# IQGAP-related protein IqgC suppresses Ras signaling during large-scale endocytosis

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**Macropinocytosis and phagocytosis are evolutionarily conserved forms of bulk endocytosis used by cells to ingest large volumes of fluid and solid particles, respectively. Both processes are regulated by Ras signaling, which is precisely controlled by mechanisms involving Ras GTPase activating proteins (RasGAPs) responsible for terminating Ras activity on early endosomes. While regulation of Ras signaling during large-scale endocytosis in WT *Dictyostelium* has been, for the most part, attributed to the *Dictyostelium* ortholog of human RasGAP NF1, in commonly used axenic laboratory strains, this gene is mutated and inactive. Moreover, none of the RasGAPs characterized so far have been implicated in the regulation of Ras signaling in large-scale endocytosis in axenic strains. In this study, we establish, using biochemical approaches and complementation assays in live cells, that *Dictyostelium* IQGAP-related protein IqgC interacts with active RasG and exhibits RasGAP activity toward this GTPase. Analyses of *iqgC*$^{-}$ and IqgC-overexpressing cells further revealed participation of this GAP in the regulation of both types of large-scale endocytosis and in cytokinesis. Moreover, given the localization of IqgC to phagosomes and, most prominently, to macropinosomes, we propose IqgC acting as a RasG-specific GAP in large-scale endocytosis. The data presented here functionally distinguish IqgC from other members of the *Dictyostelium* IQGAP family and call for repositioning of this genuine RasGAP outside of the IQGAP group.**

IqgC | RasGAP | Ras | macropinocytosis | phagocytosis

Large-scale endocytosis (i.e., macropinocytosis and phagocytosis) is a mechanism by which cells ingest liquid and solid nutrients. These are ancient ways of feeding conserved from amoebae to humans. However, with the development of advanced forms of multicellularity when food uptake and processing was transferred into extracellular compartments inside the organism, the need for all cells to perform bulk endocytosis ceased. In mammals, only specialized cells still use large-scale endocytosis, although for new purposes. Cells of the innate immune system, such as macrophages, neutrophils, and dendritic cells, are professional phagocytes that clear pathogens and remnants of apoptotic cells from the organism (1). Dendritic cells also perform nonspecific bulk uptake of soluble antigens by constitutive macropinocytosis, which has a key role in antigen presentation to T cells (2). Neurons perform bulk endocytosis during intense synaptic activity to retrieve and recycle synaptic vesicles' membranes and proteins (3). Macropinocytosis has also been linked with several pathological states, such as neurodegenerative diseases, where it participates in the spread of prions and disease-specific misfolded proteins between cells (4). Cancer cells, similar to amoebae, use macropinocytosis as a nutrient supply pathway. In particular, Ras-driven cancers utilize extracellular serum albumin as a source of glutamine via Ras-induced up-regulated macropinocytic uptake to sustain tumor growth (5). Ras-transformed cancer cells also use macropinocytosis as a route for uptake of extracellular ATP (6). Bulk uptake mechanisms are also widely abused by intracellular pathogens for their entry into host cells (7).

Although macropinocytosis and phagocytosis are mechanistically similar processes that utilize common signaling and structural components to reorganize the actin cytoskeleton, still there are important differences between them (8). While the phagosome formation is triggered by the contact with a particle via localized activation of cell surface receptors, macropinosomes develop spontaneously or in response to soluble growth factors. Macropinosome formation is intimately linked to membrane ruffling and requires highly localized protrusion of the plasma membrane achieved by the actin assembly along the nascent macropinocytic cup promoted by the WAVE-activated Arp2/3 complex and formin G (9–11). The whole process is directed by small GTPases from the Ras superfamily: e.g., Ras, Rac, Cdc42, Arf6, and Rab5 (12). Roles of the Rho family GTPases in the regulation of actin dynamics in endocytic pathways are already well-established (13). Although a positive correlation between Ras expression and membrane ruffling coupled to macropinocytosis has been demonstrated more than three decades ago (14), detailed regulatory mechanisms mediated by Ras are still under investigation. Results obtained by studying macropinocytosis in *Dictyostelium* revealed involvement of Ras family GTPases in

## Significance

***Dictyostelium* WT amoebae live in soil and feed by phagocytosis. Macropinocytosis is largely suppressed in natural strains and cannot ensure sufficient nutrient uptake for survival. On the other hand, axenic laboratory strains have strongly up-regulated macropinocytosis and can grow in nutritive liquid media. This difference is attributed to the RasGAP protein NF1 that suppresses Ras signaling, thereby down-regulating macropinocytosis in WT amoebae. Since the gene encoding NF1 is inactive in axenic strains, other GAPs must be involved in the control of bulk fluid uptake. Here, we identify IqgC as a RasGAP responsible for regulation of large-scale endocytosis in the widely used axenic laboratory strain AX2 and show that it acts as a RasG-specific GAP to suppress RasG signaling.**

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Data deposition: Mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository (dataset identifier PXD006311).

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early events, upstream of Rho-induced actin rearrangements, during macropinosome formation. *Dictyostelium* RasG and RasS have been identified as upstream regulators of three class I phosphoinositide 3-kinases (PI3Ks), where PI3K1 and PI3K2 were implicated in the activation of PI(3,4,5)$P_3$-rich plasma membrane patches primed for macropinocytosis whereas PI3K4 effected the subsequent closure of these patches into macropinosomes (15, 16). Of note, although the predominant inositol phospholipids present in *Dictyostelium* are plasmanylinositols instead of phosphatidylinositols, the same abbreviations are used, regardless of the hydrocarbon chains composition and linkage to the glycerol backbone, and there are no known differences regarding the polar head interactions (17). It is becoming increasingly clear that Ras activity is important for both macropinocytosis and phagocytosis of large particles in *Dictyostelium* (18). For example, we recently demonstrated a direct regulatory role of RasG and RasB in activating the actin polymerization factor formin G at the base of nascent macropinocytic and phagocytic cups, respectively (9). In addition to RasB, RasS and Rap1 have also been implicated in regulation of phagocytosis (9, 19, 20).

Ras activity during large-scale endocytosis has to be temporally and spatially restricted to the plasma membrane region belonging to the endocytic cup. It was indicated that a diffusion barrier prevents the lateral leaking of signaling molecules from the circular ruffle delineating the incipient cup to the adjacent regions of the plasma membrane (21). In addition, Ras signaling must be terminated in a timely fashion, and this is achieved by the action of Ras GTPase activating proteins (RasGAPs) that normally turn off Ras activity (22). RasGAPs stimulate the weak intrinsic GTP hydrolytic activity of Ras GTPases, thus converting them to their inactive GDP-bound forms. *Dictyostelium* RasGAPs are still poorly characterized. Out of the 15 genes encoding RasGAP domain-containing proteins, as yet, only three genuine RasGAPs have been identified (18, 23, 24). The product of *nfaA*, NfaA or DdNF1, was identified as a negative regulator of *Dictyostelium* RasG and RasB proteins during chemotaxis and cytokinesis (23). Recently, C2GAP1 protein was characterized as a G protein-coupled receptor (GPCR)-regulated RasGAP that plays a key role in cAR1-mediated chemotaxis (24). The *Dictyostelium* ortholog of human NF1, the product of *axeB*, was identified as the RasGAP present in WT *Dictyostelium* isolates responsible for the inability of WT amoebae to grow axenically in liquid medium (18).

Here, we investigate the role of IQGAP-related protein IqgC in growth-phase *Dictyostelium* cells. IQGAP proteins are large, multidomain proteins conserved from yeast to human, which act as scaffolds that integrate different signaling pathways to regulate diverse cellular processes (25). Despite the presence of a GAP-related domain (GRD) that is highly homologous to the catalytic domain of RasGAPs, IQGAP proteins do not exhibit GAP activity toward small GTPases and generally do not bind Ras (26–29). However, they interact with the Rho-family GTPases Cdc42 and Rac1. Since IQGAPs phylogenetically belong to the RasGAP domain-containing proteins (30), from this point on, we will use the term RasGAP instead of GRD for this domain. *Dictyostelium discoideum* encodes four IQGAP-like proteins, DGAP1/DdIQGAP1, GAPA/DdIQGAP2, IqgC/DdIQGAP3, and DdIQGAP4, with the latter two members virtually uncharacterized (31). IqgC has been implicated in controlling cell polarity during chemotaxis toward cAMP (32), but its biological role in vegetative cells is unknown. All IQGAP-related proteins in *Dictyostelium* contain the RasGAP domain whereas DGAP1 and GAPA also bind *Dictyostelium* Rho-family GTPases and do not exhibit GAP activity (33–36).

Here, we show that the third IQGAP-related protein in *Dictyostelium*, IqgC, has a profoundly different function in comparison with DGAP1 and GAPA. IqgC localizes to the patches of the plasma membrane that develop into endocytic cups and remains there until cup closure. Interaction studies demonstrate no interaction with Rho-family GTPases but instead identify IqgC as a binding partner of RasG. In vitro GAP assay further revealed IqgC acting as a RasG-specific GAP. Enhanced fluid and particle uptake by *iqgC*$^{-}$ cells and diminished uptake by IqgC-overexpressing cells characterize this protein as a negative regulator of macropinocytosis and phagocytosis. Taken together, we have identified a RasGAP that regulates RasG activity specifically during large-scale endocytosis in vegetative cells.

