A $G_\alpha$-Stimulated RapGEF Is a Receptor-Proximal Regulator of Dictyostelium Chemotaxis

Highlights

- The Rap1GEF GflB is a receptor-proximal regulator of chemotaxis in Dictyostelium
- GflB localizes to the leading edge and is stimulated directly by $G_\alpha$-GTP binding
- GflB directly links $G_\alpha$ activation to monomeric G-protein signaling
- GflB provides a mechanism to discriminate between distinct chemoattractants

Authors

Youtao Liu, Jesus Lacal, Douwe M. Veltman, Fabrizia Fusetti, Peter J.M. van Haastert, Richard A. Firtel, Arjan Kortholt

Correspondence

a.kortholt@rug.nl

In Brief

G-protein-coupled receptors (GPCRs) mediate chemotaxis via activation of linked G proteins. Although many signaling pathways downstream of $G_\beta\gamma$ are known, less is known about $G_\alpha$. Liu, Lacal et al. identify a Dictyostelium Rap1GEF that binds $G_\alpha$ and thereby links the cAMP GPCR to Rap1, regulating cytoskeletal dynamics and chemotaxis.
A Gα-Stimulated RapGEF Is a Receptor-Proximal Regulator of Dictyostelium Chemotaxis

Youtao Liu,1,5 Jesus Lacal,2,5 Douwe M. Veltman,3 Fabrizia Fusetti,4 Peter J.M. van Haastert,1 Richard A. Firtel,2,6 and Arjan Kortholt1,6,*

1Department of Cell Biochemistry, University of Groningen, Nijenborgh 7, 9747 AG Groningen, the Netherlands
2Section of Cell and Developmental Biology, Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92093, USA
3MRC Laboratory of Molecular Biology, Cambridge CB2 0QH, UK
4Department of Biochemistry, Netherlands Proteomics Centre, Groningen Biological Sciences and Biotechnology Institute, University of Groningen, 9747 AG Groningen, the Netherlands
5Co-first author
6Co-senior author
*Correspondence: a.kortholt@rug.nl

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SUMMARY

Chemotaxis, or directional movement toward extracellular chemical gradients, is an important property of cells that is mediated through G-protein-coupled receptors (GPCRs). Although many chemotaxis pathways downstream of Gβγ have been identified, few Gα effectors are known. Gα effectors are of particular importance because they allow the cell to distinguish signals downstream of distinct chemoattractant GPCRs. Here we identify GfIB, a Gα2 binding partner that directly couples the Dictyostelium cyclic AMP GPCR to Rap1. GfIB localizes to the leading edge and functions as a Gα2-stimulated, Rap1-specific guanine nucleotide exchange factor required to balance Ras and Rap signaling. The kinetics of GfIB translocation are fine-tuned by GSK-3 phosphorylation. Cells lacking GfIB display impaired Rap1/Ras signaling and actin and myosin dynamics, resulting in defective chemotaxis. Our observations demonstrate that GfIB is an essential upstream regulator of chemoattractant-mediated cell polarity and cytoskeletal reorganization functioning to directly link Gα activation to monomeric G-protein signaling.

INTRODUCTION

Chemotaxis, or directional movement toward an extracellular chemical gradient, is fundamentally important for processes as diverse as innate immunity, food foraging, and organogenesis (Artemenko et al., 2014). During the last decade, important progress has been made by elucidating a complex network of interconnecting signaling pathways controlling ameboid cell chemotaxis, mainly through the use of Dictyostelium and mammalian neutrophils as experimental systems (Artemenko et al., 2014; Nichols et al., 2015). Activation of G-protein-coupled receptor (GPCR)-linked heterotrimeric G protein by guanosine diphosphate/triphosphate (GDP/GTP) exchange in the Gα subunit results in the dissociation into Gα-GTP and a Gβγ subunit, and the activation of the Rho/Rac and Ras families of monomeric G proteins (Faix and Weber, 2013; Jin, 2013; Kortholt et al., 2013; Sasaki and Firtel, 2009). Positive and negative feedback and feedforward loops lead to the intracellular amplification of the extracellular chemoattractant gradient that includes the preferential localization of activated Rac, Ras, and Rap1 at the leading edge, which results in major changes in the cytoskeleton with actin polymerization at the leading edge and actin-myosin filament assembly at the rear and sides of the cell (Artemenko et al., 2014).

Many chemotaxis pathways that are directly regulated by Gβγ have been identified, including: human phosphatidylinositol 3-kinase γ (PI3Kγ; phosphatidylinositol (3,4,5)-trisphosphate [PIP3] production) ( Stephens et al., 2008); Dictyostelium ElmoE, which, with Dock proteins, activates RacB (Yan et al., 2012); Dock2, which activates Rac1 and Rac2 (Kunisaki et al., 2006); and phospholipase Cβ1, which hydrolyzes phosphatidylinositol (4,5)-bisphosphate (PIP2) to DAG and inositol trisphosphate ( Tang et al., 2011). However, we are only beginning to understand whether Gα-GDP/GTP exchange mediates downstream signaling mainly through the release of Gβγ and/or whether distinct signaling pathways are regulated through different Gα subunits. Recently, some mammalian Gα effectors important for chemotaxis were identified, including Dock180, a Rac activator highly homologous to Dock2 (Li et al., 2013), the scaffolding protein mlnsC (Kamakura et al., 2013), and Homer3, a Gα2-binding protein that spatially helps organizes actin assembly (Wu et al., 2015).

Using a combination of proteomic approaches in Dictyostelium (Kataria et al., 2013; Kölsch et al., 2013), we identify GfIB as a Gα2-GTP interacting protein and GSK-3 substrate that is required for efficient directional sensing and cell movement during chemotaxis. Our findings indicate that GfIB is a Gα-stimulated, Rap1-specific guanine nucleotide exchange factor (GEF) that plays a central role for the balance between Ras and Rap signaling at the leading edge of chemotaxing cells.

RESULTS AND DISCUSSION

GfIB Plays a Role in Gα2-Mediated Chemotaxis

We identified Dictyostelium GfIB (GEF-like protein B), with six unique and specific peptides that were not present in control
Figure 1. GflB Is a Gα2-Specific Effector Important for Chemotaxis

(A) Domain topology and schematic representation of GflB and its truncated versions used in this study. The GSK-3 phosphorylation site (197 and 201 amino acids) is indicated with an asterisk.

(B) Physical association of full-length GflB and Gα(s). Glutathione Sepharose beads coated with GST and GST-Gα1, 2, 4, 5, 8, and 9 were incubated with GFP-GflB cell lysate. GFP and GST tags were detected by western blotting as described in Experimental Procedures. A representative western blot of three independent experiments is shown.

(C) GflB binds to Gα2 in vivo. Lysates of GFP-GflB/GST-Gα2, or GFP-GflB/GST co-expressed gflB/C0 cells were subjected to GSH beads in the presence of either 100 μM Gpp(NH)p or 100 μM GDP. Bound proteins were resolved by stained SDS-PAGE and visualized by Coomassie blue (left panel) or western blot (right panel) with anti-GST and anti-GFP antibodies. Representative images of three independent experiments are shown.

(D) GflB binds preferentially to GTP-bound Gα2 using GFP-GflB as bait. GFP-GflB was bound to GFP antibody pre-coupled protein A magnetic beads, followed by the incubation of GST-Gα2 cell lysate in the presence of either 100 μM Gpp(NH)p or 100 μM GDP. Proteins were detected by western blot with anti-GST and anti-GFP antibodies. A representative western blot of three independent experiments is shown.

(legend continued on next page)
samples (pulled down with glutathione S-transferase [GST] protein), in a proteomic analysis of potential Gas2 binding partners (Kataria et al., 2013) and independently as a potential GSK-3 substrate through a comparison of the phosphoproteome of wild-type and gskA− (GSK-3) null cells (Kolsch et al., 2013). Interestingly, GflB contains both a putative Rho GTPase-activating protein (RhoGAP) domain (663–813 amino acids) and a putative Ras guanine nucleotide exchange factor (RasGEF) domain (855–1,256 amino acids) (Figure 1A).

To confirm GflB binding to and specificity for Gas2, we expressed GFP-tagged GflB full-length protein in Dictyostelium and performed pull-down experiments with recombinant, purified GST-fused Gas2 proteins. Figure 1B illustrates that GFP-GflB binds to GST-Gas2 but not to the other tested Dictyostelium Gas2 proteins, including Gas4, which controls chemotaxis downstream of the Dictyostelium folate chemoattractant GPCR (Hadwiger et al., 1994), nor to free GST, suggesting that GflB interacts specifically with Gas2 (Figure 1B). To address binding in vivo and determine the nucleotide specificity, we co-expressed GFP-GflB and GST-Gas2 in Dictyostelium cells and performed pull-down experiments in the presence of GDP or GppNP (Figure 1C). Importantly, western blots show that GFP-GflB preferentially binds to active GST-Gas2 (GppNP-bound) compared with inactive (GDP-bound) GST-Gas2. Pull-downs with GFP-GflB as bait consistently revealed much stronger binding to GppNP-loaded GST-Gas2 compared with GDP-loaded Gas2 (Figure 1D). To investigate which GflB domain interacts with Gas2, we generated truncated constructs that either contain the N-terminal fragment (GFP-GflBP1) or the GflB GAP and GEF domains (GflBP2) (Figure 1A). Immunoprecipitation analyses showed that active GST-Gas2 (GppNP-bound) preferentially binds to GFP-GflBP1, while we observed no detectable binding of GFP-GflBP2 to active or inactive GST-Gas2 (Figures 1E and 1F).

Dictyostelium Gas2 binds to the high-affinity cyclic AMP (cAMP) receptor (cAR1) and is responsible for almost all cAMP-mediated responses (Kumagai et al., 1989). To characterize the function of GflB in vivo, we generated a gflB-null (gflB−) strain by homologous recombination. Consistent with the specific interaction of GflB with Gas2, GflB does not play a major role during vegetative growth; gflB− cells do not show any defect in cell growth, macropinoscytosis, Rac activation, or folate chemotaxis (Figures S1A–S1C). Interestingly, cells expressing GflBP2 show multiple patches of active Rac and severely reduced growth, and become multi-nucleated (Figures S1A–S1C, see also below). Next we quantified the ability of wild-type and gflB− cells to chemotax to cAMP using a micropipette assay (Meili et al., 1999). gflB− cells show only slightly reduced cAR1 expression compared with wild-type cells, indicating that they are sufficiently developed to respond to cAMP (Figure S1D). Whereas wild-type cells chemotax efficiently up a gradient of cAMP, gflB− cells exhibit considerable chemotaxis defects, including poorer directionality, more directional changes, and slower speed compared with wild-type cells (Figure 1G). Overexpression of GFP-tagged GflB complements the gflB− cells’ chemotaxis defects (Table 1; Figure 1G; Movies S1, S2, and S3). As expected from the chemotaxis defects, gflB− cells exhibit severe developmental defects (Figure S1E). While wild-type cells form streams and aggregate between 5 and 8 hr after plating on non-nutrient agar plates, gflB− cells form delayed, smaller aggregates that mostly do not form fruiting bodies (Figure S1E). In contrast, overexpressing GFP-tagged GflB in gflB− results in slightly faster development compared with wild-type cells.

GflB Is a Gas2-Dependent Rap1-Specific GEF

One of the first downstream responses of heterotrimeric G-protein signaling is the activation of small G proteins (Kae et al., 2004; Kortholt and van Haastert, 2008; Sasaki and Firtel, 2006) that rapidly switch between an inactive (GDP-bound) and active (GTP-bound) state with only the GTP-bound form being able to bind to downstream effectors (Bourne et al., 1991). For small G proteins, the switch between the GTP-bound active and GDP-bound inactive states is controlled by GEFs and GAPs, which stimulate the low intrinsic GTPase activity of the G proteins (Trahey and McCormick, 1987). GflB contains both a putative RhoGAP domain and a putative RasGEF domain (Figure 1A). However, the RasGEF domain sequence contains notable changes in the catalytic domain (Figure S2A) compared with known GAPs, including a substitution of the invariant Arg required for GAP activity (Zhang et al., 1999).

Whereas wild-type cells chemotax efficiently up a gradient of the chemoattractant cAMP, gflB−/GFP-GflBP2T1180E cells migrating toward the tip of a needle (indicated by the asterisks) containing 10 μM of the chemoattractant cAMP. Tracks of individual cells (n = 19) are shown as lines for all strains. These tracks were generated using individual cells from five independent experiments. A total elapsed time of 15 min (150 frames; each frame was pictured every 6 s) was used to plot the x and y positions of the centroid of the cells, which were computed over the 150 frames using DIAS. See also Figures S1 and S2.
Rap1-specific GEF. bind Rap1, and that GflB is thus a G
g
auto-inhibition of the N terminus, thereby allowing GflB to

These data suggest that binding of active G

efficiently to GFP-Rap1 only in the presence of active G

experiments with full-length GflB in the presence or absence

of cold GDP was then added, and the nucleotide exchange was

measured as the decay in radioactivity associated with the G

protein. Addition of recombinant GST-GflB-GEF (644–1,282

amino acids) to [3H]GDP-labeled Rap1 results in a rapid
decrease in bound [3H]GDP, indicating an acceleration of the

intrinsic low nucleotide exchange activity of Rap1 (Figure 2A).

However, GfiB-GEF is unable to stimulate the nucleotide

exchange of functional [3H]GDP-labeled RasG (Figure 2A) (Kortholt

et al., 2006), suggesting that GfiB may have specificity for Rap1

in vitro. Consistent with this conclusion, immunoprecipitation

experiments reveal that GfiB-GEF binds to nucleotide-free Rap1,

whereas we detected no binding to nucleotide-free RasB,

RasC, RasD, RasG, or RasS (Figure S2E).

Although GflBP2 efficiently binds Rap1, we detected little

binding of full-length GflB to Rap1 (Figure 2B). As the N terminus

of GflB interacts with active Gz2, we expressed pull-down

experiments with full-length GflB in the presence or absence

of active Gz2. GST-tagged GflB was expressed in Dicyoste-

lium cells and used as bait in a lysate of Dicyostelium

cells expressing GFP-Rap1. Figure 2B shows that GflB binds

efficiently to GFP-Rap1 only in the presence of active Gz2.

These data suggest that binding of active Gz2 releases an

auto-inhibition of the N terminus, thereby allowing GflB to

bind Rap1, and that GflB is thus a Gz2-GTP stimulated,

Rap1-specific GEF.

gflB– Cells Exhibit Decreased Rap1 and Increased Ras

Activation

Figure S3A shows that N-terminal GFP-fused Rap1 and RasG

are localized mainly at the cell membrane of both wild-type

and gflB– cells. To monitor spatial activation of the protein,
rather than its localization, we quantified cAMP-stimulated

Rap1 activation in cell lysates using pull-down assays with

GST-RalGDS and imaged them in vivo using the RalGDS-GFP

reporter (Matsubara et al., 1999). As previously reported (Jeon

et al., 2007a), we found that wild-type cells exhibit a low basal

level of Rap1-GTP that rises rapidly in response to global chemo-

attractant stimulation with a peak at ~5 s and then returns

quickly to basal levels (Figures 2C and 2D). Currently it is not

known how cAMP-mediated Rap activation is regulated, since

the two identified Rap1-specific GEFs, GbpD and GEFQ, are

mainly important for Rap-mediated adhesion in vegetative

cells and cytoskeletal rearrangement at the poles of dividing

cells (Kortholt et al., 2006; Plak et al., 2014). In contrast, and consist-

tent with GfiB being a Gz2-mediated Rap1 GEF, gflB– cells

exhibit severely impaired Rap1 activation in response to cAMP

(Figures 2C and 2D).

We next quantitated Ras activation in wild-type and gflB– cells

using pull-down assays with GST-Raf1-RBD or GST-Byr2 as

bait, and Ras activation was imaged in vivo with a Rap1-RBD-

GFP marker as described previously (Kortholt et al., 2011).

GST-Raf1-RBD preferentially binds RasG and RasB, while

GST-Byr2 binds strongest to RasC (Kae et al., 2004; Zhang

et al., 2006). Wild-type cells exhibit a low basal level of Ras-

GTP, which rises rapidly in response to global chemoattractant

stimulation with a peak at ~5 s and then returns quickly to basal

levels (Figures 2E and 2F). The highly sensitive pull-down assays

showed that gflB– cells have high basal levels of active Ras in

vitro and, both in vitro and in vivo, have an elevated and

extended cAMP-mediated Ras response compared with that in

wild-type cells.

In a cAMP gradient, wild-type cells are polarized, and both

RalGDS-GFP and Raf1-RBD-GFP rapidly accumulate at the side

of the cell facing the gradient (Figures 2D and 2F, right panels).

We calculated the ratio of fluorescence at the cell
cortex in the up-gradient half of the cell to that in the down-

gradient half of the cell (Figure S3B; Kortholt et al., 2011). Wild-
type cells show 7.28 ± 2.79-fold (n = 8) and 8.73 ± 0.75-fold

(n = 8) more Rap1 and Ras activation, respectively, at the front

gradient half of the cell (Figure S3B; Kortholt et al., 2011). Wild-
type cells show 7.28 ± 2.79-fold (n = 8) and 8.73 ± 0.75-fold

(n = 8) more Rap1 and Ras activation, respectively, at the front

half compared with the back half of the cell. gflB– cells have a

less polarized Ras (2.98 ± 0.54, n = 8, p < 0.0001) and Rap1

(4.00 ± 1.31, n = 8, p < 0.05) response compared with wild-
type cells (Figure S3B). In steep gradients of cAMP, the initial

wide bell-shaped curve of membrane-bound Rap1-RBD-GFP

becomes very narrow (7.53 ± 0.55 μm), with very steep flanks.

In contrast, this confinement is absent in gflB– cells, resulting in

a much broader Ras crescent (12.46 ± 1.44 μm, n = 8, p < 0.0001)

compared with that in wild-type cells, indicating an inability to
effectively polarize in a chemoattractant gradient. Confinement of Ras signaling depends on cytoskeleton rearrange-

ment (Kortholt et al., 2013), a process that is coordinated by Rap1 (Jeon et al., 2007a).

**Table 1. DIAS Analysis of Representative Developed Cells Performing Chemotaxis to 1 μM cAMP**

<table>
<thead>
<tr>
<th>Cell Strains</th>
<th>Directionality</th>
<th>Speed (μm/min)</th>
<th>Direction Change</th>
<th>Roundness (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AX3</td>
<td>0.60 ± 0.13</td>
<td>7.65 ± 1.44</td>
<td>42.29 ± 9.29</td>
<td>39.38 ± 3.77</td>
</tr>
<tr>
<td>gflB+/GFP-GflB</td>
<td>0.20 ± 0.06**</td>
<td>4.02 ± 0.49*</td>
<td>65.40 ± 4.56**</td>
<td>47.50 ± 8.20</td>
</tr>
<tr>
<td>gflB+/GFP-GflBP2</td>
<td>0.74 ± 0.05</td>
<td>8.42 ± 1.15</td>
<td>29.65 ± 5.82*</td>
<td>47.15 ± 4.08*</td>
</tr>
<tr>
<td>gflB+/GFP-GflBP2T1180E</td>
<td>0.67 ± 0.11</td>
<td>11.26 ± 2.26**</td>
<td>37.58 ± 10.49</td>
<td>48.14 ± 7.62*</td>
</tr>
<tr>
<td>gflB+/GFP-GflBP2T1180E</td>
<td>0.39 ± 0.18**</td>
<td>7.35 ± 3.38</td>
<td>55.20 ± 9.68**</td>
<td>48.48 ± 11.09*</td>
</tr>
</tbody>
</table>

Data represent analysis performed with 12 different cells from five independent experiments ± SD, for each strain. Speed refers to the speed of the cell’s centroid movement along the total path; directionality indicates migration straightness; direction change refers to the number and frequency of turns; and roundness indicates cell polarity. *p < 0.05, **p < 0.01, Student’s t test. The chemotaxis defects shown result in severe developmental defects. See also Figure S1.

Thus far, five Ras proteins (RasB, RasC, RasD, RasG, and RasS) and one Rap (Rap1) have been characterized to some extent, and they all appear to have important roles in cell physiology (Kortholt and van Haastert, 2008). Although one cannot presently predict the specificity of putative RasGEFs based on sequence, the in vitro specificity of GEFs can be directly examined using a nucleotide exchange assay (Figure 2A). Purified GST-tagged Rap1 and RasG were loaded with [3H]GDP and an excess of cold GDP was then added, and the nucleotide exchange was measured as the decay in radioactivity associated with the G protein. Addition of recombinant GST-GfiB-GEF (644–1,282 amino acids) to [3H]GDP-labeled Rap1 results in a rapid decrease in bound [3H]GDP, indicating an acceleration of the intrinsic low nucleotide exchange activity of Rap1 (Figure 2A). However, GfiB-GEF is unable to stimulate the nucleotide exchange of functional [3H]GDP-labeled RasG (Figure 2A) (Kortholt et al., 2006), suggesting that GfiB may have specificity for Rap1 in vitro. Consistent with this conclusion, immunoprecipitation experiments reveal that GfiB-GEF binds to nucleotide-free Rap1, whereas we detected no binding to nucleotide-free RasB, RasC, RasD, RasG, or RasS (Figure S2E). Although GflBP2 efficiently binds Rap1, we detected little binding of full-length GflB to Rap1 (Figure 2B). As the N terminus of GflB interacts with active Gz2, we expressed pull-down experiments with full-length GflB in the presence or absence of active Gz2. GST-tagged GflB was expressed in Dicyostelium cells and used as bait in a lysate of Dicyostelium cells expressing GFP-Rap1. Figure 2B shows that GflB binds efficiently to GFP-Rap1 only in the presence of active Gz2. These data suggest that binding of active Gz2 releases an auto-inhibition of the N terminus, thereby allowing GflB to bind Rap1, and that GflB is thus a Gz2-GTP stimulated, Rap1-specific GEF.
Our findings suggest that Gfb is involved in the regulation of the balance between Ras and Rap signaling at the leading edge of chemotaxing cells.

**Gfb Is Required for Proper Regulation of Chemotaxis Pathways**

Both Ras and Rap1 are upstream regulators of the PI3K and TORC2 pathways that play critical roles in directional sensing, cytoskeletal reorganization, and cell movement (Arthur et al., 2004; Bolourani et al., 2006; Charest et al., 2010; Funamoto et al., 2002; Lee et al., 2005; Mun and Jeon, 2012; Plak et al., 2013; Sasaki and Firtel, 2006; Sasaki et al., 2004; Takeda et al., 2007).

We analyzed the kinetics of PI3K activation, which is an effector of RasG and Rap1 (Funamoto et al., 2002; Kortholt et al., 2010), using the PIP3 reporter PHCRAC-GFP (Insall et al., 1994). In wild-type cells, PHCRAC-GFP is predominantly cytosolic in unstimulated cells and rapidly localizes to the plasma membrane in response to uniform cAMP stimulation and to the leading edge in chemotaxing cells (Figure 3A). gfb− cells exhibit an extended plasma membrane localization of PHCRAC-GFP in response to cAMP stimulation compared with wild-type cells. Furthermore, these cells exhibit a high, uniformly distributed PHCRAC-GFP over the entire cell periphery prior to stimulation and also during chemotaxis as well as in vegetative cells, suggesting a high, uniform basal PI3K activity, consistent with elevated basal RasG activity (Figure 3A). Consistent with these observations, we find that cAMP-stimulated PI3K cortical localization is extended in gfb− cells compared with that in wild-type cells, and is found along the entire cortex already prior to stimulation and also during chemotaxis rather than localized at the leading edge (Figure 3A). These results agree with the lack of apparent polarization in developed gfb− cells, as described above.

Akt/PKB and the related enzyme PKBR1 play key roles in regulating leading edge function during chemotaxis (Meili et al., 1999, 2000; Kamimura et al., 2008). Akt/PKB and PKBR1 are activated at the plasma membrane by two phosphorylations, as are mammalian Akt/PKBs (Sarbassov et al., 2005): their activation loops (ALs) are phosphorylated by the two PKD1 isoforms (Kamimura et al., 2008; Liao et al., 2010), whereas TORC2, an effector of RasC, phosphorylates the conserved hydrophobic motif (HM) (Cai and Devreotes, 2011; Charest et al., 2010; Kamimura et al., 2008; Lee et al., 2005). Chemoattractant-mediated plasma membrane localization of Akt/PKB is mediated through the binding of its PH domain to the PI3K product PIP3 (Meili et al., 1999; Funamoto et al., 2002) while PKBR1 is constitutively localized on the plasma membrane through an N-terminal myristoyl group (Meili et al., 2000; Lee et al., 2005; Kamimura et al., 2008). In cAMP-responsive wild-type cells, the AL and HM phosphorylation of Akt/PKB and PKBR1 peak at ~10 s after chemoattractant stimulation, consistent with the peak of kinase activity (Figures 3B and 3C) (Meili et al., 2000; Lee et al., 2005; Kamimura et al., 2008). In gfb− cells, the AL phosphorylation is elevated and extended for Akt/PKB and PKBR1, and the phosphorylation of the HM is also elevated and extended (Figures 3B and 3C). Consistent with these findings, several substrates of Akt/PKB have extended phosphorylation in response to cAMP stimulation in gfb− cells (Figure 3B). These observations are consistent with elevated basal RasG and RasC activities and elevated and extended cAMP-stimulated activity.

**Gfb Regulates cAMP-Induced Myosin II Assembly and Actin Polymerization**

Spatially localized activation of Rap1 and Ras induces F-actin polymerization at the leading edge of chemotaxing cells through the Rac, PI3K, and TORC2 pathways described above (Artemenko et al., 2014; Jin, 2013). At the same time, active Rap1 inhibits myosin assembly at the leading edge through activation of its effector Phg2, while low levels of active Rap1 at the side and back of the cell allow myosin formation (Jeon et al., 2007a, 2007b). Thus, the balance in the temporal and spatial regulation of Ras and Rap1 activation is essential to control both actin and myosin rearrangements during chemotaxis (Artemenko et al., 2014). Consistent with the increased RasG response, we find that F-actin polymerization is elevated in gfb− cells compared with that in wild-type cells, whereas myosin II (MyoII) assembly is dramatically reduced compared with that in wild-type cells (Figures 4A–4C). Overexpression of GFP-Gfb or GfbP2 complements these gfb− cell phenotypes (Figures 4A–4C). Furthermore, in wild-type cells the F-actin reporter Lifeact-GFP is found at a low level along the cell cortex in unstimulated cells, predominantly at small protrusions, and is transiently localized along the whole cell cortex in response to uniform cAMP stimulation (Figure 4D). In chemotaxing wild-type cells, the reporter is enriched at the leading edge while gfb− cells exhibit a higher basal and more uniformly distributed level of Lifeact-GFP at the cortex before and after cAMP stimulation, consistent with the elevated F-actin in gfb− cells. In addition, the basal and cortically localized levels of MyoII are dramatically decreased in gfb− cells compared with those in wild-type cells (Figure 4E). As expected, GFP-MyoII is enriched at the rear and sides of the migrating wild-type cells but is not detectable at the cortex in gfb− cells (Figure 4F). Together, our findings demonstrate that Gfb has an important role in regulating Ras- and Rap1-mediated F-actin and myosin dynamics at the leading edge of chemotaxing cells.

**The N Terminus Functions to Regulate Both Gfb-GEF Activity and Its Localization**

To further understand the activation mechanism of Gfb, we analyzed the function and localization of full-length, truncated, and mutated versions of Gfb. In developed, randomly moving cells, Gfb is mainly in the cytoplasm except for a few patches of increased fluorescence observed at the cell boundary at the sites of protrusions (Figure 5A). During chemotaxis, Gfb localizes at the leading edge of cells (Figure 5A), whereas in response to uniform (global) cAMP stimulation, GFP-Gfb rapidly and transiently translocates to the cortex (Figure 5A). The kinetics of the Gfb translocation are more accurately monitored by computational analysis of fluorescence intensity depletion in the cytoplasm (Figure 5B). The translocation of GFP-Gfb starts immediately upon cAMP stimulation, peaks at ~8 s, and returns to basal levels by ~18 s. Because Gfb localization seems to be thicker than that defined by plasma membrane as observed with Raf1-RBD-GFP or PHCRAC-GFP (Figure 5A), and Gfb regulates actin-myosin dynamics, we investigated a role for the cell cortex in regulation. In cells incubated with the actin-polymerization inhibitor latrunculin A (LatA), Gfb does not translocate to the cell.
Figure 2. GfIB Is a Gα2-Dependent Rap1-Specific GEF

(A) Rap1 and RasG [3H]GDP release with (red line) and without (blue line) GfIB-GEF in the presence of an excess of GDP. Data are mean and SD of at least three independent experiments; significantly different from control without GfIB-GEF at *p < 0.05 (Student’s t test).

(legend continued on next page)
boundary on uniform cAMP stimulation (Figure 5B), suggesting that localization depends on a functional cytoskeleton. Previous reports have shown that cytoskeleton-associated GAP proteins are important for the regulation of Rap1 activity (Jeon et al., 2007a). Consistent with these results, we find that addition of LatA to starved cells results in a strong uniform Rap1 response that does not increase further upon addition of cAMP (Figure S4D). In contrast, LatA does not induce a uniform Rap1 response in gflB−/− cells, suggesting that GflB is required for cytoskeletal regulation of Rap1 activation. Furthermore, addition of LatA to gflB−/− cells completely restores the prolonged cAMP-mediated Ras response in gflB-null cells, while it does not affect the amplitude or kinetics of the Ras response in wild-type cells (Figure S4E). These data show that the observed Ras phenotype of gflB−/− cells depends on a functional cytoskeleton and that GflB, consistent with our biochemical data (Figure S2E), indirectly regulates Ras activity.

Interestingly, the constitutively active GflBP2 fragment (see above) is already localized at the cortex before stimulation, and we detect no further increase of fluorescence after global stimulation with cAMP (Figure 5B). Similarly, in unstimulated cells the N terminus of GflB (GflBP1) also localizes to the cell boundary; however, the localization of GflBP1 does not change in the presence of LatA, suggesting that GflBP1 is bound to the membrane rather than the cytoskeleton (Figure 5C). The binding of GflBP1 to the membrane most likely involves interaction with lipids (Figure S5A).

Together, these results suggest that translocation of GflB is initiated by binding of activated Gαz2 to the GflB N terminus, resulting in exposure of a lipid binding site in GflB, resulting in a more stable binding to the plasma membrane at the sites of activated Gαz2. This interaction then allows the binding of a new active GflBP2 to the cell cortex via interaction with Rap1 and/or other sites on the cortex. At the same time, RapGAPs accumulate at the cortex at the back of the cell (Jeon et al., 2007b). This spatial separation of GEFs and GAPs subsequently leads to Rap activation in a large area at the front of the cell.

**GSK-3 Phosphorylation Modulates GflB Localization**

A comparative bioinformatics screening of a total cell phosphoproteomic analysis (tandem mass spectrometry array) of developed wild-type and GSK-3-null (gskA−) cells in response to cAMP stimulation shows that GflB is not phosphorylated at a potential GSK-3 site in gskA− cells (Figure S5B). GflB is simultaneously phosphorylated at Ser197 (putative GSK-3 site) and Thr201 (putative priming site) before (0 s, basal), at 10 s (the time of maximum activation of many leading edge pathways), and at 60 s (adaptation) after cAMP stimulation in wild-type, but no phosphorylation at these sites is observed in gskA− cells (Figure S5B). Indeed, we identified the same phosphorylation sites in a previous proteomics screening in wild-type cells (Char est et al., 2010). In addition, peptides containing phospho-Ser197 (but not phospho-Thr201) are only identified at 60 s after cAMP stimulation, suggesting that GSK-3 might regulate GflB adaptation rather than its activation in response to the chemoattractant.

Our study detects other phosphorylations on GflB in gskA− cells, suggesting that the lack of phosphorylation at the potential GSK-3 site is probably due to the absence of GSK-3 rather than GflB not being detected. We verified that GflB is a direct target of GSK-3 by examining the ability of immunoprecipitated T7-GflB expressed in gskA− cells to be phosphorylated in an in vitro kinase assay by recombinant human GSK-3β (Figure 5D). Wild-type GflB and GflB carrying Ala substitutions at Ser197 and Thr201 (GflB5197A/T201A) show similar low phosphorylation during the first 4 min, suggesting that GSK-3β can phosphorylate additional sites in vitro. However, at later time points GflB5197A/T201A is less phosphorylated than the wild-type protein, suggesting that Ser197 and Thr201 are the major sites of GSK-3β phosphorylation in vitro. We observed smaller differences in the phosphorylation of GflB and GflB5197A/T201A for incubation times longer than 10 min (Figure S5C). The in vitro kinase assay observations, together with the phosphoproteomics findings, identified a GSK-3 phosphorylation site in GflB, suggesting that GflB is a direct in vivo target for GSK-3, which is known to regulate MyoII function (Kölsch et al., 2013).

We examined the function of GflB phosphorylation by GSK-3 by expressing GFP-GflB in gskA− cells. In response to uniform cAMP concentration, the cortical translocation of GFP-GflB peaks more rapidly, and the level of accumulation is more elevated and extended than that of GFP-GflB in wild-type cells (Figure 5E, left panel). To determine whether phosphorylation of the identified GSK-3 site in GflB mediates this response, we expressed the non-phosphorylatable (GFP-GflB5197A/T201A).
and the phosphomimetic form of GflB (GFP-GflB{S197D/T201D}) in wild-type and gflB<sup>-</sup> cells. The cortical translocation of GFP-GflB<sup>S197D/T201D</sup> in gflB<sup>-</sup> cells is similar to that of wild-type GFP-GflB expressed in gflB<sup>-</sup> cells (Figure 5E, right panel). In contrast, we found that GFP-GflB<sup>S197A/T201A</sup> peaks more rapidly and the peak is slightly elevated and more extended than in wild-type GFP-GflB, similar to, but not as extreme as, our observations when GFP-GflB is expressed in gskA<sup>-</sup> cells (Figure 5E, right panel). We made similar observations for GFP-GflB<sup>S197A/T201A</sup> expressed in wild-type cells (Figure 5E). Together, these findings suggest that phosphorylation of the N terminus of GflB by GSK-3 results in less, delayed, and shorter GflB recruitment to the cortex. Thus, GSK3 phosphorylation is not essential for activation but helps modulate the extent and timing of the translocation.

Model for the Function of GflB
We demonstrate that GflB binds to and is activated by G<sub>a2</sub>-GTP and directly links the cAMP receptor to Rap1 activation at the leading edge and, thus, one of the most receptor-proximal regulators of chemotaxis in this system. GflB is required for proper cell polarization, integration of Rap1 and Ras pathways, and the spatiotemporal regulation of the F-actin/myosin cytoskeleton (Figure 6A). GflB is a Rap1 GEF that is stimulated by direct binding to Gs. Interestingly, GflB appears to be specific to the...
Figure 4. GflB Regulates Myosin II Assembly and Actin Polymerization

(A and B) 1 μM cAMP induced F-actin polymerization (A) and MyoII assembly (B) in AX3, gflB−, and gflB− cells expressing GFP-GflB and GFP-GflBP2.

(C) Quantification of relative F-actin and MyoII from at least three independent experiments, representing the mean ± SD normalized to unstimulated AX3 at time 0. *p < 0.05, Student’s t test.

(legend continued on next page)
cAMP chemotaxis receptor pathway (involved in aggregation and development). GflB binds to Gα2 and not Gα4, which couples to the folate GPCR used as a food-forging receptor. The activation of GflB through a specific Gα2, but not the sole Gβγ subunit, provides a mechanism for Dictyostelium cells to respond differently to distinct chemoattractants.

Binding of active Gα2-GTP to the N terminus of GflB initiates recruitment of GflB to the leading edge and releases the auto-inhibition (Figure 6B), allowing the GEF domain to bind and stimulate the exchange activity of Rap1. In an extracellular gradient, GflB strongly localizes at the leading edge, while the activation of Gα2 is only proportional to the steepness of the gradient (Janetopoulos et al., 2001; Ueda et al., 2001). It is therefore unlikely that only binding of Gα2 to the N terminus is responsible for the accumulation of GflB to the leading edge. Furthermore, the constitutively active GflB2P2 fragment and the N terminus of GflB are exclusively localized at the cortex and cell membrane, respectively. Together, these data suggest that the leading edge localization of GflB is initiated by Gα2-mediated lipid binding of the N-terminal domain of GflB, followed by localization to the cell cortex via binding of GflB2P2 domain. Subsequently, active GflB regulates cytoskeletal reorganization, resulting in recruitment of additional GflB to the cortex. Interestingly, F-actin also regulates PI3K localization at the leading edge via a similar positive feedback loop mechanism (Funamoto et al., 2002). So far, we have been unable to determine whether the inactive RhoGAP domain or the RasGEF domain is responsible for binding to the cell cortex. However, it is tempting to speculate that, by analogy to human OCRL (Mehta et al., 2014), binding to Rac protein via the catalytic inactive RhoGAP domain plays an important role in this process. The extent and timing of the GflB translocation to the cortex and leading edge is further fine-tuned by the phosphorylation state of the N terminus of GflB by GSK-3; non-phosphorylatable GFP-GflB protein translocation exhibits a more rapid translocation upon cAMP stimulation, consistent with constitutive inhibitory phosphorylation by GSK-3. Furthermore, our proteomic observations suggest that the Ser197 GSK-3 site of GflB shows increased phosphorylation at 60 s after cAMP stimulation, which might suggest that GSK-3 regulates GflB inactivation rather than activation in response to cAMP at the leading edge. The exact mechanism by which phosphorylation of the N terminus of GflB attenuates localization remains to be determined but might involve allosteric regulation of exposure of the lipid binding and/or RhoGAP domain.

Although human Rap1 was initially identified as a suppressor of Ras signaling, recent studies in various model systems have revealed several downstream pathways that are activated by both Rap1 and Ras or are activated by Rap1 independent of Ras (Frische and Zwartruis, 2010; Kitayama et al., 1989; Kortholt et al., 2010; Mishra et al., 2005; Schwamborn and Püschel, 2004). Furthermore, studies in both mammals and Dictyostelium have also shown that Ras and Rap1 activation are strongly interconnected (Bolourani et al., 2008; Lee et al., 2011). Importantly, not only the individual levels of, but also the balance between, Ras and Rap1 activation are important for many processes (Ye and Carew, 2010; Ye et al., 2008) (Figure 6B). GflB is key for the balance between Ras and Rap1 activation at the leading edge of chemotaxing Dictyostelium cells. GflB functions as a Rap-specific GEF whose activation is mediated by direct binding of Gα2-GTP. The exact mechanism by which GflB regulates Ras activation is not completely understood but requires a functional cytoskeleton. Therefore, cells lacking GflB have impaired spatial and temporal Rap1 and Ras activation, which together results in uncoordinated and/or extended activation of the downstream pathways and impaired coordination of cytoskeletal rearrangements (Figure 6A). Rap1 acts as a global regulator of a large number of processes crucial for Dictyostelium, including actin, myosin and microtubule filament formation, adhesion, and protrusion formation (Jeon et al., 2007b; Kortholt et al., 2006, 2010; Plak et al., 2013) (Figure 6B). During chemotaxis, Rap1 activation is restricted to a broad patch at the leading edge, whereby activation of the Rap-effector Phg2 it inhibits myosin filament formation. The spatial activation of Rap1 thus allows myosin filament formation only at the sites with lowest Rap1 activity (the back and sides of the cell) (Jeon et al., 2007a, 2007b). Ras, which is activated in a narrow patch at the leading edge, and Rap both stimulate actin filament formation at the front of the cell and thereby facilitate directional movement (Figure 6C).

Together, our findings indicate that GflB provides a direct link from Gα2 activation to localized monomeric G-protein signaling and localized cytoskeletal rearrangement, and is therefore important for Dictyostelium development and chemotaxis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

All GflB mutants were derived from the axenic Dictyostelium discoideum AX3 strain, designated here as wild-type. Dictyostelium cells were maintained in HLS-C medium including glucose either on plastic Petri dishes or shaking suspension at 150 rpm at 21°C to a density of no more than 2 x 10⁶ cells/ml. For selection, the respective antibiotics were added at a concentration of 10 μg/ml. For acquisition of developmentally competent cells capable of responding to cAMP as a chemoattractant, log-phase vegetative cells were harvested by low-speed centrifugation (300 x g for 3 min), washed twice, and resuspended at a density of 5 x 10⁶ cells/ml with 12 mM Na/K phosphate buffer (PB), and pulsed with 7.5 μM CAMP solution for 5.5 hr at 6-min intervals. Subsequently, cells were collected and suspended in PB buffer. The density of cells grown in suspension was determined every 12 hr over a 7-day period, and the doubling times were obtained from growth rates that occurred during log-phase growth. For measurement of the rate of macrophagocytosis, cells were shaken in 8 ml of HLS medium at a density of 10⁶ cells/ml. 20 μg of fluorescein isothiocyanate-dextran was added and 500-μl samples

(D) Translocation kinetics of the F-actin reporter Lifeact-GFP in AX3 and gflB−/C0 cells in response to uniform (1 μM) and gradient (10 μM) cAMP stimulation. Scale bar represents 5 μm. Quantitation of the relative fluorescence intensity of membrane-localized GFP-Lifeact is shown in the right panel, taking the starting point in AX3 cells as 1.0. Data represent mean ± SD of cells from three independent experiments. **p < 0.01, Student’s t test.

(E) Live imaging of GFP-MyoII expressed in AX3 and gflB−/C0 cells in response to 1 μM uniform cAMP. Images captured at 1-s intervals and frames at selected time points are shown. Scale bar represents 5 μm. Quantitation of the relative fluorescence intensity of membrane-localized GFP-MyoII is shown in the right panel, taking the starting point in AX3 cells as 1.0. Data represent mean ± SD of cells from three independent experiments. **p < 0.01, Student’s t test.

(F) Live imaging of translocation of GFP-MyoII expressed in AX3 and gflB−/C0 cells upon gradient cAMP (10 μM) stimulation. Asterisks indicate the place of the CAMP source. Scale bar represents 5 μm.
were taken at t = 0, 15, 30, 45, 60, 120, and 180 min. Samples were pelleted, washed once in PB, and resuspended in lysis buffer (50 mM KCl, 10 mM Tris [pH 8.3], 2.5 mM MgCl2, 0.45% Triton X-100, 0.45% Tween 20). Fluorescence was measured using a fluorimeter (470 nm excitation, 515 nm emission).

**Plasmid and Protein Preparation**

The GFP-PI3K, PHCRAC-GFP, GFP-Lifeact, GFP-MyoII, Raf1-RBD-GFP, and RaGDS-GFP cellular markers were reported previously (Kortholt et al., 2011; Plak et al., 2014; Sasaki et al., 2004). The indicated GflB overexpressing and knockout constructs were generated (Supplemental Experimental Procedures) and subsequently transformed into Dictyostelium cells by electroporation. Three independent, clonally isolated gflB strains were used for the subsequent analysis. GflB-GEF (amino acids 644–1,282), Rap1, and RasG were expressed from a pGEX4T1 plasmid containing an N-terminal GST (GE Healthcare) in Rosetta2 (DE3) Escherichia coli (Novagen). Protein expression was induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside at room temperature for 16 hr. Bacterial cell pellets were resuspended in buffer containing 50 mM Tris (pH 7.5), 50 mM NaCl, 5 mM MgCl2, and 1 mM phenylmethylsulfonyl fluoride, supplemented with 1 mg/ml crushed protease inhibitor tablets (Roche), and lysed by sonication. Proteins were isolated by SDS-PAGE, and the concentration was determined by Bradford's method (Bio-Rad).

**Co-immunoprecipitation Experiments and Phospholipid Binding Assay**

Co-immunoprecipitation assays are described in detail in Supplemental Experimental Procedures and were performed as described previously (Kataaria et al., 2013; Kortholt et al., 2012). Lipid dot-blot assays were done using PIP
In Vitro Guanine Nucleotide Exchange Assay

In brief, 1 \times 10^8 GFP-GflBP1-expressing cells were resuspended in 1 ml of lysis buffer. The PIP strips were pre-blocked with Tris-buffered saline and Tween 20 (TBST) containing 3% fatty acid free BSA (Sigma-Aldrich) for 1 hr at room temperature and subsequently incubated with 5 ml of TBST containing 50 ng of protein of the cleared GFP-GflBP1 lysate. Proteins were visualized by western blotting using an anti-GFP antibody (Santa-Cruz Biotechnology, 1:2,000 dilution) with 5 ml of TBST containing 3% fatty acid free BSA (Sigma-Aldrich) for 1 hr at room temperature and subsequently incubated with 5 ml of TBST containing 50 ng of protein of the cleared GFP-GflBP1 lysate. Proteins were visualized by western blotting with the anti-Pan Ras (Pierce, Thermo Fisher Scientific) and anti-Rap1 antibodies. Image analysis was carried out using ImageJ software (NIH). All assays were repeated at least three times.

Ras and Rap1 Activation Assays

Rap1 and Ras activation was quantified in cell lysates using the previously described GST-RalGDS (Jeon et al., 2007b) and GST-RBD (Kae et al., 2007) pull-down assays, respectively. In brief, pre-cleared Dictyostelium cell lysates (see above) were mixed with 100 μg of recombinant purified GST-RBD or GST-RBD (RalGDS) (Kortholt et al., 2006). The samples were incubated with GSH beads for 1 hr at 4°C and subsequently washed three times with lysis buffer. Bound proteins were eluted by boiling in 1× SDS buffer and resolved on SDS-PAGE gels. Ras and Rap1 proteins were visualized by western blotting with the anti-GFP antibody (Santa-Cruz Biotechnology, 1:2,000 dilution) or anti-GST antibody.

Development, Chemotaxis, and Live Imaging

Cells were harvested from plates at a log-phase density, washed, and suspended at a density of 3 \times 10^8 cells/ml in PB buffer to monitor the development of Dictyostelium. Serial dilutions of 30-μl drops were placed on PB non-nutrition agar plates and development was monitored for 24 hr at room temperature using a stereo microscope (SMZ-U, Nikon). Chemotaxis of Dictyostelium cells toward cAMP was analyzed as described previously (Sasaki et al., 2004) and quantified by DIAS (dynamic image analysis system) software (Wessels et al., 1998). Chemotaxis toward 1 μM cAMP was measured as previously described (Katariya et al., 2013). Live confocal images were recorded using a Zeiss LSM 510 Metano confocal laser scanning microscope equipped with a Zeiss plan-apochromatic 63× numerical aperture 1.4 objective. The quantification of fluorescence intensity was done as described previously (Kortholt et al., 2011). Experiments were repeated independently at least three times, always assaying wild-type cells as a control for comparison in each experiment.

Figure 6. Model for the Activation Mechanism and Function of GflB

(A) Overview of the spatial and temporal cAMP-mediated responses of the indicated markers in AX3 and gflB-Δ cells.

(B) Cartoon depicting the activation cycle of GflB.

(C) Schematic of the GflB-mediated signaling pathways. Gs2-mediated GflB activation is key for the balance between Ras and Rap1 activation at the leading edge of chemotaxing Dictyostelium cells. Activated Rap promotes myosin disassembly at the leading edge through various downstream pathways. At the same time lower levels of active Rap1 at the side and back of the cell allow myosin assembly, thereby establishing forces for the contraction of the cell’s posterior. Ras and Rap both stimulate actin filament formation at the front of the cell and thereby facilitate directional movement.
In Vitro PKB/PKB1 Phosphorylation Assay
Phosphorylation immunoblot assays of PKB and PKB1 were performed as described previously (Meili et al., 1999). In brief, samples of unstimulated and stimulated (1 μM cAMP) developed cells were taken at the indicated time points, lysed, run on SDS-PAGE gels, and transferred to nitrocellulose membranes. Phosphorylation of Akt/PKB and PKB1 at the AL was detected using α-phospho-p70S6 kinase antibody (Cell Signaling Technology), and phosphorylation at the AL was detected using α-phospho-protein kinase C (pan) antibody (Cell Signaling) (Kamimura et al., 2003). Western blots were quantified using ImageJ. Experiments were repeated independently at least three times, always assaying wild-type cells as a control for comparison in each experiment.

F-Actin Polymerization and MyoII Assembly
Developmentally competent cells were stimulated with 1 μM cAMP, samples were taken at the indicated time points, and cytoskeletal proteins were isolated as described previously (Steimle et al., 2001). The samples were separated on SDS-PAGE and stained with Coomassie blue. Protein amounts were quantified using ImageJ. Experiments were repeated independently at least five times, always assaying wild-type cells as a control for comparison in each experiment.

In Vitro GSK-3 Kinase Assay
Extracts from gsk4Δ (GSK-3 null) cells expressing T7-GflB or T7-GflB<sup>B197A/T201A</sup> were mixed and immunoprecipitated with T7-tag antibody agarose beads (Novagen). The immune complexes were washed three times with cold lysis buffer and twice with cold kinase buffer (5 mM MOPS [pH 7.2], 2.5 mM β-glycerophosphate, 1 mM EGTA, 0.4 mM EDTA, 4 mM MgCl<sub>2</sub>, 0.05 mM DTT, 40 ng/μl BSA) and then incubated with 30 ng of human recombinant GSK-3β (Cell Signaling). The kinase reactions were performed for up to 30 min at room temperature in the presence of radioactively labeled [γ-<sup>32</sup>P]ATP (250 μM, 2 μCi/μl). The reactions were terminated by the addition of 2× SDS sample buffer. Samples were boiled and loaded on SDS-PAGE gels. The results were visualized by exposing to HyBlotCL autoradiography film (Denville Scientific).

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, five figures, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2016.05.001.

AUTHOR CONTRIBUTIONS
R.A.F. and A.K. designed the experiments and wrote the paper. Y.L. and J.L. performed most biochemical and cellular experiments, made figures, and actively contributed to writing the paper. P.J.M.v.H. quantified in vivo Ras activation, and F.F. per- formed most biochemical and cellular experiments, made figures, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2016.05.001.

REFERENCES


Note Added in Proof
During the final revision of our manuscript, Senoo et al. identified GflB as a RacE binding protein with a role in chemotaxis. These findings are now published: