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RESEARCH ARTICLE

An atypical MAPK regulates translocation of a GATA transcription factor in response to chemoattractant stimulation

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ABSTRACT

The *Dictyostelium* atypical mitogen-activated protein kinase (MAPK) Erk2 is required for chemotactic responses to cAMP as amoeba undergo multicellular development. In this study, Erk2 was found to be essential for the cAMP-stimulated translocation of the GATA transcription factor GtaC as indicated by the distribution of a GFP–GtaC reporter. Erk2 was also found to be essential for the translocation of GtaC in response to external folate, a foraging signal that directs the chemotaxis of amoeba to bacteria. Erk1, the only other *Dictyostelium* MAPK, was not required for the GtaC translocation to either chemoattractant, indicating that GFP–GtaC is a kinase translocation reporter specific for atypical MAPKs. The translocation of GFP–GtaC in response to folate was absent in mutants lacking the folate receptor Far1 or the coupled G-protein subunit Gα4. Loss of GtaC function resulted in enhanced chemotactic movement to folate, suggesting that GtaC suppresses responses to folate. The alteration of four Erk2-preferred phosphorylation sites in GtaC impacted the translocation of GFP–GtaC in response to folate and the GFP–GtaC-mediated rescue of aggregation and development of *gtaC*$^{-}$ cells. The ability of different chemoattractants to stimulate Erk2-regulated GtaC translocation suggests that atypical MAPK-mediated regulation of transcription factors can contribute to different cell fates.

KEY WORDS: MAPK, Transcription factor, Chemotaxis, *Dictyostelium*, GATA transcription factor, Folate, Kinase translocation reporter, Nuclear-to-cytoplasmic translocation

INTRODUCTION

Environmental signals can regulate eukaryotic cell function and fate through signaling pathways that control cellular processes, such as gene expression and cell movement (Groom, 2019). These signaling pathways typically involve cell surface receptors coupled to G proteins and a variety of protein kinases that serve as molecular switches for a wide range of cellular responses (Cargnello and Roux, 2011; Goldsmith and Dhanasekaran, 2007; Luttrell, 2006). Mitogen-activated protein kinases (MAPKs) play important roles in many different signaling pathways in most eukaryotes, including animals, plants, fungi and protists (Bogoyevitch and Court, 2004; Chen and Thorner, 2007; Dóczi et al., 2012; Hadwiger and Nguyen, 2011; Yildiz and Arslanyolu, 2014). These protein kinases typically function downstream of other protein kinases in G-protein-coupled receptor and tyrosine kinase receptor signaling pathways, and they facilitate the regulation of many types of signaling proteins including other protein kinases, phosphodiesterases and transcription factors that modulate cellular activities, such as second messenger signaling, metabolism, chemotaxis and gene expression (Cargnello and Roux, 2011; MacKenzie et al., 2000; Maeda et al., 2004; Nichols et al., 2019; Roskoski, 2012; Schwebs et al., 2018). Canonical MAPK regulation involves kinase cascades that include MAPK kinases (MAP2Ks), MAPKK kinases (MAP3Ks) and scaffold proteins. However, one subgroup of MAPKs, referred to as atypical MAPKs, do not appear to be regulated through conventional MAP2Ks but instead have another regulatory mechanism that remains to be fully understood (Coulombe and Meloche, 2007). Compared to other MAPKs, relatively little is known about the function of atypical MAPKs in animals, but several reports have indirectly associated atypical MAPKs with stress responses (Chia et al., 2014; Colecchia et al., 2018; Groehler and Lannigan, 2010; Hasygar and Hietakangas, 2014; Iavarone et al., 2006; Klevernic et al., 2009; Liwak-Muir et al., 2016; Rossi et al., 2011; Saelzler et al., 2006; Zacharogianni et al., 2011). Major challenges to understanding the roles of atypical MAPKs have been identifying specific signals that directly stimulate atypical MAPK activation and characterizing the phenotypes that result from losses of atypical MAPK function, but the analysis of atypical MAPKs in genetically amenable organisms has begun to provide insight into both function and regulation of atypical MAPKs (Chen and Segall, 2006; Gaskins et al., 1996; Maeda et al., 1996; Maeda and Firtel, 1997; Schwebs et al., 2018; Segall et al., 1995).

Atypical MAPKs have been found in animals and protists, such as amoebae, and they share some structural similarities that make them distinct from other MAPKs (Abe et al., 2002; Ellis et al., 2004; Segall et al., 1995; Valenciano et al., 2016). The absence of atypical MAPKs in fungi has excluded their analysis in yeast, which have been important for understanding the function and regulation of other types of MAPKs (Chen and Thorner, 2007). However, the amoeba *Dictyostelium discoideum* encodes an atypical MAPK and genetic analyses have provided many clues to atypical MAPK function and regulation (Schwebs et al., 2018). The *Dictyostelium* atypical MAPK, Erk2 (also known as ErkB), is rapidly activated (within 30 s) in response to chemoattractants and mediates chemotactic cell movement, consistent with the presence of atypical MAPKs in organisms with cell motility (Maeda et al., 1996; