An Autocrine Negative Feedback Loop Inhibits Dictyostelium discoideum Proliferation through Pathways Including IP3/Ca2+^1

Yu Tang,a Ramesh Rijal,a David E. Zimmerhanzel,a Jacquelyn R. McCullough,a Louis A. Cadena,a Richard H. Gomer^a

aDepartment of Biology, Texas A&M University, College Station, Texas, USA

Abstract

Little is known about how eukaryotic cells can sense their number or spatial density and stop proliferating when the local density reaches a set value. We previously found that Dictyostelium discoideum accumulates extracellular polyphosphate to inhibit its proliferation, and this requires the G protein-coupled receptor GrlD and the small GTPase RasC. Here, we show that cells lacking the G protein component Gb, the Ras guanine nucleotide exchange factor GefA, phosphatase and tensin homolog (PTEN), phospholipase C (PLC), inositol 1,4,5-trisphosphate (IP3) receptor-like protein A (IplA), polyphosphate kinase 1 (Ppk1), or the TOR complex 2 component PiaA have significantly reduced sensitivity to polyphosphate-induced proliferation inhibition. Polyphosphate upregulates IP3, and this requires GrlD, GefA, PTEN, PLC, and PiaA. Polyphosphate also upregulates cytosolic Ca^{2+}, and this requires GrlD, Gb, GefA, RasC, PLC, IplA, Ppk1, and PiaA. Together, these data suggest that polyphosphate uses signal transduction pathways including IP3/Ca^{2+} to inhibit the proliferation of D. discoideum.

Importance

Many mammalian tissues such as the liver have the remarkable ability to regulate their size and have their cells stop proliferating when the tissue reaches the correct size. One possible mechanism involves the cells secreting a signal that they all sense, and a high level of the signal tells the cells that there are enough of them and to stop proliferating. Although regulating such mechanisms could be useful to regulate tissue size to control cancer or birth defects, little is known about such systems. Here, we use a microbial system to study such a mechanism, and we find that key elements of the mechanism have similarities to human proteins. This then suggests the possibility that we may eventually be able to regulate the proliferation of selected cell types in humans and animals.

Keywords

Dictyostelium, cell density sensing, polyphosphate, cell proliferation, PLC/IP3/Ca^{2+}, calcium signaling, inositol trisphosphate, quorum sensing

A longstanding idea in developmental biology is that the size of a tissue or group of cells, or the spatial density of a specific cell type, could be limited by an autocrine proliferation inhibitor, where the concentration of the inhibitor increases as the size of the tissue or cell group, or the density of cells, increases (1–8). The existence of autocrine proliferation inhibitors has been reported in mammalian tissues and organs, including skin (2), muscle (8), spleen (1), and liver (4), and the eukaryotic microorganism Dictyostelium discoideum (9). Although a considerable amount is known about signals and signal transduction pathways that promote cell proliferation, relatively little is known about autocrine proliferation-inhibiting signals and their signal transduction pathways.

Polyphosphate is a linear polymer of phosphate residues and is present in all kingdoms of life (10–12). In bacteria, polyphosphate functions in energy and phosphate storage (10) and potentiates both survival under some high-stress conditions (13) and biofilm formation (14, 15). In mammals, polyphosphate inhibits bone calcification (16).
and the proliferation of leukemia cells (17), potentiates proinflammatory responses (18) and mTOR activation of plasma cells (19), accelerates blood coagulation (20), and induces apoptosis (21).

*D. discoideum* grows on soil surfaces and eventually overgrows its food supply and starves. *D. discoideum* accumulates extracellular polyphosphate as cells grow and proliferate (9). At cell densities corresponding to mid-log phase, the extracellular polyphosphate causes some cells to store rather than digest phagocytosed bacteria, possibly in anticipation of possible starvation (22). At very high cell densities, when the cells are about to starve, the accumulated extracellular polyphosphate reaches ~150 μM. This concentration of polyphosphate contributes to the inhibition of cytokinesis (and, thus, cell proliferation) (9), possibly to prevent the formation of small cells. Therefore, just before starvation, the percentage of large cells with relatively large reserves of stored nutrients is increased (9).

Polyphosphate regulates the proliferation of *D. discoideum* by different signaling pathways depending on nutrient levels (23). In rich media, the loss of the G protein-coupled receptor GrlD, a metabotropic glutamate receptor-like receptor, partially reduced the sensitivity of cells to polyphosphate, and the loss of the small GTPase RasC did not reduce the sensitivity of cells to polyphosphate (23). However, under low-nutrient conditions, the loss of GrlD or RasC blocked the sensitivity of cells to polyphosphate (23).

The above-mentioned results suggest that polyphosphate uses a signal transduction pathway to inhibit *D. discoideum* proliferation under low-nutrient conditions. To elucidate additional signaling components in the polyphosphate proliferation inhibition pathway, we screened 52 available signal transduction pathway mutants for insensitivity to polyphosphate-induced proliferation inhibition under low-nutrient conditions. In combination with biochemical assays, we found evidence for a pathway involving inositol 1,4,5-trisphosphate (IP3) and cytosolic calcium that may mediate autocrine proliferation inhibition in *Dictyostelium*.

**RESULTS**

In addition to a G protein-coupled receptor and a Ras protein, a Ras GEF potentiates polyphosphate inhibition of cell proliferation. We previously observed that polyphosphate inhibits the proliferation of wild-type *D. discoideum* cells and that the loss of GrlD, RasC, or polyphosphate kinase 1 (Ppk1) reduces the ability of polyphosphate to inhibit proliferation (9, 23), suggesting the existence of a polyphosphate signal transduction pathway. To identify additional components of the polyphosphate proliferation inhibition pathway, 52 available mutants were screened for sensitivity to polyphosphate-induced proliferation inhibition under the low-nutrient condition of 25% HLS. The data were graphed in 9 groups: commonly used parental/wild-type cells (Ax2 to HPS400) and previously reported polyphosphate signal transduction pathway components (Fig. 1A), G protein subunits (Fig. 1B), AprA pathway components (Fig. 1C), selected cAMP pathway components (Fig. 1D), phospholipase C (PLC)/IP3 pathway components (Fig. 1E), mitogen-activated protein kinase (MAPK) pathway/polyphosphate synthesis pathway components (Fig. 1F), *D. discoideum* development-related proteins (Fig. 1G), TOR complex components/protein kinases (Fig. 1H), and mechanotransduction components (Fig. 1I). The initial cell density was $1.5 \times 10^6$ cells/ml, and cells were counted 24 h later. The data were plotted as 100 × (density with polyphosphate $- 1.5 \times 10^6$ cells/ml)/(density with no added polyphosphate $- 1.5 \times 10^6$ cells/ml). This value would then be 100 if the polyphosphate had no effect on cell proliferation and 0 if the polyphosphate completely inhibited cell proliferation. Compared to no added polyphosphate, 125 μM and 150 μM polyphosphate reduced the increase in the cell density of Ax2 wild-type cells to ~30% and ~18%, respectively (Fig. 1A). At 24 h, the density of the Ax2 cells with no polyphosphate was $3.9 \times 10^6 \pm 0.1 \times 10^6$ cells/ml (mean ± standard error of the mean [SEM] ($n = 7$) (see Table S2 in the supplemental material), so the 18% cell density increase at 24 h represents a change in the doubling time from the control value of 17.7 ± 0.7 h to 81.3 ± 16.7 h. Polyphosphate
also reduced the proliferation of all the other commonly used parental/wild-type strains (Fig. 1A). The proliferation of these strains in the absence of added polyphosphate, and all of the mutant strains described below, is shown in Table S2. As previously reported, compared to Ax2 cells, cells lacking the putative polyphosphate receptor GrlD (23) or the Ras protein RasC (24) showed abolished sensitivity (no significant difference compared to no added polyphosphate by a t test) to 125 and 150 µM polyphosphate (Fig. 1A). Cells lacking GefA, a Ras guanine nucleotide exchange factor (GEF) for RasC but not RasB, RasD, or Rap1 (25), also showed reduced sensitivity to polyphosphate (Fig. 1A). The density of cells lacking RasG after 24 h was 80% ± 18% (mean ± SEM) (n = 3) of the initial cell density, suggesting that cells lacking rasG (rasG− cells) do not grow in 25% HL5.

The Gβ subunit potentiates polyphosphate inhibition of cell proliferation. Cells lacking the heterotrimeric G protein subunit Gβ (26) showed reduced sensitivity to polyphosphate inhibition of cell proliferation (Fig. 1B). Cells lacking Ga2, -3, -4, -5, -7, -8, or -9 did not have significantly abnormal sensitivity to polyphosphate. Cells lacking Ga1 (27) showed increased sensitivity to polyphosphate at 150 µM compared to their

---

**FIG 1** Some signal transduction pathway components are needed for polyphosphate (polyP) inhibition of proliferation in 25% HL5. The indicated cell lines were tested for proliferation with 0, 125, or 150 µM polyphosphate for 24 h. The increase in cell density over 24 h was normalized to the value with no added polyphosphate for the indicated strain. For each strain, the left bar is with 125 µM, and the right bar is with 150 µM polyphosphate. All values are means ± SEM (n ≥ 3 independent experiments). * indicates a P value of <0.05 compared to the parental wild-type cells with the same concentration of polyphosphate (by 2-way ANOVA, with multiple comparisons with Dunnett's test within the panel). 0 indicates not significantly different from 100, and thus, the associated concentration of polyphosphate does not significantly inhibit proliferation in that mutant (by a two-tailed one-sample t test).
parental strain HPS400. Whereas cells lacking the putative receptor GrlD appeared to be completely insensitive to polyphosphate, none of the Gα mutants showed complete insensitivity. Comparing the values for grlD− cells in Fig. 1A to those for the G protein mutants in Fig. 1B, although at 125 μM, the difference for gβ− was not significant, at 150 μM, the differences for gβ− were significant, with a P value of <0.01 (by t tests). These results suggest that there is an additional pathway downstream of GrlD that does not involve the single characterized Gβ in Dictyostelium (26) and that GrlD may activate multiple Gα subunits or untested Gα subunits.

The AprA pathway component PakD potentiates polyphosphate inhibition of cell proliferation. AprA is a secreted autocrine proliferation repressor and chemorepellent (28). Compared to their parental Ax2 cells, cells lacking the AprA pathway component PakD (a p21-activated kinase family member) (29) showed reduced sensitivity to polyphosphate (Fig. 1C). Compared to wild-type cells, cells lacking AprA, CfaD (a secreted factor that binds to AprA and then slows cell proliferation) (30), RblA (a retinoblastoma ortholog) (31), CnrN (a phosphatase and tensin homolog [PTEN]-like phosphatase involved in AprA sensing) (32–34), QkgA (a leucine-rich repeat [LRR] kinase family protein that is required for AprA-induced proliferation inhibition and chemorepulsion) (35), or BzpN (a transcription factor that is required for AprA-induced proliferation repression) (36) did not show significantly abnormal sensitivities to polyphosphate (Fig. 1C), indicating that many AprA pathway components are not used by polyphosphate to inhibit proliferation.

The cAMP response component PlaA potentiates polyphosphate inhibition of cell proliferation. Gβ and Gα2 mediate cAMP signaling in developing cells (37, 38). Compared to their parental Ax3 cells, cells lacking the cAMP chemoattraction pathway component phospholipase A2 PlaA (39) showed reduced sensitivity to 125 μM or 150 μM polyphosphate (Fig. 1D). Cells lacking ScrA (an adaptor protein that regulates actin polymerization) (40), ElmoE (an engulfment and cell motility protein, which transduces signals from chemoattractant receptors to the cytoskeleton) (41), GcA and SgcA (membrane-bound and soluble guanylyl cyclases, respectively) (42), WasA (an adaptor protein that regulates actin polymerization) (43), PikA and -B phosphatidylinositol kinases (44), or DagA (the cytosolic regulator of adenylate cyclase) (45) did not show significantly abnormal sensitivity to polyphosphate (Fig. 1D), indicating that many of the components that mediate cAMP chemoattraction are dispensable for polyphosphate to inhibit proliferation.

The PLC/IP3 pathway components PTEN, PLC, IplA, and DdSP4 potentiate polyphosphate inhibition of cell proliferation. Cells lacking PTEN (46), PLC (47), the inositol 1,4,5-trisphosphate (IP3) receptor-like protein IplA (48), or the inositol 5-phosphatase 4 DdSP4 (49) showed abolished sensitivity to 125 μM polyphosphate, and compared to their parental Ax2 or DH1 cells, cells lacking PTEN, PLC, or IplA showed reduced sensitivity to 150 μM polyphosphate (Fig. 1E). PTEN catalyzes the conversion of phosphatidylinositol (3-5)-trisphosphate (PIP3) to phosphatidylinositol (4,5)-bisphosphate (PIP2) (50), and PLC catalyzes the hydrolysis of PIP2 to diacylglycerol (DAG) and IP3 (50). IplA is a potential IP3 receptor in D. discoideum (48). DdSP4 dephosphorylates PIP3, PIP2, and IP3 (51). These results suggest that the PLC/IP3 pathway affects or is involved in polyphosphate inhibition of cell proliferation and that IP3 might be a second messenger in the polyphosphate signal transduction pathway.

The MAPK/Erk pathway components Erk1 and MekA potentiate polyphosphate inhibition of cell proliferation. Compared to their parental KAx3 or JH10 cells, cells lacking the extracellular signal-regulated kinase Erk1 (52) or the Erk1 kinase MekA (53) showed reduced sensitivity to 125 μM or 150 μM polyphosphate inhibition of cell proliferation (Fig. 1F). Deleting the suppressor of MekA, SmkA (53), did not significantly alter sensitivity to polyphosphate (Fig. 1F). These results suggest that the MekA-Erk1 pathway is involved in polyphosphate proliferation inhibition.

The polyphosphate synthesis pathway components I6kA and Ppk1 potentiate polyphosphate inhibition of cell proliferation. The inositol phosphate kinase I6kA does not appear to affect intracellular polyphosphate levels at cell densities below
~1 × 10^7 cells/ml but plays a role in upregulating intracellular polyphosphate at cell densities of ≥2 × 10^7 cells/ml (9). The polyphosphate kinase Ppk1 is essential for intracellular polyphosphate production at all cell stages (12). Compared to their parental Ax2 cells, cells lacking I6kA showed reduced sensitivity to 125 μM polyphosphate. Cells lacking Ppk1 showed abolished sensitivity to 125 μM polyphosphate and strongly reduced sensitivity to 150 μM polyphosphate (Fig. 1F). The correlation between intracellular polyphosphate synthesis and sensitivity to extracellular polyphosphate suggests that intracellular polyphosphate plays a role in polyphosphate inhibition of cell proliferation.

The development-related Gdt proteins potentiate polyphosphate inhibition of cell proliferation. Members of the growth-differentiation transition family of proteins (Gdts) are Dictyostelium-specific tyrosine kinase-like proteins, classified by their sequence similarity and their participation in development (54). Gdt1 and Gdt2 are negative regulators of the Dictyostelium growth-differentiation transition process (54, 55), but there is no report about the function of Gdt4 yet. Compared to their parental Ax4 cells, cells lacking growth-differentiation transition family member 2, or both Gdt1 and Gdt2, showed reduced sensitivity to both 125 μM and 150 μM polyphosphate (Fig. 1G). Cells lacking Gdt4 had reduced sensitivity to 125 μM polyphosphate. Cells lacking the protein contact site A CsaA (56) or the ammonium transporter AmtA (57) did not show significantly altered sensitivity to polyphosphate. These results suggest that Gdt2 and Gdt4 may play a role in cell proliferation.

The cell aggregate size regulator SmlA attenuates polyphosphate inhibition of cell proliferation. The small-aggregate formation protein SmlA regulates the size of cell aggregates and fruiting bodies during development by inhibiting the extracellular accumulation of the group size-regulating factor counting factor (58, 59). Compared to their parental strain DH1, for unknown reasons, cells lacking SmlA showed increased sensitivity to 125 μM polyphosphate and appeared to be hypersensitive to 150 μM polyphosphate (after 24 h, this polyphosphate concentration caused the cell density to decrease from 1.5 × 10^6 cells/ml to 1.2 × 10^6 ± 0.2 × 10^6 cells/ml [mean ± SEM] [n = 4]) (Fig. 1G).

The TORC2 component PiaA and the protein kinase PKA-C potentiate polyphosphate inhibition of cell proliferation. Dictyostelium Tor complex 2 (TORC2), composed of Tor, PiaA, Lst8, and Rip3, regulates adenylyl cyclase ACA (60, 61) and protein kinase B/Akt activation (60, 62) and is essential for cell aggregation (60, 63). Cells lacking the TORC2 component PiaA (Rictor) but not Lst8 showed abolished sensitivity to both 125 μM and 150 μM polyphosphate, suggesting that PiaA is an essential component of the polyphosphate proliferation inhibition pathway (Fig. 1H). Compared to their parental JH10 cells, cells lacking the cAMP-dependent protein kinase catalytic subunit PKA-C (64) showed reduced sensitivity to polyphosphate inhibition of cell proliferation, suggesting that cAMP might be a messenger in the polyphosphate proliferation inhibition pathway (Fig. 1H). Compared to wild-type cells, cells lacking Lst8 or protein kinase C (PKCA) did not show significantly abnormal sensitivities to polyphosphate (Fig. 1H), indicating that some components of the PKCA pathway are dispensable for polyphosphate to inhibit proliferation.

Four mechanotransduction components do not significantly affect polyphosphate inhibition of cell proliferation. Testing a variety of other signal transduction pathway components, we observed that cells lacking the mechanotransduction components SibA (an integrin beta-like protein) (65), TPC2 (two-pore calcium channel protein 2) (65), TrpP (the transient receptor potential cation channel protein) (65), or Mcln (an ortholog of mucolipin) (65) did not show significantly altered sensitivities to polyphosphate compared to their parental DH1 cells (Fig. 1I). These results suggest that many components of the mechanotransduction pathway are dispensable for polyphosphate to inhibit proliferation.

Gβ, GefA, PTEN, PLC, IplA, Ppk1, and PiaA potentiate polyphosphate inhibition of cell proliferation in both 25% and 100% HL5. With two-way analysis of variance (ANOVA) (multiple comparisons with Dunnett’s test), cells lacking GrbD, Gβ, GefA, RasC,
PTEN, PLC, IplA, Ppk1, and PiaA showed strongly reduced sensitivity to both 125 and 150 μM polyphosphate (indicated by * in Fig. 1A, B, and E to H) and showed abolished sensitivity (no statistical difference [by a one-sample t test] with 100% proliferation) to 125 μM polyphosphate (indicated by 0) (Fig. 1A, B, E, F, and H). They were thus chosen for further tests.

To further test the effects of the genes encoding Gβ, GefA, PTEN, PLC, IplA, Ppk1, and PiaA on the cells’ sensitivity to polyphosphate, mutant and available complemented strains were tested for sensitivity to polyphosphate with a more extensive dose-response curve in 25% HL5 (Fig. S1) (these assays were previously done for GrlD and RasC [17]). Compared to their respective parental wild-type cells, cells lacking Gβ, GefA, PTEN, PLC, IplA, Ppk1, or PiaA showed reduced sensitivity to physiological levels of polyphosphate (150 μM or lower) (Fig. S1). The 50% inhibitory concentrations (IC50s) of polyphosphate proliferation inhibition of these knockout mutant strains were higher than that of parental wild-type cells in 25% HL5 (Table 1). Expressing PTEN in pten−/cells and PLC in pIC−/cells rescued or partially rescued the decreased sensitivity to polyphosphate (Table 1 and Fig. S1C and D).

To determine if these proteins are also involved in the polyphosphate signal transduction pathway under nutrient-rich conditions, the corresponding knockout strains were tested for sensitivity to polyphosphate with dose-response curves in 100% HL5 (Fig. S2). In 100% HL5, compared to parental wild-type cells, cells lacking Gβ, GefA, PTEN, PLC, IplA, Ppk1, or PiaA also showed reduced sensitivity to polyphosphate (Fig. S2). In 100% HL5, the proliferation inhibition curve fits for gβ− and pten− cells could not be generated, and the curve fits for gefA−, pIC−, iplA−, ppp1−, and piaA− cells were ambiguous. The IC50s of polyphosphate proliferation inhibition of these knockout mutant strains were higher than that of parental wild-type cells (Table 1). Expressing PTEN in pten− cells and PLC in pIC− cells appeared to partially rescue or rescue the decreased sensitivity to polyphosphate (Table 1 and Fig. S2C and D). Together, these results support the idea that Gβ, GefA, PTEN, PLC, IplA, Ppk1, and PiaA affect the polyphosphate proliferation inhibition signal transduction pathway under both low- and high-nutrient conditions.

**Gβ, PTEN, PLC, IplA, Ppk1, and PiaA affect cell proliferation.** To assess the effect of the disruption of these genes on general cell proliferation, we assayed proliferation curves of the above-described strains in 100% HL5 in a shaking culture (Fig. 2), except for gβ− cells, which were assayed previously (66). The doubling times at a low cell

---

**TABLE 1** Deletion of some potential polyphosphate pathway components increases the IC50 for polyphosphate inhibition of proliferation

| Strain       | Mean IC50 (μM) ± SEM in 25% HLS5 | Mean IC50 (μM) ± SEM in 100% HL5 |
|--------------|----------------------------------|----------------------------------|
| Ax2          | 106 ± 3                          | 117 ± 10                         |
| DH1          | 121 ± 16                         | 102 ± 9                          |
| gβ−          | 173 ± 13                         | >200                             |
| gefA−        | 160 ± 5                          | 177 ± 35                         |
| pten−        | 178 ± 15**                       | >200                             |
| pten−/pten-GFP| 127 ± 8@                         | 91 ± 7                           |
| plC−         | 168 ± 6**                        | >200                             |
| plC−/pIC     | 124 ± 3@                         | 130 ± 3                          |
| iplA−        | 192 ± 8***                       | 188 ± 41                         |
| ppk1−        | 168 ± 5***                       | 189 ± 48                         |
| piaA         | 167 ± 10***                      | >200                             |

*IC50s were calculated from the data in Fig. S1 and S2 in the supplemental material, using Prism with nonlinear regression (sigmoidal dose-response, variable slope, and the top constrained to 100). All values are means ± SEM (n ≥ 3 independent experiments). *, P < 0.05; **, P < 0.01; ***,***, P < 0.001 (compared to the parental wild-type strain Ax2 or DH1 [by a two-tailed t test or one-way ANOVA followed by Tukey’s test among DH1, pIC−, and pIC−/cells or among Ax2, pten−, and pten−/pten-GFP cells]). @, P < 0.001 (compared to pIC− or pten− cells [by one-way ANOVA with Tukey’s test among DH1, pIC−, and pIC−/pIC cells or among Ax2, pten−, and pten−/pten-GFP cells]).

---
density (\(\sim 0.5 \times 10^6\) to \(6 \times 10^6\) cells/ml) and a high cell density (\(6 \times 10^6\) cells/ml to the maximal cell density or plateau) were calculated. At low cell densities, where the extracellular polyphosphate concentration is expected to be low, cells lacking PTEN or Ppk1 had a longer doubling time than Ax2 cells (Table 2), and cells lacking Gβ or PLC had a shorter doubling time than the parental wild-type DH1 cells (66). Expressing PTEN in \(pten^{-}\) cells rescued the long-doubling-time phenotype, and expressing PLC in \(plC^{-}\) cells further shortened the doubling time (Table 2), possibly because too little or too much PLC potentiates cell proliferation. At high cell densities, where the extracellular polyphosphate concentration is expected to be high, cells lacking IplA, Ppk1, or PiaA had shorter doubling times than Ax2 cells (66), and cells lacking PLC had a longer doubling time than DH1 cells (Table 2). Expressing PLC in \(plC^{-}\) cells caused a shorter doubling time than in DH1 cells (Table 2). These data suggest that PTEN and Ppk1 promote cell proliferation at low cell densities; PLC promotes cell proliferation, and IplA, | A | DH1 | AX2 | \(pten^{-}\) | \(pten^{-}/pten-GFP\) |
|---|---|---|---|---|
| B | AX2 | \(pten^{-}\) | \(pten^{-}/pten-GFP\) |
| C | DH1 | \(plC^{-}\) | \(plC^{-}/plC\) |
| D | Ax2 | \(iplA^{-}\) |
| E | Ax2 | \(ppk1^{-}\) |
| F | Ax2 | \(piaA^{-}\) |
Ppk1, and PiaA slow cell proliferation at high cell densities. The maximal cell density is abnormally high in cells lacking Gβ, GefA, IplA, Ppk1, or PiaA (66) (Fig. 2A, D, E, and F and Table 2) and is abnormally low in cells lacking PTEN or PLC (Fig. 2B and C and Table 2). Expressing PTEN in pten− cells and PLC in plC− cells rescued or reversed the phenotype (Fig. 2B and C and Table 2). These data suggest that these genes affect the proliferation of D. discoideum cells.

**Polyphosphate upregulates inositol 1,4,5-trisphosphate.** PLC catalyzes the hydrolysis of PIP2 to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (50, 67). PLC and the putative IP3 receptor IplA potentiate polyphosphate inhibition of cell proliferation, suggesting that IP3 might mediate polyphosphate proliferation inhibition. To examine this, we measured the effect of polyphosphate on IP3 levels with an IP3 assay. Polyphosphate significantly increased IP3 for ptiA− cells, and expressing PTEN in pten− cells and PLC in plC− cells partially rescued the response (Fig. 3C). The upregulation of IP3 for ptiA− cells is slightly but statistically significant. Polyphosphate did not significantly affect IP3 levels in grlD−, gefA−, pten−, plC−, and piaA− cells, and expressing PTEN in pten− cells and PLC in plC− cells partially rescued the response (Fig. 3C), possibly because the complementation, with the expression of the cDNA from an actin promoter, causes abnormally high or low levels of the complementing mRNA. Compared to Ax2 cells, the baseline IP3 levels of grlD−, plC−/plC−, and pppk1− cells were significantly higher, and the baseline IP3 level of piaA− was significantly lower (Fig. 3C). These results indicate that polyphosphate upregulates IP3 in D. discoideum; that this upregulation requires GrlD, GefA, PTEN, PLC, and PiaA; and that Gβ, RasC, IplA, or Ppk1 is dispensable for polyphosphate-induced upregulation of IP3.

**Polyphosphate upregulates cytosolic free Ca2+.** IP3 activates IP3 receptors on the endoplasmic reticulum, leading to Ca2+ release from the endoplasmic reticulum lumen to the cytosol in many organisms (50). In D. discoideum, the putative IP3 receptor IplA is localized mostly in cytoplasmic organelles and at very low levels at the plasma membrane and is involved in Ca2+ entry into the cytosol in response to chemoattractants (48, 68). As a partial test of the hypothesis that the GrlD-PLC-IP3-IP3-Ca2+ pathway is required for the inhibition of proliferation by polyphosphate, we examined the effect of polyphosphate on cytosolic Ca2+. 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetate acid (BAPTA-1) dextran, which shows increased fluorescence in the presence of Ca2+ (69), was loaded into Dictyostelium cells by electroporation. This technique loads BAPTA-dextran into the cytosol (69, 70). The BAPTA-1 dextran-loaded cells

---

**TABLE 2** Deletion of some potential polyphosphate pathway components alters the doubling time and maximal cell density

| Strain | Mean doubling time (h) ± SEM | Maximal density (10^6 cells/ml) |
|--------|------------------------------|---------------------------------|
|        | Low density                  | High density                    |                                |
| Ax2    | 16.3 ± 1.1                   | 32.4 ± 1.7                      | 21.8 ± 0.7                     |
| DH1    | 29.3 ± 2.1                   | 26.0 ± 2.7                      | 16.1 ± 1.2                     |
| gefA−  | 26.3 ± 1.2                   | 27.2 ± 1.2                      | 31.8 ± 0.5**                   |
| pten−  | 29.4 ± 4.4*                  | 33.8 ± 4.0                      | 13.8 ± 1.1***                  |
| pten−/pten-GFP | 17.6 ± 1.3@ | 32.9 ± 3.5                      | 22.5 ± 0.7@                   |
| plC−/plC | 21.2 ± 0.9*,@               | 41.8 ± 5.0@                     | 11.0 ± 0.6*                    |
| iplA−  | 9.0 ± 0.6***,@              | 20.9 ± 1.2                      | 33.0 ± 3.1*,@                  |
| ppk1−  | 14.9 ± 0.5                   | 25.2 ± 1.7*                     | 52.0 ± 1.3***                  |
| piaA−  | 19.9 ± 0.9*                  | 25.3 ± 1.1*                     | 27.3 ± 0.7**                   |
|        | 19.4 ± 1.6                   | 23.7 ± 2.2*                     | 38.4 ± 9.1                     |

*For the data in Fig. 2, doubling times were calculated for low cell densities (0.5 x 10^6 to 6 x 10^6 cells/ml) and high cell densities (6 x 10^6 cells/ml to the maximal density reached). Values are means ± SEM (n ≥ 3 independent experiments). *, P < 0.05; **, P < 0.01; ***, P < 0.001 (compared to their parental strains by a t test or one-way ANOVA with Dunnett’s test among DH1, plC−, and plC−/plC− cells or among Ax2, pten−, and pten−/pten-GFP cells). @, P < 0.001 (compared to plC− or pten− cells by one-way ANOVA with Dunnett’s test among DH1, plC−, and plC−/plC− cells or among Ax2, pten−, and pten−/pten-GFP cells).
were then incubated with or without polyphosphate, and Ca\(^{2+}\) levels were analyzed based on the total fluorescence per cell (representing the total Ca\(^{2+}\) amount) (Fig. 4A and C) and the mean fluorescence per square micrometer of cells (Fig. 4B and D) to exclude the impact of cell size/surface area. By both measurements, polyphosphate increased cytosolic free Ca\(^{2+}\) in Ax2 cells (Fig. 4 and Fig. S3). The polyphosphate-induced Ca\(^{2+}\) increase happened in 1 h and was maintained for at least 8 h (Fig. 4A and B). These data suggest that polyphosphate upregulates the resting Ca\(^{2+}\) level of cells.

To test if GrlD, G\(\beta\), GefA, RasC, PTEN, PLC, IplA, Ppk1, and PiaA affect the polyphosphate-induced Ca\(^{2+}\) increase, we measured the Ca\(^{2+}\) levels of the related mutant cells with or without polyphosphate for 4 h. Polyphosphate did not significantly affect cytosolic free Ca\(^{2+}\) in cells lacking GrlD, GefA, RasC, IplA, Ppk1, or PiaA (Fig. 4C and D); increased Ca\(^{2+}\) in cells lacking PTEN; and reduced Ca\(^{2+}\) in cells lacking G\(\beta\) or PLC (Fig. 4C and D). Expressing PLC in \(plC^{-}\) cells rescued the response to polyphosphate (Fig. 4C and D). Overall, these data suggest that polyphosphate upregulates cytosolic free Ca\(^{2+}\) of \(D.\) discoideum, and this requires GrlD, G\(\beta\), GefA, RasC, PLC, IplA, Ppk1, and PiaA.

**Polyphosphate inhibits cytokinesis.** Polyphosphate inhibits the proliferation of cells by inhibiting cytokinesis, causing an increased number of multinucleated cells (9). To determine if the signal transduction components identified above are needed for the effect of polyphosphate on cytokinesis, we measured the number of nuclei per cell in the presence or absence of polyphosphate. For wild-type cells (Ax2, Ax3, KAx3, Ax4, DH1, and JH10), polyphosphate increased the number of nuclei per cell (Table 3). This effect was not observed in cells lacking GrlD, G\(\beta\), RasC, PTEN, PLC, IplA, Ppk1, and PiaA (Table 3). Expressing PTEN in \(pten^{-}\) cells and PLC in \(plC^{-}\) cells rescued or partially rescued the sensitivity to polyphosphate (Table 3). These data suggest that most of the

---

**FIG 3** Polyphosphate upregulates inositol 1,4,5-trisphosphate (IP3) levels. (A) Cells were cultured with 0, 125, or 150 \(\mu\)M polyphosphate in 25% HLS for 1, 2, 4, 8, or 24 h and collected by centrifugation, and IP3 in the cells was measured. (B) For each assay, values were normalized to zero polyphosphate. (C) The indicated cell lines were assayed at 4 h as described above for panel B. All values are means ± SEM (n = 4 independent experiments for Ax2 and n ≥ 3 for mutants). *, \(P < 0.05\); **, \(P < 0.01\) (by a two-tailed paired \(t\) test). X indicates a \(P\) value of <0.05 compared to Ax2 with no added polyphosphate (by a two-tailed \(t\) test).
potential signaling components identified above are needed for polyphosphate inhibition of cytokinesis.

**Polyphosphate does not upregulate total Ras activity.** Ras is activated when it binds to GTP and inactivated when it binds to GDP (71). As RasC is required for the polyphosphate effect on proliferation, we hypothesized that polyphosphate might affect RasC activation. Due to the lack of a RasC-specific detection method, we tested the effect of polyphosphate on the total Ras activity of Ax2 cells. There are 11 Ras proteins in Dictyostelium (72). We did not observe any significant difference in active-Ras levels between cells cultured with 0 and those cultured with 150 μM polyphosphate for 1, 4, and 24 h (Fig. S4). This suggests that the RasC activity needed for the polyphosphate proliferation inhibition pathway might be only a small fraction of the total Ras activity.

**DISCUSSION**

We screened 52 signal transduction pathway mutants for sensitivity to polyphosphate-induced proliferation inhibition. We found that in addition to the previously reported GrlD receptor and RasC (17), Gβ, GefA, PakD, PlaA, PTEN, PLC, IpIA, Dd5p4, Erk1, MekA, I6kA, Ppk1, Gdt1, Gdt2, Gdt4, PlaA, and PKA-C potentiate polyphosphate inhibition of cell proliferation, suggesting that a complex signal transduction pathway mediates this example of an autocrine proliferation inhibition mechanism (Fig. 5). Compared to their respective parental cells, gβ−, gefA−, rasC−, pten−, plc−, ipIA−, ppk1−, and piaA− cells showed strongly reduced sensitivity to polyphosphate proliferation inhibition but not as abolished as that of grlD− cells. This suggests that there might be branched pathways downstream of the receptor GrlD. We observed that the lack of any tested Gα subunit did not abolish the cells’ sensitivity to polyphosphate inhibition of cell proliferation (Fig. 1B). This is possibly because multiple Gα subunits are
TABLE 3 The potential polyphosphate pathway components are needed for polyphosphate induced cell multinucleationa

| Cell type  | Polyphosphate concn (µM) | Mean no. of nuclei/100 cells ± SEM | Mean % of cells with no. of nuclei ± SEM |
|------------|--------------------------|-----------------------------------|-----------------------------------------|
|            |                          | 1       | 2       | 3+       |
| Ax2        | 0                        | 108 ± 2 | 93.2 ± 1.5 | 6.5 ± 1.3 | 0.3 ± 0.2 |
|            | 150                      | 123 ± 3*** | 79.4 ± 2.1*** | 18.7 ± 1.5*** | 1.9 ± 0.8 |
| Ax3        | 0                        | 114 ± 2 | 88.5 ± 1.5 | 10.0 ± 1.0 | 1.5 ± 0.6 |
|            | 150                      | 138 ± 5*** | 71.7 ± 2.6*** | 21.5 ± 1.3*** | 6.8 ± 1.7* |
| KAxF       | 0                        | 103 ± 2 | 97.0 ± 1.3 | 2.7 ± 1.1 | 0.3 ± 0.3 |
|            | 150                      | 127 ± 3*** | 75.2 ± 2.6*** | 23.1 ± 2.6*** | 1.7 ± 0.9 |
| Ax4        | 0                        | 102 ± 1 | 98.3 ± 0.6 | 1.6 ± 0.6 | 0.2 ± 0.2 |
|            | 150                      | 131 ± 4*** | 75.0 ± 3.1*** | 20.8 ± 3.2*** | 4.2 ± 0.8* |
| DH1        | 0                        | 118 ± 2 | 84.3 ± 1.1 | 12.8 ± 1.0 | 2.6 ± 1.0 |
|            | 150                      | 135 ± 3** | 69.2 ± 2.2*** | 27.1 ± 2.1*** | 3.7 ± 0.5 |
| JH10       | 0                        | 124 ± 4 | 82.1 ± 2.4 | 14.5 ± 2.0 | 3.5 ± 0.6 |
|            | 150                      | 147 ± 5* | 64.5 ± 4.2*** | 27.5 ± 3.5** | 8.0 ± 1.0* |
| giri<sup>+</sup> | 0                       | 104 ± 1 | 96.2 ± 1.2 | 3.8 ± 1.2 | 0 ± 0 |
|            | 150                      | 105 ± 2 | 95.3 ± 1.4 | 4.7 ± 1.2 | 0 ± 0 |
| gβ<sup>+</sup> | 0                       | 101 ± 1 | 98.9 ± 0.3 | 1.1 ± 0.3 | 0 ± 0 |
|            | 150                      | 102 ± 1 | 98.3 ± 0.7 | 1.4 ± 0.6 | 0 ± 0 |
| gefA<sup>-</sup> | 0                       | 109 ± 2 | 91.2 ± 1.6 | 8.5 ± 1.5 | 0.3 ± 0.3 |
|            | 150                      | 120 ± 4* | 81.0 ± 3.1* | 17.9 ± 2.8* | 1.1 ± 0.4* |
| rasC<sup>-</sup> | 0                       | 116 ± 4 | 86.8 ± 2.2 | 11.3 ± 1.5 | 1.9 ± 0.7 |
|            | 150                      | 117 ± 5 | 85.9 ± 3.0 | 12.5 ± 2.5 | 1.6 ± 0.5 |
| pten<sup>-</sup> | 0                       | 120 ± 2 | 82.4 ± 1.9 | 16.0 ± 1.9 | 1.6 ± 0.1 |
|            | 150                      | 116 ± 4 | 86.4 ± 2.6 | 12.1 ± 2.0 | 1.5 ± 0.7 |
| pten<sup>-</sup>/pten-GFP | 0                     | 114 ± 6 | 87.2 ± 4.2 | 12.1 ± 3.9 | 0.7 ± 0.3 |
|            | 150                      | 123 ± 5 | 79.9 ± 3.2*** | 17.7 ± 2.9 | 2.3 ± 0.3* |
| plC<sup>-</sup> | 0                       | 106 ± 3 | 94.1 ± 2.5 | 5.9 ± 2.5 | 0 ± 0 |
|            | 150                      | 110 ± 4 | 89.7 ± 3.0 | 10.3 ± 3.0 | 0 ± 0 |
| plC<sup>-</sup>/plC<sup>-</sup> | 0                     | 106 ± 1 | 94.6 ± 1.0 | 5.3 ± 0.9 | 0.2 ± 0.2 |
|            | 150                      | 121 ± 4* | 81.4 ± 2.6** | 16.6 ± 2.0** | 2.0 ± 0.7** |
| iplA<sup>-</sup> | 0                       | 103 ± 1 | 97.3 ± 0.5 | 2.7 ± 0.5 | 0 ± 0 |
|            | 150                      | 104 ± 1 | 96.5 ± 1.0 | 3.5 ± 1.0 | 0 ± 0 |
| ppk1<sup>-</sup> | 0                       | 106 ± 2 | 94.9 ± 0.9 | 4.7 ± 0.8 | 0.4 ± 0.2 |
|            | 150                      | 111 ± 2 | 90.6 ± 1.8 | 8.5 ± 2 | 0.9 ± 0.4 |
| piaA<sup>-</sup> | 0                       | 102 ± 1 | 98.4 ± 0.5 | 1.6 ± 0.5 | 0 ± 0 |
|            | 150                      | 104 ± 1 | 96.0 ± 1.0 | 4.0 ± 1.0 | 0 ± 0 |

*aThe number of nuclei and percentage of cells with 1, 2, and 3 or more nuclei were measured by counts of DAPI (4′,6-diamidino-2-phenylindole)-stained cells. Cells were examined by using an epifluorescence microscope with a 40× lens, and for each condition, at least 100 cells were counted. Values are means ± SEM (n = 3 independent experiments). *, P < 0.05; ***, P < 0.01; ***, P < 0.001 (compared to no polyphosphate [by a two-tailed t test]).

The number of nuclei and percentage of cells with 1, 2, and 3 or more nuclei were measured by counts of DAPI (4′,6-diamidino-2-phenylindole)-stained cells. Cells were examined by using an epifluorescence microscope with a 40× lens, and for each condition, at least 100 cells were counted. Values are means ± SEM (n = 3 independent experiments). *, P < 0.05; ***, P < 0.01; ***, P < 0.001 (compared to no polyphosphate [by a two-tailed t test]).

involved in the polyphosphate pathway, and the loss of a single Gα subunit could be compensated for by other Gα subunits, or the Gα subunit(s) activated by polyphosphate is among the untested Gα subunits. Many mutants with abnormal proliferation (see Table S2 in the supplemental material) do not appear to be part of the polyphosphate signal transduction pathway, indicating that, as expected, many other factors besides polyphosphate affect proliferation.
The polyphosphate signal transduction pathway appears to use components that regulate proliferation in other systems. Ras-, PLC-, and IP3-induced Ca\(^{2+}\) release promotes proliferation, and PTEN and PKA inhibit proliferation in mammalian systems (73–79). Inhibition of Ras-, PLC-, or IP3-induced Ca\(^{2+}\) release inhibits cell proliferation in various cell types (75, 80–82). The overexpression of PTEN inhibits cell proliferation in many cancer cell lines (76, 77, 83), and the activation of PKA inhibits vascular smooth cell proliferation induced by injury (78, 79).

Consistent with the observation that polyphosphate induces Erk phosphorylation (17), we found that cells lacking Erk1 showed reduced sensitivity to polyphosphate. Polyphosphate-induced Erk phosphorylation requires RasC (17). Combined with the data in this report, this suggests that RasC-Erk1 is part of a pathway involved in polyphosphate inhibition of proliferation.

Polyphosphate is a prestarvation factor that primes Dictyostelium cells for development (17). Polyphosphate induces the expression of the early-onset development protein CsaA (17). Cells lacking the polyphosphate receptor GrlD showed an impaired response to the starvation-induced expression of the aggregation markers CsaA, Car1 (cyclic AMP receptor 1), and AcaA (adenyl cyclase A) and could not perform normal development (23). Many signal transduction pathway components affecting the Dictyostelium growth-development transition also affect polyphosphate inhibition of proliferation (Fig. 1G). As starvation causes both the cessation of proliferation and the initiation of development, many components involved in the initiation of development might also affect the proliferation inhibition response. Inducing these development-related components could be part of the mechanism whereby high concentrations of extracellular polyphosphate allow cells to anticipate starvation. How bacteria, by either consuming the polyphosphate secreted by Dictyostelium cells or secreting their own polyphosphate, interfere with Dictyostelium polyphosphate signaling is unclear. An intriguing observation is that Dictyostelium cells can proliferate on lawns.
of *Pseudomonas aeruginosa* bacteria that lack the bacterial polyphosphate kinase PPK1 but not on lawns of wild-type *P. aeruginosa* cells (84). One possibility for this result is that the polyphosphate from wild-type *P. aeruginosa* cells causes *Dictyostelium* cells to stop proliferating.

Many components of the AprA and cAMP signal transduction pathways (some components, such as the cAMP receptor CAR1 [85], were not examined) did not affect polyphosphate inhibition of cell proliferation. For those components in these two pathways that potentiated polyphosphate-induced proliferation inhibition, the effect on polyphosphate inhibition was relatively mild. PiaA and Lst8 are both Tor complex 2 components (60), but piaA− cells showed some impairment of polyphosphate signaling, while lst8− cells showed no significant inhibition, suggesting that PiaA and Lst8 have independent functions.

We tested the effect of the mutants that attenuate polyphosphate-mediated inhibition of proliferation in a shaking culture. *pten*− and *ppk1*− cells proliferated abnormally slowly and *gβ*− (66) and *plc*− cells proliferated abnormally quickly at low cell densities, and *iplA*−, *ppk1*−, and *piaA*− cells proliferated abnormally quickly at high cell densities. The maximal cell densities of *gβ*−, *gefA*−, *iplA*−, *ppk1*−, and *piaA*− cells were abnormally high, and those of *pten*− and *plc*− cells were abnormally low. Compared to wild-type cells, we expected that mutant cells with reduced sensitivity to polyphosphate would proliferate faster and reach higher maximal cell densities. However, *pten*− cells proliferated slower, and *pten*− and *plc*− cells had a lower maximal cell density. The accumulated extracellular polyphosphate levels of these four mutants might be abnormally high, or the genes knocked out could be required for regulating proliferation through other pathways. Compared to DH1 or *plc*− cells, *plc*−/−*plc* cells (overexpressing PLC in *plc*− cells) proliferate faster and reach a higher maximal density (Table 2).

Polyphosphate upregulated both IP3 levels and cytosolic Ca2+ levels in cells lacking the inositol 1,4,5-trisphosphate receptor-like protein IplA. In cells lacking RasC or Ppk1, polyphosphate upregulated IP3 but did not affect cytosolic Ca2+. In cells lacking Gβ, polyphosphate upregulated IP3 but downregulated cytosolic Ca2+. A possible explanation is that Gβ, RasC, and Ppk1 are required for IP3 to activate the IplA receptor to release Ca2+ to the cytosol and that GrlD might use components in addition to G proteins to transduce extracellular signals. Unexpectedly, the IP3 levels of cells lacking PTEN or PLC were not altered by polyphosphate, but the cytosolic Ca2+ of cells lacking PTEN or
PLC was upregulated or downregulated, respectively. These results suggest that polyphosphate can regulate cytosolic Ca\textsuperscript{2+} levels through a pathway not involving IP3.

Ppk1 mediates intracellular polyphosphate production, and the intracellular polyphosphate of ppk\textsuperscript{1} cells is undetectable (12). How intracellular (as opposed to extracellular) polyphosphate or Ppk1 affects extracellular polyphosphate-induced proliferation inhibition is unclear. As polyphosphate can bind to free divalent cations such as Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (10), one hypothesis is that intracellular polyphosphate might bind to the extracellular polyphosphate-induced elevated cytosolic free Ca\textsuperscript{2+}, and the intracellular polyphosphate-Ca\textsuperscript{2+} complex could then function as a second messenger. If this is the case, compared to Ax2 cells, cells lacking Ppk1 should show a higher increase of the fluorescence signal with the BAPTA-1 dextran method after stimulating cells with polyphosphate, as polyphosphate-bound Ca\textsuperscript{2+} could not be detected by BAPTA-1. However, cells lacking Ppk1 lost the polyphosphate-induced cytosolic free Ca\textsuperscript{2+} increase (Fig. 4) while still showing a polyphosphate-induced IP3 increase (Fig. 3). This result disproves the hypothesis of a polyphosphate-Ca\textsuperscript{2+} elevation and a Ca\textsuperscript{2+}-bound polyphosphate pathway. This indicates that Ppk1/intracellular polyphosphate functions downstream of IP3 and upstream of Ca\textsuperscript{2+} elevation.

Besides proliferation inhibition, polyphosphate inhibits proteasome activity, promotes aggregation, and regulates actin polymerization in D. discoideum cells (23). In both 25% and 100% HL5, polyphosphate reduces proteasome activity, and this requires GrlD and RasC (23). However, in 25% HL5 but not 100% HL5, MG132-induced inhibition of proteasome activity inhibits proliferation (23). In human colon cancer HCT116 cells, the proteasome inhibitor MG132 increases intracellular Ca\textsuperscript{2+} levels (88), and in mouse embryonic fibroblasts, chelating calcium by BAPTA-acetoxymethyl ester (AM) decreases proteasome activity, while increasing intracellular Ca\textsuperscript{2+} with 2 mM extracellular Ca\textsuperscript{2+} and ionomycin treatment increases proteasome activity (89). In Dictyostelium, whether there is cross talk in the polyphosphate signal transduction pathway between proteasome activity and IP3/Ca\textsuperscript{2+} levels is unclear.

In this report, we identified 7 signaling components in the polyphosphate pathway and showed that polyphosphate appears to inhibit Dictyostelium proliferation through pathways including the IP3/Ca\textsuperscript{2+} pathway. An intriguing possibility is that similar mechanisms may be used in other eukaryotes for autocrine proliferation inhibition and group and tissue size regulation.

**MATERIALS AND METHODS**

**Cell culture and strains.** Dictyostelium discoideum strains were obtained from the Dictyostelium stock center (90) and were parental/wild-type strains Ax2 (Dictybase identifier DBS0237699) (91), Ax3 (DBS0235542) (92), KAx3 (DBS0266758) (93), Ax4 (DBS0302402) (94), DH1 (DBS0235700) (85), JH8 (DBS0236545) (95), HJ10 (DBS0236449) (95), and HPS400 (DBS0235027) (23), ras\textsuperscript{C} (DBS0236853) (24), gef\textsuperscript{A} (DBS0236896) (25), ras\textsuperscript{G} (DBS0236862) (97), g\textsuperscript{B} (DBS0236531) (26), gat\textsuperscript{1} (DBS0236088) (27), gat\textsuperscript{2} (DBS0236575) (37), gat\textsuperscript{3} (DBS0235986) (98), gat\textsuperscript{4} (DBS0235984) (99), gat\textsuperscript{5} (DBS0236451) (100), gat\textsuperscript{6} (DBS0236106) (101), gat\textsuperscript{7} (DBS0236107) (101), gat\textsuperscript{8} (DBS0236109) (102), papa1 (DBS0235590) (29), calD (DBS0302444) (30), pak\textsuperscript{D} (DBS0350281) (29), rbA (DBS0236877) (31), cnr\textsuperscript{N} (DBS0302655) (32), qkA (DBS0236839) (35), bapN (DBS0349965) (36), scrA (DBS0236926) (40), etnoE (DBS0350065) (41), gat\textsuperscript{A} /gat\textsuperscript{A} (DBS0236279) (42), ras\textsuperscript{C} (DBS0350272) (103), plA2 (DBS0238068) (39), pka\textsuperscript{A} /pka\textsuperscript{B} (DBS0236766) (44), dag\textsuperscript{A} (DBS0235559) (45), ptenA (DBS0236830) (46), ptenA /pten-GFP (DBS0236831) (46), p1c\textsuperscript{A} /p1c\textsuperscript{B} (DBS0236793) (47), p1c\textsuperscript{C} /p1c\textsuperscript{D} (DBS0236795) (104), gol\textsuperscript{A} /gol\textsuperscript{B} (DBS0236260) (48), DbsSo4 (DBS0266692) (49), erk\textsuperscript{1} (DBS0350622) (52), erk\textsuperscript{1} /erk\textsuperscript{2} (DBS0351256) (105), mekA (DBS0236541) (53), smkA (DBS0235983) (53), itkA (DBS0236426) (106), ppk1 (DBS0350686) (12), csA (DBS0236957) (56), snk\textsuperscript{A} (DBS0236939) (58), paa (DBS0349879) (107), list\textsuperscript{A} (DBS0236517) (60), pka (DBS0236783) (64), pkcA (DBS0350916) (108), antA (DBS0235497) (57), xibA (DBS0236935) (109), pct2 (65), and tpp5 (65) (gifts from Pierre Coisson, University of Geneva, Geneva, Switzerland); mutants mcln1 (DBS0350059) (110) and wasA (gifts from Robert Insall, Beatson Institute for Cancer Research, Glasgow, UK) (43); and mutants gdt1 /gdt2 (65), gdt1 /gdt2 (65), and gdt4 (gifts from Adam Kuspa, Baylor College of Medicine) (see Table S1 in the supplemental material). As described previously, all mutants were confirmed by PCR (33). Cells were cultured at 21°C in a shaking culture at 175 rpm in HL5 (Formedium Ltd., Norwich, England). Cells were counted by a hemocytometer.

**Proliferation inhibition and counts of nuclei.** Polyphosphate was prepared by dissolving 0.474 g of –46–mer (average length) S0169 sodium polyphosphate (Spectrum, New Brunswick, NJ) in 10 ml of PB (20 mM KH\textsubscript{2}PO\textsubscript{4}, 0.01 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2} [pH 6.1]) (23) to make a 10 mM stock; the final pH was 6.1, and the pH was thus not adjusted. Mid-log-phase cells (1 × 10\textsuperscript{6} to 4 × 10\textsuperscript{6} cells/ml) cultured in HL5 were collected by centrifugation at 1,000 × g for 3 min, washed once by resuspension of the cells in May/June 2021 Volume 12 Issue 3 e01347-21 mbio.asm.org
PBM and centrifugation at 1,000 × g for 3 min, and then resuspended in fresh HLS to 6 × 10^6 cells/ml. Cell cultures were started by mixing 100 μl of these cells with 300 μl of PBM or HLS containing the indicated concentrations (adjusted for the dilution with cells) of polyphosphate in the well of a type 353047 24-well plate (Corning, Corning, NY) and incubated in a humid box for 24 h at 21°C. For work with cells in 25% HLS, HLS was diluted by mixing 1 volume of HLS with 3 volumes of PBM. Cells were counted at 24 h, and the cell density normalized to the density with no added polyphosphate was calculated. The doubling time and maximal density of each strain were calculated as described previously (23), and the numbers of nuclei per cell were counted as described previously (28). Curve fits and IC50 calculations were done using Prism (GraphPad, San Diego, CA) with nonlinear regression (sigmoidal dose-response, variable slope, and top constrained to 100).

**Extraction and measurement of inositol (1,4,5)-trisphosphate.** Cells were grown to mid-log phase and counted, and ~2 × 10^6 cells were collected by centrifugation, washed with PBM as described above, and then resuspended and incubated in 10 ml 25% HLS (diluted with PBM) with 0 or 150 μM polyphosphate in a shaking culture at 175 rpm. After 1, 2, 4, 8, or 24 h, cells were collected by centrifugation at 1,000 × g for 3 min and resuspended in 110 μl of the supernatant from the centrifugation step in 1.7-ml Eppendorf tubes. From the resuspended cells, 10 μl was taken out for cell counts, and the remaining cells were mixed with 100 μl 3.5% perchloric acid and incubated on ice for 15 min as described previously (111). Half-saturated KHCO3 (50 μl) was then added to the 200-μl mix to neutralize the lysates, and CO2 was allowed to escape. The material was then clarified by centrifugation at 14,000 × g for 5 min at 4°C. The supernatant (200 μl) of each tube was transferred to new prechilled 1.7-ml tubes and stored at 0°C. The IP3 levels in the clarified lysates were measured with a type 2515875 IP3 ELISA kit (MyBioSource, San Diego, CA) less than 1 week after extraction. The baseline IP3 levels that we measured (Fig. 3A and C) are far lower than the levels previously reported using an isotope dilution kit that has been discontinued by the manufacturer (picograms versus micrograms per 10^7 cells) (112, 113). Both kits detect IP3 levels based on a competitive binding strategy, but the isotope kit used an IP3 binding protein prepared from bovine adrenal cortex, and the ELISA kit uses an anti-IP3 antibody. We hypothesize that the difference between the measured IP3 levels could be caused by the specificity of the anti-IP3 antibody being much higher than that of the bovine IP3 binding protein.

**Measurement of cytosolic free Ca^{2+}.** Mid-log-phase cells (3 × 10^6) were collected by centrifugation at 1,000 × g for 3 min, washed with ice-cold Sorensen’s buffer (14.7 mM KH2PO4, 2 mM Na2HPO4 [pH 6.1]) twice (each time collecting cells by centrifugation and resuspension), and then resuspended in 95 μl ice-cold Sorensen’s buffer. As described previously (70), 90 μl of washed cells was then mixed with 10 μl 25 mg/ml BAPTA-1 dextran at a 10,000 molecular weight (MW) (Invitrogen, Eugene, OR), loaded into an Eppendorf tubes. From the resuspended cells, 10^5 cells were collected by centrifugation, washed with PBM as described above, and then resuspended in 1 ml 25% HLS, HL5 was diluted by mixing 1 volume of HL5 with 3 volumes of PBM. Cells were counted at 24 h, and the cell density normalized to the density with no added polyphosphate was calculated. The doubling time and maximal density of each strain were calculated as described previously (23), and the numbers of nuclei per cell were counted as described previously (28). Curve fits and IC50 calculations were done using Prism (GraphPad, San Diego, CA) with nonlinear regression (sigmoidal dose-response, variable slope, and top constrained to 100).

**Measurement of active Ras.** Cells were grown to mid-log phase (1 × 10^6 to 4 × 10^6 cells/ml) and counted, and 1 × 10^6 cells were collected by centrifugation, washed with PBM as described above, and then resuspended and incubated in 1 ml 25% HLS (diluted with PBM) with 0 or 150 μM polyphosphate. After 1, 4, or 24 h, cells were lysed, and the active Ras levels in the lysates were measured with a Ras activation assay kit (Cytoskeleton, Denver, CO). All the procedures were performed according to the manufacturer’s manual except that the cell lysate with 30 μg protein was mixed with 30 μg Raf-RBD protein beads for active Ras pulldown.

**Statistics.** Statistical analyses were done using Prism (GraphPad). Significance was defined as a P value of <0.05.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1, TIF file, 1.4 MB.**

**FIG S2, TIF file, 1.4 MB.**

**FIG S3, TIF file, 0.4 MB.**

**FIG S4, TIF file, 2 MB.**

**TABLE S1, DOCX file, 0.02 MB.**

**TABLE S2, DOCX file, 0.02 MB.**

**ACKNOWLEDGMENTS**

We thank Kristen Consalvo and Sara Kirolos for helpful comments. We thank the Dictybase stock center for the cell strains.

We declare no competing interests.

This work was supported by NIH grant GM118355.
REFERENCES

1. Metcalf D. 1964. Restricted growth capacity of multiple spleen grafts. Transplantation 2:387–392. https://doi.org/10.1097/00007890-196405000-00008.

2. Bullough WS, 1975. Mitotic control in adult mammalian tissues. Bio Rev Camb Philos Soc 59:509–127. https://doi.org/10.1111/j.1469-185X.1975.tb00990.x.

3. Bullough WS. 1975. Chalone control mechanisms. Life Sci 16:323–330. https://doi.org/10.1016/0024-3205(75)90253-2.

4. Nadal C. 1979. Control of liver growth by growth inhibitors (chalones). Arch Toxicol Suppl 1979:131–142. https://doi.org/10.1007/978-3-642-67265-1_11-31:42.

5. Roisin-Bouffay C, Gomer RH. 2004. How to reach the right size? Med Sci (Paris) 20:219–224. (In French) https://doi.org/10.1051/medsci:200420219.

6. Suess PM, Gomer RH. 2016. Extracellular polyphosphate inhibits proliferation in an autocrine negative feedback loop in Dictyostelium discoideum. J Biol Chem 291:20260–20269. https://doi.org/10.1074/jbc.M116.737825.

7. Kim J, Koga T, Namba A, Kornberg A, Gomer RH. 2006. Inorganic polyphosphate stimulates mammalian TOR, a kinase involved in the proliferation of mammalian protein kinase that regulates its rate of growth and development. Nucleic Acids Res 31:e107. https://doi.org/10.1093/nar/gng095.

8. Phillips JE, Huang EY, Shaulsky G, Gomer RH. 2011. The putative BZIP transcription [sic] factor BzipN slows proliferation and functions in the regulation of cell density by autocrine signals in Dictyostelium. PLoS One 6:e21765. https://doi.org/10.1371/journal.pone.0021765.

9. Kumagai A, Pupillo M, Gundersen R, Miake-Lye R, Devreotes PN. 1989. Regulation and function of Galpha protein subunits in Dictyostelium. Cell 57:265–275. https://doi.org/10.1016/0022-8482(89)90064-1.

10. Wu L, Vakalena R, Van Haastert PJ, Devreotes PN. 1995. The G protein beta subunit is essential for Dictyostelium development. Eukaryot Cell 13:1667. https://doi.org/10.1083/jcb.129.6.1667.

11. Chen L, Iijima M, Tang M, Landree MA, Huang YE, Xiong Y, Iglesias PA, Devreotes PN. 2007. PLA2 and PI3K/PTEN pathways act in parallel to mediate chemotaxis. Dev Cell 12:603–614. https://doi.org/10.1016/j.devcel.2007.05.005.

12. Blagg SL, Stewart M, Sambles C, Insall RH. 2003. PI3K regulates pseudopod dynamics and SCAR activity in Dictyostelium. Curr Biol 13:1480–1487. https://doi.org/10.1016/S0960-9822(03)00580-3.

13. Yan J, Mihaylov V, Xu X, Bzostowski LA, Li H, Li L, Veenstra TD, Parent CA, Jin T. 2012. A Gbetagamma effector, ElmoE, transduces GPCR signaling to the actin network during chemotaxis. Dev Cell 22:92–103. https://doi.org/10.1016/j.devcel.2011.11.007.

14. Roelofs J, Snipe H, Kleineidam RG, Van Haastert PJ. 2001. Guanylate cyclase in Dictyostelium discoideum with the topology of mammalian adenylate cyclase. Biochem J 354:679–706. https://doi.org/10.1042/0264-6021:3540097.

15. Davidson AJ, Amato C, Thomas PA, Insall RH. 2018. WASP family proteins and formins compete in pseudopod- and bleb-based migration. J Cell Biol 217:701–714. https://doi.org/10.1083/jcb.201705160.
44. Chung CY, Potliyan G, Firtel RA. 2001. Control of cell polarity and che- motaxis by Akt/PKB and PI3 kinase through the regulation of PAKa. Mol Cell 7:937–947. https://doi.org/10.1016/S1097-2765(01)00247-7.

45. Lilly PJ, Devreotes PN. 1995. Chemotactic receptor and GTP gamma S-medi- ated stimulation of adenyl cyclase in Dictyostelium requires translocation of CRAC to membranes. J Cell Biol 129:1659–1665. https://doi.org/10.1083/jcb.129.6.1659.

46. Iijima M, Devreotes P. 2002. Tumor suppressor PTEN mediates sensing of chemotactic receptor gradients. Cell 109:599–610. https://doi.org/10.1016/S0092-8674(02)00704-Y.

47. Keizer-Gunnink I, Kortholt A, van Haastert PJ. 2007. Chemoattractants and chemorepellents act by inducing opposite polarity in phospholipase C and PI3-kinase signaling. J Cell Biol 177:579–585. https://doi.org/10.1083/jcb.200611046.

48. Traynor D, Milne JL, Insall RH, Kay RR. 2000. Ca(2+)-mediated activation of the TORC2-PKB pathway is critical for chemotaxis. Cell 109:599–608. https://doi.org/10.1016/S0092-8674(00)00922-5.

49. Loovers HM, Veenstra K, Snippe H, Pesesse X, Erneux C, van Haastert PJ. 2007. PTEN controls the transition of CRAC to membranes in Dictyostelium. Cell 129:1659–1669. https://doi.org/10.1016/j.cell.2007.05.010.

50. Bruce A, Johnson A, Lewis J, Raff M, Roberts K, Walter P. 2007. Molecular biology of the cell, 5th ed. Garland Science, New York, NY.

51. Loovers HM, Kortholt A, de Groot J, Whitby L, Hussaum RL, van Haastert PJ. 2007. Regulation of phagocytosis in Dictyostelium by the inositol 5-phosphatase OCRG holomodull SP4. Traffic 8:618–628. https://doi.org/10.1111/j.1600-0854.2007.00546.x.

52. Nguyen HN, Raisley B, Hadwiger JA. 2010. MAP kinases have different functions in Dictyostelium G protein-mediated signaling. Cell Signal 22:836–847. https://doi.org/10.1016/j.cellsig.2010.01.008.

53. Mendoza MC, Du F, Iranfar N, Tang N, Ma H, Loomis WF, Firtel RA. 2005. Loss of SMEK, a novel, conserved protein, suppresses MEK1 null cell polar- ity, chemotaxis, and gene expression defects. Mol Cell Biol 25:7839–7853. https://doi.org/10.1128/MCB.25.17.7839–7853.2005.

54. Chibalina MV, Anjard C, Insall RH. 2004. Gt2 regulates the transition of Dictyostelium cells from growth to differentiation. BMC Dev Biol 4:8. https://doi.org/10.1186/1471-213X-4-8.

55. Zeng C, Anjard C, Riemann K, Konzok A, Nellen W. 2000. gdt1, a new sig- naling pathway in Dictyostelium discoideum. Eukaryot Cell 5:991–996. https://doi.org/10.1089/1083/jcb.200611046.

56. Caterina MJ, Devreotes PN. 1994. Mutation of the third intracel- lular loop of the cAMP receptor, cAR1, of Dictyostelium yields mutants with altered chemotaxis. Oncol Rep 3:779–783. https://doi.org/10.1080/1742733X.2005.1161328.

57. Cordova-Alarcon E, Centeno F, Reyes-Esparza J, Garcia-Carranca A, Garrido E. 2005. Effects of HRAS oncogene on cell cycle progression in a cervical cancer-derived cell line. Arch Med Res 36:311–316. https://doi.org/10.1016/j.jcb.200611046.

58. Huang H, Berk A, Kaiser CA, Krieger M, Scott MP, Brescher A, Ploegh H, Matsudarra P. 2008. Molecular cell biology. Macmillan, New York, NY.

59. Stallings JD, Zeng YX, Narvaez F, Rebecchi MJ. 2008. Phospholipase C-delta1 expression is linked to proliferation, DNA synthesis, and cyclin E levels. J Biol Chem 283:13992–14001. https://doi.org/10.1074/jbc.M800752200.

60. Zhao H, Dupont J, Yakar S, Karas M, LeRoith D. 2004. PTEN inhibits cell proliferation and induces apoptosis by downregulating cell surface IGFR expression in prostate cancer cells. Oncogene 23:786–794. https://doi.org/10.1038/sj.onc.1207162.

61. Truong TV, Marchand ME, Atkinson C. 2004. PTEN regulatory functions in tumor sup- pression and cell biology. Med Sci Monit 10:RA235–RA241.

62. Indolfi C, Avvedimento EV, Di Lorenzo E, Esposito G, Rapacciuolo A, Gianolio P, Grieco D, Cavuto L, Stingone AM, Ciullo I, Condorelli G, Chiarelli M. 1997. Activation of cAMP–PKA signaling in vivo inhibits smooth muscle cell proliferation by vascular injury. Nat Cell Biol 3:775–779. https://doi.org/10.1038/383775a0.

63. Hewer RC, Sala-Newby GB, Wu YJ, Newby AC, Bond M. 2011. PKA and Epac synergistically inhibit smooth muscle cell proliferation. J Mol Cell Cardiol 50:87–98. https://doi.org/10.1016/j.jmcc.2010.10.010.

64. Indolfi C, Avvedimento EV, Rapacciuolo A, Di Lorenzo E, Esposito G, Stabile E, Feliciello A, Mele E, Gianolio P, Condorelli G, Chiarelli M. 1995. Inhibition of cellular ras prevents smooth muscle cell proliferation after vascular injury in vivo. Nat Med 1:541–545. https://doi.org/10.1038/36095-541.

65. Karpöth-Jolin KA, Li X, Reks SE, Kelley GG. 2008. Phospholipase C delta 1 regulates cell proliferation and cell-cycle progression from G1 to S phase by control of cyclin E-CDK2 activity. Biochem J 415:439–448. https://doi.org/10.1042/BJ20080233.

66. Szatkowski C, Pavy JB, Ouadid-Ahidouch H, Matifat F. 2010. Inositol 1,4,5-trisphosphate-induced Ca2+ signaling is involved in estradiol- induced breast cancer epithelial cell growth. Mol Cancer 9:156. https://doi.org/10.1186/1476-4598-9-156.

67. Sun Y, Tian H, Wang J. 2015. Effects of PTEN on the proliferation and ap- optosis of colorectal cancer cells via the phosphoinositol-3-kinase/Akt pathway. Oncol Rep 33:1828–1836. https://doi.org/10.3892/or.2015.3804.

68. Zhang H, Gómez-Garcia MR, Brown MR, Kornberg A. 2005. Inorganic poly- phosphates in Dictyostelium discoideum: influence on development, sporulation, and predation. Proc Natl Acad Sci U S A 102:2731–2735. https://doi.org/10.1073/pnas.0500233102.

69. Caterina MJ, Milne JL, Devreotes PN. 1994. Mutation of the third intracel- lular loop of the cAMP receptor, cAR1, of Dictyostelium yields mutants impaired in multiple signaling pathways. J Biol Chem 269:1523–1532. https://doi.org/10.1006/jbcl.2005.110129.
86. Rivet-Bastide M, Imbert N, Cognard C, Dupont G, Rideau Y, Raymond G. 1993. Changes in cytosolic resting ionized calcium level and in calcium transients during in vivo development of normal and Duchenne muscular dystrophy myotubes contracting in vitro. J. Cell Biol 124:443–455. https://doi.org/10.1083/jcb.124.2.443

87. Imbert N, Vandebrouck C, Constanttin B, Dupont G, Guillou C, Cognard C, Raymond G. 1996. Hypoosmotic shocks induce elevation of resting calcium level in Duchenne muscular dystrophy myotubes contracting in vitro. Neuromuscul Disord 6:351–360. https://doi.org/10.1073/pnas.93.9.3900

88. Williams JA, Hou Y, Ni HM, Ding WX. 2013. Role of intracellular calcium in protein-mediated signal transduction pathway inhibits anterior prestalk cell development in Dictyostelium. Differentiation 64:195–204. https://doi.org/10.1016/j.diff.2013.02.015

89. Uvarov AV, Meseale N. 2008. Enhanced ubiquitin-proteasome activity in calcium deficient cells: a compensatory mechanism for cell survival. Biochim Biophys Acta 1783:1237–1247. https://doi.org/10.1016/j.bbamar.2008.03.004

90. Fey P, Dodson RJ, Basu S, Chisholm RL. 2013. One stop shop for everything Dictyostelium: dictyBase and the Dicty Stock Center in 2012. Methods Mol Biol 983:59–80. https://doi.org/10.1007/978-1-62703-302-2_4

91. Watts DJ, Ashworth JM. 1970. Growth of myxamoebae of the cellular slime mould Dictyostelium discoideum in axenic culture. Biochim J 119:171–174. https://doi.org/10.1014/2bj1190171

92. Loomis WF Jr. 1971. Sensitivity of Dictyostelium discoideum to nucleic acid analogues. Exp Cell Res 64:484–486. https://doi.org/10.1016/0014-4827(71)90107-8

93. Neilen W, Silan C, Firtel RA. 1984. DNA-mediated transformation in Dictyostelium discoideum: regulated expression of an actin gene in cell survival. Mol Cell Biol 4:2890–2898. https://doi.org/10.1128/mcb.4.12.2890

94. Knecht DA, Cohen SM, Loomis WF, Lodish HF. 1986. Developmental regulation of Dictyostelium discoideum actin gene fusions carried on low-copy and high-copy transformation vectors. Mol Cell Biol 6:3973–3983. https://doi.org/10.1128/mcb.6.11.3973

95. Hadwiger JA, Firtel RA. 1992. Analysis of G alpha 4, a G-protein subunit required for multicellular development in Dictyostelium. Genes Dev 6:38–49. https://doi.org/10.1101/gad.6.1.38

96. Podgorski G, Deering RA. 1984. Thymidine-requiring mutants of Dictyostelium discoideum. Mol Cell Biol 4:2784–2791. https://doi.org/10.1128/mcb.4.12.2784

97. Bolourani P, Spiegelman GB, Weeks G. 2006. Delineation of the roles played by RasG and RasC in cAMP-dependent signal transduction during the early development of Dictyostelium discoideum. Mol Biol Cell 17:4543–4550. https://doi.org/10.1091/mbc.e05-11-1019

98. Brandon MA, Podgorski GJ. 1997. G alpha 3 regulates the CAM signaling system in Dictyostelium. Mol Biol Cell 8:1677–1685. https://doi.org/10.1091/mbc.8.9.1677

99. Hadwiger JA, Srivivasan J. 1999. Folic acid stimulation of the Galpha4 G protein-mediated signal transduction pathway inhibits anterior prestalk cell development in Dictyostelium. Differentiation 64:195–204. https://doi.org/10.1016/j.diff.1999.640577

100. Hadwiger JA, Natarajan K, Firtel RA. 1996. Mutations in the Dictyostelium heterotrimeric G protein alpha subunit G alpha5 alter the kinetics of tip morphogenesis. Development 122:1215–1224. https://doi.org/10.1242/dev.122.4.1215

101. Wu L, Gaskins C, Zhou K, Firtel RA, Devreotes PN. 1994. Cloning and targeted mutations of G alpha 7 and G alpha 8, two developmentally regulated G protein alpha-subunit genes in Dictyostelium. Mol Biol Cell 5:691–702. https://doi.org/10.1091/mbc.5.6.691

102. Brzostowski JA, Johnson C, Kimmel AR. 2002. Galpha-mediated inhibition of developmental signal transduction. Curr Biol 12:1199–1208. https://doi.org/10.1016/s0960-9822(02)00953-3

103. Han JW, Leeper L, Rivera F, Chung CY. 2006. Role of RasC for the regulation of WASP and phosphatidylinositol 3-kinase during chemotaxis of Dictyostelium. J Biol Chem 281:35224–35234. https://doi.org/10.1074/jbc.M605979200

104. Drayer AL, Meima ME, Derks MW, Tuik R, van Haastert PJ. 1995. Mutation of an EF-hand Ca2+-binding motif in phospholipase C of Dictyostelium discoideum: inhibition of activity but no effect on Ca2+-dependence. Biochim J 311(Par 2):505–510. https://doi.org/10.1042/bj3110505

105. Schwebs DJ, Pan M, Adhikari N, Kuburich NA, Jin T, Hadwiger JA. 2018. Dictyostelium Erk2 is an atypical MAPK required for chemotaxis. Cell Signal 46:154–165. https://doi.org/10.1016/j.cellsig.2018.03.006

106. Luo HR, Huang YE, Chen JC, Saiardi A, Iijima M, Ye K, Huang Y, Nagata E, Devreotes P, Snyder SH. 2003. Inositol pyrophosphates mediate chemotaxis in Dictyostelium via pleckstrin homology domain-PTPlsins(3,4,5)P3 interactions. Cell 114:559–572. https://doi.org/10.1016/s0092-8674(03)00640-8

107. Tang M, Iijima M, Kamimura Y, Chen L, Long Y, Devreotes P. 2011. Disruption of PKB signaling restores polarity to cells lacking tumor suppressor PTEN. Mol Biol Cell 22:437–447. https://doi.org/10.1091/mbc.e10-06-0522

108. Mohamed M, Ray S, Brazill D, Baskar R. 2015. Absence of catalytic domain in a putative protein kinase C (PKcA) suppresses tip dominance in Dictyostelium discoideum. Dev Biol 405:10–20. https://doi.org/10.1016/j.ydbio.2015.05.021

109. Cornillon S, Gebbie L, Benghezal M, Nair P, Keller S, Wehrle-Haller B, Charrette SJ, Bruckert F, Letourneur F, Cosson P. 2006. An adhesion molecule in free-living Dictyostelium amoebae with integrin beta features. J Cell Sci 119:1215–1224. https://doi.org/10.1242/jcs.100362

110. Van Haastert PJ. 1989. Determination of inositol 1,4,5-trisphosphate levels in Dictyostelium by isotope dilution assay. Anal Biochem 187:265–272. https://doi.org/10.1016/0003-2697(89)90024-9

111. Brazill DT, Lindsey DF, Bishop JD, Gomer RH. 1998. Cell density sensing by Dictyostelium medullosa cells is mediated by a G protein-coupled receptor activating phospholipase C. J Biol Chem 273:8161–8168. https://doi.org/10.1074/jbc.273.14.8161

112. Williams RS, Eames M, Ryves WJ, Viggars J, Harwood AJ. 1999. Loss of a prolyl oligopeptidase confers resistance to lithium by elevation of inositol(1,4,5)trisphosphate. EMBO J 18:2734–2745. https://doi.org/10.1093/embj/18.10.2734