensor expression was induced 48 h before imaging through trypsinization and culturing without doxycycline. On the day of imaging, cells were replated using Accumax (Innovative Cell Technologies) onto coverslips coated with collagen I (10 $\mu\text{g ml}^{-1}$ 37 °C overnight) and allowed to attach in DMEM/10%FBS. After 2 h, the media was replaced with Hams/F12 with 0.2% BSA, 10 ng μl^{-1} EGF (R and D systems) 10 mM HEPES, 100 μM trolox and 0.5 mM ascorbate and cells were allowed to equilibrate. After a further 2–4 h, cells were imaged in a closed chamber with media treated with Oxyfluor (1/100). For single biosensor experiments, cells were imaged using the filters listed above. For dual biosensor experiments, the excitation filters used were FF-434/17 for Cerulean3/mTFP and LSSmOrange, and FF-546/6 for Cherry (Semrock) combined with a custom zt440/545 dichroic (Chroma). For emission, a TuCam was fitted with a FF560-FDi01 imaging flat dichroic and a Gemini dual view (Hamamatsu) was added to each emission port. For the short wavelength Gemini, the filters used were donor, FF-482/35; FRET, FF-520/15 and a FF509-FDi01 imaging flat dichroic mirror. For the red-shifted Gemini, the filters used were orange, FF01-575/15; FRET/mCherry, FF01-647/57 and a FF580-FDi01 imaging flat dichroic. For the shRNA experiments, multiple shRNA constructs using microRNA backbones (mir-E)⁵⁰ were tested for knockdown using quantitative PCR with reverse transcription. The most efficient was inserted downstream of iRFP670 (ref. ⁵¹) in a tet-off inducible retroviral expression system and stable lines were produced. When imaging, cells expressing the highest levels of iRFP670 were chosen to ensure knockdown.

Image processing and analysis. Biosensor activation levels were measured in living cells by monitoring the ratio of FRET to donor emission on a pixel by pixel basis. Donor and FRET images were aligned using fluorescent beads as fiducials to produce a transformation matrix using the MATLAB function 'cp2tform'. This was then applied to the Donor image using the MATLAB function 'imtransform'. The camera dark current was determined by obtaining images for each camera without excitation, and the dark current was subtracted from all images. Images were corrected for shading due to uneven illumination by taking images of a uniform dye solution under conditions used for each wavelength, normalizing this image to an average intensity of one to produce a reference image for each wavelength and then dividing the images corrected for dark current by the shading correction reference image. Background fluorescence was removed by subtracting, at each frame, the intensity of a region containing no cells or debris. Images were segmented into binary masks separating cell and noncell regions using the segmentation package 'MovThresh', which is based on the Otsu algorithm. The Donor channel was used for segmentation, as it had the highest signal to noise, particularly at the cell edge. The masks were then applied to all channels, setting noncell regions to zero intensity. For dual biosensor imaging these masked images were then corrected for bleed-through of Cerulean3/mTFP and YPet into the orange/red channels.

For RhoGEF biosensors, activation maps were obtained by dividing the corrected donor image by the FRET image. For the GTPase biosensors, the images were corrected for bleed-through and ratios were obtained using the following equation (using data from control cells expressing donor or acceptor alone to obtain the bleed-through coefficients α and β): $R = (\text{FRET} - \alpha(\text{donor}) - \beta(\text{acceptor})) / \text{donor}$ where R is the ratio, FRET is the total FRET intensity as measured, α is the bleed-through of the donor into the FRET signal, β is the bleed-through of acceptor into the FRET signal, and donor and acceptor are the donor and acceptor intensities as measured through direct excitation. These ratio images were then corrected for photobleaching. For

stimulation experiments, the ratios were divided by a reference curve derived from mock-stimulation experiments. For constitutive migration the whole cell average was fitted to a double exponential curve and this curve was used to normalize. Pseudocolor scales were produced without considering the lowest and highest 5% of ratio values to eliminate spurious pixels, and normalizing so the lowest value is 1. For the dual chain biosensors, there is ideally no FRET in the off state, so dynamic range cannot be defined as $\text{FRET}_{\text{on}}/\text{FRET}_{\text{off}}$. The lowest ratio values are in fact determined by the level of noise in the image and any binding in the off state.

Cell windowing analysis. After the corrections described, the cell images of the ratiometric biosensor activity were compartmentalized into layers of rectangular windows along the entire cell edge. To construct the sampling windows at a constant distance from the cell edge we computed a distance map (MATLAB function: `bwdist`) to the segmented cell edge. The distance map yielded equidistant contours at either 0.7 μm (2 pixels) or 1.4 μm (4 pixels) from the cell edge. At the cell edge the first contour was divided into segments of 1.8–3 μm width (Fig. 4a). Sampling windows located at the cell edge derived a time course of edge velocity and biosensor intensity as the windows tracked the morphological changes of the cell over time. For windows placed in layer 2 and higher, only the biosensor intensities were sampled. However, each of these windows maintained unambiguous correspondence to a window at the cell edge, allowing correlation of biosensor intensity fluctuations inside the cell with cell edge movements. The ability to maintain unique correspondences depended on the cell morphology. Windows for which the correspondence was lost at one or several time points of a video because of particularly strong cell morphological changes were eliminated from the analysis.

This *in silico* compartmentalization of the cell allowed us to represent the biosensor activity into a cell-shape invariant space. For each frame of the videos, the biosensor activity was averaged within the area of each sampling window, resulting in a set of matrices representing the biosensor activity of a layer of windows with a fixed distance from the cell edge. Rows correspond to windows and columns to time (Fig. 4b). This method has shown to be an efficient way to spatiotemporally sample the activity of sensors expressed by the cell. For more information, see ref. ⁵².

Windows selection. Migrating cells usually display regions along the cell edge that are active with protrusion–retraction cycles, but they also exhibit quiescent regions with little morphodynamic activity. We implemented window selection criteria based on the autocorrelation function of the protrusion–retraction speed estimated by the windowing algorithm described above. The autocorrelation function of a random variable X can be described as the cross-correlation between X and its time-delayed version $X(\text{lag})$ where lag is the duration of the delay. It can measure an average duration of ‘memory’ in the signal that can be described as the maximum time spacing between samples that still exhibit a linear association. No significant linear correlation can be measured when taking samples further than this duration apart. Consequently, the autocorrelation function of a signal with structure has a much slower decay compared with the autocorrelation function of a signal with samples independently drawn from a uniform random distribution. We used the full width at half maximum (FWHM) of the window speed autocorrelation as a measure of information in the signal that our analysis methods can make use of. Only windows with autocorrelation FWHM equal to or higher than 0.6 frames were chosen for analysis, that is the speed time series in such windows showed on average some temporal coherence in motion. For reference, a white noise time series with no coherence has a FWHM of <0.5 frames. The effect of this strategy was the elimination of quiescent regions. For the example in Fig. 2, all windows are shown in Supplementary Fig. 7.

A second method of window selection was implemented to avoid a phenomenon we observed when cells were expressing two biosensors. This problem arose due to a subpixel segmentation error that appears when two biosensors have opposing gradients. See Supplementary Fig. 8. This transient error causes a strong negative cross-correlation between the activities of the two imaged biosensors. To minimize the impact of this artifact on our final analysis, we excluded windows that presented a sharp negative cross-correlation between two biosensors at lag zero. It is worth mentioning that windows with a negative cross-correlation trend that resulted in a negative score at lag zero were not excluded from analysis. The selection algorithm starts by decomposing the cross-correlation between the biosensors for a given window using empirical mode decomposition⁵³. This decomposition technique recursively extracts components of the signal from the fastest, or higher frequency content, to slower component of the signal. The fastest component or first intrinsic mode function (IMF) solves all fast variations present in the signal. The window is excluded from analysis if after its cross-correlation decomposition, the amplitude of the first IMF at lag zero exceeds a threshold and shows change in derivative for the lag zero neighborhood. The threshold is estimated as three standard deviations away from the mean using the first IMF points to build the distribution.

In our further analysis of the red-shifted biosensors, we used the Ljung–Box statistical test to exclude the quiescent subcellular regions automatically. The test provides a P value where the time series of interest, here that is cell edged motion, can be considered as a white noise. For the implementation of the method, we

used the MATLAB function `lbqtest()` and checked the statistical significance of auto-correlations up to 20 lags of the velocity time series for each window. Using a significance level of 0.05, certain subsets of the windows were identified as white noise signals, equivalent to our previous quiescent velocities. The selected windows were further excluded from our analysis.

Pearson correlation analysis. The main aim of the Pearson correlation analysis is to determine the strength of the linear relationship between two random variables. For instance, it can be applied to find time-dependent linear relationship of two measured parameters, which is the case in this work. The correlation value ranges from $(-1,1)$ where score value one represents perfect linear correlation, 0 represents no correlation and -1 perfect anticorrelation. Additionally, one time series can be time-shifted in relation to the second by a value usually referred to as lag. This lag analysis can be useful when two variables are correlated with a time delay. That means there will a value of lag where the correlation score is maximum. The lag at peak correlation can then be interpreted as the time needed for one variable to process upstream information or as the time taken for information to be transferred or a combination of both. Because correlation analysis requires a stationary signal, few preprocessing steps are required before the correlation coefficient is calculated. All time series data used to calculate a correlation score in this work were mean subtracted and linear trend removed.

Pearson's correlation coefficient $\rho(a_1(t), a_2(t))_\tau$ between two activity time courses $a_1(t)$ and $a_2(t)$ was computed as a function of the time lag τ using the MATLAB function (`xcov`). This function implements the mean corrected and normalized correlation functions as follows:

$$\rho(a_1(t), a_2(t))_\tau = \frac{\text{Cov}(a_1(t) - \bar{a}_1, a_2(t - \tau) - \bar{a}_2)}{\sqrt{\text{Var}(a_1(t) - \bar{a}_1)} \times \sqrt{\text{Var}(a_2(t) - \bar{a}_2)}}$$

The operators `Cov(.)` and `Var(.)` denote the covariance and the variance of the mean corrected time courses, respectively. The variables $\bar{a}_1$ and $\bar{a}_2$ denote the mean value of the respective time course. Correlation functions were first calculated for pairs of time courses per window (either between edge velocity and biosensor intensity, or between two biosensor intensities in the case of simultaneous imaging GEF and GTPase activity). For each correlation curve we computed the level, which is exceeded by no more than 5% of correlations between two random time series. This level (~ 0.1 for all our data) depended on the duration of the videos and the number of sampling windows. Regions of the correlation curve that extend above this confidence interval are therefore considered significant with a confidence of 95%. In addition, through bootstrap analysis of the variation among all correlation curves, we computed confidence intervals for the mean cross-correlation. This reflected the number and consistency of sampling windows across experiments. This computation is performed by applying the Fisher transformation to the correlation values with a hyperbolic tangent and bringing back the distribution parameters with the hyperbolic tangent inverse⁵⁴.

Partial correlation analysis. Although Pearson correlation analysis has been shown to be useful when only two variables are at hand, it can result in misleading outcomes when trying to identify linear relationships between random variables where the data generating system has confounding structure⁵⁵. A simple example of this case is when a random variable A drives two other unrelated random variables B and C . There is a flow of information going from A to B and A to C but no exchange of information between B and C . A simple correlation analysis would identify the confounding information from A shared by B and C as a direct connection between B and C . However, the partial correlation between B and C control for A , or partial (p) ($B, C|A$), would statistically return zero. For this simple three variable example, the partial correlation can be computed as:

$$\rho(B, C|A)_\tau = \frac{\rho(B, C)_\tau - \rho(B, A)_\tau \times \rho(C, A)_\tau}{\sqrt{1 - \rho(B, A)_\tau^2} \times \sqrt{1 - \rho(C, A)_\tau^2}}$$

The time dependency of the random variables was omitted for simplicity. The equation here shows that partial correlation removes the influence of the variable A from B and C , and renormalizes it such that the final value lies within the $(-1,1)$ interval. Although simple, using the inverse of the correlation matrix is a more efficient way to calculate the partial correlation when more than three variables are present⁵⁶.

Because of the stationary requirement, all time series were mean subtracted and linear trend corrected as was done for the simple correlation analysis. Confidence intervals and levels were calculated in the same fashion as the Pearson's correlation.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. Figures 4–6 have associated raw data used to produce the correlation plots.

Code availability

All code used for the data analysis was written in MATLAB 2014b. All the code can be downloaded from the Danuser Lab github: <https://github.com/DanuserLab> under Biosensor, Windowing-Protrusion and Time-Series-Tools repositories.

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Author contributions

D.J.M., J.S. and K.M.H. designed the biosensors. D.J.M. carried out all experiments, except for work by J.R. in building Tiam1, Tim, B-Pix and LARG biosensors. G.G. and M.A. contributed to initial design of Tim and LARG biosensors, respectively. M.V., J.H. and G.D. developed and carried out computational analysis. G.D., J.S. and K.M.H. provided intellectual input in all phases of the study and directed the work. The paper was written by D.J.M., M.V., G.D., J.S. and K.M.H., with input from all authors.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at <https://doi.org/10.1038/s41589-020-0542-9>.

Correspondence and requests for materials should be addressed to G.D., J.S. or K.M.H.

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Software and code

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Data collection

Metamorph version 7.8.10

Data analysis

All code used for the data analysis was written in Matlab 2014b. There are three major packages: Biosensor toolbox, Windowing-Protrusion toolbox and a collection of scripts for the estimation of partial and simple cross-correlation. The Biosensor toolbox was used for image processing, where a series of corrections were applied to the time-lapsed microscopy images. The Windowing-Protrusion toolbox was used for image analysis, where the corrected images were digitally segmented into windows, cell edge velocity were estimated and the biosensor signal sampled throughout the cell. Once sampled, the time-lapsed images can be represented as a collection of time series each sampled from a specific window. A collection of scripts was then used to pre-process the signal and subsequently estimation Pearson partial and simple cross-correlation analysis. All the code can be downloaded from the Danuser Lab github: <https://github.com/DanuserLab> under Biosensor, Windowing-Protrusion and Time-Series-Tools repositories.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample size is a combination of many (hundreds) of windows across multiple cells across different acquisition days. Due to the variability of window numbers between cells, sample size is not predictable. Confidence intervals are calculated to show variability and significance.
Data exclusions	Migrating cells usually display regions along the cell edge that are active with protrusion/retraction cycles, but they also exhibit quiescent regions with little morphodynamic activity. We implemented window selection criteria based on the autocorrelation function of the protrusion/retraction speed estimated by the windowing algorithm described above. The autocorrelation function of a random variable X can be described as the cross-correlation between X and its time-delayed version X (lag) where lag is the duration of the delay. It can measure an average duration of “memory” in the signal that can be described as the maximum time spacing between samples that still exhibit a linear association. No significant linear correlation can be measured when taking samples further than this duration apart. Consequently, the autocorrelation function of a signal with structure has a much slower decay compared with the autocorrelation function of a signal with samples independently drawn from a uniform random distribution. We used the full width at half maximum (fwhm) of the window speed autocorrelation as a measure of information in the signal that our analysis methods can make use of. Only windows with autocorrelation fwhm equal to or higher than 0.6 frames were chosen for analysis, i.e. the speed time series in such windows showed on average some temporal coherence in motion. For reference, a white noise time series with no coherence has a fwhm < 0.5 frames. The effect of this strategy was the elimination of quiescent regions. For the example in Figure 2, all windows are shown in Figure S7. A second method of window selection was implemented in order to avoid a phenomenon we observed when cells were expressing two biosensors. This problem arose due to a sub-pixel segmentation error that appears when two biosensors have opposing gradients. See Figure S8. This transient error causes a strong negative cross-correlation between the activities of the two imaged biosensors. In order to minimize the impact of this artifact on our final analysis, we excluded windows that presented a sharp negative cross-correlation between two biosensors at lag zero. It is worth mentioning that windows with a negative cross-correlation trend that resulted in a negative score at lag zero were not excluded from analysis. The selection algorithm starts by decomposing the cross-correlation between the biosensors for a given window using empirical mode decomposition – EMD (Huang et al., 1998). This decomposition technique recursively extracts components of the signal from the fastest, or higher frequency content, to slower component of the signal. The fastest component or first intrinsic mode function (IMF) absorbs all fast variations present in the signal. The window is excluded from analysis if after its cross-correlation decomposition, the amplitude of the first IMF at lag zero exceeds a threshold and shows change in derivative for the lag zero neighborhood. The threshold is estimated as three standard deviations away from the mean using the first IMF points to build the distribution.
Replication	Data acquisition was repeated on multiple cells and different days to show reproducibility
Randomization	Allocation was not random, however all imaging experiments were done under identical conditions
Blinding	Investigators were not blinded. However all imaging experiments were done under identical conditions and were analyzed using the same code

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☒	☐ Animals and other organisms
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☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Anti-phospho Vav antibody (ECM Research VP2641), Anti-Vav2 antibody (Epitomics 1993-1).
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Validation

Anti-phospho-Vav: From Manufacturer: This antibody was cross-adsorbed to unphosphorylated Vav2 (Tyr-172) peptide before affinity purification using phospho-Vav2 (Tyr-172) peptide (without carrier). The antibody detects a 100 kDa* band corresponding to Vav on SDS-PAGE immunoblots of human A431 cells treated with EGF and detects Vav1, Vav2, and Vav3 in Jurkat cells treated with pervanadate. The antibody does not detect these Vavs after treatment with alkaline phosphatase. Anti-Vav2 antibody: From the manufacturer- was shown to specifically react with VAV2 in wild-type HAP1 cells as signal was lost in VAV2 knockout cells. Wild-type and VAV2 knockout samples were subjected to SDS-PAGE.

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