## Results

**IqgC Does Not Interact with Rho GTPases and Localizes to Macropinosomes and Phagosomes.** According to its polypeptide sequence, IqgC was classified as an IQGAP protein family member, together with DGAP1 and GAPA (31). These two *Dictyostelium* proteins, similar to their mammalian counterparts, bind small GTPases from the Rho family and participate in actin cytoskeleton remodeling (33, 35, 36). Specifically, both DGAP1 and GAPA are crucial for efficient cytokinesis and localize to the cleavage furrow of dividing cells, and to the rear cortex of interphase cells (36, 37). Thus, to elucidate the biological function of IqgC, we first screened Rho-family GTPases from *Dictyostelium* for interaction with IqgC. We tested a set of Rac GTPases in their constitutively active form using a yeast two-hybrid screen and, unexpectedly, failed to identify an interactor (*SI Appendix*, Fig. S1*A*). To ensure that lack of an interaction is not a consequence of, for instance, an autoinhibitory conformation or dimerization of the IqgC protein that precludes Rho binding, we repeated the screen using the IqgC RasGAP domain alone and again detected no interaction partner among Rho GTPases (*SI Appendix*, Fig. S1*B*). Next, we investigated the subcellular localization of IqgC in vegetative *Dictyostelium* cells. Vegetative cells of axenic strains are known to alternate between two types of behavior: They either take up fluid medium via large macropinosomes while their movement is restricted or crawl on the surface while essentially not performing macropinocytosis (38, 39). Our results show that localization of fluorescently labeled IqgC is dependent on the cell's current state. In randomly moving cells, IqgC did not localize to any particular structure, and YFP fluorescence was distributed homogenously in the cytosol and the F-actin–rich hyaline zones (Fig. 1*A* and Movie S1). On the other hand, in cells that actively macropinocytose, IqgC was highly enriched at membrane patches that form into macropinosomes and dispersed soon after the macropinosome closure (Fig. 1*B*, *SI Appendix*, Fig. S2, and Movie S2). Next, we analyzed IqgC distribution during phagocytosis of large particles. Although IqgC was faintly visible at phagocytic cups during yeast particle engulfment, it was recruited there sporadically and transiently, with lower signal intensity compared with macropinosomes (Fig. 1*C* and Movie S3). We next examined localization of IqgC during phagocytosis of small particles and again observed a modest increase in YFP fluorescence during ingestion of bacteria (*SI Appendix*, Fig. S3). Finally, IqgC did not localize to the cleavage furrow nor show any other prominent localization during cytokinesis (Fig. 1*D* and Movie S4).

**IqgC Interacts Specifically with the Constitutively Active Q61L Mutant Form of RasG.** Our findings clearly discriminate IqgC from already characterized IQGAP family members in *Dictyostelium*. In contrast to DGAP1 and GAPA, IqgC does not localize to the trailing edge of a polarized interphase cell or to the cleavage furrow of a dividing cell. Moreover, its localization strongly suggests its involvement in large-scale endocytosis. In addition, a detailed analysis of the IqgC RasGAP domain revealed that it harbors conserved arginine residues inside specific regions, which are essential for RasGAP catalytic activity (*SI Appendix*, Fig. S4). Exactly these motifs are changed in mammalian IQGAPs and *Dictyostelium* DGAP1 and GAPA, which have lost the ability

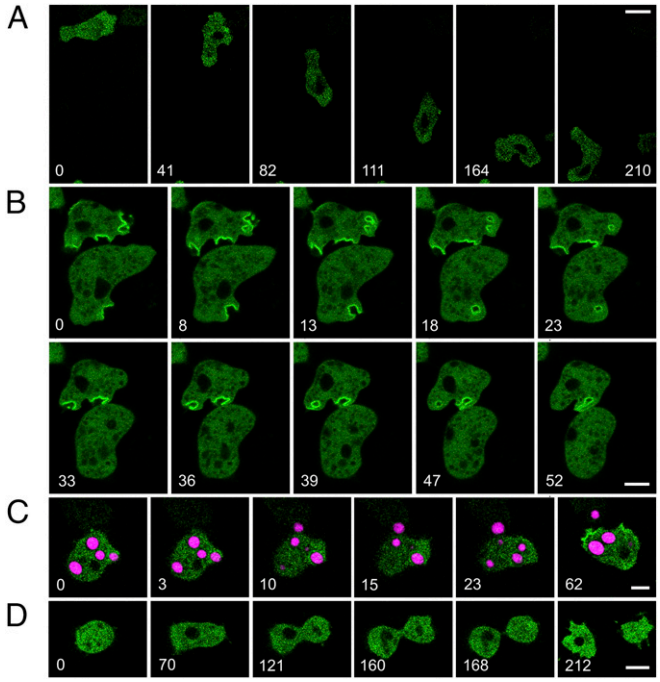


**Fig. 1.** IqgC accumulates strongly on macropinosomes and weakly on phagosomes of growth-phase cells. Image sequences of vegetative WT cells ectopically expressing YFP-IqgC during (*A*) random motility, (*B*) macropinocytosis, (*C*) engulfment of TRITC-labeled yeast particles, and (*D*) cytokinesis. *A*, *C*, and *D* correspond to Movies S1, S3, and S4. Time is given in seconds. (Scale bars: *A*, 10 μm; *B*–*D*, 5 μm.)

to promote GTP hydrolysis catalyzed by small GTPases (26, 33, 35, 40). Since an involvement of Ras GTPases in large-scale endocytosis is already well-established (9, 15, 19, 41), we hypothesized that IqgC could have a conserved RasGAP activity exerted during macropinocytosis and phagocytosis. This hypothesis was further supported by pull-down experiments aimed at identifying binding partners of IqgC. GST-tagged IqgC was used to precipitate potential interactors from total lysates of WT laboratory *Dictyostelium* strain AX2 and analyze them by mass spectrometry (42). This approach identified RasB, RasC, and RasG as potential IqgC binding partners (Fig. 2 and Dataset S1). To further validate direct interaction of IqgC with the identified Ras GTPases, we again employed a yeast two-hybrid assay. This time, we included constitutively active Ras Q61L variants in addition to G12V activating mutations. Namely, although RasGAPs are not able to promote GTP hydrolysis in either of these two Ras mutant forms, they bind the Q61L mutants with much higher affinity compared with the G12V mutants (43, 44). Indeed, IqgC bound only to the Q61L-activated form of RasG, which further supported our hypothesis of IqgC being a RasGAP (Fig. 3). We also performed a yeast two-hybrid assay with the Q61L variant of Rac1A since this Rho-family GTPase was also identified in the interactome. However, a direct interaction of active Rac1A with IqgC was not confirmed.

**IqgC Interacts with GTP-Bound RasG in Live Cells.** To confirm an interaction between IqgC and RasG biochemically, we first performed a GST pull-down assay. GST-IqgC bound to glutathione-agarose was used to pull down RasG from lysates of WT AX2 cells expressing HA-tagged RasG from an extrachromosomal vector. The assay confirmed that recombinant IqgC forms a complex with RasG, but, unexpectedly, the interaction was stronger with the constitutively inactive S17N form (Fig. 4*A*). Coimmunoprecipitation experiments demonstrated that endogenous IqgC also binds RasG from the cell lysate, and again preferentially the inactive mutant (Fig. 4*B*, *Upper*). We also detected a faint band of coimmunoprecipitated WT RasG (Fig. 4*B*, *Lower*).

Since these results were not in accord with the yeast two-hybrid data with respect to the nucleotide status of RasG, we decided to test interactions between IqgC and WT, G12V, Q61L, and S17N variants of RasG in live cells using the bimolecular fluorescence complementation assay (BiFC). Split fragments of fluorescent protein Venus were used as a reporter of BiFC, as already described (9, 45). As a positive control, we first monitored fluorescence complementation with the Ras-binding domain (RBD) from a well-established Ras effector, Raf1 kinase, with RasG (46, 47). As expected, Raf1(RBD) bound WT and both variants of constitutively active RasG but did not show any interaction with its inactive form (Fig. 5, *Upper*). BiFC with IqgC was tested in the *iqgC*$^{-}$ background to avoid unproductive competing interactions between RasG fused to the N-terminal part of Venus, VN-RasG, and endogenous IqgC. An *iqgC*$^{-}$ cell line (strain IW013) was generated by homologous recombination in the laboratory WT AX2 background, and successful genetic inactivation of this gene was confirmed by PCR and Southern and Western blots (*SI Appendix*, Fig. S5). Fluorescence complementation was observed in *iqgC*$^{-}$ cells coexpressing IqgC fused to the C-terminal part of Venus, VC-IqgC, and VN-RasG (wt) or VN-RasG(Q61L) but was never observed when using VN-RasG(G12V) or VN-RasG(S17N) mutants (Fig. 5, *Lower*). These data corroborated the higher affinity of IqgC toward Q61L mutated Ras determined by the yeast two-hybrid assay and demonstrated that IqgC interacts with the active, GTP-bound RasG in live cells.

**IqgC Exhibits RasGAP Activity Toward Human H-Ras and *Dictyostelium* RasG.** Next, we examined RasGAP activity of recombinant IqgC using a luminescence-based GAP assay in vitro. In this assay, the GTP remaining in solution after completion of GTPase reaction is converted to ATP, which is then used in a luciferase reaction to

A

| Proteins | Accession number | Molecular weight | Unique peptides (#1) | Unique peptides (#2) | Unique peptides (#3) |
|---|---|---|---|---|---|
| RasG | gi\|66800415 | 21 kDa | 8 | 5 | 6 |
| RasB | gi\|66800749 | 22 kDa | 3 | 3 | 2 |
| RasC | gi\|66813008 | 21 kDa | 1 | 1 | 3 |

B

**RasG** (45% coverage)

MTEYKLVIVGGGGVGKSALTIQLIQNHFIDEYDPTIEDSYRKQVTIDEETCLLDI
LDTAGQEEYSAMRDQYMRTGQGFLCVYSITSRSSFDEIASFREQILRVKDKD
RVPMIVVGNKCDLESDRQVTTGEGQDLAKSFGSPFLETSAKIRVNVEEAFYS
LVREIRKDLKGDSKPEKGKKKRPLKACTLL

**RasB** (32% coverage)

MSVSNEYKLVVMGGGGVGKSALTIQFIQNHFIEEYDPTIEDSYRRQCQVDED
TCLLDILD TAGQDDYSAMRDQYMRTGQGFLCVYDVTSRTSFEEINVVREQIIR
VKDNDKVPIVLVGNK CDLENLREVTEGEGSELAKSFSVPFLETSAKKRLNVD
ECFFEVVREIKKSLKEPGRSKKDKKGGILKKFKGGDCLIL

**Ras C** (21% coverage)

MSKLLKLVIVGDGGVGKSALTIQLTQNQFIAEYDPTIENSYRKQVNIDEEVYML
DILDTA GQEEYSAMRDQYIRSGRGFLIVYSIISRASFEAVTTFREQILRVKDLST
YPIVIIGNKAD LPDKDRKVPPMEGKELAKSFGAPFLETSAKSRVNVEEAFFTL
VREIKRWNQNPQNEEMLPPKKRGCIIL

**Fig. 2.** Proteomic identification of Ras proteins as binding partners of IqgC. (*A*) Table showing the number of identified unique peptides of RasG, -B, and -C proteins in each of the three biological replicates. (*B*) Amino acid coverage of Ras GTPases obtained from all identified peptides.

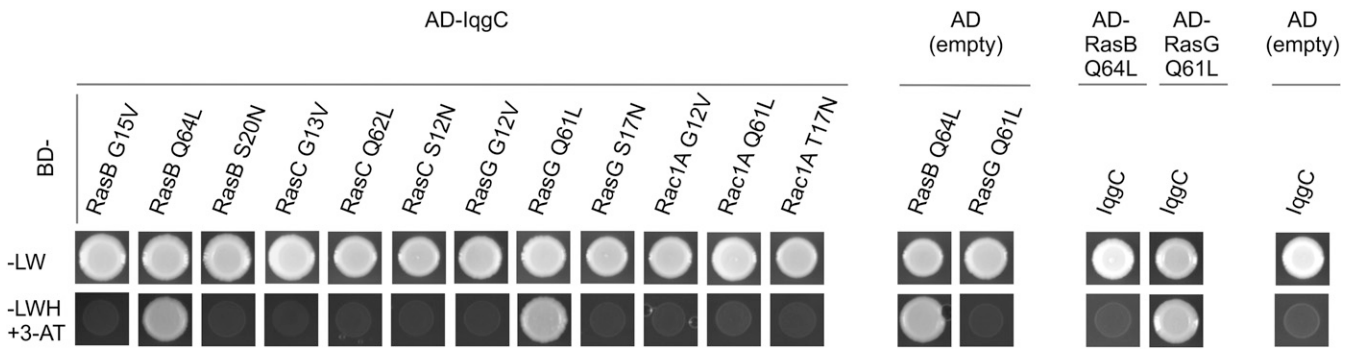


**Fig. 3.** IqgC interacts specifically with the constitutively active RasG(Q61L) mutant, but neither with the active G12V nor with the inactive S17N mutant in the yeast two-hybrid assay. IqgC was fused to the Gal4 activation domain (AD), and Ras and Rac proteins to the Gal4 DNA binding domain (BD). Yeast transformants carrying both indicated expression vectors were selected by growth on plates with selective media lacking leucine (L) and tryptophan (W). Interactions were assayed on plates lacking leucine, tryptophan, and histidine (H) in the presence of 3 mM 3-amino-1,2,4-triazole (3-AT). Interaction between IqgC and RasB(Q64L) was unspecific since yeast expressing RasB(Q64L) also grew on –LWH +3-AT plates in the absence of IqgC bait. Specific interaction of active RasG(Q61L) with IqgC was independently confirmed by exchanging bait and prey proteins.

produce light. Hence, the higher the GTP consumption, the lower is the luminescence output. First we used purified WT human H-Ras, which shares 68% overall identity and an identical effector domain with *Dictyostelium* RasG. Purified IqgC was titrated against a fixed concentration of H-Ras in a GTPase reaction, and the detected drop in luminescence demonstrated the ability of IqgC to stimulate GTP hydrolysis catalyzed by human H-Ras (Fig. 6*A*). We next examined putative IqgC RasGAP activity toward its endogenous interaction partner, *Dictyostelium* RasG, and found that IqgC promoted GTPase activity of recombinant GST-tagged RasG(wt) (Fig. 6*B*). Finally, we checked whether IqgC affects GTP hydrolysis of RasG(Q61L). As expected, addition of IqgC to the GTPase reaction did not have any effect on intrinsic GTPase activity of this constitutively active mutant (Fig. 6*C*). Of note, we could detect a weak GTP hydrolytic activity of RasG(Q61L) alone. This observation is in agreement with previously published data showing that the Q61L mutant does not hydrolyze GTP in vivo but still has a weak residual activity in vitro (48).

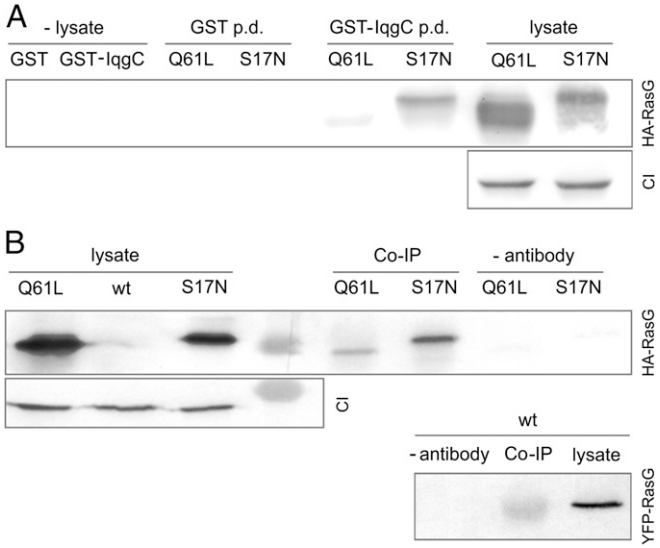


**Fig. 4.** Recombinant and endogenous IqgC interacts with RasG from cell lysates. (*A*) GST-fusion protein binding assay demonstrates that recombinant GST-IqgC forms a complex with both constitutively active (Q61L) and inactive (S17N) HA-tagged RasG ectopically expressed in WT cells. *Upper* lanes show anti-HA blot as follows: after incubation of GST-bound and GST-IqgC–bound agarose with the lysis buffer alone (- lysate); after incubation of GST-agarose [GST pull-down (p.d.)] and GST-IqgC-agarose (GST-IqgC p.d.) with lysates of cells expressing active (Q61L) and inactive (S17N) HA-RasG; input lysates (lysate). *Lower* lanes show anti-cortexillin I (CI) blot as a loading control. (*B*, *Upper*) Both active and inactive RasG coimmunoprecipitate from cell lysates with endogenous IqgC. *Upper* lanes show anti-HA blot as follows: of expression of HA-RasG(Q61L) and HA-RasG(S17N) in AX2 cells (lysate); after incubation of protein A with anti-IqgC serum and the respective cell lysates [coimmunoprecipitation (Co-IP)]; negative control without antiserum (- antibody). *Lower* lanes show the blot of constitutively expressed CI as a loading control. (*B*, *Lower*) Anti-GFP blot showing: coimmunoprecipitated YFP-RasG (wt) with endogenous IqgC (Co-IP); control Co-IP without antiserum (- antibody); and expression level of YFP-RasG(wt) in WT cells (lysate). CI loading control can be seen on the *Upper* (lysate, wt).

**IqgC Negatively Regulates the Fluid-Phase Uptake.** After demonstrating the RasGAP activity of IqgC toward its endogenous binding partner RasG, we proceeded to evaluate the physiological consequences of the genetic elimination and overexpression of IqgC. Prompted by the strong localization of IqgC to macropinosomes, we first evaluated macropinocytosis efficiency using fluid-phase marker TRITC-dextran since more than 90% of the fluid uptake occurs by macropinocytosis in axenic *Dictyostelium*

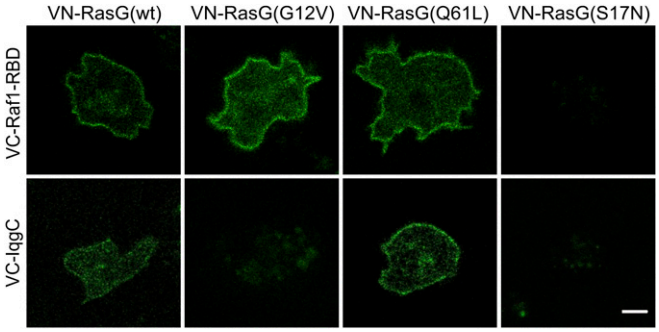


**Fig. 5.** IqgC interacts with WT and constitutively active RasG(Q61L) in live cells. RasG variants were C-terminally fused to the N-terminal portion of the fluorescent protein Venus (VN). The Ras interactors Raf1(RBD) and full-length IqgC were C-terminally fused to the C-terminal part of Venus (VC). Pairs of fusion proteins were coexpressed in WT cells for testing interactions with Raf1(RBD), and in *iqgC*$^{-}$ cells for testing interactions with IqgC. Fluorescence complementation was monitored by confocal microscopy. Raf1 kinase RBD, used as a positive control, binds to WT and both active forms of RasG (*Upper*). IqgC also binds WT, but only the active Q61L variant of RasG (*Lower*). Neither of the tested proteins showed fluorescence with the constitutively inactive form of RasG. (Scale bar: 5 μm.)

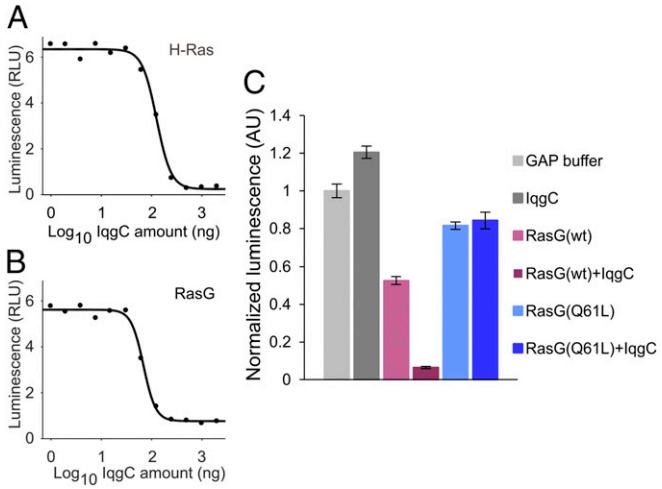


**Fig. 6.** IqgC acts as a RasGAP toward WT human H-Ras and *Dictyostelium* RasG. (*A*) To assess the RasGAP activity of IqgC, the protein was serially diluted in a GAP buffer containing 10 μM GTP and mixed with H-Ras so that final concentrations of H-Ras and GTP in a GTPase reaction were 1 μM and 5 μM, respectively. The GTP that remained in the solution after 2 h was converted to ATP by nucleoside-diphosphate kinase and was subsequently detected in a luciferase reaction. (*B*) To analyze RasGAP activity for RasG, GTPase reactions were set up with the same serial dilutions of IqgC and mixed with a constant amount of GST-RasG(wt) immobilized to glutathione-agarose. Increasing amounts of IqgC in the GTPase reaction resulted in decreasing luminescence. Both curves show representative experiments. (*C*) Histogram showing that IqgC promotes Ras GTPase activity on WT RasG, but not on mutant Q61L. All GTPase reactions containing IqgC were performed with 1,000 ng of IqgC. Luminescence of GAP buffer was set to 1, and other values were rescaled accordingly (mean ± SD, $n = 3$). AU, arbitrary unit; RLU, relative luminescence unit.

strains (49, 50). We determined an increase in the fluid-phase uptake of *iqgC*$^-$ cells compared with WT in the late phase of macropinocytosis whereas an opposite effect was observed in IqgC-overexpressing cells (Fig. 7*A*). These results provide further evidence for our hypothesis that GAP activity of IqgC suppresses macropinocytosis by local deactivation of RasG. Moreover, our measurements clearly underestimate the effect of IqgC overexpression on the rate of macropinocytosis due to the highly variable level of YFP-IqgC expression from the extrachromosomal vector. In fact, we noticed that strongly fluorescent cells tended to accumulate less TRITC-dextran in the cytosol than weakly fluorescent and unlabeled cells (Fig. 7*B*). This effect was analyzed by correlating the fluorescence intensities of YFP and TRITC in the population of 500 randomly chosen cells transfected with YFP-IqgC expression vector, 90 min after the addition of TRITC-dextran. Using the data from 286 cells with discernible YFP fluorescence, a negative correlation between the IqgC protein level and the fluid-phase uptake was corroborated (Fig. 7*C*). Next, we tested possible differences in the size of macropinosomes and the frequency of their occurrence between parental AX2 and *iqgC*$^-$ cells, which were previously demonstrated for the WT *Dictyostelium* DdB strain and its *axeB*$^-$ derivative deficient in RasGAP NF1 (18). A small, but statistically significant increase in the length of the major macropinosome axis was determined in *iqgC*$^-$ cells compared with WT cells whereas a similar decrease was determined in IqgC-overexpressing cells (Fig. 7*D*). No significant difference in the frequency of macropinosome occurrence between the three cell lines was found (Fig. 7*E*). Since the difference in TRITC-dextran uptake between WT and *iqgC*$^-$ cells was prominent only after 60 min in the bulk pinocytosis assay (Fig. 7*A*), we suspected that mutant cells have somewhat delayed endosomal trafficking due to a slower inactivation of active Ras on internalized macropinosomes. This prompted us to compare the retention times of active Ras and $PI(3,4,5)P_3/PI(3,4)P_2$ after macropinosome closure, using Raf1(RBD) and CRAC(PH) as reporters, respectively (46, 51). Interaction of a widely used probe for active Ras, mammalian Raf1(RBD), with constitutively active *Dictyostelium* RasG was verified in yeast two-hybrid (9) and BiFC assays (Fig. 5, *Upper*), and RasG was shown to localize to macropinosomes (9). Contrary to expectations, we did not detect a prolonged activation of RasG or lifetime of $PI(3,4,5)P_3$ patches in *iqgC*$^-$ cells (*SI Appendix*, Fig. S6*A*). We were also not able to detect delays in the acidification of early endosomes or the onset of proteolytic degradation of vesicles' content (*SI Appendix*, Fig. S6*A*). To cover the entire endocytic pathway, we analyzed the exocytosis kinetics and failed to detect a difference between WT and *iqgC*$^-$ cells (*SI Appendix*, Fig. S6*B*). Taken together, the obtained evidence indicates that a slight increase of the macropinosome size in mutant cells accounts for an enhanced amount of TRITC-dextran accumulated in these cells over time.

Finally, we compared the dynamics of fluorescently labeled IqgC with active RasG during macropinosome closure. We first showed that IqgC colocalizes with active RasG on macropinosomes using WT cells simultaneously expressing YFP-IqgC and mRFP-Raf1(RBD) (Fig. 7*F* and Movie S5). Then, we analyzed YFP-IqgC and mRFP-Raf1(RBD) fluorescence during macropinocytosis and, interestingly, found that the active Ras probe disappears from the closed macropinosome before IqgC [$\Delta t = 1.7$ (1.0 to 2.3) s, $n = 9$, (median, interquartile range)] (Fig. 7 *F* and *G*). These results are consistent with the assumed role of IqgC in terminating RasG signaling on the macropinosome.

***iqgC*$^-$ Cells Are Mildly Multinucleated and Show Enhanced Phagocytosis Efficiency.** We next investigated whether *iqgC*$^-$ cells display any phenotypic features in addition to a mildly enhanced macropinocytosis efficacy. We noticed that *iqgC*$^-$ cells appear to be slightly larger than WT cells when cultivated attached to a solid surface. To verify this, we analyzed the distribution of cells according to the number of nuclei per cell for WT and three independent *iqgC*$^-$ clones. As shown in Fig. 8*A*, *iqgC*$^-$ cells showed a modest shift toward a multinucleated phenotype that could be reverted by overexpression of IqgC, and this effect was exaggerated when cells were grown to full confluency. Next, we examined the growth of *iqgC*$^-$ cells in suspension over 5 d but found no defect (Fig. 8*B*). However, when cells sampled from suspension on the third and fourth day of growth were stained with DAPI, again we noticed a mild increase in the number of nuclei per cell (Fig. 8*C* and *SI Appendix*, Table S1). In addition, a comparison of protein contents between WT and *iqgC*$^-$ cells in the midexponential growth phase showed on average a 26% increase in the volume of mutant cells. Altogether, a mild cytokinesis defect in combination with an increased liquid-nutrient uptake did not change the overall growth rate in shaken suspension. We also analyzed growth on bacterial lawns and found that *iqgC*$^-$ cells formed modestly, but significantly, smaller plaques (Fig. 8*D*). Finally, since microscopy analyses demonstrated that IqgC localizes to phagocytic cups during ingestion of both small (*SI Appendix*, Fig. S3) and large particles (Fig. 1*C* and Movie S3), we examined the phagocytosis of bacteria and yeast. *iqgC*$^-$ cells were more efficient in the uptake of bacteria while the opposite was determined for IqgC-overexpressing cells (Fig. 8*E*). Moreover, *iqgC*$^-$ cells showed considerably enhanced uptake of yeast particles compared with WT cells, reaching the maximum in internalized material already 60 min after addition of yeast, and the greatest difference of 37% at the 45-min time point (Fig. 8*F*). IqgC-overexpressing cells were again less efficient than WT cells. Altogether, cells deficient for IqgC have enhanced fluid- and solid-phase uptake from suspension, while exhibiting WT growth rates in the shaken suspension and a modest decrease in growth on bacterial lawns.

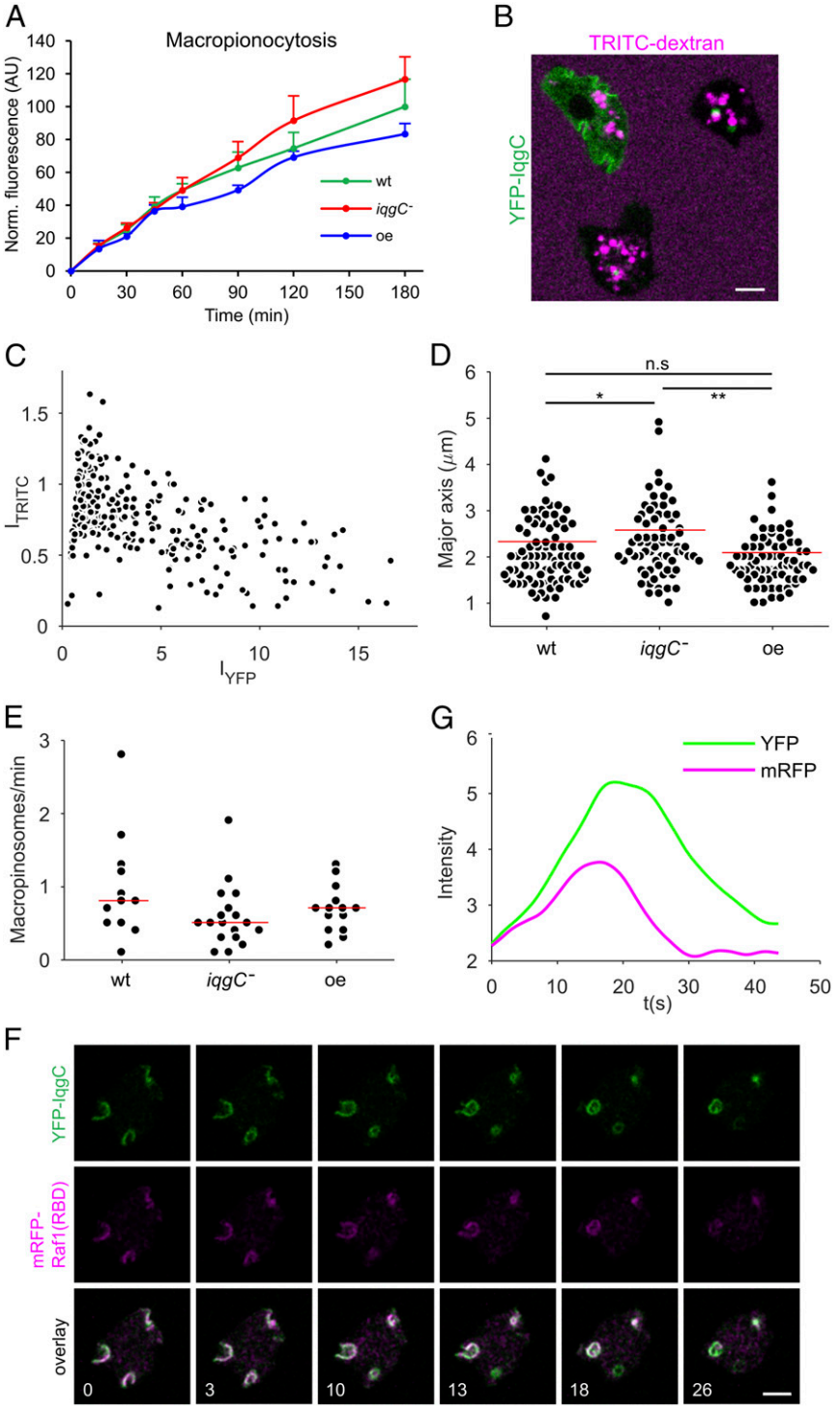


**Fig. 7.** IqgC negatively regulates TRITC-dextran uptake and the size of macropinosomes. (*A*) Quantification of TRITC-dextran uptake demonstrates increased macropinocytosis efficiency of *iqgC*$^{-}$ cells compared with WT (wt) and IqgC-overexpressing cells (oe) (mean ± SD, $n = 3$). (*B*) Confocal section showing three cells with notably different YFP-IqgC expression levels illustrates the diminished accumulation of TRITC-dextran in the cytosol of strong overexpressors. (Scale bar: 5 μm.) (*C*) Scatter plot showing negative correlation between the fluorescence intensities corresponding to YFP-IqgC ($I_{YFP}$) and TRITC-dextran ($I_{TRITC}$) signals in 286 analyzed cells (Pearson correlation coefficient equal to −0.52). (*D*) Cells devoid of IqgC form larger macropinosomes compared with WT and IqgC-overexpressing cells as judged by the length of the longest axis of the macropinosome upon closure, the major axis (*a*): $a(iqgC^{-}) = 2.2$ (1.9 to 2.8) μm, $n = 70$; $a$(wt) = 2.0 (1.5 to 2.6) μm, $n = 86$; $a$(oe) = 1.8 (1.5 to 2.2) μm, $n = 72$ (median, interquartile range); $^{*}P < 0.01$, $^{**}P < 0.001$, n.s., not significant. (*E*) The frequency of the macropinosome formation does not differ between WT, IqgC-deficient, and IqgC-overexpressing cells ($\chi^2 = 4.15$, $P = 0.1258$). (*F*) IqgC and the active Ras probe, Raf1(RBD), colocalize on the macropinosomes, from the activation of a membrane patch until macropinosome closure. Confocal sections correspond to Movie S5. Time is given in seconds. (Scale bar: 5 μm.) (*G*) Probe for active Ras disappears from the macropinosome before the IqgC probe. The graph shows YFP-IqgC and mRFP-Raf1(RBD) signals over time for one monitored macropinosome, with $\Delta t = 2.2$ s time difference between peak intensities of the two signals.

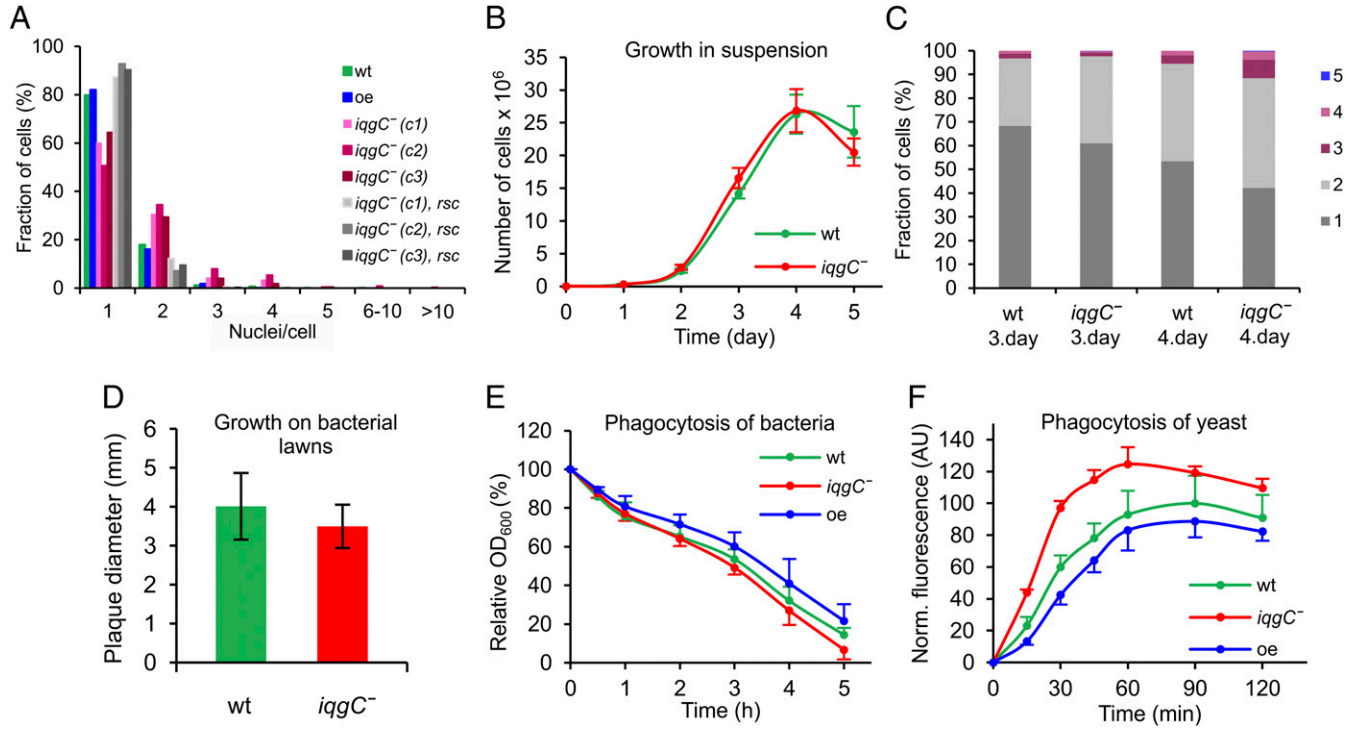


**Fig. 8.** *iqgC*⁻ cells exhibit a mild cytokinesis defect and increased phagocytosis. (*A*) WT AX2 cells (wt), AX2 cells overexpressing IqgC (oe), three *iqgC*⁻ clones (*iqgC*⁻, *c1–c3*) and *iqgC*⁻ clones expressing YFP-IqgC (*iqgC*⁻, *c1–c3, rsc*) were harvested at subconfluent density, fixed, and stained with DAPI. The number of nuclei per cell was determined for more than 500 cells from each cell line. (*B*) *iqgC*⁻ and WT cells grow at the same rate in shaken suspension (mean ± SD, $n \geq 3$). (*C*) WT and *iqgC*⁻ cells were harvested after 3 and 4 d from the shaken suspension, fixed, and stained with DAPI. The distribution of cells according to the number of nuclei per cell was evaluated for more than 500 cells per cell line. Numbers of nuclei per cell are designated in different colors. (*D*) *iqgC*⁻ cells show reduced growth rate on *Klebsiella aerogenes* lawns compared with WT cells, according to the plaque diameter (*d*) after 5 d of growth: $d(\text{wt}) = 4.01 \pm 0.85$ mm, $n = 198$; $d(iqgC^-) = 3.49 \pm 0.55$, $n = 244$ (mean ± SD); $P < 0.001$. (*E*) *iqgC*⁻ cells are more efficient at the uptake of bacteria from suspension than WT and IqgC-overexpressing cells (mean ± SD, $n = 4$). (*F*) WT, *iqgC*⁻, and IqgC-overexpressing cells were challenged with TRITC-labeled yeast particles, demonstrating a marked increase in the phagocytosis efficiency of mutant cells (mean ± SD, $n = 3$).

**IqgC Translocates to the Cortex in Response to Chemoattractant Pulses, but Not During Migration and Chemotaxis.** Since Ras signaling is known to be involved in the regulation of chemotaxis in *Dictyostelium* (52), we checked the localization of IqgC in cells during unstimulated migration and during directed migration in a chemotactic gradient. Neither vegetative (Fig. 1*A*) nor aggregation-competent cells (Fig. 9*A* and Movie S6) showed any cortical enrichment or polarization of YFP-IqgC during random migration. Also, no cortical localization of IqgC was found in aggregation-competent cells during directed migration in the radial gradient of chemoattractant cAMP diffusing from a micropipette (Fig. 9*B* and Movie S7). Next, we checked for possible intracellular translocation of YFP-IqgC upon exposure to isotropic pulses of chemoattractants. Remarkably, both stimulation of vegetative cells with folic acid (Fig. 9*C* and Movie S8) and of aggregation-competent cells with cAMP (Fig. 9*D* and Movie S9) induced a transient cortical recruitment of IqgC resembling the well-established cortical recruitment of the pan-probe for active Ras GTPases, Raf1(RBD) (24, 53, 54), but the response to folic acid was stronger than to cAMP (Fig. 9 *E* and *F*).

## Discussion

Regulation of Ras signaling by RasGAPs in large-scale endocytosis is still poorly understood in *Dictyostelium* axenic strains. Characterized RasGAPs include C2GAP1 and NfaA, which are important for directional sensing, cell polarity, and chemotaxis (23, 24). Besides its role in chemotaxis, NfaA was also implicated in random cell motility and cytokinesis (23). In this study, we investigated the molecular and cellular functions of *Dictyostelium* IQGAP-related protein IqgC during vegetative growth and showed that it is an atypical IQGAP protein that exhibits GAP activity. We employed a yeast two-hybrid assay, biochemical approaches, a bimolecular fluorescence complementation assay in live cells, and a GAP assay in vitro to establish that IqgC interacts with GTP-bound RasG in live cells and promotes its intrinsic GTPase activity. Microscopy studies indicated an involvement of IqgC in large-scale endocytosis, with strong accumulation on macropinosomes and notably weaker on phagosomes. Functional assays with knock-out and overexpressing cells corroborated the localization data, but, unexpectedly, the effect on the intake efficiency in mutants was more pronounced in phagocytosis. Hence, we identified a RasGAP that regulates Ras activity specifically during large-scale endocytosis in vegetative cells.

*Dictyostelium* cells are preferentially phagocytes with a facultative ability to up-regulate and perform macropinocytosis if the appropriate nutrients that support growth are available in liquid medium (49). It is well-established that Ras signaling regulates macropinocytosis in *Dictyostelium* and mammalian cells alike (16). For instance, expression of both proto-oncogenic and oncogenic Ras positively affects nutrient uptake by macropinocytosis (5, 14). In line with Ras-induced macropinocytosis in mammalian cells, RasG and RasS proteins seem to be the main positive regulators of macropinocytosis in *Dictyostelium*. Initial phenotypic characterization of *rasG*⁻ cells reported a growth defect in shaken suspension that was linked to a cytokinesis defect (55), but a recent study also demonstrated reduced fluid-phase uptake (15). Disruption of *rasS* also induced a macropinocytosis defect, and even a strain-dependent inability of mutant cells to grow in axenic medium (15, 19). We observed an increased macropinocytosis

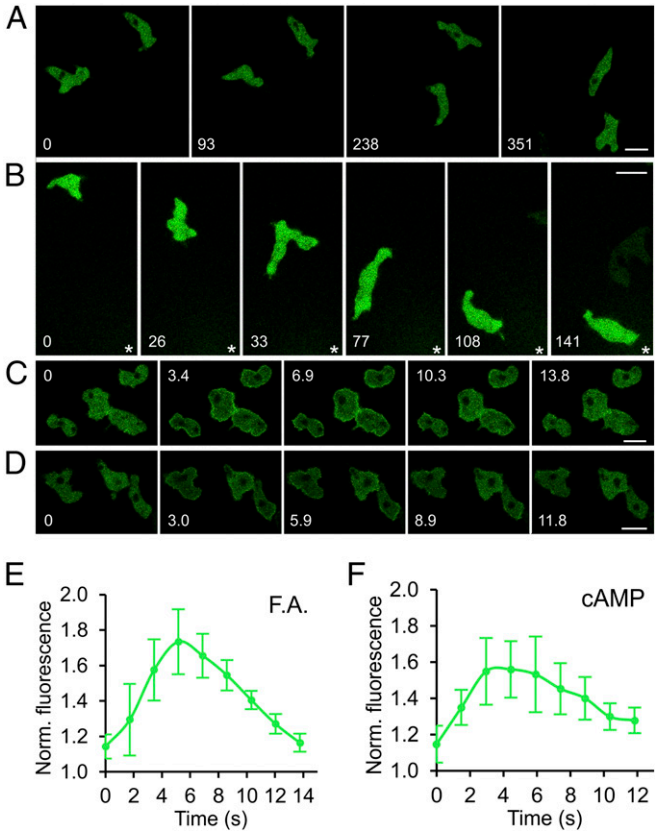


**Fig. 9.** IqgC does not polarize during migration and chemotaxis but translocates to the cortex in response to chemoattractant pulses. Localization of YFP-IqgC is shown during (*A*) unstimulated migration of aggregation-competent cells, (*B*) chemotaxis toward the 100 μM cAMP-filled micropipette (designated with asterisk), (*C*) stimulation of vegetative cells with a pulse of 50 μM folic acid, and (*D*) stimulation of aggregation-competent cells with a pulse of 50 μM cAMP. Time course of the normalized cortical fluorescence intensity (mean cortical intensity/mean cytoplasmic intensity) of YFP-IqgC is shown for (*E*) folic acid pulse stimulation, corresponding to experiment shown in *C*, and (*F*) cAMP pulse stimulation, corresponding to experiment shown in *D*. *A*–*D* correspond to Movies S6–S9. Time is given in seconds. (Scale bars: 10 μm.)

efficiency of *iqgC*$^{-}$ cells and a consistent negative correlation between the level of IqgC expression and both the TRITC-dextran uptake and the macropinosome size, thus identifying IqgC as a RasGAP that inhibits RasG signaling during macropinocytosis.

The increase in fluid-phase uptake in the absence of IqgC is relatively small probably because of a compensatory regulation of macropinocytosis via RasS. It was shown that RasS and RasG bind with similar affinities to RBDs of PI3K1 and PI3K2, which both control the ruffle formation in the initial phase of macropinocytosis (15). In addition, a partial redundancy between RasG and RasS was further suggested by the findings that PI(3,4,5)$P_3$ patches form at, and YFP-PI3K1(RBD) localizes to, the sites of fluid-phase uptake in both *rasS*$^{-}$ and *rasG*$^{-}$ mutants (15). Ruffle closure at the later stage of macropinocytosis, on the other hand, appears to be governed mostly by RasS-regulated PI3K4 (15). The relatively small impact that up-regulation of RasG activity in the absence of IqgC has on macropinocytosis efficiency suggests that Ras signaling in macropinocytosis is already operating near the saturation level in WT axenic cells. Interestingly, down-regulation of RasG activity in IqgC-overexpressing cells appears to have a more profound influence on macropinocytosis, suggesting yet a unique role for RasG that cannot be compensated by RasS, and possibly other Ras proteins.

The negative regulatory role of IqgC on macropinocytosis is reminiscent of the role played by the *Dictyostelium* ortholog of the human RasGAP NF1 in natural WT amoebas, which are not able to grow axenically in chemically defined nutrient medium due to a poor fluid-phase uptake (18). The gene encoding *Dictyostelium* NF1, *axeB*, is largely deleted in axenic laboratory strains, thus enabling these cells to feed by constitutive macropinocytosis. Although this study did not directly demonstrate increased levels of activated Ras in *axeB*$^{-}$ cells, an increased FITC-dextran uptake in parallel with more frequent generation of larger macropinosomes was observed (18). We detected a small, but statistically significant, increase in the macropinosome size in *iqgC*$^{-}$ compared with WT cells, which is even more pronounced compared with overexpressors, but no change in the macropinosome occurrence rate in *iqgC*$^{-}$ cells. Of note, the frequency of macropinosome formation in WT AX2 cells was lower in our experiments compared with the data published in a previous work (18). This is probably due to the fact that we substituted the glucose in HL5 medium with 50 mM maltose. Namely, it was recently shown that 55 mM glucose induces almost a twofold increase in the rate of macropinocytosis compared with the same concentration of maltose in the nutrient medium (49). Further, live cell imaging showed that IqgC resides on the macropinosome from the activation of the membrane patch until its closure, but slightly longer than the pan-probe for active Ras. We also investigated sequential stages of the endocytic pathway to test the possibility that mutant cells have a delay in vesicle trafficking due to a prolonged retention of active RasG on internalized macropinosomes. Since no delay was detected at any step of vesicle processing, we propose that the role of IqgC is restricted to regulation of macropinosome size. Taken together, our results indicate that IqgC finely tunes spatial, rather than temporal, aspects of RasG signaling during macropinosome formation and closure.

Although IqgC was only weakly and/or sporadically present at the phagosome, *iqgC*$^{-}$ cells exhibited a markedly increased phagocytosis rate, thus identifying IqgC as a negative regulator of phagocytosis as well. Hitherto, however, no published data clearly support RasG involvement in phagocytosis. As far as we are aware, only one study tested the phagocytosis rate of *rasG*$^{-}$ cells in the AX3 background, but reported no difference in comparison with parental cells (56). On the other hand, RasG was identified as a part of the phagosome proteome (57). Furthermore, it was shown that the increased macropinocytosis efficiency of *axeB*$^{-}$ cells correlates with increased phagocytosis of large particles and that NF1 restricts the size of the endocytic cup via inhibition of Ras in both processes (18, 49). In addition, the phosphoinositide dynamics is similar on both macropinosomes and phagosomes (58) although PI3Ks responsible for the formation of these two classes of endocytic cups are not the same. In particular, PI3Ks that are possibly activated by RasG on the phagosome are yet to be identified (59, 60). However, since class I PI3K activity is dispensable for phagocytosis of bacteria-sized particles (15, 61, 62), it is conceivable that IqgC influences phagocytosis of small particles through interaction with a GTPase other than RasG. Our IqgC interactome analysis revealed several GTPases from the Ras superfamily, including RasB, Rac1A, Rap1, Sar1, and Rab11A, which were also identified in the proteome of the *Dictyostelium* phagosome (57). The yeast two-hybrid screen failed to identify a direct interaction of RasB and Rac1A with IqgC. A likely target of the IqgC RasGAP activity in phagocytosis is Rap1, an important regulator of early stages in large-scale endocytosis. Overexpression of WT and constitutively active Rap1 induced more than a twofold increase in the phagocytosis rate, but a marked decrease in the macropinocytosis rate (20). *Dictyostelium* Rap1 is an ortholog of human Rap1A/Rap1B, with a 77% identity to both isoforms (63). Furthermore, human Rap1 is highly similar to human H-Ras, and these GTPases can

interact with the same regulatory proteins, such as RasGAPs. For instance, $p120^{GAP}$ was shown to bind Rap1A preferentially in its GTP-bound form, but it does not stimulate Rap1A GTPase activity (64, 65). On the other hand, RasGAPs from GAP1 and SynGAP families display dual GAP activity toward Ras and Rap (66, 67). Consequences of these interactions on Rap1 regulation in cells might be either its direct inactivation or sequestration by RasGAP, which would also reduce the amount of RasGAP available for interaction with Ras. It would be worthwhile to conduct experiments evaluating the interaction between IqgC and Rap1 and testing whether IqgC promotes GTPase activity of Rap1. Finally, we cannot exclude the possibility that IqgC affects phagocytosis through interaction with other binding partners or by performing roles distinct from its GAP activity, such as scaffolding to integrate signaling pathways, as already proposed for other *Dictyostelium* IQGAP proteins (36). In addition, IqgC is ubiquitously expressed throughout the *Dictyostelium* life cycle, with only a minor drop in the early development (*SI Appendix*, Fig. S7). Since RasG is only expressed during vegetative growth and early development when macropinocytosis is attenuated (68, 69), IqgC must operate in distinct, RasG-independent processes during late development.

Based on the absence of its polarized cortical enrichment in moving cells, IqgC appears not to be involved in localized regulation of cell migration, neither in vegetative nor in aggregation-competent cells during random motility and chemotaxis. Nevertheless, since IqgC is homogenously distributed in the cytoplasm and the hyaline, actin-rich zones of migrating cells, its role in global regulation of directed cell migration, possibly inhibitory and mediated by small GTPases other than RasG, cannot be excluded (70, 71). A previous quantitative analysis of chemotaxis parameters, however, demonstrated no major differences in the chemotaxis of *iqgC*$^{-}$ cells compared with WT (32). Interestingly, cortical recruitment of YFP-IqgC can be induced using pulsed stimulation by chemoattractants folic acid and cAMP. Three arguments support the notion that IqgC is corecruited with RasG, as shown recently for C2GAP1 (24). First, the temporal profile of the IqgC response to both chemoattractants corresponds to the response of the active Ras pan-probe (24, 53, 54). Second, the response to folic acid is more prominent than the response to cAMP, consistent with the temporal expression profile of RasG (72). Third, based on the similarity of the reduced ERK2 phosphorylation response to both chemoattractants in cells expressing constitutively active RasG, it was suggested that RasG is involved in the regulation of signaling pathways initiated by both folic acid and cAMP receptors (73). Thus, IqgC might be corecruited with RasG in response to strong and rapid stimuli whereas it is retained in the cytoplasm during milder stimulation in the chemoattractant gradient.

The *iqgC*$^{-}$ strain displayed a slight increase in the number of multinucleated cells when grown both attached to the surface and in the shaken suspension, suggesting a mild cytokinesis defect. This defect, however, did not affect the growth rate in the shaken suspension, possibly because of a compensating increase in the fluid uptake. Interestingly, both *rasG*$^{-}$ cells, and *nfaA*$^{-}$ cells that have increased levels of activated RasG and RasB, exhibited a defect in cytokinesis that was coupled to significantly impaired growth in suspension (23, 55). Similarly, cells overexpressing WT, constitutively active, or dominant negative RasG, all exhibited decreased growth rates in the shaken suspension (74). *iqgC*$^{-}$ cells also exhibited lower growth rates on bacterial lawns, consistent with greatly reduced growth of AX3 cells overexpressing activated RasG on bacterial plates (75). It appears, therefore, that cytokinesis and growth are affected by deregulated RasG signaling in general. Additional experiments will be necessary to delineate the physiological functions of IqgC in cytokinesis and development.

Phylogenetic analysis of RasGAP proteins from 64 diverse eukaryotic genomes revealed five clusters, and two of them, GAP1-like and IQGAP-like RasGAPs, probably have a common origin predating the last eukaryotic common ancestor (30). Human IQGAPs and the *Schizosaccharomyces pombe* GAP1 family share a conserved RasGAP-RasGAP_C domain architecture that is not present in any other RasGAP family. Although reports about an interaction between human IQGAP3 and H-Ras are contradictory (76, 77), there is no evidence for a RasGAP activity of IQGAP proteins. On the other hand, *S. pombe* GAP1 negatively regulates Ras1 activity, but no GAP1-like RasGAPs were found in higher animals (78). Consistent with the functional data, *Dictyostelium* DGAP1 and GAPA that have lost GAP activity are positioned on the phylogenetic tree within the IQGAP group while IqgC that has a conserved RasGAP activity is closer to the GAP1 family members (30). Together with the results presented here, these findings call for repositioning of IqgC, also designated as DdIQGAP3, outside of the *Dictyostelium* IQGAP group.

## Materials and Methods

A complete description of the methods is provided in *SI Appendix, Supplementary Materials and Methods*. It includes the description of all used vectors and *Dictyostelium* cell lines, construction of the *knock-out* strain, the GST-fusion protein binding assay and mass spectrometry analysis, yeast two-hybrid, BiFC, GAP, and endocytosis assays, confocal microscopy, and image analyses with statistical analysis. Mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD006311 (79).

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1. Conner SD, Schmid SL (2003) Regulated portals of entry into the cell. *Nature* 422:37–44.
2. Liu Z, Roche PA (2015) Macropinocytosis in phagocytes: Regulation of MHC class-II-restricted antigen presentation in dendritic cells. *Front Physiol* 6:1.
3. Clayton EL, Cousin MA (2009) The molecular physiology of activity-dependent bulk endocytosis of synaptic vesicles. *J Neurochem* 111:901–914.
4. Zeineddine R, Yerbury JJ (2015) The role of macropinocytosis in the propagation of protein aggregation associated with neurodegenerative diseases. *Front Physiol* 6:277.
5. Commisso C, et al. (2013) Macropinocytosis of protein is an amino acid supply route in Ras-transformed cells. *Nature* 497:633–637.
6. Qian Y, et al. (2014) Extracellular ATP is internalized by macropinocytosis and induces intracellular ATP increase and drug resistance in cancer cells. *Cancer Lett* 351:242–251.
7. Cossart P, Helenius A (2014) Endocytosis of viruses and bacteria. *Cold Spring Harb Perspect Biol* 6:a016972.
8. Swanson JA (2008) Shaping cups into phagosomes and macropinosomes. *Nat Rev Mol Cell Biol* 9:639–649.
9. Junemann A, et al. (2016) A Diaphanous-related formin links Ras signaling directly to actin assembly in macropinocytosis and phagocytosis. *Proc Natl Acad Sci USA* 113:E7464–E7473.
10. Buckley CM, King JS (2017) Drinking problems: Mechanisms of macropinosome formation and maturation. *FEBS J* 284:3778–3790.
11. Veltman DM, et al. (2016) A plasma membrane template for macropinocytic cups. *eLife* 5:e20085.
12. Egami Y, Taguchi T, Maekawa M, Arai H, Araki N (2014) Small GTPases and phosphoinositides in the regulatory mechanisms of macropinosome formation and maturation. *Front Physiol* 5:374.
13. Croisé P, Estay-Ahumada C, Gasman S, Ory S (2014) Rho GTPases, phosphoinositides, and actin: A tripartite framework for efficient vesicular trafficking. *Small GTPases* 5:e29469.
14. Bar-Sagi D, Feramisco JR (1986) Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. *Science* 233:1061–1068.
15. Hoeller O, et al. (2013) Two distinct functions for PI3-kinases in macropinocytosis. *J Cell Sci* 126:4296–4307.
16. Bloomfield G, Kay RR (2016) Uses and abuses of macropinocytosis. *J Cell Sci* 129:2697–2705.
17. Clark J, et al. (2014) Dictyostelium uses ether-linked inositol phospholipids for intracellular signalling. *EMBO J* 33:2188–2200.

18. Bloomfield G, et al. (2015) Neurofibromin controls macropinocytosis and phagocytosis in Dictyostelium. *eLife* 4:e04940.
19. Chubb JR, Wilkins A, Thomas GM, Insall RH (2000) The Dictyostelium RasS protein is required for macropinocytosis, phagocytosis and the control of cell movement. *J Cell Sci* 113:709–719.
20. Seastone DJ, et al. (1999) The small Mr Ras-like GTPase Rap1 and the phospholipase C pathway act to regulate phagocytosis in Dictyostelium discoideum. *Mol Biol Cell* 10: 393–406.
21. Welliver TP, Chang SL, Linderman JJ, Swanson JA (2011) Ruffles limit diffusion in the plasma membrane during macropinosome formation. *J Cell Sci* 124:4106–4114.
22. Hennig A, Markwart R, Esparza-Franco MA, Ladds G, Rubio I (2015) Ras activation revisited: Role of GEF and GAP systems. *Biol Chem* 396:831–848.
23. Zhang S, Charest PG, Firtel RA (2008) Spatiotemporal regulation of Ras activity provides directional sensing. *Curr Biol* 18:1587–1593.
24. Xu X, et al. (2017) GPCR-controlled membrane recruitment of negative regulator C2GAP1 locally inhibits Ras signaling for adaptation and long-range chemotaxis. *Proc Natl Acad Sci USA* 114:E10092–E10101.
25. White CD, Erdemir HH, Sacks DB (2012) IQGAP1 and its binding proteins control diverse biological functions. *Cell Signal* 24:826–834.
26. Weissbach L, et al. (1994) Identification of a human rasGAP-related protein containing calmodulin-binding motifs. *J Biol Chem* 269:20517–20521.
27. Hart MJ, Callow MG, Souza B, Polakis P (1996) IQGAP1, a calmodulin-binding protein with a rasGAP-related domain, is a potential effector for cdc42Hs. *EMBO J* 15: 2997–3005.
28. Brill S, et al. (1996) The Ras GTPase-activating-protein-related human protein IQGAP2 harbors a potential actin binding domain and interacts with calmodulin and Rho family GTPases. *Mol Cell Biol* 16:4869–4878.
29. Briggs MW, Sacks DB (2003) IQGAP proteins are integral components of cytoskeletal regulation. *EMBO Rep* 4:571–574.
30. van Dam TJ, Bos JL, Snel B (2011) Evolution of the Ras-like small GTPases and their regulators. *Small GTPases* 2:4–16.
31. Rivero F, Xiong H (2016) Rho signaling in Dictyostelium discoideum. *Int Rev Cell Mol Biol* 322:61–181.
32. Lee S, Shen Z, Robinson DN, Briggs S, Firtel RA (2010) Involvement of the cytoskeleton in controlling leading-edge function during chemotaxis. *Mol Biol Cell* 21:1810–1824.
33. Adachi H, et al. (1997) Dictyostelium IQGAP-related protein specifically involved in the completion of cytokinesis. *J Cell Biol* 137:891–898.
34. Faix J, Dittrich W (1996) DGAP1, a homologue of rasGTPase activating proteins that controls growth, cytokinesis, and development in Dictyostelium discoideum. *FEBS Lett* 394:251–257.
35. Faix J, et al. (1998) The IQGAP-related protein DGAP1 interacts with Rac and is involved in the modulation of the F-actin cytoskeleton and control of cell motility. *J Cell Sci* 111:3059–3071.
36. Faix J, et al. (2001) Recruitment of cortexillin into the cleavage furrow is controlled by Rac1 and IQGAP-related proteins. *EMBO J* 20:3705–3715.
37. Filić V, Marinović M, Faix J, Weber I (2012) A dual role for Rac1 GTPases in the regulation of cell motility. *J Cell Sci* 125:387–398.
38. Veltman DM, Lemieux MG, Knecht DA, Insall RH (2014) $PIP_3$-dependent macropinocytosis is incompatible with chemotaxis. *J Cell Biol* 204:497–505.
39. Veltman DM (2015) Drink or drive: Competition between macropinocytosis and cell migration. *Biochem Soc Trans* 43:129–132.
40. Scheffzek K, Lautwein A, Kabsch W, Ahmadian MR, Wittinghofer A (1996) Crystal structure of the GTPase-activating domain of human p120GAP and implications for the interaction with Ras. *Nature* 384:591–596.
41. Lim CJ, et al. (2005) Loss of the Dictyostelium RasC protein alters vegetative cell size, motility and endocytosis. *Exp Cell Res* 306:47–55.
42. Fonović M (2018) Proteomic identification of IqgC binding partners. PRIDE Archive. Available at https://www.ebi.ac.uk/pride/archive/projects/PXD006311. Deposited December 21, 2018.
43. Vogel US, et al. (1988) Cloning of bovine GAP and its interaction with oncogenic ras p21. *Nature* 335:90–93.
44. Gideon P, et al. (1992) Mutational and kinetic analyses of the GTPase-activating protein (GAP)-p21 interaction: The C-terminal domain of GAP is not sufficient for full activity. *Mol Cell Biol* 12:2050–2056.
45. Ohashi K, Kiuchi T, Shoji K, Sampei K, Mizuno K (2012) Visualization of cofilin-actin and Ras-Raf interactions by bimolecular fluorescence complementation assays using a new pair of split Venus fragments. *Biotechniques* 52:45–50.
46. Chuang E, et al. (1994) Critical binding and regulatory interactions between Ras and Raf occur through a small, stable N-terminal domain of Raf and specific Ras effector residues. *Mol Cell Biol* 14:5318–5325.
47. Herrmann C, Martin GA, Wittinghofer A (1995) Quantitative analysis of the complex between p21ras and the Ras-binding domain of the human Raf-1 protein kinase. *J Biol Chem* 270:2901–2905.
48. Buhrman G, Wink G, Mattos C (2007) Transformation efficiency of RasQ61 mutants linked to structural features of the switch regions in the presence of Raf. *Structure* 15: 1618–1629.
49. Williams TD, Kay RR (2018) The physiological regulation of macropinocytosis during Dictyostelium growth and development. *J Cell Sci* 131:jcs213736.
50. Hacker U, Albrecht R, Maniak M (1997) Fluid-phase uptake by macropinocytosis in Dictyostelium. *J Cell Sci* 110:105–112.
51. Huang YE, et al. (2003) Receptor-mediated regulation of PI3Ks confines PI(3,4,5)P3 to the leading edge of chemotaxing cells. *Mol Biol Cell* 14:1913–1922.
52. Charest PG, Firtel RA (2007) Big roles for small GTPases in the control of directed cell movement. *Biochem J* 401:377–390.
53. Kortholt A, et al. (2011) Dictyostelium chemotaxis: Essential Ras activation and accessory signalling pathways for amplification. *EMBO Rep* 12:1273–1279.
54. Srinivasan K, et al. (2013) Delineating the core regulatory elements crucial for directed cell migration by examining folic-acid-mediated responses. *J Cell Sci* 126: 221–233.
55. Tuxworth RI, et al. (1997) Dictyostelium RasG is required for normal motility and cytokinesis, but not growth. *J Cell Biol* 138:605–614.
56. Khosla M, Spiegelman GB, Insall R, Weeks G (2000) Functional overlap of the dictyostelium RasG, RasD and RasB proteins. *J Cell Sci* 113:1427–1434.
57. Gotthardt D, et al. (2006) Proteomics fingerprinting of phagosome maturation and evidence for the role of a Galpha during uptake. *Mol Cell Proteomics* 5:2228–2243.
58. Dormann D, Weijer G, Dowler S, Weijer CJ (2004) In vivo analysis of 3-phosphoinositide dynamics during Dictyostelium phagocytosis and chemotaxis. *J Cell Sci* 117: 6497–6509.
59. Buczynski G, et al. (1997) Inactivation of two Dictyostelium discoideum genes, DdPIK1 and DdPIK2, encoding proteins related to mammalian phosphatidylinositide 3-kinases, results in defects in endocytosis, lysosome to postlysosome transport, and actin cytoskeleton organization. *J Cell Biol* 136:1271–1286.
60. Chen C-L, Wang Y, Sesaki H, Iijima M (2012) Myosin I links PIP3 signaling to remodeling of the actin cytoskeleton in chemotaxis. *Sci Signal* 5:ra10.
61. Peracino B, Balest A, Bozzaro S (2010) Phosphoinositides differentially regulate bacterial uptake and Nramp1-induced resistance to Legionella infection in Dictyostelium. *J Cell Sci* 123:4039–4051.
62. Schlam D, et al. (2015) Phosphoinositide 3-kinase enables phagocytosis of large particles by terminating actin assembly through Rac/Cdc42 GTPase-activating proteins. *Nat Commun* 6:8623.
63. Caron E (2003) Cellular functions of the Rap1 GTP-binding protein: A pattern emerges. *J Cell Sci* 116:435–440.
64. Frech M, et al. (1990) Inhibition of GTPase activating protein stimulation of Ras-p21 GTPase by the Krev-1 gene product. *Science* 249:169–171.
65. Cullen PJ, et al. (1995) Identification of a specific Ins(1,3,4,5)P4-binding protein as a member of the GAP1 family. *Nature* 376:527–530.
66. Kupzig S, et al. (2006) GAP1 family members constitute bifunctional Ras and Rap GTPase-activating proteins. *J Biol Chem* 281:9891–9900.
67. Krapivinsky G, Medina I, Krapivinsky L, Gapon S, Clapham DE (2004) SynGAP-MUPP1-CaMKII synaptic complexes regulate p38 MAP kinase activity and NMDA receptor-dependent synaptic AMPA receptor potentiation. *Neuron* 43:563–574.
68. Robbins SM, Williams JG, Jermyn KA, Spiegelman GB, Weeks G (1989) Growing and developing Dictyostelium cells express different ras genes. *Proc Natl Acad Sci USA* 86: 938–942.
69. Katoh M, Chen G, Roberge E, Shaulsky G, Kuspa A (2007) Developmental commitment in Dictyostelium discoideum. *Eukaryot Cell* 6:2038–2045.
70. Tang M, et al. (2014) Evolutionarily conserved coupling of adaptive and excitable networks mediates eukaryotic chemotaxis. *Nat Commun* 5:5175.
71. Devreotes PN, et al. (2017) Excitable signal transduction networks in directed cell migration. *Annu Rev Cell Dev Biol* 33:103–125.
72. Khosla M, Robbins SM, Spiegelman GB, Weeks G (1990) Regulation of DdrasG gene expression during Dictyostelium development. *Mol Cell Biol* 10:918–922.
73. Kosaka C, Khosla M, Weeks G, Pears C (1998) Negative influence of RasG on chemoattractant-induced ERK2 phosphorylation in Dictyostelium. *Biochim Biophys Acta* 1402:1–5.
74. Khosla M, Spiegelman GB, Weeks G (1996) Overexpression of an activated rasG gene during growth blocks the initiation of Dictyostelium development. *Mol Cell Biol* 16: 4156–4162.
75. Chen CF, Katz ER (2000) Mediation of cell-substratum adhesion by RasG in Dictyostelium. *J Cell Biochem* 79:139–149.
76. Wang S, et al. (2007) IQGAP3, a novel effector of Rac1 and Cdc42, regulates neurite outgrowth. *J Cell Sci* 120:567–577.
77. Nojima H, et al. (2008) IQGAP3 regulates cell proliferation through the Ras/ERK signalling cascade. *Nat Cell Biol* 10:971–978.
78. Imai Y, Miyake S, Hughes DA, Yamamoto M (1991) Identification of a GTPase-activating protein homolog in Schizosaccharomyces pombe. *Mol Cell Biol* 11: 3088–3094.
79. Vizcaíno JA, et al. (2016) 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res* 44:D447–D456.