Our recent work uncovered novel roles for activated Gαi signaling in the regulation of neutrophil polarity and adhesion. Gαi-GTP-mediated enhancement of neutrophil polarization was dependent on inhibition of cAMP/PKA signaling, whereas reversal of Gβγ-stimulated adhesion was cAMP/PKA independent. To uncover the mechanism for Gαi regulation of adhesion, we analyzed the effects of constitutively active Gαi1(Q204L) expression on adhesion driven by constitutively active Rap1a(G12V) or its downstream effector Radil in neutrophil-like HL-60 cells, or in HT-1080 fibrosarcoma cells. In HT-1080 cells, Rap1a(G12V) or Radil cause an increase in cell spreading and adhesion to fibronectin, which are both reversed by Gαi1(Q204L) but not WT Gαi1. In contrast, Gαi3(Q204L) did not reverse Rap1-GTP–interacting adaptor molecule (RIAM)–dependent increases in cell adhesion. This indicates that adhesion regulation by Gαi–GTP occurs downstream of Rap1a and Radil, but is upstream of components such as integrins and talin that are regulated by both Radil and RIAM. HL-60 neutrophil-like cells expressing Rap1a(G12V) or Radil have an elongated phenotype because of enhanced uropod adhesion as they attempt to migrate on fibronectin. This elongated phenotype driven by Rap1a(G12V) or Radil is reversed by Gαi1(Q204L), but not by WT Gαi1 expression, suggesting that Gαi–GTP also regulates adhesion in immune cells at the level of, or downstream of, Radil. These data identify a novel role of Gαi–GTP in regulation of cell adhesion and migration. Cell migration involves cycles of adhesion and de-adhesion, and we propose that the dynamic spatiotemporal balance between Gβγ-promoted adhesion and Gαi-GTP reversal of adhesion is important for this process.

Chemotaxis is the directional movement of cells in response to a concentration gradient of extracellular signals such as chemokines (1). Chemokines are small peptides secreted by various cell types and play a crucial role in immune surveillance, inflammation, cancer metastasis, and development. These molecules bind to their cognate receptor(s), which belong to the class A, rhodopsin-like family of G protein–coupled receptors (GPCR)2 (2). Downstream effects orchestrated by chemokines include directional migration, reactive oxygen species (ROS) production, proliferation, and survival. Migrating cells respond to a chemokine gradient by protrusion of the leading edge forming a pseudopod and retraction of the uropod tail (1). This morphology is driven by biochemical polarization within the cell that ultimately leads to F-actin polymerization in the leading edge and myosin II assembly in actomyosin filaments at the tail (3).

Chemokine receptors primarily couple to the Gi/o family of Gα proteins. Their activation triggers dissociation of the heterotrimeric G protein, Gα and Gβγ dimer, both of which can initiate downstream signal transduction pathways. The role of Gβγ in chemokine signaling has been well studied and characterized. Gβγ is thought to be the main transducer of chemokine signaling by recruiting and activating multiple effectors via protein–protein interactions. In contrast, Gαi has been proposed to not play a downstream role in regulation of neutrophil chemotaxis (4). Studies of Gαi in this process have been hampered by the fact that perturbations that inhibit Gαi signaling, such as pertussis toxin treatment or Gαi siRNA, also inactivate signaling of the obligate Gβγ dimer by disruption of the G protein cycle (5).

Our laboratory recently published evidence that activated Gαi has a direct role in migration of neutrophils (5). A key observation was that activated Gαi regulates adhesion through a cAMP-independent mechanism. Acute activation of Gβγ using small molecule 12155 resulted in increased migration, loss of polarity, and enhanced adhesion of neutrophils. Overexpression of the constitutively active mutant Gαi1(Q204L) reversed these phenotypes caused by global Gβγ activation suggesting a direct role for Gαi–GTP in chemokine signaling. Gβγ subunits, activated by chemokine receptors, promote cell adhesion through the small GTP-binding protein Rap1a. Gβγ activates Rap1a through a pathway involving direct activation of phospholipase β (PLCβ) by Gβγ resulting in increased intracellular calcium and diacylglycerol (DAG) leading to activation of the diacylglycerol-regulated Rap1 guanine nucleotide exchange factor CalDAG-GEF (Fig. 1A) (6). Rap1 is a small GTP-binding protein that belongs to the Ras family of small

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2 The abbreviations used are: GPCR, G protein–coupled receptors; PLCβ, phospholipase β; DAG, diacylglycerol; CalDAG-GEF, calcium and diacylglycerol-binding guanine nucleotide exchange factor; PH, pleckstrin homology; RIAM, Rap1-GTP–interacting adaptor molecule; ArhGAP29, Rho GTPase-activating protein 29; ROCK, Rho-associated protein kinase.
GTPases. It plays an important role in cell adhesion, spreading, and migration by mediating integrin inside-out signaling (6–9). Rap1 can regulate both integrin activity (affinity) or clustering (avidity) through various downstream effectors (10). Active GTP-bound Rap1 associates with RA domain-containing proteins such as regulator for cell adhesion and polarization enriched in lymphoid tissues (RAPL), Rap1-GTP–interacting adaptor molecule (RIAM), a protein containing Arf GAP, Rho GAP, ankyrin repeat, RA, and five PH domains (ARAP3), and Ras association and DIL domain (RADIL) (9, 11, 12). Furthermore, Rap1a and its downstream effector Radil were shown to be positive regulators of integrin activation, cell-matrix adhesion, and migration in neutrophils and HT-1080 fibrosarcoma cells (13).

Cell migration is a dynamic process requiring cycles of adhesion and de-adhesion to allow the cells to move forward on an extracellular matrix substrate. We hypothesize that activated Gαi is required to inhibit integrin-mediated adhesion to counteract integrin activation driven by free Gβγ. In this study, we determined where in the chemokine receptor–Gβγ–PLCβ–Rap1–Radil–integrin signaling pathway Gαi acts to ultimately understand the mechanism for Gαi action in cell adhesion. The data we present provides evidence for a novel role of Gαi-GTP in regulating integrin-dependent adhesion and cell migration in both cancer cells and neutrophils.

Results

Gαi-GTP inhibits cell spreading and adhesion

Neutrophils and neutrophil–like cell lines where Gβγ-dependent integrin regulation is critical for cell adhesion and migration are difficult to manipulate genetically. In HT-1080 fibrosarcoma cells an fMLP–Rap1a–Radil–β1 integrin pathway analogous to that operating in neutrophils regulates adhesion to fibronectin, providing a tractable model to dissect Gαi effects.

Figure 1. Gαi1-GTP inhibits Rap1a(G12V) and Radil-mediated cell spreading. A, diagram showing key components of the pathway involved in integrin activation and adhesion downstream of Gβγ. The role of Gαi-GTP in this pathway is not known. B, expression of constitutively active Gαi1(Q204L) in HT-1080 fibrosarcoma cells inhibited the Rap1a(G12V) and Radil-dependent increase in cell spreading. The degree of cell spreading was quantified by measuring the area of each cell using Image J software. At least 15 cells per condition per experiment were analyzed. Data are mean ± S.E. of pooled data from three independent experiments and analyzed by one-way ANOVA with Bonferroni posttest. ***, p < 0.001; **, p < 0.01.
*Gα∗ regulation of cell adhesion*

**A** Control | Rap1a(G12V) | Rap1a(G12V) + Gαi1(Q204L) | Rap1a(G12V) + Gαi1 | Radil + Gαi1(Q204L) | Radil + Gαi1

*Washed*

*Input*

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**B**

![Cell adhesion graph](image)

**C**

![Cell adhesion graph](image)

**D**

![Cell adhesion graph](image)

**E**

![Cell adhesion graph](image)

**F**

![Western blot](image)

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**Figure 2. Adhesive phenotype driven by Rap1a(G12V) or Radil is reversed by Gαi1(Q204L) expression.** A, representative images of HT-1080 cells stained with DAPI in response to overexpression as indicated. Top panels show attached cells remaining after washes and bottom panels show cells’ input before washing. Cells were visualized with 10× objective epifluorescence microscope. B, expression of constitutively active Gαi1(Q204L) in HT-1080 cells inhibited Rap1a(G12V) and/or Radil-dependent increase in cell adhesion on fibronectin-coated surface. Cell adhesion was quantified using a Celigo cell imaging cytometer and quantified as -fold over control. C, same as B except both Rap1a and Radil were expressed in the presence or absence of Gαi1(Q204L) or Gαi1(WT). D, same as B except the effects of transfection of Gαi1 or Gαi1(Q204L) on basal adhesion were measured. E, same as B except the effects of inhibition of PKA on Gαi1(Q204L) regulation of adhesion was tested. F, a representative Western blot to show the relative expression of the various constructs in HT-1080 cells. Data are mean ± S.E. of at least three independent experiments and analyzed by one-way ANOVA with Bonferroni posttest. ***, p < 0.001; **, p < 0.01; *, p < 0.05.

Previous studies have shown that overexpression of either active Rap1a-GTP or Radil promote cell spreading and adhesion of HT-1080 cells on extracellular matrix by enhancing β1 integrin activation (13). To determine where Gα∗ regulation of cell adhesion was tested.

First, we tested if expression of constitutively active Gαi1(Q204L) could reverse Rap1a or Radil-dependent cell spreading that results from increased cell adhesion to extracellular matrix (Fig. 1A). HT-1080 cells were transfected with either constitutively active Rap1a(G12V) or Radil in the presence or absence of Gαi1(Q204L). Cells were cotransfected with YFP to identify positively transfected cells. Consistent with published data, overexpression of Rap1a(G12V) or Radil enhances cell spreading on fibronectin-coated slides as measured by cell area. Co-expression of Gαi1(Q204L) reversed spreading of HT-1080 cells driven by expression of either Rap1a(G12V) or Radil (Fig. 1B).

To directly measure adhesion to extracellular matrix, transfected cells were plated on fibronectin and washed repeatedly to remove weakly attached cells before quantifying residual adherent cells. Rap1a(G12V) or Radil expression enhanced cell adhesion relative to YFP transfected control (Fig. 2, A–C). Co-expression of active Gαi1(Q204L) blocked Rap1a(G12V) or Radil-dependent adhesion (Fig. 2, A–C). Concurrent expression of both Rap1a(G12V) and Radil enhanced cell adhesion to the same extent as their individual expression (Fig. 2C). Even so, coexpression of Gαi1(Q204L) reversed adhesion of cells...
expressing both Rap1a(G12V) and Radil (Fig. 2C). Representative Western blots show that levels of expression of Go1 (Fig. 2F) are considerably higher than that of endogenous Go1, but this is likely required to overcome the overexpression of upstream Rap1a or Radil inherent in the experimental design. Expression of wildtype Go1 had no effect in this assay, indicating that the GTP-bound form of Go1 is responsible for these effects (Fig. 2, A–C). Transfection of wildtype Go1 or Go1(Q204L) had no effect on cell adhesion in the absence of expression of upstream components indicating that Go1 does not have general effects on adhesion (Fig. 2D). Consistent with our previously published results in neutrophils, Go1-dependent regulation of adhesion is independent of cAMP, because Go1-dependent and Rap1a-dependent regulation of adhesion is not affected by inhibition of PKA with PKI (Fig. 2E). Overall, our results suggest that Go1-GTP regulates adhesion downstream of the receptor G protein cycle, activation of phospholipase Cβ, and Rap1a, at the level of, or downstream of Radil in a cAMP-independent manner.

**Go1-GTP does not regulate RIAM-dependent adhesion and is Rap1GAP independent**

RIAM is a downstream effector of Rap1 that can induce cell spreading and adhesion by interacting with talin to activate β1 and β2 integrins (14, 15). To determine whether inhibition by Go1-GTP is specific for the Radil signaling pathway, we expressed RIAM in HT-1080 cells and measured adhesion. RIAM expression stimulated adhesion of HT-1080 cells on fibronectin (Fig. 3A). However, coexpression of Go1(Q204L) did not inhibit RIAM-dependent adhesion. This indicates that regulation by Go1-GTP is upstream of components such as integrin and talin, which are downstream of both Radil and RIAM.

Rap1GAPII is a Rap1 GTPase-activating protein containing a GPR motif that mediates direct interaction with Go1-GDP. This association stimulates the membrane localization and activation of Rap1GAPII, which could decrease the amount of GTP-bound Rap1 to produce the observed effects (16). Although our results suggest the involvement of Go1-GTP not Go1-GDP, it is important to address whether activation of a RapGAP by Go1 could explain our results. To test this we transfected HT-1080 cells with Rap1a(F64A), which is a constitutively active, and a GAP-insensitive mutant of Rap1a (17). Rap1a(F64A) stimulates adhesion of HT-1080 cells on fibronectin similar to Rap1a(G12V) (Fig. 3B). Coexpression of Go1(Q204L) inhibited the adhesion of Rap1a(F64A) expressing cells. This indicates that the mechanism for Go1-GTP-dependent inhibition of adhesion is independent of interaction with Rap1GAP.

**Effects of Go1 on Rap-dependent Radil plasma membrane recruitment**

Rap1-GTP regulates the activity of Radil at least in part by promoting plasma membrane association of Radil. We tested the possibility that Go1 may inhibit this translocation by examining constitutively active Rap1(Q63E)-dependent Venus-Radil translocation in HT-1080 cells. When Venus-Radil and HA–Rap1a(Q63E) were cotransfected there was significant colocalization of Venus and HA staining at the periphery of the cell (Fig. 4). This colocalization was unaffected by coexpression of Go1(Q204L). This indicates that Go1(Q204L) does not affect the interactions between Rap1a and Radil nor its ability to localize Radil to the plasma membrane.

We tested for direct interactions between Go1(Q204L) and Radil using both communoprecipitation and chemical cross-linking approaches when Radil and Go1(Q204L) were coexpressed in Cos7 cells and were unable to detect any interaction suggesting that activated Go1 does not directly interact with Radil (data not shown).

**Inhibition of Rho signaling attenuates Go1-GTP-negative regulation**

The mechanism by which Radil regulates adhesion is unknown because direct interactions with talin or integrin subunits have not been demonstrated. Mass spectrometry proteomics analysis revealed that Radil interacts with Rho GTPase-activating protein 29 (ArhGAP29), which is a GAP for RhoA (18). Rap1-GTP promotes the plasma membrane localization of a Radil and ArhGAP29 complex to spatially inhibit Rho signaling (19). Rho-GTP regulates cytoskeletal dynamics via its downstream effector, Rho-associated protein kinase (ROCK), which in turn activates myosin light chain 2 (MLC2) and inactivates myosin phosphatase via phosphorylation, thereby pro-
moting actomyosin contractile force generation (20, 21). Consequently, inhibition of Rho activity by Rap1 and Radil reduces actomyosin cytoskeletal contraction, which can impinge on cell spreading and adhesion (22).

We hypothesized that if the effect of Gαi1-GTP is dependent on Rho signaling, inhibition of Rho signaling should attenuate the inhibitory effects of Gαi1(Q204L) expression. To address this, we treated HT-1080 cells with the ROCK inhibitor Y-27632 (10 μM) to inhibit Rho activity in the adhesion assay (Fig. 5). Indeed, treatment with Y-27632 reversed the inhibitory effect of Gαi1(Q204L) on Rap1a(G12V) and Radil-mediated adhesion on fibronectin. In contrast, inhibition of Rho signaling by Y-27632 had no significant effect on R1AM-dependent adhesion, with or without Gαi1(Q204L) expression. Taken together, we conclude that Rho signaling is required for Gαi1-GTP to inhibit Rap1a and Radil-mediated adhesion.

**Gαi1-GTP regulates uropod retraction in differentiated HL-60 cells during migration**

HL-60 human promyelocytic leukemia cells can be differentiated into neutrophil-like cells and are a useful model system to study cell migration. It has been reported that Rap1 and Radil regulate tail retraction of migrating neutrophils (3). To determine whether there is a role for active Gαi in regulating tail retraction, we transfected differentiated HL-60 cells with plasmids encoding wildtype Gαi1 or constitutively active Gαi1(Q204L). Cells were cotransfected with YFP and individual fluorescent cells were identified and analyzed. Overexpression of Rap1a(G12V) or

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**Figure 4. Gαi1(Q204L) does not affect Rap1a-dependent recruitment of Radil to the plasma membrane.** A, HT-1080 cells stably expressing Venus-Radil were transiently transfected with HA-Rap1a(G12V) with or without Gαi1(Q204L). Cells were fixed, stained with α-HA monoclonal antibody, followed by secondary detection with goat anti-mouse IgG (H+L) secondary antibody, Alexa Fluor 594. Samples were imaged for Venus (green) and HA (red) by confocal fluorescence microscopy. B, relative membrane fluorescence was quantified using ImageJ software by measuring the membrane over the cytosolic fluorescence intensity. Three cells were analyzed for each condition for each experiment. Data are mean ± S.E. of pooled data from three independent experiments and analyzed by one-way ANOVA with Bonferroni posttest. *, p < 0.05.
Radil caused the cells to adopt an elongated phenotype because of enhanced uropod adhesion as they attempt to migrate on fibronectin (Fig. 6). This elongated phenotype by Rap1a(G12V) and Radil expression is reversed by Ga\textsubscript{i1}(Q204L), but not by wildtype Ga\textsubscript{i1} expression. Thus, Ga\textsubscript{i1}-GTP is required for uropod retraction which is required proper migration of neutrophils.

**Ga\textsubscript{i1}-GTP promotes migration of HT-1080 cells downstream of Rap and Radil activation**

Because of the low transfection efficiency of HL-60 neutrophil-like cells it was difficult to quantify cell migration in cells doubly transfected with Radil or Rap1 and Ga\textsubscript{i1}. As a surrogate we measured random migration of HT-1080 cells by live cell video microscopy. Cells were transfected with Radil or Rap1 with or without wildtype Ga\textsubscript{i1} or Ga\textsubscript{i1}(Q204L). YFP was also cotransfected and migration of individual YFP fluorescent cells was tracked over 6 h (see Movies S1–S4 for representative cell migration). Rap1a(G12V) (Fig. 7, A and B) or Radil (Fig. 7, C and D) significantly inhibited velocity and distance traveled of the cells presumably because they stimulate strong adhesion. Full migratory capacity was rescued in the presence of Ga\textsubscript{i1}(Q204L) but wildtype Ga\textsubscript{i1} was without effect. Thus under conditions of integrin activation because of Rap1a and Radil activation, Ga\textsubscript{i1}-GTP is able to maintain the migratory capacity of these cells likely through promoting de-adhesion.

**Discussion**

In our previous work we used a chemical biology approach to demonstrate that activation of G\textsubscript{\beta}\textgamma without coincident activation of Ga\textsubscript{i} leads to a strongly adhesive cellular phenotype that prevents cell migration. Migration was partially rescued by activated Ga\textsubscript{i}(Q204L) supporting a model where Ga\textsubscript{i} activation and signaling is required for neutrophil migration, in part because of its ability to inhibit strong adhesion driven by free G\beta\gamma. Cell migration requires cycles of forward protrusion and adhesion of the leading edge to the substrate followed by de-adhesion of the trailing edge allowing the cell to move forward. We propose that during cell GPCR-directed cell migration G\beta\gamma drives adhesion of the leading edge and Ga\textsubscript{i} promotes de-adhesion of the trailing edge. During chemokine-GPCR activation, G proteins are continuously cycling through activated-deactivated states; as GTP is hydrolyzed by the Ga subunit, the G\beta\gamma heterotrimer is reformed, and followed by reactivation. This dynamically cycling G protein system may be well suited to the dynamic cycles of processes that occur as cells move. Our results do not directly address how these
functions may be spatiotemporally regulated but define a previously unappreciated role for G\(_i\)-GTP signaling in adhesion and migration.

Chemotactic receptors stimulate G\(_i\) proteins leading to G\(_i\)-mediated stimulation of PLC\(_{\beta}\). PLC\(_{\beta}\) activation generates DAG which in turn activate CalDAG-GEF, a Rap1 exchange factor. Activated Rap1a and G\(_i\)-GTP bind to the Rap1 effector Radil to promote cell adhesion because of increased integrin activation (3, 13). Our results demonstrate a direct and novel role of G\(_i\)-GTP in signaling to regulate cell adhesion and migration that is downstream of the GPCR, the G protein cycle, and any component of the signaling pathway upstream of Rap1.

Radil directly interacts with active Rap1a and G\(_i\)-GTP downstream of the fMLP receptor in HT-1080 cells and neutrophils to regulate integrin activation (13). We could not detect a direct interaction between G\(_i\) and Radil in cross-linking coimmunoprecipitation assays (data not shown), suggesting that G\(_i\)-GTP does not directly interact with Radil, and is likely acting downstream of Radil. How Radil controls integrin activation is unclear because it does not interact directly with talin or integrin subunits. Radil contains a PDZ domain that has been shown to interact with ArhGAP29 (18). Rap1 effectors such as Rasip1 and its homolog Radil have been shown to stimulate cell spreading by activation of ArhGAP29, which negatively regulates Rho signaling (19, 22). Here we show that inhibition of Rho signaling by the ROCK inhibitor Y-27632 attenuated the inhibitory action of G\(_i\)(Q204L) in Rap1a(G12V) and Radil-dependent adhesion. Conversely, inhibiting Rho signaling had no effect on RIAM-dependent adhesion in the presence or absence of G\(_i\)(Q204L). This suggests that G\(_i\)-GTP

Figure 7. G\(_i\)(Q204L) expression, but not wildtype G\(_i\), rescues migration of Rap1a(G12V) and Radil expressing HT-1080 cells. A, spider plots of video-tracked HT-1080 cell transfected with Rap1a(G12V) and the indicated constructs and YFP. YFP-positive cells were tracked and this is a representative experiment. (See Movies S1–S4 for representative videos.) B, data obtained from at least 100 cells were pooled from three independent experiments as in A and are represented as a box and whisker plot. The box shows the interquartile range that contains values between 25th and 75th percentile. The line inside the box shows the median and the two whiskers go down to the smallest value and up to the largest value observed. Data were analyzed by one-way ANOVA with Bonferroni posttest. ****, p < 0.0001; ns, not significant. C, same as A except Radil was used to inhibit migration. D, same as B except Radil was used.
requires active Rho downstream of the Rap1-Radil pathway to regulate adhesion.

RiAM is a well-studied downstream effector of Rap1 known to promote cell adhesion and spreading by facilitating talin interaction with β1 and β2 integrins, a final essential step for integrin activation (10, 14, 15). Activated RiAM coimmunoprecipitates with talin and forms a tertiary complex with talin and activated integrins. In this study, we show that Goαi(Q204L) does not inhibit RiAM-dependent cell adhesion, suggesting that Goαii-GTP regulates adhesion upstream of talin and integrin.

Rap1α and Radil have been reported to play a critical role in neutrophil migration by regulating tail retraction and back detachment (3). Neutrophils overexpressing Rap1α(G12V) and/or Radil adopt an elongated phenotype as they attempt to migrate. This dramatic phenotype is a consequence of activated integrins. In this study, we show that Goαi(Q204L) but not wildtype Goαi is sufficient to restore the circularity of neutrophils from an elongated phenotype demonstrate a role for active Goαi in regulation of tail retraction of neutrophils, a critical process involved in neutrophil migration.

Because Goαi-dependent regulation of adhesion appears to be independent of cAMP modulation it raises the exciting possibility that Goαi is regulating adhesion through a previously unappreciated target. Rap1GAPII is a GTPase-activating protein that negatively regulates Rap1 signaling and is known to directly bind to Goαi via its GPR motif (16), however Goαi(Q204L) rescues adhesion driven by expression of a RapGAP-resistant Rap1α(F64A) mutant indicating the observed effects are independent of RapGAP. Thus, although we have been able to identify a site of action for Goαi in the adhesion pathway, we have not identified the direct target for the Goαi-dependent effects.

Overall, we have shown a novel role for Goαii-GTP in regulating adhesion and migration of neutrophils and a cancer cell line. This inhibitory role is specific for the Rap1α-Radil pathway. Identification of the direct target of Goαii-GTP that regulates this pathway will require further investigation.

**Experimental procedures**

**DNA constructs and reagents**

Rap1α (RAP1A00000), Rap1α(G12V) HA3-tagged (RAP1A000C0), and G protein αi1 (Q204L)(GNA1000C0) were from cDNA Resource Center. FLAG-human Radil and Venus-human Radil were kindly provided by Dr. Carol Parent. His-RiAM (32803) was from Addgene. The Rap1α(F64A) mutation was introduced into Rap1α via the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Y-27632 was from (Sigma).

**Cell culture and transfection**

HL-60 cells (ATCC) were grown in RPMI 1640 medium with 10% FBS and 1% penicillin/streptomycin and differentiated at 0.2 × 10⁶ cells/ml for 4–6 days with 1.2% DMSO. HT-1080 cells (ATCC) were grown in DMEM with 5% FBS and 1% penicillin/streptomycin. HT-1080 cells were transfected using Lipofectamine 2000 (Invitrogen) at a 1:2 ratio (cDNA:Lipofectamine 2000) at 70% cell confluency.

**Nucleofection of HL-60 cells**

Differentiated HL-60 cells (5 × 10⁶ cells) were harvested by centrifugation at 80 × g for 10 min. The cells were resuspended in 100 µl of Ingenio transfection solution (Mirus Bio LLC) and 2 µg of DNA were added. The mixture was transferred to a 0.2-cm nucleofection cuvette, and the cells were nucleofected using Amaxa Nucleofector II (Lonza) on the recommended setting (T-019). 500 µl of recovery media (RPMI supplemented with 10% FBS and 1.2% DMSO) were immediately added and incubated cells were incubated in an microfuge tube at 37 °C for 30 min. The cells were then transferred to a dish containing 1.5 ml recovery media in a 35-mm dish and incubated overnight at 37 °C 5% CO₂.

**HL-60 cell migration imaging and circularity**

35-mm glass bottom dishes were coated with 5 µg of fibronectin (Sigma) overnight at 4 °C. Differentiated HL-60 cells nucleofected with different constructs along with YFP were allowed to adhere to the dish for 30 min and nonadherent cells were washed off before microscopic analysis in Hanks’ balanced salt solution (HBSS) with 10 mM HEPES (pH 7.4). Cells coexpressing YFP were imaged at 60× magnification by bright field differential interference contrast (DIC) microscopy. Circularity of YFP-positive cells was calculated in ImageJ software using the formula for circularity where a value of 1.0 represents a perfect circle and 0.0 approaches an elongated polygon (4π × area/ perimeter²).

**Cell spreading assay**

HT-1080 cells were plated in 35 mm dishes and transfected with different constructs along with YFP. 48 h after transfection, cells coexpressing YFP and the overexpressed protein of interest were identified and captured with an epifluorescence microscope (Nikon) at 40× magnification. Data were pooled and the area of each cell was quantified using ImageJ software.

**Adhesion assays**

96-well flat bottom plates were coated with 5 µg/ml of fibronectin (Sigma) overnight at 4 °C. Wells were washed with PBS and blocked with 2% BSA in PBS for 1 h at room temperature. 24 h after transfection, HT-1080 cells were dissociated with 0.25% trypsin, washed, and resuspended in serum-free DMEM with 0.1% BSA and 20 mM HEPES. In some cases, cells were treated with the ROCK inhibitor Y-27632 for 30 min at room temperature in a rotator. 2.5 × 10⁵ HT-1080 cells per well were plated in triplicate and allowed to adhere for 8 min at 37 °C then washed with warm 0.2% BSA to remove nonadhered cells. Unwashed total cell input control per condition was allowed to adhere for 30 min at 37 °C. Cells were fixed with 4% paraformaldehyde for 15 min and stained with 1 µg/ml DAPI for 10 min at room temperature. Cell adhesion was quantified using Ceglo Image Cytometer or CellInsight CX5 High-Content Screening (HCS) Platform to count the number of GFP-expressing DAPI-labeled cells adhered to the plate.

**HT-1080 cell migration**

Microslide 8-well glass bottoms were coated with 5 µg/ml of fibronectin overnight at 4 °C. HT-1080 cells were plated on
Go4, regulation of cell adhesion

fibronectin and transfected with different constructs, along with YFP, for 48 h. Cells were imaged at 10-min intervals for 6 h. Tracks of individual YFP-positive cells from three separate experiments were analyzed with the Chemotaxis and Migration Tool from Ibidi using ImageJ software to determine velocity and distance traveled.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and Student’s t test using GraphPad Prism software’s Bonferroni functionality. Statistical significance is indicated as *, p < 0.05; **, p < 0.01; ***p, < 0.001; ****p, < 0.0001; n.s., not significant.

Author contributions—J. Y. T. and A. V. S. conceptualization; J. Y. T. and A. V. S. data curation; J. Y. T. and A. V. S. formal analysis; A. V. S. supervision; J. Y. T. and A. V. S. writing-original draft; A. V. S. project administration; J. Y. T. and A. V. S. writing-review and editing.

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