Phosphorylated Rho–GDP directly activates mTORC2 kinase towards AKT through dimerization with Ras–GTP to regulate cell migration

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mTORC2 plays critical roles in metabolism, cell survival and actin cytoskeletal dynamics through the phosphorylation of AKT. Despite its importance in biology and medicine, it is unclear how mTORC2-mediated AKT phosphorylation is controlled. Here, we identify an unforeseen principle by which a GDP-bound form of the conserved small G protein Rho GTPase directly activates mTORC2 in AKT phosphorylation in social amoebae (Dictyostelium discoideum) cells. Using biochemical reconstitution with purified proteins, we demonstrate that Rho–GDP promotes AKT phosphorylation by assembling a supercomplex with Ras–GTP and mTORC2. This supercomplex formation is controlled by the chemoattractant-induced phosphorylation of Rho–GDP at S192 by GSK-3. Furthermore, Rho–GDP rescues defects in both mTORC2-mediated AKT phosphorylation and directed cell migration in Rho-null cells in a manner dependent on phosphorylation of S192. Thus, in contrast to the prevailing view that the GDP-bound forms of G proteins are inactive, our study reveals that mTORC2-AKT signalling is activated by Rho–GDP.

G proteins are molecular switches that control a wide range of biological processes, including signal transduction, protein and membrane trafficking, cytoskeletal and organelle remodelling, and cell growth and proliferation[1–3]. The G-protein family consists of two major groups: small monomeric GTPases and heterotrimeric G proteins. Both types of G proteins are regulated by a GDP/GTP cycle in which GTP binding activates them[1,4,7–9]. The majority of G proteins are associated with GDP in cells and are considered to be in an inactive form.

G proteins control chemotaxis, that is, directed cell migration towards chemoattractants. Following ligand binding, chemoattractant receptors, such as G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases, activate small GTPase Ras by generating its GTP-bound form. The activated Ras GTPase in turn leads to the phosphorylation of a critical serine/threonine kinase, AKT (also known as protein kinase B), to regulate the actin cytoskeleton[10–15]. This AKT phosphorylation is controlled by two evolutionarily-related kinases, target of rapamycin (TOR) and phosphoinositide 3-kinase (PI3K)[14]. One of the TOR-containing serine/threonine kinase complexes, mTORC complex 2 (mTORC2), phosphorylates AKT directly[16–18].

This mTORC2-mediated AKT phosphorylation requires the recruitment of AKT to the plasma membrane. Following chemoattractant stimulation, PI3K generates phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and PIP3 recruits AKT to the plasma membrane through its interaction with a PIP3-binding pleckstrin homology domain of AKT[19–21]. Ras–GTP interacts directly with PI3K through its Ras-binding domain and this interaction stimulates PIP3 production by PI3K. It has been shown that Ras–GTP also interacts with mTORC2 and stimulates AKT phosphorylation when overexpressed in cells[22–24]; however, how Ras regulates the enzymatic activity of mTORC2 against AKT is unknown. To address this critical question, it is essential to determine the function of Ras in mTORC2 activity by separating its function in the PI3K pathway.

In addition, PIP3 stimulates the reorganization of the actin cytoskeleton, probably through the activation of members of the Rho family GTPases, Rac and Rho[25–28]. Rac controls actin polymerization and network formation at the front of the migrating cells that extend pseudopods. Coordinating with pseudopod extension, Rho regulates actomyosin contraction at the rear end of the cell to move the cytoplasm forward[15]. However, it is unknown whether Rac and Rho have a role in controlling mTORC2 in addition to their known roles downstream of mTORC2 and PI3K signalling.

In the current study, we found that Rho forms a signalling supercomplex with Ras and mTORC2 and activates mTORC2-mediated AKT phosphorylation in vivo and in vitro. A critical regulatory step in this activation mechanism is not GTP binding to Rho, but rather the chemoattractant-stimulated serine phosphorylation of Rho–GDP by glycogen synthase kinase-3 (GSK-3). Our findings provide a principle that controls G proteins through a combination of GDP binding and phosphorylation, and identifies a direct activation mechanism of mTORC2 towards AKT.

Results
RacE–GDP promotes directed cell migration. In Dictyostelium, all 20 members of the Rho family GTPases have been named Rac ( Supplementary Fig. 1a). We have previously shown that in Dictyostelium discoideum cells the closest homologue of human RhoA, RacE29 (Supplementary Fig. 1b), is required for directed cell migration towards the chemoattractant CAMP but not for random cell migration (Fig. 1a,b and Supplementary Fig. 2)30,31. To investigate the mechanism by which RacE controls chemotaxis, we expressed green fluorescent protein (GFP) fused to wild-type (WT) RacE, GDP-bound RacE T23N or GTP-bound RacE G20V in RacE-knockout cells.
**Fig. 1 | RacE–GDP functions in directed cell migration.** a–b, Cell migration towards the cAMP was analysed in WT and RacE-KO Dictyostelium cells carrying WT RacE, GDP-bound RacET25N, GTP-bound RacEG20V, or effector-domain-defective RacET43A for 60 min in a microfluidic chamber (a). Scale bar, 100 µm. Chemotaxis efficiency was quantified by measuring the number of cells that moved towards a higher concentration of cAMP (observation window; b). The chemotaxis efficiency in RacE-KO cells expressing KO + RacE was set as 100% (n = 4 independent experiments). P values are shown for the comparisons to KO + RacE. c–e, Guanine-nucleotide binding to RacE. Differentiated Dictyostelium cells carrying GFP, GFP–RacE, GFP–RacET25N or GFP–RacEG20V were metabolically labelled using P32 for 1 h. c, GFP fusion proteins were immunopurified using GFP-Trap beads and analysed using SDS-PAGE and Coomassie Brilliant Blue (CBB) staining. d, Bound guanine nucleotides were analysed by thin-layer chromatography and phosphoimaging. e, Quantification of GDP (left) and GTP (right; n = 5 independent experiments for GFP–RacE and GFP–RacEG20V, and n = 4 independent experiments for GFP–RacET25N). P values are shown for comparison of GFP–RacE with others. Data in b,e are shown as the average ± s.d. Significance was calculated using analysis of variance (ANOVA) with a Tukey’s post-hoc test.

(KO) cells that normally grow on solid substrates. We examined the chemotactic cell migration towards extracellular cAMP using a microfluidic chamber (Fig. 1a). Surprisingly, GDP-bound RacET25N almost completely rescued the migration defects in RacE-KO cells, similar to WT RacE (Fig. 1a,b), whereas GTP-bound RacEG20V or effector-domain-defective RacET43A failed to do so. Metabolic labelling using P32 showed that WT RacE and RacET25N are mainly associated with GDP, whereas RacEG20V is predominantly bound to GTP (Fig. 1c–e). These data suggest that RacE–GDP mediates directed cell migration.

**RacE–GDP interacts with mTORC2.** Chemoattractants stimulate signal transduction pathways involving mTORC2 and AKT via GPCRs in Dictyostelium cells and human neutrophils. Using proteomic analysis of RacE-binding proteins, we identified two subunits of mTORC2, Tor (TOR homologue) and PiaA (Rictor homologue; Supplementary Figs. 1c, 3 and Supplementary Tables 1, 2). Subsequent co-immunoprecipitation assays showed that both Tor and PiaA specifically co-precipitated WT RacE or GDP-bound RacET25N but not GTP-bound RacEG20V or effector-domain-defective RacET43A (Fig. 2a,b). As described above, WT RacE is primarily GDP-bound; therefore, RacE–GDP specifically interacts with mTORC2 and this interaction requires the effector domain. Unlike RacE, a homologue of human Rac1, Dictyostelium RacA (Supplementary Fig. 1b) failed to interact with mTORC2 regardless of its guanine-nucleotide-binding status (Fig. 2c). To determine whether GDP-dependent interactions between Rho and mTORC2 are conserved in humans, we performed co-immunoprecipitation of yellow fluorescent protein (YFP)–RhoA and YFP–Rac1 with mTORC1 and -2 in human embryonic kidney (HEK) 293T cells. GDP-bound RhoA bound most strongly to the two mTORC2 subunits, Tor and Rictor, but did not bind the mTORC1 subunit Raptor (Fig. 2d,e).

**mTORC2-mediated AKT phosphorylation requires RacE–GDP in cells.** To determine the function of RacE in mTORC2 signalling in cells, we first examined the impact of RacE loss on the phosphorylation of AKT, a major substrate of mTORC2. Two AKT homologues (PkbA and PkbB1) are phosphorylated by mTORC2 in the hydrophobic motif and by PDK in the activation loop (Fig. 2f and Supplementary Fig. 4). Both sites were transiently phosphorylated in response to GPCR activation by cAMP in WT cells but not in cells lacking the Rictor homologue PiaA (Fig. 2g,h), as previously reported. Similarly to observations in PiaA-KO cells, chemoattractant-induced AKT phosphorylation was greatly decreased in...
Fig. 2 | RacE-GDP specifically interacts with mTORC2. a, Dictyostelium cell lysates carrying GFP fused to the indicated forms of RacE were incubated with cell lysates carrying FLAG-Tor and subjected to immunoprecipitation with GFP-Trap (left). Quantification of the interactions is shown (right). The band intensity of FLAG–Tor in the immunoprecipitates of cells expressing WT RacE was set as 100% (n = 6, 6, 6, and 3 independent experiments for RacE, RacE\textsubscript{G20V}, RacE\textsubscript{T25N}, and RacE\textsubscript{E43A}, respectively). P values are shown for the comparisons to RacE. b, Dictyostelium cell lysates carrying the indicated forms of GFP–RacE were subjected to immunoprecipitation with GFP–Tor (left) to analyse its association with endogenous PiaA (right). The band intensity of PiaA in the immunoprecipitates of the cells expressing WT RacE was set as 100% (n = 3 independent experiments). c, Dictyostelium cell lysates carrying GFP fused to the indicated forms of Rac1A and RacE were incubated with cell lysates carrying FLAG–T or and subjected to immunoprecipitation using GFP–Tor (left). d, HEK293T cells were transfected with YFP fused to the indicated constructs of human Rac1 and RhoA, and subjected to immunoprecipitation using GFP–Tor (left). The band intensities of Tor and Rictor in the immunoprecipitates of the cells expressing WT RhoA was set as 100% (right; n = 3 and n = 4 independent experiments for Tor (top) and Rictor (bottom), respectively). P values are shown for the comparisons to YFP–RhoA. e, Summary of the data in a–d. f, AKTs are phosphorylated in the hydrophobic motif by mTORC2 and in the activation loop by PDK. g, h, WT, RacE-KO and PiaA-KO Dictyostelium cells were stimulated with cAMP (1 μM; n = 3 independent experiments). The total quantities of two AKT homologues (PkbR1 and PkbA) and their phosphorylation (hydrophobic motif in red and activation loop in green) were analysed by immunoblotting (g). PVDF membranes were stained with CBB as a loading control (g, bottom). h, Quantification of the band intensity of phosphorylated AKTs. WT cells at 30 s were set at 100%. Data in a,b,d,h are shown as the average ± s.d. Significance in a,b,d was calculated using ANOVA with a Tukey’s post-hoc test. IP, immunoprecipitation.
RacE–KO cells (Fig. 2g,h). The total quantities of endogenous PkbR1 and PkbA did not change in either PiaA-KO or RacE–KO cells in the presence or absence of chemoattractant simulation (Fig. 2g). Second, we expressed WT RacE and various RacE mutants in RacE–KO cells, and then stimulated these cells with cAMP. Intriguingly, WT RacE and GDP-bound RacE$_{T25N}$ but not GTP-bound RacE$_{G20V}$ or effector-domain-defective RacE$_{F168A}$ restored mTORC2-mediated AKT phosphorylation in response to chemoattractants (Fig. 3a–d). It has been shown that a mutation (G17V) in human RhoA, which dissociates GTP from RhoA, is a prevalent driver mutation in T-cell lymphoma$^{36,41}$; in cells carrying RhoA$_{G17V}$, AKT phosphorylation is increased. To test whether an equivalent mutation promotes AKT phosphorylation in Dictyostelium, we expressed RacE$_{G20V}$ in WT and RacE–KO cells and found that RacE$_{G20V}$ increases the phosphorylation of PkbR1 and PkbA in both cell types (Fig. 3c–f). The expression levels of the GFP–RacE proteins were comparable in cells (Supplementary Fig. 5). Third, the mTORC2 inhibitor PP242 completely blocked cAMP-induced AKT phosphorylation in WT and RacE–KO cells expressing WT RacE (Fig. 3g,h). Fourth, AKT phosphorylation requires RasC and is increased by ectopic expression of GTP-bound RacC$_{G20V}$ (Fig. 3i). Fifth, consistent with the function of RacC–GDP and RasC–GTP in chemotactic signalling, metabolic labelling with $^{32}$P showed that GDP binding to RacE and GTP binding to RasC significantly increased in response to cAMP (Supplementary Fig. 6).

GSK-3 phosphorylates RacE at S192 in response to chemotactic stimulation. Previous phosphoproteomics have suggested that chemoattractant stimulation induces RacE phosphorylation at S192 in Dictyostelium cells$^{42}$. Using antibodies raised to S192-phospho-RacE, we found that RacE indeed undergoes transient phosphorylation following chemoattractant stimulation (Fig. 4a). To confirm the specificity of our S192-phospho-RacE antibodies, we expressed GFP–RacE, C-terminal phospho-defective GFP–RacE$_{G20A}$ and phospho-mimetic GFP–RacE$_{G20D}$ in WT cells and stimulated these cells with cAMP. We found that GFP–RacE was transiently phosphorylated at S192, similarly to endogenous RacE (Fig. 4b). In contrast, phospho-defective GFP–RacE$_{G20A}$ was not detected by anti-phospho–RacE(S192) antibodies (Fig. 4b). As a positive control, phospho-mimetic GFP–RacE$_{G20D}$ was detected by anti-phospho–RacE(S192) antibodies even in the absence of chemotactic stimulation.

