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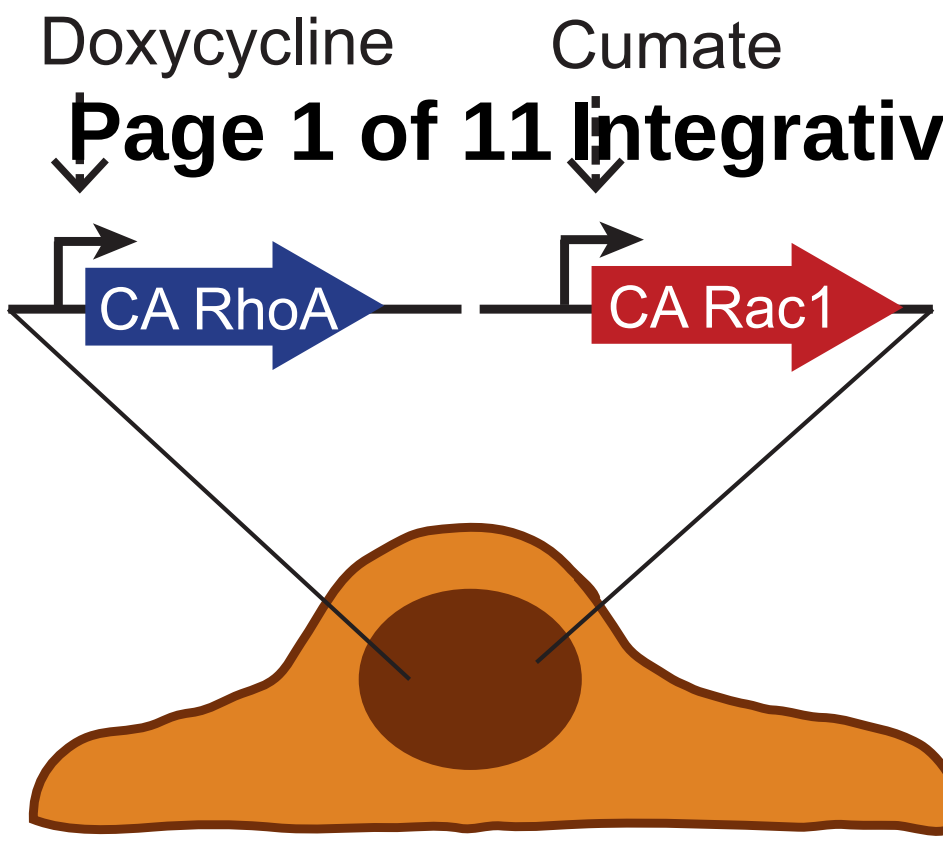


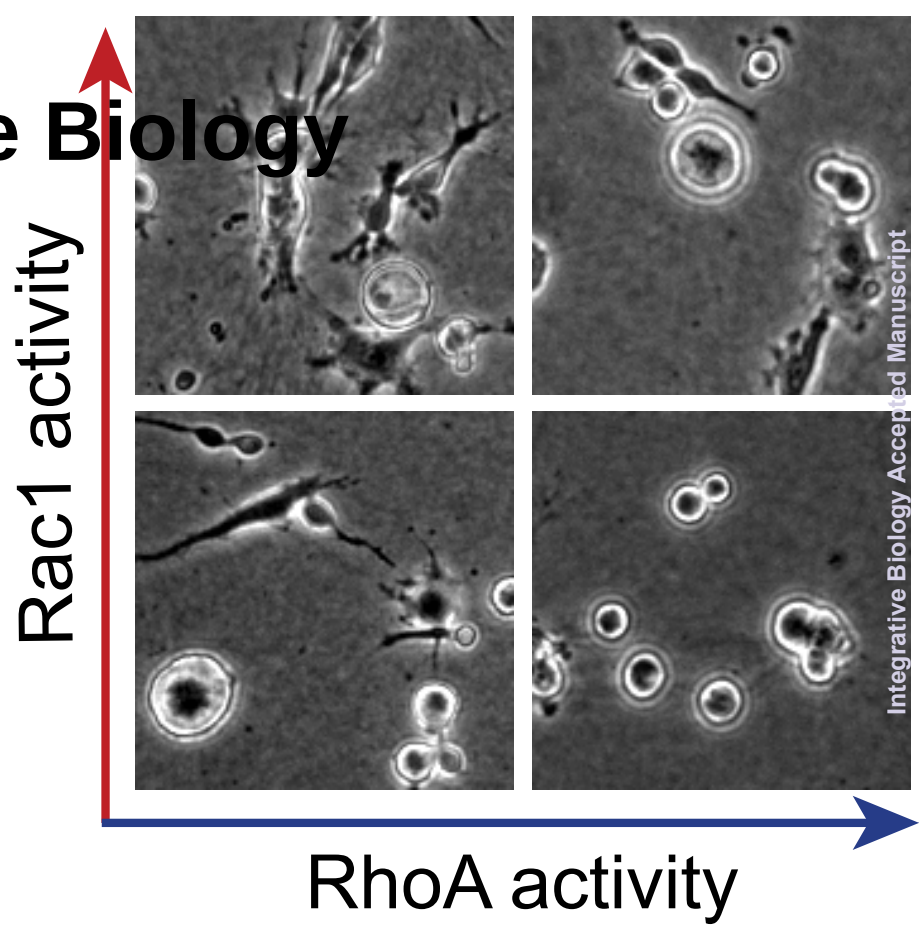

Integrative Biology

PAPER

# Simultaneous and independent tuning of RhoA and Rac1 activity with orthogonally inducible promoters

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The GTPases RhoA and Rac1 are key regulators of cell spreading, adhesion, and migration, and they exert distinct effects on the actin cytoskeleton. While RhoA classically stimulates stress fiber assembly and contraction, Rac1 promotes branched actin polymerization and membrane protrusion. These competing influences are reinforced by antagonistic crosstalk between RhoA and Rac1, which has complicated efforts to identify the specific mechanisms by which each GTPase regulates cell behavior. We therefore wondered whether RhoA and Rac1 are intrinsically coupled or whether they can be manipulated independently. To address this question, we placed constitutively active (CA) RhoA under a doxycycline-inducible promoter and CA Rac1 under an orthogonal cumate-inducible promoter, and we stably introduced both constructs into glioblastoma cells. We found that doxycycline addition increased RhoA activity without altering Rac1 and similarly cumate addition increased Rac1 activity without altering RhoA. Furthermore, co-expression of both mutants enabled high activation of RhoA and Rac1 simultaneously. When cells were cultured on collagen hydrogels, RhoA activation prevented cell spreading and motility, whereas Rac1 activation stimulated migration and dynamic cell protrusions. Interestingly, high activation of both GTPases induced a third phenotype, in which cells migrated at intermediate speeds similar to control cells but also aggregated into large, contractile clusters. In addition, we demonstrate dynamic and reversible switching between high RhoA and high Rac1 phenotypes. Overall, this approach represents a unique way to access different combinations of RhoA and Rac1 activity levels in a single cell and may serve as a valuable tool for multiplexed dissection and control of mechanobiological signals.

## Introduction

The Rho-family GTPases control rearrangement of the actin cytoskeleton and are known to regulate many fundamental cell behaviors, such as cell proliferation, apoptosis, migration, and differentiation.[1-3] Consequently, aberrant activation of Rho GTPases has been implicated in various human diseases, including cancer,[4-5] cardiovascular disease,[6] hypertension,[7] asthma,[8] and neurodegenerative diseases.[9] The most well studied Rho GTPases are RhoA and Rac1, which regulate distinct processes within the actin cytoskeleton necessary for cell shape maintenance, tension generation, and motility.[10-11] Rac1 stimulates polymerization of branched actin networks at the cell periphery directly through its effectors WAVE and Arp2/3, and also indirectly by inhibiting cofilin-mediated severing of actin filaments.[1] In contrast, RhoA induces the formation of actomyosin stress fibers and other contractile structures by activating mDia, which stimulates actin polymerization in the context of bundle formation,[12-13] and Rho-associated kinase (ROCK), which facilitates myosin activation.[1] In this way, RhoA is typically associated with cell contraction, while Rac1 is associated with membrane protrusion. Thus RhoA and Rac1 often have opposing effects on cell behavior, which has been observed in numerous studies on cell spreading,[14-22] adhesion,[23-24] and migration.[25-26] For example, when cells initially spread on a surface, integrin engagement leads to activation of Rac1 and suppression of RhoA,[27-29] which relaxes cellular tension and promotes membrane protrusion outwards. After nascent adhesions are formed, Rac1 activity then decreases and RhoA activity increases, which leads to maturation of adhesions into focal complexes.[23] Similarly, neurite outgrowth is first promoted by Rac1 and inhibited by RhoA[15, 30] but later requires a balance of both GTPases to stabilize point contacts.[31] In addition, cell-cell adhesions are initiated by local Rac1 activation and RhoA inhibition, but are later strengthened by RhoA-mediated contractility.[24] Consistent with these functional distinctions, RhoA and Rac1 activation also localize to distinct cellular compartments, suggesting that this competition is locally regulated and varies significantly within a single cell.[25, 32-34]

The opposing phenotypic effects of RhoA-mediated contraction and Rac1-mediated protrusion are reinforced by

molecular mechanisms through which the activity of one GTPase reduces the activity of the other.[2, 35-36] This antagonistic crosstalk predominantly occurs through two types of upstream regulatory proteins; guanine nucleotide exchange factors (GEFs) serve to activate GTPases by replacing GDP with GTP, and GTPase activating proteins (GAPs) serve to inactivate GTPases by promoting GTP hydrolysis to GDP. For example, several studies have shown that Rac1 signaling through its effector p21-activated kinase (PAK1) can lead to RhoA inhibition by deactivating RhoA GEFs, including PDZ-RhoGEF[37], P115-RhoGEF[37-39], NET1-RhoGEF[39], and GEF-H1[40-41]. Rac1 can also inhibit RhoA signaling by recruiting and activating p190RhoGAP.[42-44] In the opposite direction, RhoA signaling through ROCK has been shown to inhibit Rac1 by activating Rac1 GAPs, including ARHGAP22[26] and FilGAP[45]. In addition, myosin II activation can locally reduce Rac1 activity by preventing recruitment of the Rac1 GEFs DOCK180[46] and β-PIX[46-47]. There is also evidence that RhoA and Rac1 can antagonize one another through competitive binding to Rho-specific guanine nucleotide-dissociation inhibitors (GDIs), which sequester GTPases in the cytoplasm and protect them from degradation.[35, 48]

Mutual antagonism between RhoA and Rac1 has made it difficult to determine the specific mechanisms by which RhoA or Rac1 activation leads to changes in cell behavior, since a particular phenotype could be directly mediated by activation of a given GTPase or could instead result from deactivation of the opposite GTPase. For example, several studies have shown that increasing the activity of RhoA in glioblastoma cells reduces cell motility,[49-54] which could be due to RhoA-mediated increases in cellular contractility and stiffness.[49-50] However, an alternative explanation is that RhoA activation could indirectly reduce glioblastoma motility by decreasing Rac1 activity and preventing Rac1-dependent membrane protrusion.[49, 55-56] Given this confusion and the expectation that RhoA and Rac1 both play important roles in regulating cell behaviors, we wondered whether RhoA/Rac1 antagonism mandates that activation of RhoA necessarily leads to deactivation of Rac1, and vice versa, or whether their activity levels could somehow be manipulated independently. We reasoned that developing a strategy to separately and quantitatively tune RhoA and Rac1 activity could be useful both to investigate how RhoA and Rac1 signaling individually contribute to changes in cell behavior and also as a synthetic biological design tool to engineer more precise control over cell mechanics and motility in cell and tissue engineering applications. We therefore explored whether we could vary the activity levels of RhoA and Rac1 independently from each other by expressing constitutively active (CA) mutants in the same cell under orthogonal conditional promoters that are induced by either doxycycline (a tetracycline analog) or cumate. We found that this strategy provided orthogonal control over RhoA and Rac1 activation, and we used this capability to investigate how simultaneously varying the activity levels of both GTPases alters cell spreading and motility on collagen hydrogels.

## Experimental Methods

### Cell lines and reagents

Myc-tagged CA RhoA (Q63L) was subcloned into the lentiviral vector pSLIK[57] (Addgene plasmids 25755 and 25734) containing the TRE-tight doxycycline-inducible promoter, the reverse tetracycline transactivator (rtTA), and the YFP variant Venus. CA Rac1 was subcloned into the lentiviral SparQ expression vector (QM516B-1, System Biosciences, Mountain View, CA) containing the cumate-inducible promoter[58], puromycin resistance, and RFP. This promoter system requires simultaneous expression of the cumate repressor (CymR) from a separate lentiviral vector containing neomycin resistance (QM400PA/VA-1). Viral particles were packaged in 293T cells as previously described[59] and used to infect U373-MG human glioma cells at a multiplicity of infection of 1 IU/cell. Cells were first sorted on a DAKO-Cytomation MoFlo High Speed Sorter based on Venus fluorescence, and then selected for expression of the SparQ and CymR vectors with 1 μg/ml puromycin and 400 μg/ml G418 for two weeks. Control cell lines were created in the same manner with empty vectors. All U373-MG cell lines were maintained at 37°C in a 5% $CO_2$ humidified chamber and cultured in high glucose DMEM supplemented with 10% calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1x MEM non-essential amino acids, and 1 mM sodium pyruvate. To induce expression from the doxycycline- and cumate-inducible promoters, cells were incubated for at least three days in doxycycline (Fisher Bioreagents, Waltham, MA) and cumate (System Biosciences), respectively. As a technical note, we used U373-MG cells from the UC Berkeley Tissue Culture Facility, which sources its stocks from the American Type Culture Collection (ATCC). ATCC U373-MG cells have recently been recognized to be a subclone of the human glioblastoma line U251-MG, with the two lines having subsequently diverged to exhibit distinct karyotypes and chemosensitivities.[60]

### Western blots

Cells were lysed in RIPA buffer with protease and phosphatase inhibitors (EMD Millipore, Billerica, MA). Protein content was measured by BCA assay and used to normalize samples before loading. Lysates were boiled, run on a 4-12% Bis-Tris gel, and transferred onto a PVDF membrane. The following primary antibodies were used: anti-RhoA (#2117, Cell Signaling Technology, Danvers, MA), anti-Myc (#2276, Cell Signaling Technology), anti-phosphorylated (s199) PAK1 (#2605, Cell Signaling Technology), anti-GAPDH (#G8795, Sigma-Aldrich, St. Louis, MO), and anti-Rac1 (#ARC03, Cytoskeleton Inc., Denver, CO). HRP-conjugated secondary antibodies (Life Technologies, Carlsbad, CA) and ECL reagent (Thermo Fisher Scientific, Waltham, MA) were used for detection on X-ray film. Films were scanned and quantified with the built-in gel analyzer tool in ImageJ. Band intensities were normalized by GAPDH levels.



 

### RhoA and Rac1 activity assays

To measure levels of GTP-bound RhoA or Rac1, colorimetric G-LISA assays (Cytoskeleton Inc.) were performed according to the manufacturer's directions.

### Collagen gels

Hydrogels of 1 mg/ml collagen I were created by mixing media with PureCol (3 mg/ml, Advanced BioMatrix, San Diego, CA) and incubating at 37°C for 1 hour before plating cells on top at a density of 5000 cells/cm$^2$. Cells were also seeded at a higher density of 20,000 cells/cm$^2$. For experiments in which one inducer molecule (doxycycline or cumate) was washed out and replaced with the other, the cultures were washed 12 times with each wash consisting of a 30-minute incubation in fresh media containing the new inducer.

### Microscopy

Live cell imaging was performed within a 37°C and 5% CO2 atmosphere using TE2000-E2 and Ti-E microscopes equipped with motorized stages and NIS Elements software (Nikon Corporation, Tokyo, Japan). Phase contrast images were captured at 10x magnification in several fields of view every 15 minutes for 6 hours. Cell migration speed was quantified using the Manual Tracking plug-in for ImageJ by clicking on the center of the cell body in consecutive frames and by using the x-y position to calculate the average displacement of each cell over time. To quantify cell spreading, cells were classified as either spread or rounded based on their morphology in the phase-contrast images.

### Statistical analysis

To determine whether the average cell migration speed or the ratios of spread and rounded cells were significantly different across conditions at $p < 0.05$, one-way analysis of variance (ANOVA) tests were performed with a Tukey post-hoc analysis. Within each test, samples contained equal numbers and appeared normally distributed when graphed as box plots.

## Results

To investigate whether the activity levels of RhoA and Rac1 can be independently modulated, we chose to utilize a conditional gene expression strategy, given that several pairs of promoter systems have been shown to operate orthogonally and could potentially be combined in the same cell to control expression of RhoA and Rac1 mutants. Promoter pairs that have been previously used together in mammalian cells include those induced by tetracycline/streptogramin,[61] tetracycline/IPTG,[62] and macrolides/streptogramin/tetracycline,[63] which were all introduced into cells by transient transfection. Since viral transduction provides more stable transgene expression with higher efficiency and well-controlled copy numbers, we decided to use lentiviral vectors to express RhoA/Rac1 mutants from either a doxycycline-inducible promoter[57, 64] or a cumate-inducible promoter[58], which we have previously shown can operate orthogonally when transduced into different cell populations in a mixed culture system.[65] Specifically, we placed a CA RhoA mutant under the doxycycline-inducible promoter and a CA Rac1 mutant under the cumate-inducible promoter, and we used both lentiviruses at a low multiplicity of infection to transduce the human glioblastoma cell line U373-MG, which is a highly motile and mechanosensitive cell type.[50, 66] We refer to these dually-transduced cells as RhoRac cells. We also created a control cell line by transducing cells with empty versions of both constructs.

To first confirm that RhoRac cells can inducibly express CA RhoA with addition of doxycycline and express CA Rac1 with addition of cumate, we compared RhoA and Rac1 expression levels by Western blot (Fig. 1). The CA RhoA

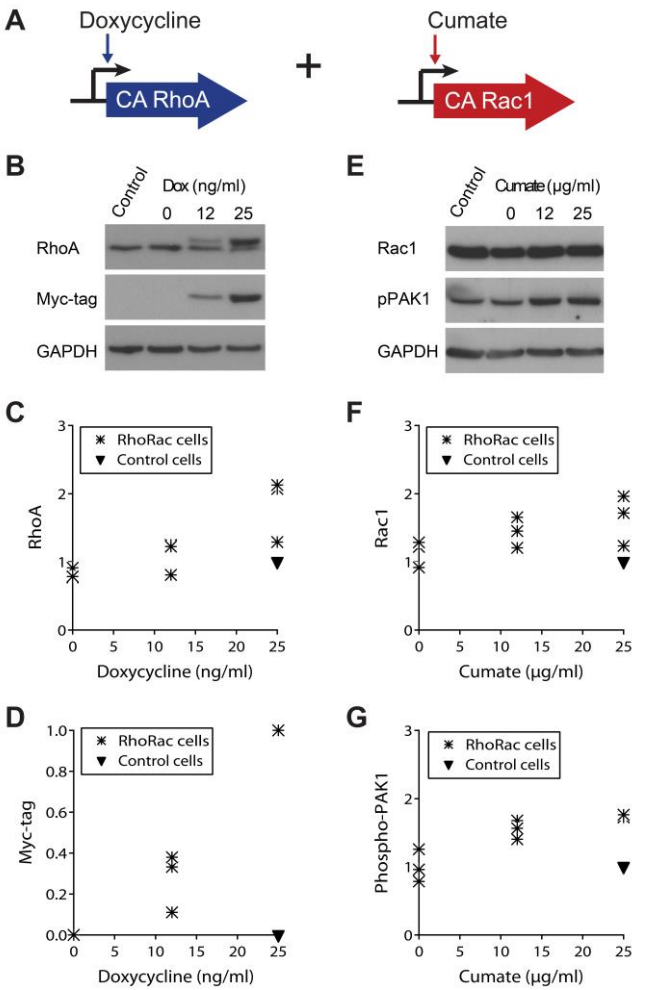


**Fig. 1** CA RhoA and CA Rac1 were expressed in the same cell from orthogonal inducible promoters. (A) Schematic showing that cells were transduced with both doxycycline-inducible CA RhoA and cumate-inducible CA Rac1, which we refer to as RhoRac cells. (B-D) RhoRac cells were cultured in 0, 12, or 25 ng/ml doxycycline, and expression of the Myc-tagged RhoA mutant was detected with antibodies against RhoA and Myc by western blot. Control cells containing empty vectors were cultured in 25 ng/ml doxycycline. (E-G) RhoRac cells were cultured in 0, 12, or 25 µg/ml cumate, and expression of the Rac1 mutant was detected with antibodies against Rac1 and phosphorylated (S199) PAK1. Control cells were cultured in 25 µg/ml cumate. Data points represent independent samples for each condition (n = 3 blots) and are displayed relative to control cells from the same blot, except in (D), where the data points are displayed relative to the 25 ng/ml doxycycline condition.

mutant contains a 1 kDa Myc-tag that enables us to distinguish it from endogenous protein, and we found that addition of 12 and 25 ng/ml doxycycline caused a graded increase in CA RhoA expression (Fig. 1B-D). For the CA Rac1 mutant, which was not analogously tagged, we measured both total Rac1 expression and the phosphorylation levels of PAK1, which undergoes auto-phosphorylation at serine 199 when activated by Rac1.[67] We found that addition of 12 and 25 µg/ml cumate caused only a slight increase in total Rac1 expression, but also led to increased PAK1 phosphorylation (Fig. 1E-G). This suggests that CA Rac1 expression and effector activation can be induced in a cumate-dependent manner. As expected, RhoRac cells cultured without doxycycline or cumate had similar RhoA, Rac1, and phosphorylated PAK1 expression levels as control cells.

We then investigated how simultaneously varying the expression of both CA RhoA and CA Rac1 leads to changes in RhoA and Rac1 activity levels. Since GTPases cycle between active and inactive states, we used an enzyme-linked immunosorbent assay (ELISA) to measure active (GTP-bound) RhoA or Rac1 protein. We found that culturing RhoRac cells in increasing concentrations of doxycycline above 6 ng/ml caused a graded increase in RhoA activity, whereas simultaneously varying the cumate concentration had little effect (Fig. 2A). Similarly, culturing cells in increasing concentrations of cumate up to 12 µg/ml caused a graded increase in Rac1 activity, independent of the doxycycline concentration (Fig. 2B). Since doxycycline addition (and thus CA RhoA expression) appeared to alter only RhoA activity and cumate addition (and thus CA Rac1 expression) appeared to alter only Rac1 activity, we surmised that antagonistic crosstalk between the two mutant GTPases does not occur and that their activation states can be decoupled from each other. Moreover, we found that simultaneous addition of doxycycline and cumate enabled cells to exhibit both high RhoA and high Rac1 activity simultaneously, thus overcoming mutual inhibition between these two signals (Fig. 2C). Importantly, treatment of control cells with doxycycline and cumate at the maximum working dosages did not intrinsically alter RhoA or Rac1 activity (Fig. 2D-E).

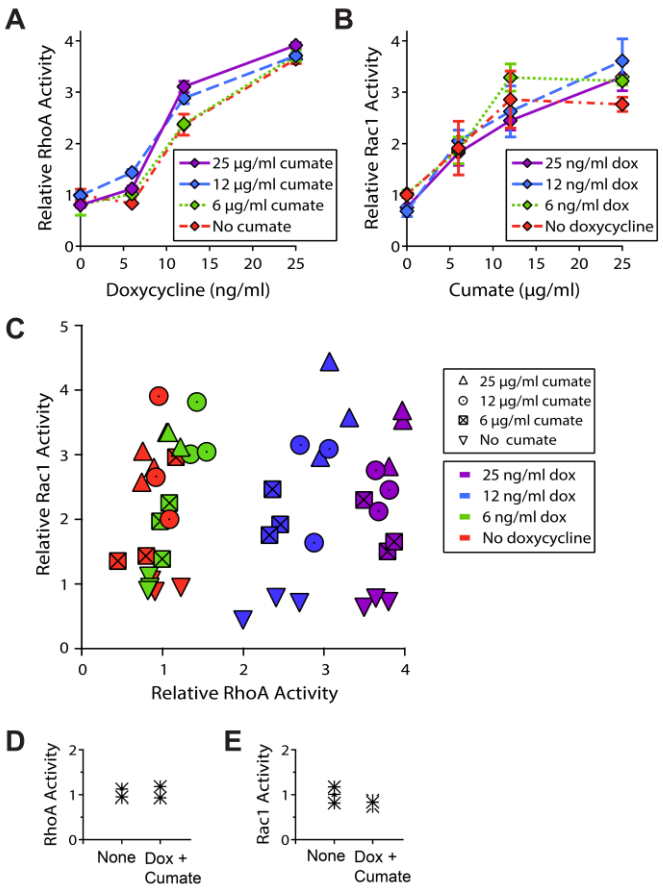


**Fig. 2** RhoA and Rac1 activity levels can be independently modulated. RhoRac cells were cultured in different concentrations of doxycycline and cumate, and the activity levels of RhoA and Rac1 were measured by ELISA. (A) RhoA activity is shown relative to cells cultured without doxycycline/cumate and depends primarily on the doxycycline concentration. Mean ± s.e. (n = 3 independent samples). (B) Rac1 activity is shown relative to cells cultured without doxycycline/cumate and depends primarily on the cumate concentration. Mean ± s.e. (n = 3 independent samples). (C) Replotting Rac1 activity versus RhoA activity for each combination of doxycycline/cumate shows that cells can have both high RhoA and high Rac1 activity. (D) RhoA activity of control cells cultured with or without doxycycline (25 ng/ml) and cumate (25 µg/ml). (E) Rac1 activity of control cells cultured with or without doxycycline (25 ng/ml) and cumate (25 µg/ml). For (C-E) data points represent independent samples for each condition (n = 3), displayed relative to cells cultured without doxycycline/cumate.

RhoA and Rac1 are known to have opposite effects on cell spreading, whereby Rac1 promotes cell spreading through actin polymerization, and RhoA restricts cell spreading through myosin-mediated contraction.[14-22, 27-29] In addition, we have previously shown that U373-MG cells are rendered more sensitive to high RhoA activity when cultured on soft matrices as opposed to stiff matrices, and that CA RhoA expression completely inhibits cell spreading and migration on soft collagen I gels.[50] In contrast, we have also shown that CA Rac1 expression enhances U373-MG cell spreading and motility within 3D collagen gels.[65] Therefore to confirm that CA RhoA and CA Rac1 expression in the RhoRac cell line leads to these expected changes in cell behavior, we cultured RhoRac cells on soft collagen gels in different concentrations of doxycycline and cumate. We first found that increasing concentrations of doxycycline induced cell rounding and significantly decreased cell motility (Fig. 3A-C, Supplementary Movies 1 and 2), which is likely due to overly high cell contractility from RhoA activation.[50] On the other hand, increasing concentrations of cumate caused a dramatic increase in cell spreading and motility (Fig. 3D-F), with cells extending dynamic processes and visibly exerting force on the collagen gel (Supplementary Movie 3). Interestingly, we found that expressing both CA RhoA and CA Rac1 simultaneously (by adding doxycycline and cumate) caused cells to adopt a mixed morphology in which they were partially spread and migrated at the same speed as cells cultured without doxycycline or cumate (Fig. 4A-C). However, at higher cell densities (seeded at 20,000 cells/cm$^2$ instead of 5000 cells/cm$^2$), simultaneous CA RhoA and CA Rac1 expression surprisingly caused cells to aggregate into large clusters that were highly contractile (Fig. 4D). In fact, the contractile tension was clearly visible in the

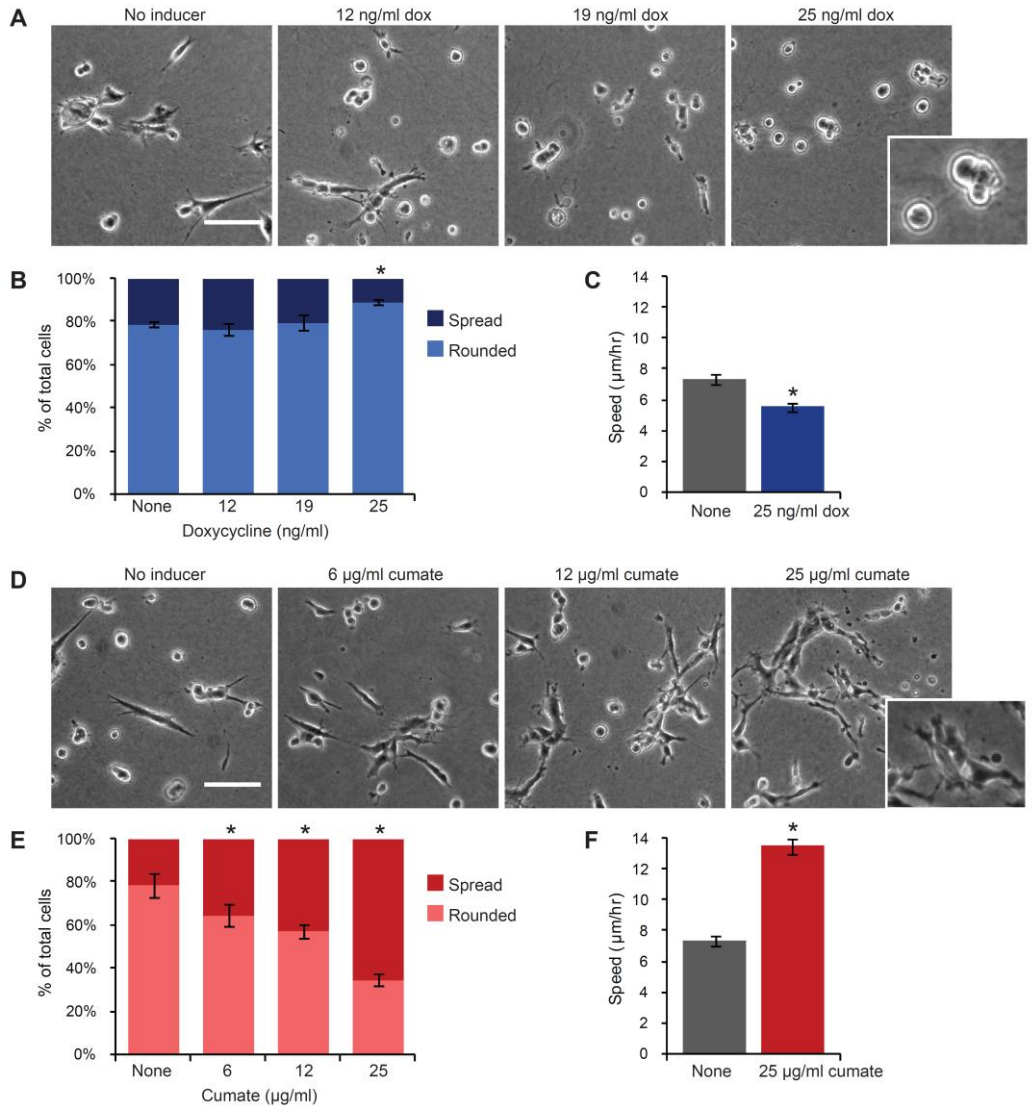


**Fig. 3** Expression of CA RhoA or CA Rac1 has opposite effects on cell spreading and migration. RhoRac cells were cultured on 1 mg/ml collagen gels in different concentrations of doxycycline or cumate. (A) Phase contract images show that cell spreading is inhibited by increasing CA RhoA expression. (B) Quantification of (A) in which cells were categorized as either spread or rounded. Data is shown as percentage of total cells counted ± s.e. (at least 300 cells counted per sample, n = 3 independent samples). (C) Migration speed of cells cultured without inducers or with 25 ng/ml doxycycline; mean ± s.e. (n = 142 cells per condition). (D) Phase contract images show that cell spreading is enhanced by CA Rac1 expression. (E) Quantification of (D) in which cells were categorized as either spread or rounded. Data is shown as percentage of total cells counted ± s.e. (at least 300 cells counted per sample, n = 3 independent samples). (F) Migration speed of cells cultured without inducers or with 25 μg/ml cumate; mean ± s.e. (n = 142 cells per condition). Scalebars = 100 μm; * indicates $p < 0.05$ compared to no inducer condition (ANOVA).

matrix as collagen fibers were bundled and pulled between nearby clusters (Supplementary Movie 4). Since this behavior was not observed in the other culture conditions of doxycycline alone, cumate alone, or neither inducer, it appears that the effects of high RhoA and high Rac1 do not simply cancel each other out, but instead may interact in complex and potentially synergistic ways to promote multicellular matrix engagement.

Since inducible promoters can be dynamically turned on and off by adding or removing the inducers, we then explored whether we could use this dual induction strategy to dynamically switch cells from a state of high RhoA activity to one of high Rac1 activity, and vice versa. We first cultured RhoRac cells on collagen gels in either doxycycline or cumate for several days and then replaced the media so that it contained only the opposite inducer, effectively turning one promoter off and the other promoter on. We found that both the high RhoA and high Rac1 phenotypes were reversible, and we could switch back and forth between them, with profound consequences for migration speed. Switching from the high RhoA to high Rac1 phenotype stimulated cell spreading within approximately 24 hours of replacing doxycycline with cumate, and cells began migrating at the same speed as cells cultured in cumate continuously (Fig. 5A and C, Supplementary Movie 5). Switching cells in the opposite direction from the high Rac1 to high RhoA phenotype caused cells to become rounded and produced comparable reductions in migration speed on a similar time scale (Fig. 5B and C, Supplementary Movie 6).

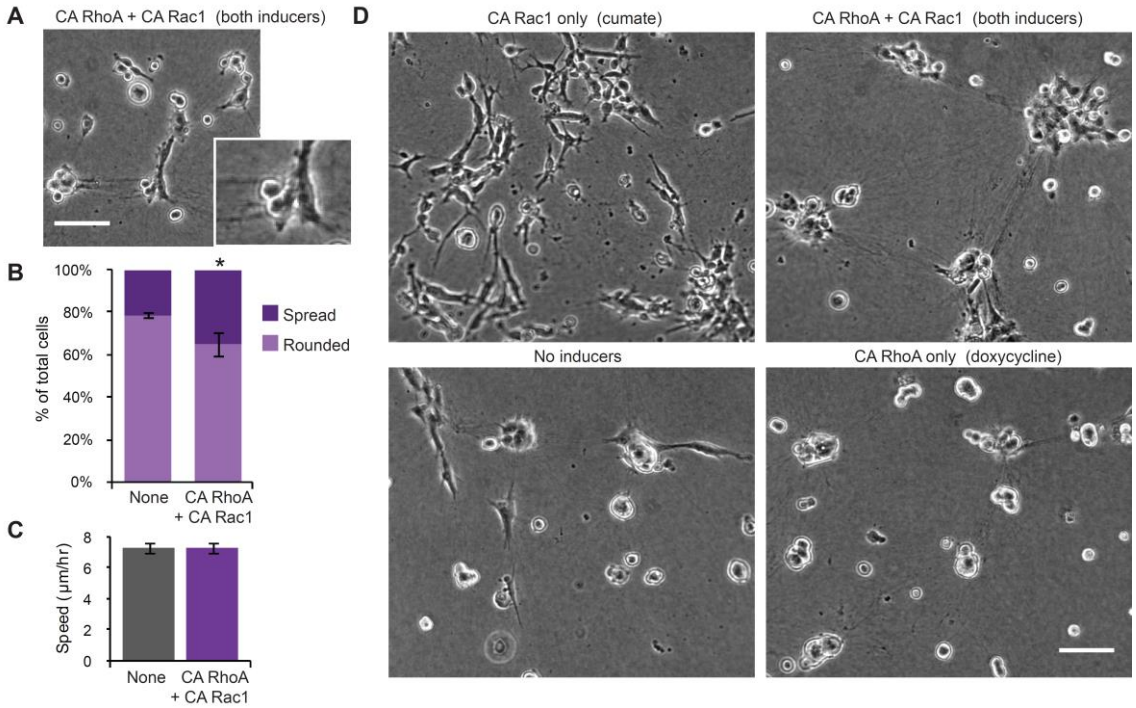


**Fig. 4** Simultaneous expression of CA RhoA and CA Rac1 leads to intermediate cell spreading and migration speeds, but induces cell clustering. RhoRac cells were cultured on 1 mg/ml collagen gels in 25 ng/ml doxycycline and 25 μg/ml cumate. (A) Phase contrast image showing that CA RhoA and CA Rac1 expression leads to partial cell spreading. (B) Quantification of (A) in which cells were categorized as either spread or rounded. Data is shown as percentage of total cells counted ± s.e. (at least 300 cells counted per sample, n = 3 independent samples). (C) Migration speed of cells cultured either without inducers or with both doxycycline and cumate. Mean ± s.e. (n = 142 cells per condition). (D) Phase contrast images showing that at higher cell densities, only cells expressing both CA RhoA and CA Rac1 aggregated together into large contractile clusters. Scalebars = 100 μm. For (B) and (C), data points for the no inducer condition were duplicated from Figure 3; * indicates $p < 0.05$ compared to the no inducer condition (ANOVA).

## Discussion

Many studies have shown that RhoA and Rac1 GTPase are mutually antagonistic and that altering the activity of one GTPase leads to a reciprocal change in the activity of the other through a variety of crosstalk mechanisms.[35] Since this antagonism has complicated efforts to elucidate the specific molecular mechanisms by which RhoA and Rac1 each regulate cell behavior, we have explored whether expressing CA mutants of both GTPases simultaneously from orthogonal inducible promoters would enable us to independently manipulate RhoA and Rac1 activity. The CA mutants RhoAQ63L and Rac1Q61L are deficient in GTP hydrolysis and are known to bind and sequester their respective GAPs, leading to sustained activation of endogenous protein.[68-71] We hypothesized that since antagonistic crosstalk between RhoA and Rac1 is thought to occur through regulation of GAPs and GEFs,[35] expression of both CA mutants would potentially supersede RhoA/Rac1 antagonism by sequestering RhoA and Rac1 GAPs and by rendering GEF-based inhibition ineffective since the GTPases would be less dependent on these effectors for activation. In contrast, if the CA mutants were incapable of overcoming mutual inhibition of RhoA and Rac1, the effects of CA RhoA and CA Rac1 expression would be expected to cancel each other, perhaps resulting in close-to-endogenous levels of RhoA and Rac1 activity. We found that expressing CA RhoA from a doxycycline-inducible promoter in U373-MG glioblastoma cells increased RhoA activity over three-fold without significantly altering Rac1 activity, while expressing CA Rac1 in the same cell from a cumate-inducible promoter similarly increased Rac1 activity without altering RhoA. Therefore, RhoA and Rac1 activity levels can be independently varied in these cells, which enabled us to explore the phenotypic consequences of activating RhoA and Rac1 in different pairwise combinations, including the previously inaccessible regime of high RhoA and high Rac1 activation. It would be valuable to revisit this approach in other cell types, particularly those in which RhoA and Rac1 drive even more profound phenotypic endpoints, e.g. in the context of development or stem cell differentiation, and identify "tipping points" in the RhoA/Rac1 phase space where specific phenotypes are induced.

Inducing expression of CA RhoA or CA Rac1 in U373-MG cells had opposite effects on cell spreading and migration. CA RhoA expression caused cell rounding and reduced cell motility on collagen gels, while CA Rac1 expression promoted cell spreading and increased cell migration speed. We found that simultaneously expressing both CA RhoA and CA Rac1 caused cells to migrate at the same speed as cells in which both constructs were kept off. While this might seemingly indicate that the downstream effects of high RhoA and high Rac1 activity may simply offset each other, we found that overexpression of both CA mutants produced a unique clustering behavior not seen at endogenous levels of RhoA and Rac1. The mechanistic origin of this phenotype remains to be elucidated, though it likely results from a combination of



 

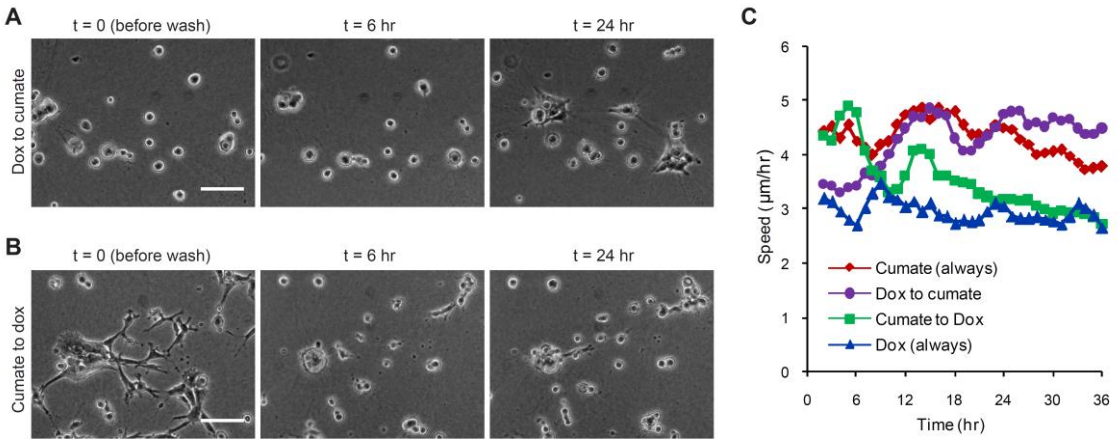


**Fig. 5** Cells can be dynamically switched from high RhoA activity to high Rac1 activity and vice versa. RhoRac cells were cultured on 1 mg/ml collagen gels in either 25 ng/ml doxycycline or 25 μg/ml cumate for 3 days. The media was then changed at t = 0 to either keep the same inducer or to switch to the opposite inducer. (A) Cells cultured in doxycycline and then switched to cumate began spreading within 24 hours. (B) Cells cultured in cumate and then switched to doxycycline became rounded within 6 hours. (C) The average cell migration speed for each condition was tracked over time and indicates when cells switched between high RhoA and high Rac1 phenotypes (n = 236 cells per condition). Scalebars = 100 μm.

altered cell-cell and cell-matrix adhesion as well as increased contractility. It will be interesting to investigate whether this RhoA/Rac1-dependent clustering behavior also occurs in other cell types that exhibit varying degrees of intrinsic contractility and motility.

While conditional gene expression is a powerful tool that enables orthogonal and dynamic control over the expression of multiple proteins simultaneously (perhaps up to 5 through the combined use of doxycycline-, cumate-, streptogramin-, macrolide-, and IPTG-inducible promoters), there are some important limitations. First, the switching kinetics are relatively slow, because turning protein activity 'on' requires transcription of new mutant protein and turning protein activity 'off' requires degradation of old mutant protein. Second, this strategy is limited to changes in total protein levels and cannot induce spatially localized changes in RhoA/Rac1 activity. On the other hand, this genetic strategy avoids limitations of existing methods that do offer more precise spatiotemporal control over protein activity. For example, while rapamycin-induced dimerization can be used to rapidly stimulate protein activity, the process is essentially irreversible since rapamycin dissociation is very slow.[72-73] In contrast, photo-activatable proteins such as PA-Rac1 are completely reversible, but they require constant illumination to maintain high activation.[74] Furthermore, most photo-activatable mutants are engineered to respond to blue light, and orthogonal red light-induced GTPase mutants have yet to be developed. Inducible gene expression may therefore be an optimal choice for sustained, independent manipulation of multiple proteins in the same cell; however, combining this genetic strategy with more acute, localized techniques could enable one to explore the effects of altering RhoA and Rac1 activity with better temporal and spatial resolution.

Germane to the above discussion, it is also important to note that different inducer-promoter systems may vary in their sensitivity. For example, the doxycycline-inducible and cumate-inducible promoter systems considered here exhibited different dose-dependent behavior with respect to inducer concentration (Fig. 2). While Rac1 activity increased linearly with cumate concentration up to 12 μg/ml before reaching a plateau, RhoA activity did not increase linearly with doxycycline concentration. Instead there appeared to be a threshold between 6 and 12 ng/ml doxycycline above which RhoA activity greatly increased. This may be due to the design of the doxycycline-inducible system used here, in which all components are introduced on a single lentiviral vector with the doxycycline-responsive element (rtTA) located downstream of the inducible promoter. While expression of rtTA is driven by a separate constitutive promoter, others have shown with this vector that the doxycycline-inducible promoter can also enhance rtTA expression, thereby creating a feed-forward loop that leads to even higher transgene expression.[57] In contrast, the cumate-inducible promoter system used here consists of two lentiviral vectors in which the cumate-responsive element (CymR) is not coupled to the cumate-inducible promoter since it is located on a separate vector. Other vector formats are also available for these promoter systems, and each have their advantages and disadvantages. For example, the cumate-inducible promoter system can be introduced into cells on a single, larger lentiviral vector (System Biosciences, QM800/QM812), which reduces the number of selective markers needed to select for transduced cells. However, larger vectors are more difficult to design and generally lead to lower viral titers. Additionally, instead of viral transduction, these vectors could be introduced into cells through transient plasmid transfection to achieve higher (yet less stable) transgene expression, although many cell types are difficult to transfect.

## Conclusions

In conclusion, we have transduced U373-MG cells with doxycycline-inducible CA RhoA and cumate-inducible CA Rac1 to demonstrate that mutual antagonism between RhoA and Rac1 activation may be overcome by simultaneously

expressing both CA mutants from orthogonal inducible promoters in the same cell. This dual-induction strategy also enables dynamic switching between high RhoA and high Rac1 phenotypes, and could in principle be used to explore phenotypic trajectories as cells are steered through the phase space of different RhoA and Rac1 activity levels. We expect that this new tool will be valuable for investigating the complex relationships between RhoA and Rac1 signaling in various cell types, and furthermore, the approach should be applicable to other pairs of interdependent signaling proteins.

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