Evaluation of active Rac1 levels in cancer cells: A case of misleading conclusions from immunofluorescence analysis

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A large number of aggressive cancer cell lines display elevated levels of activated Rac1, a small GTPase widely implicated in cytoskeleton reorganization, cell motility, and metastatic dissemination. A commonly accepted methodological approach for detecting Rac1 activation in cancer cells involves the use of a conformation-sensitive antibody that detects the active (GTP-bound) Rac1 without interacting with the GDP-bound inactive form. This antibody has been extensively used in fixed cell immunofluorescence and immunohistochemistry. Taking advantage of prostate and pancreatic cancer cell models known to have high basal Rac1-GTP levels, here we have established that this antibody does not recognize Rac1 but rather detects the intermediate filament protein vimentin. Indeed, Rac1-null PC3 prostate cancer cells or cancer models with low levels of Rac1 activation still show a high signal with the anti-Rac1-GTP antibody, which is lost upon silencing of vimentin expression. Moreover, this antibody was unable to detect activated Rac1 in membrane ruffles induced by epidermal growth factor stimulation. These results have profound implications for the study of this key GTPase in cancer, particularly because a large number of cancer cell lines with characteristic mesenchymal features show simultaneous up-regulation of vimentin and high basal Rac1-GTP levels when measured biochemically. This misleading correlation can lead to assumptions about the validity of this antibody and inaccurate conclusions that may affect the development of appropriate therapeutic approaches for targeting the Rac1 pathway.

The GTPase Rac1, a member of the Rho family of small G-proteins, plays key roles in the regulation of cellular functions, including cell migration, proliferation, differentiation, and gene expression. This GTPase is ubiquitously expressed and has been established as a master regulator of actin cytoskeletal membrane structures implicated in cell migration and invasion (1, 2). The importance of this protein has also been observed in human disease, including its misregulation or mutation in a number of cancer types (2–4).

Rac1 is a molecular switch and cycles between active GTP-bound and inactive GDP-bound states. The GTP-bound Rac1 adopts a conformation that is capable of binding effector proteins that mediate downstream signaling responses, most prominently the Pak kinases (5). Indeed, the biochemical assessment of Rac1 activation, determined from cell lysates, relies on the binding of the GTP-bound form to a Pak1-binding domain (PBD), allowing the “pulldown” of the active form of the GTPase and therefore separation from the inactive GDP-bound form. A drawback of this assay, however, is that it only provides information about global levels of active Rac1 in a cell population. Visualization of the Rac1-GTP state in single cells and the assessment of subcellular localization is difficult due to the transient nature of the Rac1 activation and the noncovalent interaction with GTP. The use of Rac-FRET reporters has, to a certain extent, overcome these obstacles by allowing for the spatiotemporal analysis of Rac1 activation in living cells (6, 7) or in transgenic organisms (8, 9). However, this approach requires ectopic expression of the reporter, therefore limiting its physiological relevance and making it an unsuitable tool for studies with fixed tissues, such as patient specimens. Furthermore, the low resolution of this technique can make studying the subcellular distribution of Rac1-GTP difficult.

One of the promising solutions to visualize the activation of endogenous Rac1 in cells or patient samples has been the development of a conformation-sensitive antibody capable of specifically detecting Rac1-GTP. This antibody purportedly provides a unique resource for visualization and quantification of the endogenous active Rac1 in fixed samples, circumventing the need presented by FRET approaches to overexpress probes, while also achieving greater resolution. Indeed, the anti-Rac1-GTP antibody has become a widely used tool for immunofluorescence (10–41), proximity ligation (41), immunohistochemistry (19, 35, 38, 42–52), flow cytometry (53), and immunoprecipitation assays (10, 11, 22, 33, 54, 55).

The extensive interest of our laboratory in the role of Rac1 signaling in tumorigenesis and metastasis (3, 56, 57) prompted us to use the anti-Rac1-GTP antibody in a number of cancer cell models. In the course of these studies, we stumbled upon a number of inconsistencies that persuaded us to pursue a deeper characterization of this antibody. We took advantage of our recent characterization of models displaying diverse levels of Rac1 activation (56), which we complemented with the use of
Rac1 knockout cells. This analysis led us to question the validity of this widely used antibody, unambiguously demonstrating that it is not specific for Rac1-GTP but rather reacts with vimentin, a marker of the mesenchymal cancer cell phenotype. Furthermore, here we show two examples in which misleading information could be derived from its use.

Results

The Rac1-GTP antibody staining colocalizes with vimentin

In a recent study (56), we characterized prostate cancer cell lines with low and high Rac1-GTP levels. Fig. 1A shows that aggressive PC3 cells display markedly higher Rac1-GTP levels relative to less aggressive LNCaP cells. To determine the intracellular localization of Rac1-GTP, we carried out immunofluorescence studies with a widely used conformation-sensitive anti-Rac1-GTP antibody. Our initial experiment revealed that, as expected, prominent staining from the anti-Rac1-GTP antibody could be observed in PC3 cells but not in LNCaP cells (Fig. 1B). Analysis of confocal images from PC3 cells revealed that the antibody recognized a prominent filamentous network localized primarily in the perinuclear region. This staining did not colocalize with filamentous actin visualized by phalloidin (Fig. 1C). These results were unexpected, because active Rac1 would normally localize at the periphery of the cell, as is consistent with its main role in the regulation of actin cytoskeleton dynamics (1, 7, 8). Further co-staining immunofluorescence analysis revealed a lack of colocalization with microtubules (as determined by α-tubulin staining), endoplasmic reticulum (as determined by PDI staining), Golgi (golglin-97 staining), or mitochondria (Mitotracker Deep Red staining). Surprisingly, we did observe a remarkable colocalization with the intermediate filament vimentin, with prominent staining on vimentin filaments close to the nucleus (Fig. 1C). It should be noted that the signal from the vimentin or Rac1-GTP staining could only be observed in the appropriate channel, confirming the absence of any bleed-through of fluorescence. Moreover, no signal could be observed from the secondary antibodies when used alone, confirming the authenticity of the observed signal being from the primary antibody staining (Fig. S1). The colocalization of Rac1-GTP staining with vimentin filaments can be better appreciated with Airyscan super-resolution microscopy (Fig. 1D).

Rac1-GTP antibody staining is not specific

To determine the specificity of the staining with the anti-Rac1-GTP antibody, we generated a Rac1 knockout (KO) PC3 cell line using a CRISPR/Cas9 approach. The knockout of Rac1 in these cells was confirmed with Western blotting and Sanger sequencing (Fig. 2, A and B). To our surprise, Rac1 KO PC3 cells display a prominent staining with the anti-Rac1-GTP antibody that has similar intensity as that observed in control PC3 cells and also colocalizes with vimentin (Fig. 2C). These results unambiguously establish that the anti-Rac1-GTP antibody is nonspecific for Rac1.

Next, we ectopically expressed in PC3 cells the EGFP-tagged Rac1Q61L mutant, which has impaired GTPase activity and therefore remains in a GTP-bound state. As expected, EGFP-Rac1Q61L displays significant expression in peripheral structures. However, we were unable to find colocalization of this active Rac1 mutant with the staining from the anti-Rac1-GTP antibody (Fig. 2D). Although we cannot rule out that the EGFP tag or Q61L mutation may interfere with the antibody binding, this result is in line with the nonspecific staining in Rac1 KO PC3 cells and supports the idea that the anti-Rac1-GTP antibody is recognizing an alternative epitope to Rac1.

In an effort to analyze in greater detail the colocalization of the anti-Rac1-GTP antibody staining with vimentin, we generated vimentin-deficient PC3 cells. PC3 cells were infected with two different vimentin shRNA lentiviruses, which caused ~80% depletion of vimentin as determined by Western blotting. The loss of vimentin had no effect on the levels of Rac1-GTP determined biochemically with a PBD pulldown assay (Fig. 3A). Interestingly, and despite the lack of changes in cellular active Rac1 levels, silencing vimentin expression essentially abolished the anti-Rac1-GTP signal detected by immunofluorescence (Fig. 3B). Therefore, the observed filamentous staining produced by this antibody depends on vimentin expression and is not representative of the levels of active, GTP-bound Rac1.

To further substantiate our conclusions, we used A549 cells, a non-small-cell lung cancer model in which EGF causes significant actin cytoskeleton rearrangement into peripheral ruffles and triggers a Rac1-dependent motility response (58). EGF stimulates the formation of large actin structures in A549 cells in a Rac1-dependent manner, as judged by the impaired response in Rac1 KO A549 cells (Fig. 5A). We then sought to determine whether Rac1 is activated at sites of peripheral protrusions by using a Rac1-FRET reporter (59). As shown in Fig. 5B and Video S1, stimulation of A549 cells with EGF caused significant activation of Rac1 detected by the FRET reporter at the site of peripheral protrusions. However, probing stimulated A549 cells with the anti-Rac1-GTP antibody resulted in no visible staining or around peripheral actin structures and instead stained vimentin filaments (Fig. 5C). Taken together, these results support the evidence that this antibody does not recognize active Rac1.

Examples of misleading results with the anti-Rac1-GTP antibody in cancer models

Despite this lack of specificity, the anti-Rac1-GTP antibody has been widely used to support results obtained with other tools and techniques, particularly in cancer cell models. Although results obtained with this antibody often fit with the expected research paradigm, it should be noted that a large number of cancer cells display elevated levels of vimentin, a marker of the mesenchymal cancer cell phenotype (60). In the
EDITORS’ PICK: Flaws in Rac1-GTP visualization

A

LNCaP PC3

Rac1-GTP

Total Rac1

Vinculin

Rac1-GTP/Total Rac1 (Fold-change)

LNCaP PC3

B

Rac1-GTP/Hoechst

LNCaP PC3

C

Rac1-GTP Phalloidin Merge

αTubulin Merge

PDI Merge

Golgin-97 Merge

Mitotracker Merge

Vimentin Merge

D

Rac1-GTP/Hoechst

Vimentin/Hoechst

Merge
mesenchymal state, cancer cells are more migratory and may therefore have enhanced active Rac1 signaling. Moreover, it has been recently shown that cancer cells undergoing epithelial-to-mesenchymal transition increase their global Rac1-GTP levels as determined biochemically (61). Therefore, it is conceivable that the observed staining often reported in cancer cells with the anti-Rac1-GTP antibody reflects an elevated vimentin expression status and is being erroneously reported as Rac1-GTP.

To test this hypothesis, we first used prostate cancer cell lines with different levels of basal Rac1-GTP. We have previously shown that androgen-independent aggressive cancer cell lines DU145, PC3, and PC3-ML have high levels of basal active Rac1 when compared with a normal prostate epithelial cell line (RWPE1), prostate hyperplastic cell line (BPH-1), and less aggressive prostate cancer cell lines (LNCaP and LNCaP variants), which all have low levels of Rac1-GTP (56) (Fig. 6A). Interestingly, only the aggressive prostate cancer cells display high levels of vimentin expression, with the notable exception of RWPE1 cells (Fig. 6B). Based on this observation, we carried out immunofluorescence analysis of three cell lines: RWPE1 (Rac1-GTP low, vimentin high), LNCaP (Rac1-GTP low, vimentin low), and PC3 (Rac1-GTP high, vimentin high). As shown in Fig. 6C, prominent staining with the anti-Rac1-GTP antibody could be observed only in those cell lines with high vimentin (RWPE1 and PC3). Again, these results indicate that this antibody does not recognize active Rac1; rather, the staining fits with the pattern of vimentin expression.

Last, we decided to evaluate this paradigm in cellular models of pancreatic cancer. Using the PBD pulldown assay, we identified pancreatic cancer cell lines with low Rac1-GTP (Capan-1 and BxPC-3) and cell lines with high Rac1-GTP levels (AsPC-1 and Panc-1). The expression of vimentin in these cell lines also correlates with the levels of Rac1-GTP (Fig. 7A), similar to the observation in prostate cancer models. When we examined the staining of these cells with the anti-Rac-1-GTP antibody, we found that it can only be detected in AsPC-1 and Panc-1, the cell lines that display high vimentin expression and visible vimentin staining (Fig. 7B). To establish a causal relationship between staining with the anti-Rac1-GTP antibody and vimentin expression, we subsequently depleted vimentin in Panc-1 cells using two different shRNA lentiviruses (Fig. 7C). Notably, there was a marked loss in anti-Rac1-GTP staining in the vimentin-knocked down cells concomitant with a loss of vimentin staining (Fig. 7D), thus recapitulating the results obtained in PC3 cells.

Discussion

Our detailed characterization of the anti-Rac1-GTP antibody clearly demonstrates that it is not specific for its stated target. Results presented in this study revealed that the staining from this antibody colocalizes with vimentin and depends on vimentin expression. The staining is not lost in PC3 cells subjected to CRISPR/Cas9-mediated KO of Rac1, therefore conclusively indicating that it does not recognize Rac1.

Previous users of this antibody have demonstrated its specificity through the use of dominant-negative Rac1 overexpression (12, 15, 22) or immunoprecipitation of endogenous Rac1 in response to stimuli (10, 11, 22, 33, 54). It may be possible that the overexpression of dominant-negative Rac1 inadvertently affected the expression of the Rac1-GTP antibody’s true target. Indeed, it has previously been reported that treatment with the Rac inhibitor NCS23766 causes disassembly of the vimentin network (62). On the other hand, the data demonstrated by others showing that the antibody successfully precipitates Rac1 does raise the possibility that the antibody can recognize Rac1-GTP in addition to its primary vimentin-dependent epitope, presumably with lower affinity (10, 11, 22, 33, 54). However, it is clear from the present work that the primary epitope is not active Rac1. Furthermore, we were unable to immunoprecipitate Rac1 from PC3 cells using the anti-Rac1-GTP antibody (data not shown).

The primary epitope of this antibody being vimentin (or alternatively a vimentin-associated protein) may explain why the antibody has been extensively used without negative reports concerning its specificity. Vimentin is an intermediate filament often used as a marker to identify mesenchymal cells (63), which are characterized by increased migratory and invasive properties, functions known to be regulated by Rac1 (64). Moreover, it has recently been reported that active Rac1 levels are elevated in cancer cells that have undergone epithelial-to-mesenchymal transition (61). Therefore, it is unsurprising that this antibody can produce false positive results by primarily staining the highly motile and invasive vimentin-rich mesenchymal cells. We demonstrated the validity of this hypothesis using cancer models displaying high and low levels of Rac1-GTP and vimentin expression. The characterization of these two scenarios both in prostate and pancreatic cancer cells represents a compelling example of how vimentin expression can cause misleading conclusions where vimentin-dependent staining by this antibody is attributed to Rac1-GTP. In conclusion, we have demonstrated that without the appropriate controls, such as the use of Rac1 KO cells, the anti-Rac1-GTP antibody could be perceived as providing legitimate information on the level and localization of active Rac1 within cells. Yet, in reality, although the use of this antibody may be producing an expected result for the researcher, it is instead contributing deceptive information to the study of Rac1 activation in a variety of settings, cell types and diseases. Current assays such as the PBD pulldown assay and FRET reporter probes do not enable the
EDITORS' PICK: Flaws in Rac1-GTP visualization

A

|            | CRISPR control | Rac1 KO |
|------------|----------------|---------|
| Rac1       |                |         |
| Actin      | -20            | -50     |
|            | -37            |         |