To identify the protein kinase that phosphorylates RacE in response to chemoattractants, we first analysed the amino acid sequence around S192 and found a GSK-3-substrate motif (Fig. 4c). A modelled structure of RacE based on human RhoA suggested that S192 is located in a C-terminal unstructured loop of RacE (Fig. 4d). To determine whether S192 phosphorylation depends on GSK-3 in cells, we pretreated Dictyostelium cells with LY2090314 and lithium, two structurally-distinct inhibitors specific to GSK-3, and stimulated these cells with cAMP. Immunoblotting showed that the chemoattractant-induced S192 phosphorylation of RacE was completely blocked (Fig. 4e,f). Conversely, other inhibitors of PI3K (LY294002) and mTORC2 (PP242) did not inhibit this RacE phosphorylation (Fig. 4g). We also found no inhibition of the RacE phosphorylation in WT cells treated with an AKT inhibitor (afuresertib; Fig. 4h) or cells lacking either PkbA or PkbR1 (Fig. 4i). To investigate whether GSK-3 directly phosphorolyses RacE at S192, we incubated purified human GSK-3β with purified FLAG–RacE, FLAG–RacE$_{T25N}$ (GDP-bound) or FLAG–RacE$_{G20A}$ (phospho-defective). We found that GSK-3β phosphorylates FLAG–RacE and FLAG–RacE$_{T25N}$ but not FLAG–RacE$_{G20A}$ on S192 in vitro (Fig. 4j). This in vitro phosphorylation of RacE by purified GSK-3β was inhibited by the GSK-3 inhibitor LY2900314 (Fig. 4k). These data suggest that GSK-3 directly phosphorylates S192 in RacE in response to chemoattractant simulation (Fig. 4j).

Chemoattractant-induced phosphorylation of RacE–GDP at S192 controls mTORC2-mediated AKT phosphorylation and directed cell migration. When ectopically expressed in RacE–KO cells, phospho-defective RacE$_{S192A}$ did not induce AKT phosphorylation in response to chemoattractant simulation (Fig. 5a,b). Conversely, phospho-mimetic RacE$_{S192D}$ increased AKT phosphorylation even without stimulation (0 s in Fig. 5a,b). The expression levels of these GFP–RacE proteins were comparable in cells (Supplementary Fig. 5). GTP-bound RacE$_{G20V}$ remained inactive even when combined with the S192D mutation (Fig. 5c,d), suggesting that the phosphorylation of RacE–GDP at S192 is crucial for the activation of mTORC2.

Both the phospho-mimetic mutants RacE$_{S192D}$ and GTP-bound RacE$_{T25N;S192D}$ rescued defects in directed cell migration towards cAMP in RacE–KO cells (Fig. 5e,f). In contrast, the phospho-defective RacE$_{S192A}$ mutation blocked the ability of WT RacE and RacE$_{T25N}$ to reverse the cell-migration defect (Fig. 5e,f). Furthermore, the mTORC2 inhibitor PP242 blocked the cell migration of RacE–KO cells expressing WT RacE or phospho-mimetic RacE$_{S192D}$ whereas LY2090314 only blocked the migration of RacE–KO cells expressing WT RacE (Fig. 5g,h). Therefore, chemoattractant stimulation induces the GSK-3-mediated S192 phosphorylation of RacE–GDP to activate mTORC2-mediated AKT phosphorylation in directed cell migration.

Reconstitution of GDP-bound RacE-regulated mTORC2 activation in vitro. To elucidate the molecular mechanism by which Rac–GDP regulates mTORC2, we reconstituted the chemoattractant-induced activation of mTORC2 using proteins purified from Dictyostelium cells (Fig. 6). To purify mTORC2 as an active kinase complex, we immunopurified FLAG–Tor under low-salt conditions in the presence of the detergent CHAPS, as described$^{34,44}$ (Supplementary Fig. 7a–c). We used inactive human AKT as a substrate and detected its phosphorylation using anti-phospho-AKT antibodies (S473)$^{34,44}$. AKT was robustly phosphorylated when incubated with mTORC2, chemoattractant-stimulated RacE and GTP-bound RasC$_{G20V}$ (Fig. 6a, lane 3). We omitted ATP, RacE, RasC and mTORC2 or used kinase-dead Tor$_{G14A}$ for the negative controls (Fig. 6a, lanes 2 and 4–8).

The Tor kinase forms mTORC2 with other subunits, including Rictor, mLst8 and mSin1 (Supplementary Fig. 1c)$^{38,44}$. To define the minimum set of subunits required for mTORC2 activation, we purified mTORC2 from WT cells or cells lacking each subunit (Rictor/PiaA-KO, Lst8-KO or mSin1/Rip3-KO) using FLAG–Tor under low-salt conditions. Although mTORC2 purified from WT cells phosphorylated AKT, mTORC2 purified from any of these KO cells failed to do so (Fig. 6b, lanes 1, 2, 5 and 8). We then added high-salt-washed purified FLAG–Lst8 or FLAG–PiaA to the purified mTORC2 (Fig. 6b and Supplementary Fig. 7f). FLAG–Lst8 restored AKT phosphorylation when added to mTORC2 purified from Lst8-KO cells but not Rip3-KO or PiaA-KO cells (Fig. 6b, lanes 4, 7 and 10). Surprisingly, FLAG–PiaA restored AKT phosphorylation when added to mTORC2 purified from Rip3-KO cells (Fig. 6b, lane 2). Whole cell lysates from WT and Rip3-KO cells contained similar quantities of PiaA (Supplementary Fig. 7d). These data suggest that PiaA and Lst8 are essential subunits of mTORC2 for AKT phosphorylation, whereas the mSin1 homologue Rip3 stabilizes the interactions between PiaA and mTORC2.

To further test this idea, we purified FLAG–Tor under high-salt conditions, which failed to phosphorylate AKT (Fig. 6c, lane 2). We added FLAG–PiaA and FLAG–Lst8 back to the FLAG–Tor. AKT phosphorylation was restored only when both subunits were added together but not individually (Fig. 6c, lanes 3–5). Therefore, the
Fig. 3 | RacE-GDP promotes chemoattractant-induced, mTORC2-mediated AKT phosphorylation in cells. The indicated Dictyostelium cell lines were stimulated with cAMP (1 μM). a–f, Analysis of WT and RacE-KO cells expressing different GFP–RacE constructs. g,h. WT and RacE-KO cells expressing GFP–RacE were pretreated with 0.5 μM of the mTORC2 inhibitor PP242 for 10 min before stimulation with cAMP. i,j. Analysis of WT and RacE-KO cells expressing FLAG-tagged RasC or GTP-bound RasC(Q62L). a–j. The total amounts of two AKT homologues (PKB1 and PKBα) and their phosphorylation (hydrophobic motif in red and activation loop in green) were analysed by immunoblotting. The PVDF membranes were stained with CBB as loading controls in a,c,e,i. The band intensity of phosphorylated AKTs was quantified in b,d,f,h,j. WT cells at 30 s (b,d,f,h) and WT cells expressing RasC at 30 s (j) were set at 100%. Data are shown as the average ± s.d. (n = 3 independent experiments).
RacET25N or phospho-defective RacES192A for 15 min. S192 phosphorylation of RacE was tested by immunoblotting. Purified human GSK-3β was incubated with purified WT, phospho-mimetic RacES192D and phospho-defective RacES192A for 10 min. The cells were then stimulated with cAMP for the indicated times. The whole cell lysates were analysed by immunoblotting with antibodies to RacE and phospho-RacE(S192).

Fig. 4 | GSK-3 phosphorylates RacE at S192 in response to chemoattractant. a, WT and RacE-KO cells were stimulated with cAMP (1 µM). The total amounts of RacE and its phosphorylation at S192 were analysed by immunoblotting with antibodies to RacE and phospho-RacE(S192). WT cells expressing GFP fused to WT RacE, phospho-defective RacE(S192) or phospho-mimetic RacE(S192) were stimulated with cAMP. The whole cell lysates prepared at the indicated time points were analysed by immunoblotting with antibodies to RacE and phospho-RacE(S192). Amino acid sequence in the vicinity of S192 in a modelled three-dimensional RacE structure.

b, WT cells expressing GFP fused to WT RacE, phospho-defective RacE(S192) or phospho-mimetic RacE(S192) were stimulated with cAMP. The whole cell lysates prepared at the indicated time points were analysed by immunoblotting with antibodies to RacE and phospho-RacE(S192). The experiments in a, b, e, f, j, k, l were repeated independently three times with similar results.

tmTORC2 components Tor, PiaA and Lst8 are sufficient to catalyse GDP-bound RacE-promoted AKT phosphorylation.

S192 phosphorylated RacE–GDP activates mTORC2-mediated AKT phosphorylation in vitro. Using this reconstitution system, we tested the effect of the guanine-nucleotide-binding status of RacE and RasC on mTORC2 activation in vitro. First, we removed guanine nucleotides from purified FLAG–RacE using EDTA and found that mTORC2 activation was completely blocked (Fig. 6d, lane 3). Second, no mTORC2 activation was observed when FLAG–RacE was loaded with the non-hydrolyzable GTP analogue GTPγS (Fig. 6d, lane 4). The subsequent replacement of GTPγS with GDP restored the ability of RacE to activate mTORC2 (Fig. 6d, lane 5). These guanine-nucleotide-switching experiments ruled out the possibility that other proteins, such as guanine-nucleotide exchange factors that might associate with GDP-bound RacE, contribute to mTORC2 activation. Third, WT RacE and GDP-bound RacE(S192N) but not GDP-bound RacE(G20V) activated mTORC2 (Fig. 6e). Fourth, GDP-bound RasG12,22, did not activate mTORC2 regardless of its guanine-nucleotide-binding status (Fig. 6f, lane 3, 4, 7 and 8).

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Chemoattractant stimulation of *Dictyostelium* cells before the purification of FLAG–RacE was required for the reconstitution of mTORC2 activation (Fig. 6d, lanes 1 and 2 and Fig. 6c, lanes 1–4). This chemoattractant stimulation was no longer necessary when phospho-mimetic GDP-bound RacE$_{T25N,S192D}$ was used (Fig. 6g, lanes 1 and 5). Conversely, phospho-defective GDP-bound RacE$_{T25N,S192A}$ failed to activate mTORC2-mediated AKT phosphorylation irrespective of the chemoattractant stimulation (Fig. 6g, lanes 3 and 4).
Fig. 6 | Phosphorylated Rac-E–GDP activates mTORC2 in vitro. Purified proteins were used to reconstitute mTORC2-mediated AKT phosphorylation. FLAG–RacE, FLAG–RacC/G and mTORC2 (FLAG–Tor, -PiaA and -Lst8) were purified from Dictyostelium cells. +cAMP, cells were stimulated by the chemoattractant. mTORC2 Activation requires PiaA and Lst8 but not Rip3. FLAG–PiaA and FLAG–Lst8 were added to FLAG–Tor or purified from the indicated KO cell lines in a, Activation of mTORC2 requires RacE–GTP but not RasG–GTP. g, Rac phosphorylation controls mTORC2 activation. The S192D mutation in GDP-bound RacET25N activates mTORC2 without chemoattractant stimulation, whereas the phospho-defective RacES192A mutation blocks it. h, Activation of mTORC2 requires RasC–GTP but not RasG–GTP. i, Activation of mTORC2 requires RasC–GTP but not RasG–GTP. j, Rac phosphorylation controls mTORC2 activation. The S192D mutation in GDP-bound RacET25N activates mTORC2 without chemoattractant stimulation, whereas the phospho-defective RacES192A mutation blocks it. h, Activation of mTORC2 requires RasC–GTP but not RasG–GTP. i, Summary of the data in a–h, RacEG23V–GDP activates mTORC2. Purified RacET25N and RacEG23V were incubated with EDTA followed by GTPγS or GDP before reconstitution. The experiments in a–h,j were repeated independently three times with similar results.
We also found that RasC must be in a GTP-bound form to stimulate mTORC2 in guanine-nucleotide-switching experiments (Fig. 6h). Together, these data show that chemoattractant-stimulated phosphorylation of RacE–GDP at S192 together with RasC–GTP directly activate mTORC2-mediated AKT phosphorylation (Fig. 6i).

RacE$_{G25N}$ is equivalent to the cancer driver mutant RhoA$_{G17N}$ (Fig. 3c–f). The G17V mutation dissociates GTP from RhoA$_{40,41,45-46}^{4}$; however, it is unknown whether RhoA$_{G17N}$ is present in a guanine-nucleotide-free or GDP-bound form. Using our reconstitution system, we tested the effect of the guanine-nucleotide-binding status of RacE$_{G25N}$ on mTORC2 activation. Similar to GDP-bound FLAG–RacE$_{G25N}$, purified FLAG–RacE$_{G25N}$ stimulated mTORC2-mediated AKT phosphorylation (Fig. 6j, lanes 1 and 2). When we treated FLAG–RacE$_{G25N}$ with EDTA, which removes guanine nucleotides that are potentially associated, this activity was lost (Fig. 6j, lane 4). Subsequent incubation of FLAG–RacE$_{G25N}$ with GDP, but not GTPγS, restored its ability to activate mTORC2 (Fig. 6j, lanes 6 and 8). Therefore, RacE$_{G25N}$ activates mTORC2 in a GDP-bound form.

**S192 phosphorylation of RacE–GDP assembles the RacE–RasC–mTORC2 supercomplex.** To understand the biochemical basis of this phosopho-mediated activation, we purified GDP-bound FLAG–RacE$_{G25N}$ from Dictyostelium cells under high-salt conditions after chemoattractant stimulation and tested its interaction with purified FLAG–RasC proteins (Fig. 7a and Supplementary Fig. 7e). The chemoattractant stimulation enabled GDP-bound RacE$_{G25N}$ to interact specifically with GTP-bound RasC$_{G12N}$, but not GDP-bound RasC$_{G12N}$ (Fig. 7a, lanes 2 and 8). The phospho-defective RacE$_{S192A}$ mutation abolished this chemoattractant-induced interaction of RacE–GDP with RasC–GTP (Fig. 7a, lanes 2 and 4). Conversely, the phosphomimetic S192D mutation bypassed the requirement for chemoattractant stimulation (Fig. 7a, lanes 1 and 5). GTP-bound RacE$_{G25N}$ did not interact with either RasC–GTP or RasC–GDP, even after chemoattractant stimulation (Fig. 7b, lanes 3 and 4). Therefore, S192 phosphorylation of RacE–GDP enables its interaction with RasC–GTP.

Phosphorylated RacE also binds Tor. GDP-bound RacE$_{G25N}$ purified after chemoattractant stimulation bound FLAG–Tor that had been purified under high-salt conditions (Fig. 7c, lane 5). Although it has been shown that recombinant GTP-loaded RasC binds the kinase domain of Tor purified from *Escherichia coli*$_{22,23}^{22}$, RacS or RasG failed to bind Tor in our binding assay, regardless of their guanine-nucleotide binding status (Fig. 7c, lanes 1–4). Interactions of phosphorylated RacE with Tor require its GDP binding but are independent of RasC (Fig. 7d). Furthermore, chemoattractant-stimulated GDP-bound RacE bridges GTP-bound RasC and Tor (Fig. 7e). Finally, interactions between RacE and RasC or Tor were sensitive to the pretreatment of chemoattractant-stimulated cells with LY2090314 before the purification of RacE (Fig. 7f,g); the S192D mutation overcame this inhibition effect (Fig. 7f,g, lanes 5 and 6). These data suggest that chemoattractant-induced S192 phosphorylation of GDP-bound RacE assembles the RacE–RasC–mTORC2 supercomplex that phosphorylates AKT (Fig. 7h).

**Discussion**

In this study, we discovered an unforeseen mechanism by which the phosphorylation of GDP-bound Rho GTPase activates mTORC2 signalling in GPCR-mediated directed cell migration. We propose that phosphorylated Rho–GDP forms the super signalling complex with Ras–GTP and mTORC2 (Fig. 7h). Our findings change the current view that switching guanine nucleotides from GDP to GTP activates G proteins$_{47-49}^{47-49}$. Because the majority of the G proteins are in a GDP-bound form, this mechanism for G-protein regulation broadens the understanding of intracellular signal transduction mediated by one of the largest protein families that control many biological processes substantially. Our findings also demonstrate a role for Rho as an upstream component that connects GPCR activation to mTORC2 to initiate a signalling cascade. This role of Rho revises the prevailing model that Rho is a downstream regulator that couples signalling events to the remodelling of the actin cytoskeleton. In our previous studies, we identified a potential GTPase-activating protein (GfpB) and guanine-nucleotide exchange factor (GxcT) for RacE$_{G12N}^{20,21}$. As both GfpB and GxcT contribute to directed cell migration, it would be important to decipher how they regulate the function of phosphorylated RacE–GDP in mTORC2 activation in future studies.

We propose that phosphorylated RacE–GDP simultaneously binds both RasC–GTP and mTORC2 (Fig. 7i). In this supercomplex, RacE probably binds RasC and mTORC2 through distinct regions. We speculate that Tor binds the effector domain of RacE. On the other hand, RasC might interact with RacE in a manner similar to human KRas dimers$_{17-19}^{17-19}$. Our data show that the activation mechanism for mTORC2 by small GTPases is distinct from that for mTORC1. The localization of mTORC1 is regulated by small GTPases, Rags. A Rag dimmer formed by RagA/B-GDP and RagC/D-GTP is anchored to the lysosomal membrane through the regulator protein complex$_{40}^{40}$. In response to increased levels of amino acids, these GTPases switch guanine nucleotides and become a RagA/B-GTP and RagC/D-GDP dimmer. This dimer associates with Raptor and localizes mTORC1 to lysosomes. On the surface of lysosomes, the kinase activity of mTORC1 is activated by interactions with GTP-bound Rheb, a Ras-related GTPase. Similarly to the Rag dimers, RacE and RasC form a dimer in the GDP- and GTP-bound forms, respectively. However, in contrast to the Rag dimers, RacE–RasC dimers directly stimulate the enzymatic activity of mTORC2.

It has been shown that GSK-3 regulates chemotaxis and differentiation in Dictyostelium cells$_{41-43}^{41-43}$; however, the role of GSK-3 in mTORC2–AKT signalling has been controversial: one group showed that GSK-3–KO cells increase this signalling in response to chemotactic stimulation$_{44,45}^{44,45}$, whereas another group reported the opposite effect$_{46}^{46}$. Significantly extending the latter findings, our current work reveals that GSK-3 directly phosphorylates the C-terminus of RacE at S192 following cAMP stimulation to promote the assembly of the mTORC2 supercomplex that phosphorylates AKTs.

In our vitro system identified a minimum set of proteins that reconstitute chemoattractant-stimulated mTORC2 activation. In this system, the kinase Tor together with the Rictor homologues PiaA and LST8, are the necessary mTORC2 subunits and sufficient for activation. The mSin1 homologue Rip3 is important for the stabilization of the interactions between Tor and PiaA in cells, but is not essential for the enzymatic activity regulated by RacE and RasC. Providing supporting to our findings, previous studies using mammalian cells have shown that mSin1 is important for the formation of mTORC2$_{22}^{22}$. In addition, mSin1 contains a lipid-binding pleckstrin homology domain and is necessary for the recruitment of mTORC2 to the plasma membrane$_{43}^{43}$. A mutation (G17V) that releases GTP from human RhoA is a prevalent driver mutation in T-cell lymphoma$_{44,45}^{44,45}$. AKT phosphorylation, the reorganization of the actin cytoskeleton and cell migration are increased in cells carrying RhoA$_{G17V}$ or a GDP-bound RhoA$_{T19N}$. These findings have been explained by the sequestration of guanine-nucleotide-exchange factors through stabilized interactions with the RhoA$_{G17V}$ and RhoA$_{T19N}$ mutant proteins. In contrast to this model, we found that RacE$_{G25N}$ and RacE$_{S192A}$—the RhoA$_{G17V}$ and RhoA$_{T19N}$ counterparts in *Dictyostelium*, respectively—induce AKT phosphorylation in cells and directly activate mTORC2-mediated AKT phosphorylation in a GDP-bound form in vitro. In light of our findings in the *Dictyostelium* Rho GTPase, it would be important to test whether the unique cancer-associated RhoA mutation (G17V) is GDP-bound in cancer cells, activates the kinase activity of mTORC2 towards AKT, and thereby promotes cancer cell proliferation and metastasis.
Fig. 7 | S192 phosphorylated RacE–GDP forms a supercomplex with Tor and Ras–GTP. a,b. The indicated GFP–RacE proteins were purified from Dictyostelium cells with or without 1 µM cAMP stimulation for 30 s. FLAG–RasC proteins were purified without cAMP stimulation. GFP–RacE was incubated with FLAG–RasC and pulled down using GFP–Tor. The pellet fraction was analysed by immunoblotting using antibodies to GFP and FLAG. c. GFP–RacE, GFP–RasC and GFP–RasG were incubated with FLAG–Tor, which was purified under high-salt conditions and pulled down with GFP–Tor. The pellet fraction was analysed by immunoblotting using antibodies to GFP and FLAG. d. GFP fused to GDP-bound RacET25N or GTP-bound RacEG20V were purified from Dictyostelium cells under high-salt conditions after stimulation with cAMP. These GFP fusion proteins were incubated with high-salt-washed FLAG–Tor and/or FLAG–RasC proteins. GFP–RacE was pulled down with GFP–Tor and the pellet fractions were analysed by immunoblotting. e. RacE forms a complex with Tor and RasC. The indicated proteins were purified under high-salt conditions and mixed for 15 min at room temperature. GFP–RasC proteins were pulled down with GFP–Tor and the pellet fraction was analysed by immunoblotting. f,g. Different GFP–RacE proteins were purified from Dictyostelium cells with or without 1 µM cAMP stimulation for 30 s in the presence or absence of LY2090314 (250 nM). GFP–RacE was incubated with FLAG–RasC (f) or FLAG–Tor (g) and pulled down using GFP–Tor. The pellet fraction was analysed by immunoblotting using antibodies to GFP and FLAG. h. Model of the GPCR-mediated mTORC2–AKT signalling. In response to GPCR activation by chemoattractant, Rho–GDP becomes phosphorylated by GSK-3 and assembles the super signalling complex with Ras–GTP and mTORC2 to promote AKT phosphorylation. The experiments in a–g were repeated independently three times with similar results.
Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41556-019-0348-8.

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References
1. Hodge, R. G. & Ridley, A. J. Regulating Rho GTPases and their regulators. Nat. Rev. Mol. Cell Biol. 17, 496–510 (2016).
2. Burridge, K. & Wennerberg, K. Rho and Rac take center stage. Nat. Rev. Mol. Cell Biol. 17, 97–109 (2016).
3. Stenmark, H. Rab GTPases as coordinators of vesicle traffic. Nat. Rev. Mol. Cell Biol. 10, 513–525 (2009).
4. Alberts, B. et al. Molecular Biology of the Cell 6th Edn (Garland Science, 2015).

18. Iden, S. & Collard, J. G. Crosstalk between small GTPases and polarity. Nat. Rev. Mol. Cell Biol. 16, 232–244 (2015).
19. Manning, B. D. & Toker, A. AKT/PKB signaling: navigating the network. Cell 169, 1172–1184 (2017).
20. Lien, E. C., Dibble, C. C. & Toker, A. PI3K signaling in cancer: beyond AKT. Nat. Rev. Mol. Cell Biol. 15, 155–162 (2014).
21. Khanna, A. et al. The small GTPases Ras and Rap1 bind to and control migration. J. Leukoc. Biol. 82, 103–116 (2007).
22. Artemenko, Y., Lampert, T. J. & Devreotes, P. N. Moving towards a paradigm: common mechanisms of chemotactic signaling in Dictyostelium and mammalian leukocytes. Cell. Mol. Life Sci. 71, 3711–3747 (2014).
23. Miao, Y. et al. Allerting the threshold of an excitable signal transduction network changes cell migratory modes. Nat. Cell Biol. 19, 329–340 (2017).
24. Gaubitz, C., Prouteau, M., Kusmider, B. & Loewith, R. TORC2 structure and function. Trends Cell Biol. 18, 401–411 (2018).
25. Mayor, R. & Etienne-Manneville, S. The front and rear of collective cell migration. Nat. Cell Biol. 16, 155–162 (2014).
26. Welch, C. M., Elliott, H., Danuser, G. & Hahn, K. M. Imaging the green fluorescent protein. Mol. Biol. Cell 11, 935–944 (1997).
27. Gaubitz, C., Prouteau, M., Kusmider, B. & Loewith, R. TORC2 structure and function. Trends Cell Biol. 18, 401–411 (2018).
28. Iden, S. & Collard, J. G. Crosstalk between small GTPases and polarity. Nat. Rev. Mol. Cell Biol. 16, 232–244 (2015).
29. Kamimura, Y., Tang, M. & Devreotes, P. Assays for chemotaxis and chemosensitization-stimulated TorC2 activation and PKB substrate phosphorylation in Dictyostelium. Methods Mol. Biol. 571, 255–270 (2009).
30. Palomero, T. et al. Recurrent mutations in epigenetic regulators, RHOA and FYN kinase in peripheral T cell lymphomas. Nat. Genet. 46, 166–170 (2014).
31. Senoo, H., Cai, H., Wang, Y., Sesaki, H. & Iijima, M. The novel Rac-binding protein GSB sharps Ras activity at the leading edge of migrating cells. Mol. Biol. Cell 27, 1596–1605 (2016).
32. Wang, Y., Chen, C. L. & Iijima, M. Signal transduction mechanisms for chemotaxis. Dev. Growth Differ. 53, 495–502 (2011).
33. Nicholls, J. M., Veltman, D. & Kay, R. R. Chemotaxis of a model organism: progress with Dictyostelium. Curr. Opin. Cell Biol. 36, 7–12 (2015).
34. Kortholt, A. & van Haastert, P. J. Highlighting the role of Ras and Rap during Dictyostelium chemotaxis. Cell. Signal. 20, 1415–1422 (2008).
35. Gerisch, G., Schroth-Diez, B., Müller-Tauberbeneger, A. & Ecke, M. Pip3 waves and PTEN dynamics in the emergence of cell polarity. Biophys. J. 103, 1170–1178 (2012).
36. Chen, M. Y., Long, Y. & Devreotes, P. N. A novel cytosolic receptor, Pianisimo, is required for chemotactant receptor and G protein-mediated activation of the 12 transmembrane domain adenylyl cyclase in Dictyostelium. Genes Dev. 11, 3218–3231 (1997).
37. Shimobayashi, M. & Hall, M. N. Making new contacts: the mTOR network in metabolism and signaling crosstalk. Nat. Rev. Mol. Cell Biol. 15, 355–366 (2014).
38. Liao, X. H., Buggy, J. & Kimmel, A. R. Chemotactic activation of Dictyostelium AGC-family kinases AKT and PKR1 requires separate but coordinated functions of PDK1 and TORC2. J. Cell Sci. 123, 983–992 (2010).
39. Kamimura, Y., Tang, M. & Devreotes, P. Assays for chemotaxis and chemosensitization-stimulated TorC2 activation and PKB substrate phosphorylation in Dictyostelium. Methods Mol. Biol. 571, 255–270 (2009).
40. Palomero, T. et al. Recurrent mutations in epigenetic regulators, RHOA and FYN kinase in peripheral T cell lymphomas. Nat. Genet. 46, 166–170 (2014).
41. Yoo, H. Y. et al. A recurrent inactivating mutation in RHOS1 GTPase in angiogenicinhibitory T cell lymphomas. Nat. Genet. 46, 371–375 (2014).
42. Charest, P. G. et al. A Ras signaling complex controls the RasC-TORC2 pathway and directed cell migration. Dev. Cell 18, 737–749 (2010).
43. Huang, J. An in vitro assay for the kinase activity of mTOR complex 2. Methods Mol. Biol. 821, 75–86 (2012).
44. Sarbasov, D. G., Bulgakova, O., Bersimbaev, R. I. & Shokan, T. Isolation of the mTOR complex by affinity purification. Methods Mol. Biol. 821, 59–74 (2012).
45. Gilbert-Ross, M., Marcus, A. I. & Zhou, W. RhoA, a novel tumor suppressor oncogene as a therapeutic target? Genes Dis. 2, 2–3 (2015).
46. Dusso, P. et al. Mechanisms of inactivation of the tumour suppressor gene RHOA in colorectal cancer. Br. J. Cancer 118, 106–116 (2018).
47. Ambrocio, C. et al. KRAS dimerization impacts MEK inhibitor sensitivity and oncogenic activity of mutant KRAS. Cell 172, 857–868 (2018).
48. Jang, H., Muratcioglu, S., Gursoy, A., Keskin, O. & Nussinov, R. Membrane-associated Ras dimers are isoform-specific: K-Ras dimers differ from H-Ras dimers. Biochem. J. 473, 1719–1732 (2016).
49. Nan, X. et al. Ras-GTP dimers activate the mitogen-activated protein kinase (MAPK) pathway. Proc. Natl Acad. Sci. USA 112, 7996–8001 (2015).
50. Harwood, A. J., Pytle, S. E., Woodgett, J., Strutt, H. & Kay, R. R. Glycogen synthase kinase 3 regulates cell fate in Dictyostelium. Cell 80, 139–148 (1993).
51. Kim, L., Liu, J. & Kimmel, A. R. The novel tyrosine kinase ZAK1 activates GSK3 to direct cell fate specification. Cell 99, 399–408 (1999).
52. Kolsch, V. et al. Daydreamer, a Ras effector and GSK-3 substrate, is important for directional sensing and cell motility. Mol. Biol. Cell 24, 100–114 (2013).
53. Lacal Romero, J. et al. The Dictyostelium GSK3 kinase GIA coordinates signal relay and chemotaxis in response to growth conditions. Dev. Biol. 345, 56–72 (2018).
54. Teo, R. et al. Glycogen synthase kinase-3 is required for efficient Dictyostelium chemotaxis. Mol. Biol. Cell 21, 2788–2796 (2010).