B

Rac1 KO

CRISPR control

C

|                | Rac1-GTP/Hoechst | Vimentin/Hoechst | Merge |
|----------------|------------------|------------------|-------|
| CRISPR control|                  |                  |       |
| CRISPR control|                  |                  |       |
| CRISPR control|                  |                  |       |
| Rac1 KO       |                  |                  |       |
| Rac1 KO       |                  |                  |       |
| Rac1 KO       |                  |                  |       |

D

|                | Rac1-GTP/Hoechst | EGFP-Rac1^{G61L}/Hoechst | Merge |
|----------------|------------------|---------------------------|-------|
|                |                  |                           |       |

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visualization of endogenous active Rac1 in patient samples via immunohistochemistry or the high-resolution imaging of subcellular Rac1-GTP localization. Therefore, the nonspecific nature of this antibody creates a major gap in the toolbox for studying this GTPase, illustrating the importance of developing alternative robust methods for studying Rac activation.

**Experimental procedures**

**Cell lines, cell culture, and reagents**

Human prostate cancer cell lines (RWPE1, BPH-1, LNCaP, LNCaP-C4, LNCaP-C4-2, DU145, and PC3), pancreatic cancer cell lines (Capan-1, BxPC-3, AsPC-1, and PANC-1), and the non-small cell lung cancer cell line A549 were obtained from ATCC (Manassas, VA). PC3-ML cells were obtained from Dr. Alessandro Fatatis (Drexel University), as described previously (65). EGF was purchased from R&D Systems (Minneapolis, MN; catalog no. AFL236).

**Plasmid transfection**

To express EGFP-Rac1Q61L, cells were transfected with a pcDNA3-EGFP-Rac1Q61L plasmid (Addgene, Watertown, MA) using Jet Optimus (Polyplus Transfection, Illkirch, France) following the manufacturer’s protocol. Experiments were carried out 24 h after transfection.

**Immunofluorescence**

Cells were grown on glass coverslips for 24 h in complete medium followed by a further 24 h in starvation medium. After fixation in 3.6% formaldehyde/PBS and blocking with 10% goat serum/PBS, cells were incubated with the anti-Rac1-GTP antibody (catalog no. 26903, New East Biosciences, King of Prussia, PA) in 10% goat serum/PBS for 1 h. After washing with PBS (three quick washes followed by three additional 10-min washes), cells were incubated with Alexa Fluor 594–conjugated goat anti-mouse IgM secondary antibody (catalog no. A21426, Thermo Fisher Scientific, Waltham, MA), used at a 1:500 dilution in 10% goat serum/PBS. For co-staining of vimentin, an alternative robust method for studying Rac activation, illustrating the importance of developing alternative robust methods for studying Rac activation.

**Western blotting**

Western blotting of Rac1 in Rac1 KO and nontarget CRISPR cell lines is shown. (A) CRISPR was used to generate a Rac1 KO PC3 cell line. Representative Western blotting of Rac1 in Rac1 KO and nontarget CRISPR cell lines is shown. B, sequencing trace of Rac1 KO PC3 cells compared with the CRISPR control showing the 16-base pair deletion. The guide sequence–binding site is indicated by a horizontal black line, and the PAM site is shown by a dashed horizontal red line. The cut site is indicated by a dashed vertical black line. C, representative images of serum-starved control and Rac1 KO cell lines stained with the anti-Rac1-GTP antibody. Cells were fixed and stained for vimentin (green), for DNA (blue), and with the anti-Rac1-GTP antibody (red). Image capture and processing procedures were identical for both images. Scale bar, 10 μm. D, representative confocal microscopy image of the ectopic expression of EGFP-Rac1Q61L in PC3 cells. Cells were transfected with a plasmid expressing EGFP-Rac1Q61L (green) and after 24 h were fixed and stained for DNA (blue) and with the anti-Rac1-GTP antibody (red).

**RESULTS**

**Figure 2. The Rac1-GTP antibody does not detect Rac1 in prostate cancer cells.** A, CRISPR was used to generate a Rac1 KO PC3 cell line. Representative Western blotting of Rac1 in Rac1 KO and nontarget CRISPR cell lines is shown. B, sequencing trace of Rac1 KO PC3 cells compared with the CRISPR control showing the 16-base pair deletion. The guide sequence–binding site is indicated by a horizontal black line, and the PAM site is shown by a dashed horizontal red line. The cut site is indicated by a dashed vertical black line. C, representative images of serum-starved control and Rac1 KO cell lines stained with the anti-Rac1-GTP antibody. Cells were fixed and stained for vimentin (green), for DNA (blue), and with the anti-Rac1-GTP antibody (red). Image capture and processing procedures were identical for both images. Scale bar, 10 μm. D, representative confocal microscopy image of the ectopic expression of EGFP-Rac1Q61L in PC3 cells. Cells were transfected with a plasmid expressing EGFP-Rac1Q61L (green) and after 24 h were fixed and stained for DNA (blue) and with the anti-Rac1-GTP antibody (red).
EDITORS' PICK: Flaws in Rac1-GTP visualization

A

B

Rac1-GTP/Hoechst  Vimentin/Hoechst  Merge

NTC

Vimentin #1

Vimentin #2
Figure 3. Loss of anti-Rac1-GTP antibody staining upon vimentin RNAi depletion.

A stable depletion of vimentin from PC3 cells was generated with two different shRNA lentiviruses (1 and 2). Active Rac1 levels were determined using a PBD pulldown assay. Left, representative Western blotting. Right, densitometric analysis of Rac1-GTP levels normalized to total Rac1. B, representative confocal microscopy images of anti-Rac1-GTP antibody staining in vimentin-depleted PC3 cells. Cells were fixed and stained for vimentin (green), for DNA (blue), and with the anti-Rac1-GTP antibody (red). Scale bar, 10 μm. Similar results were observed in three independent experiments. **, p ≤ 0.01; ns, not significant.

Figure 4. Lack of staining with the anti-Rac1-GTP antibody in EGF-stimulated prostate cancer cells.

A, LNCaP C4-2 cells were stimulated with EGF (100 ng/ml, 1 min), and Rac1-GTP levels were determined using a PBD pulldown assay. Top, representative Western blotting. Bottom, densitometric analysis of Rac1-GTP levels normalized to total Rac1 showing mean ± S.E. (error bars). B, representative confocal microscopy images of control and EGF-stimulated C4-2 cells (100 ng/ml, 1 min) stained for DNA (blue), for phalloidin (green), and with the anti-Rac1-GTP antibody (red). Scale bar, 10 μm. Similar results were observed in three independent experiments. **, p ≤ 0.01; ns, not significant.

Figure 5. The anti-Rac1-GTP antibody does not stain activated Rac1 in peripheral actin-rich protrusions.

A, control A549 cells (scrambled sgRNA) or Rac1 KO A549 cells were treated with EGF (200 ng/ml, 5 min) and subjected to Alexa Fluor 488–conjugated phalloidin staining. Left, Rac1 levels determined by Western blotting. Right, representative images of phalloidin-stained cells. B, A549 cells co-expressing a Rac-FRET biosensor and mCherry-cortactin were stimulated with EGF (added at 1:15 min) and imaged every 15 s. The top panels show the mCherry-Cortactin, and the bottom panels show the Rac1 activation reported by the FRET biosensor. Color scale bar, dynamic range of the biosensor response (1, no significant response; 1.96, strongest response throughout the time-lapse sequence). Scale bar, 5 μm. C, representative confocal microscopy image of an actin-rich protrusion in parental EGF-stimulated A549 cells stained for DNA (blue), for phalloidin (green), for vimentin (gray), and with the anti-Rac1-GTP antibody (red).

EDITORS’ PICK: Flaws in Rac1-GTP visualization

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the presence of a recombinant Pak1-binding domain tagged to GST, and lysates were clarified and then incubated for 45 min in the presence of reduced GSH beads. Beads were washed twice with lysis buffer, and precipitated Rac1-GTP was assessed by Western blotting.

**Western blotting**

Western blotting was performed as described previously (68). The following antibodies were used at 1:1000 dilution: anti-Rac1 clone 23A8 (catalog no. 05-389, EMD Millipore, Burlington, MA) and anti-vimentin (catalog no. 3390, Cell

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**Figure 6. Vimentin expression and anti-Rac1-GTP staining in prostate cancer cell lines.** A, representative Western blotting of a PBD assay showing Rac1-GTP and total Rac1 levels in prostate cancer cell lines. B, representative Western blotting of vimentin levels in prostate cancer cell lines. C, representative confocal images of anti-Rac1-GTP (red), vimentin (green), and DNA (blue) staining in the indicated prostate cancer cell lines. Capture and processing procedures were identical for all images. Scale bar, 10 μm. Similar results were observed in three independent experiments.
Signaling Technologies, Danvers, MA). The following antibodies were used at 1:5000 dilution: anti-actin (catalog no. A544, Sigma–Aldrich) and anti-vinculin (catalog no. V9131, Sigma–Aldrich). Goat anti-mouse and goat anti-rabbit (catalog nos. 172-1017 and 170-6515, respectively, Bio-Rad) were used as secondary antibodies (1:3000 dilution). Bands were visualized using enhanced chemiluminescence with the LI-COR Odyssey Fc dual mode imaging system.

**FRET acquisition and processing**
Rac1 activation status was measured using a previously characterized dimerization-optimized reporter for activation...
(DORA) single-chain Rac1 biosensor (a generous gift from Yi Wu, UConn Health, Farmington, CT) (59). A549 cells were infected with an mCherry-Cortactin–encoding adenovirus (69) and a Rac1 biosensor–encoding lentivirus to visualize membrane ruffles and Rac1 activation status, respectively. Starved cells were treated with 50 ng/ml EGF to induce ruffle formation. We used 1:100 Oxyfluor reagent (Oxyrase Inc.) and 10 mM DL-lactate (Sigma–Aldrich) to reduce oxygen free radicals. Cells were imaged every 15 s with 2 × 2 binning and 16-bit depth using a dichroic splitter to facilitate simultaneous acquisition of Cerulean3 and Venus emissions. Raw images were processed using ImageJ. Images were corrected to account for background and bleaching, and a median filter with a 2-pixel radius was applied to reduce noise. The FRET ratio was calculated, and a custom LUT was applied to allow for the visualization of Rac1 activation.

Statistical analysis

Statistical significance was tested using paired Student’s t test or, alternatively, when comparing more than two conditions, a one-way analysis of variance with Dunnett’s multiple-comparison test was performed.

Data availability

All data are contained in the article.

Author contributions—M. J. B., M. C., G. K.-L., R. G.-M., P. A. J., and M. G. K. conceptualization; M. J. B., R. G.-M., P. A. J., and M. G. K. formal analysis; M. J. B., R. G.-M., and M. G. K. funding acquisition; M. J. B., M. C., G. K.-L., and M. G. K. investigation; M. J. B. and M. G. K. visualization; M. J. B., M. C., G. K.-L., R. G.-M., P. A. J., and M. G. K. methodology; M. J. B., G. K.-L., and R. G.-M. writing–original draft; M. J. B. and M. G. K. project administration; M. J. B., P. A. J., and M. G. K. writing–review and editing; R. G.-M. resources; M. G. K. supervision.

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Abbreviations—The abbreviations used are: PBD, Pak-binding domain; EGF, enhanced green fluorescent protein; KO, knockout; PDI, protein-disulphide isomerase; Rac1, Ras-related C3 botulinum toxin substrate 1; EGF, epidermal growth factor; sgRNA, single guide RNA.

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