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Author contributions
H. Senoo, H. Sesaki and M.I. conceived the project and designed the study. H. Senoo and M.I. performed the experiments. R.K. assisted with the experiments. S.S., A.N. and Y.K. provided valuable reagents and equipment. H. Senoo, H. Sesaki and M.I. wrote the manuscript.

Competing interests
The authors declare no competing interests.

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Methods

**Cells and plasminids.** Disruption of the *RacE*, *PieA*, *Rip3* and *Lst8* genes by homologous recombination using the blasticidin resistance cassette in *D. discoideum* cells was done as previously described.\(^1\) WT, *Paa-A-KO, Rip3-KO* and *Lst8-KO* plasminid cells were cultured in HB-3 medium (1% protease peptone, 1% glucose, 0.5% yeast extract, 2.5 mM NaHPO\(_4\), and 2.5 mM KH\(_2\)PO\(_4\) (pH 6.5)) on a rotary shaker at 200 rpm and 22 °C. *Rae-C-KO* cells were cultured in HB-3 medium in Petri dishes and shaken for 1 d before use in experiments.\(^2\) Plasminid cells were then further incubated in the presence of 2 mM caffeine for 20 min.\(^3\) The cells at 10\(^8\) cells ml\(^{-1}\) were moved towards a higher concentration of cAMP by 10 µM adenylyl 3',5'-cyclic monophosphate (cAMP) pulses at intervals of 6 min for 4 h. To induce FL--RasC/G expression, cells growing exponentially were washed with development buffer (2 mM MgSO\(_4\), 0.2 mM CaCl\(_2\), 5 mM NaHPO\(_4\), and 5 mM KH\(_2\)PO\(_4\) (pH 6.5)), resuspended at 2 × 10\(^8\) cells ml\(^{-1}\), starved for 1 h and shaken with 100 nM cAMP pulses at intervals of 6 min for 4 h. To induce FL--RasC/G expression, cells growing exponentially were washed with development buffer (DB; 2 mM MgSO\(_4\), 0.2 mM CaCl\(_2\), 20 mM 2-(N-morpholino)ethanesulfonic acid (MES; pH 6.5)), washed in DB-MES and resuspended at 10\(^8\) cells ml\(^{-1}\) and 40 ng inactive human AKT1 (Sigma, cat. no. 14-279) in 80 µl CHAPS lysis buffer, snap frozen in liquid nitrogen and stored at −80 °C. In the experiments described in Fig. 6b,c, in which individual subunits of mTORC2 were tested for AKT phosphorylation in vitro, FL--Paa and FL--Lst8 were purified as described above and then washed twice in high-salt conditions (500 mM NaCl and 50 mM HEPES (pH 7.4)).

**Immunoblotting.** Proteins were separated using SDS--PAGE and transferred onto PVDF membranes. The antibodies used were to: GFP\(^{-}\),\(^1\) FL-- (Sigma, F7425 and F7426), *Paa* (Tor) (Cell Signaling, cat. no. 2981), Lst8 (Cell Signaling, cat. no. 2114), Raptor (Cell Signaling, cat. no. 2280), G3P (Bio-Rad), phospho-p85β (Cell Signaling, cat. no. 9025), phospho-p53 (Cell Signaling, cat. no. 9722) and phospho-AKT (S473) (Cell Signaling, cat. no. 9271). Polyclonal rabbit antibodies to PKB and PDK1 were raised against the peptides KNSD3RRKVKG and KCKGNKNDIETT, respectively. Polyclonal rabbit antibodies to RacE and phospho-RacE(S192) were raised against the peptide RQQHPDPNS/SGF and the phosphopeptide GMDKKS(pS) QDGSS. Immunocomplexes were visualized using fluoroblotted secondary antibodies and detected using a Bio-Rad ChemiDoc XRS Plus molecular imager. Images were analysed using NIH ImageJ. The detailed information on the antibodies used in this study is presented in Supplementary Table 4.

**Protein purification.** *Dictyostelium* cells carrying the epitope-tagged proteins were differentiated, washed twice with ice-cold DB and resuspended at 1 × 10\(^8\) cells ml\(^{-1}\). To generate a stable cAMP gradient, 100 mM cAMP pulses at intervals of 6 min for 4 h. To induce FLAG--RasC/G expression, cells growing exponentially were washed with development buffer (DB; 2 mM MgSO\(_4\), 0.2 mM CaCl\(_2\), 20 mM 2-(N-morpholino)ethanesulfonic acid (MES; pH 6.5)) at room temperature for 5 min with gentle mixing. The reactions were stopped by adding 80 µl 2×SDS--PAGE sample buffer. AKT phosphorylation was detected using immunoblotting with antibodies to RacE and phospho- RacE(S192).

Reconstitution of chemoattractant-stimulated mTORC2-mediated AKT phosphorylation. The kinase activity of mTORC2 was measured as described previously\(^4\) with some modifications. RacE, RasC, RacG and mTORC2 were eluted from anti-FLAG beads in high-salt buffer and incubated with 1× DB-MES overnight and lysed in an equal volume of ice-cold 2×CHAPS lysis buffer (0.6% CHAPS, 250 mM NaCl, 2 mM EDTA, 100 mM HEPES (pH 7.4), protease inhibitors and phosphatase inhibitors) for 10 min on ice. After clarification by centrifugation, 1-mL volumes of the cell lysates were incubated with 15 µl anti-FLAG agarose beads for 2 h at 4 °C with gentle agitation to immunoprecipitate the proteins. The beads were washed using 1×CHAPS lysis buffer, snap frozen in liquid nitrogen and stored at −80 °C. In the experiments described in Fig. 6b,c, in which individual subunits of mTORC2 were tested for AKT phosphorylation in vitro, FL--Paa and FL--Lst8 were purified as described above and then washed twice in high-salt conditions (500 mM NaCl and 50 mM HEPES (pH 7.4)). To purify FL--GFP–RacE and FL-- or GFP–RasC/G, cells at 1 × 10\(^8\) cells ml\(^{-1}\) were lysed in an equal volume of ice-cold 2×NP-40 lysis buffer (2% NP-40, 250 mM NaCl, 2 mM EDTA, 10 mM sodium phosphate (pH 7.0), protease inhibitors and phosphatase inhibitors) for 10 min on ice. After clarification by centrifugation, 1-mL volumes of the cell lysates were incubated with 15 µl anti-FLAG agarose beads for 2 h at 4 °C with gentle agitation to immunoprecipitate the proteins. The beads were washed three times in 1×NP-40 lysis buffer, twice in high-salt wash buffer (500 mM NaCl and 10 mM sodium phosphate (pH 7.0)) and three times in 1×NP-40 lysis buffer. The washed beads were snap frozen in liquid nitrogen and stored at −80 °C.

In vitro phosphorylation of RacE by GSK-3β. Purified FL--RacE (40 ng) and GSK-3β (50 ng, Sigma, G4296) were mixed in 40 µl kinase reaction buffer 1 (10 mM MgCl\(_2\), 0.1% 2-mercaptoethanol and 50 mM Tris–HCl (pH 7.5)) for 15 min at room temperature in the presence or absence of 100 µM ATP. Phosphorylation of RacE at S192 was assessed by phosphoprotein blotting with antibodies to RacE and phospho- RacE(S192).

**Immunoprecipitation.** Protein–protein interactions were assayed as performed previously\(^5\) with some modifications. *Dictyostelium* cell lysates, cells carrying different plasmids were differentiated in DB and incubated with 2 mM caffeine at 22 °C for 20 min. The cells were washed in DB, resuspended at 1 × 10\(^8\) cells ml\(^{-1}\) and lysed in an equal volume of ice-cold 2×lysis buffer (2% NP-40, 300 mM NaCl, 20 mM sodium phosphate (pH 7.0), protease inhibitors and phosphatase inhibitors) on ice for 10 min. The cell lysates were clarified by centrifugation at 4 °C and incubated with GFP–Trap agarose beads at 4 °C for 2 h to immunoprecipitate the GFP–RacE proteins. After washing with 1×lysis buffer, the bound fractions were eluted with 2×SDS--PAGE sample buffer and analysed by SDS--PAGE and CBB staining.

Chemoattractant-induced AKT phosphorylation in cells. Differentiated *Dictyostelium* cells were incubated for 20 min at 22 °C in DB with 2 mM caffeine, washed twice with ice-cold DB and resuspended at 5 × 10\(^7\) cells ml\(^{-1}\). The cells were shaken at 200 rpm at 22 °C and stimulated with 1 µM cAMP.\(^6\) Aliquots were taken at different time points and lysed in SDS--PAGE sample buffer. Proteins were analysed by immunoblotting with antibodies to PKB and PDK1. Similar to what has been extensively described in many studies,\(^7\) phosphorylation of PKB and PDK1 was detected using anti-phospho-PS6Kβ1 antibodies (Cell Signaling, cat. no. 9441) and anti-phospho-PI3K antibodies (Cell Signaling, cat. no. 2060) because of the similar amino acid sequences around the phosphorylation sites (Supplementary Fig. 4).

**Immunofluorescence microscopy.** Cells were cultured in HL5 medium (1% protease inhibitors and phosphatase inhibitors) on ice for 10 min. After clarification by centrifugation, 1-mL volumes of the cell lysates were incubated with 15 µl anti-FLAG agarose beads for 2 h at 4 °C with gentle agitation to immunoprecipitate the proteins. The beads were washed using 1×lysis buffer, snap frozen in liquid nitrogen and stored at −80 °C.
times in the same buffer and the bound proteins were analysed by immunoblotting. To examine the interactions of RasC/G with RacE and Tor, GFP-Trap beads carrying Ras proteins (40 ng) were incubated with FLAG-tagged RacE (40 ng) and Tor (200 ng).

Statistics and reproducibility. Unpaired, two-tailed Student’s t-tests were performed using GraphPad Prism7 to determine the statistical significance of the experiments described in Supplementary Fig. 2. For the multiple group comparisons, one-way ANOVA analysis followed by a Tukey’s test was performed using Prism7 (Figs. 1b,e, 2b,d and 3f,h). The statistical analyses and P values are described in each figure and figure legend. The number of independent experiments performed is described in each figure legend.

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Data availability
Mass spectrometry data have been deposited in ProteomeXchange with the primary accession code PXD014014 and provided in Supplementary Tables 1 and 2. Unprocessed images of all blots and gels are provided in Supplementary Fig. 8. The source data for all graphical representations and statistical descriptions are provided in Supplementary Table 5 for Figs. 1b,e, 2a,b,d,h, 3b,d,f,h,i, and 5b,d,f,h and Supplementary Figs. 2 and 6c. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

References
55. Lee, S. et al. TOR complex 2 integrates cell movement during chemotaxis and signal relay in Dictyostelium. Mol. Biol. Cell 16, 4572–4583 (2005).
56. Nakajima, A., Ishida, M., Fujimori, T., Wakamoto, Y. & Sawai, S. The microfluidic lighthouse: an omnidirectional gradient generator. Lab Chip 16, 4382–4394 (2016).
57. Iijima, M., Huang, Y. E., Luo, H. R., Vazquez, F. & Devreotes, P. N. Novel mechanism of PTEN regulation by its phosphatidylinositol 4,5-bisphosphate binding motif is critical for chemotaxis. J. Biol. Chem. 279, 16606–16613 (2004).
58. Castro, A. F., Rebhun, J. F. & Quilliam, L. A. Measuring Ras-family GTP levels in vivo—running hot and cold. Methods 37, 190–196 (2005).
59. Iijima, M. & Devreotes, P. Tumor suppressor PTEN mediates sensing of chemoattractant gradients. Cell 109, 599–610 (2002).
60. Futatsumori-Sugai, M. et al. Utilization of Arg-elution method for FLAG-tag based chromatography. Protein Expr. Purif. 67, 148–155 (2009).
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  - The commercial antibodies used in this study were validated by the manufacturer (links are provided for each antibody in Supplementary Table 4). The antibodies generated in this study were validated by knockout cell lines in Fig. 4a for anti-RacE and anti-phospho-RacE(Ser192) antibodies and Supplementary Fig. 4 for anti-PKBA and anti-PKBR1 antibodies.

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- Policy information about [cell lines](#)
- Cell line source(s)
  - WT, PiaA-KO, Rip3-KO and Lst8-KO Dictostelium cells were obtained from Dr. Peter Deverotes (Johns Hopkins University). RacE-KO, PkbA-KO and PkbR1-KO Dictostelium cells were generated by Miho Iijima. HEK293T cells were purchased from ATCC.
- Authentication
  - Gene knockouts were confirmed by PCR and Southern blotting.
- Mycoplasma contamination
  - HEK293T cells were tested negative for mycoplasma contamination. Dictostelium cells were not tested for mycoplasma contamination.
- Commonly misidentified lines (See [ICLAC](#) register)
  - No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample.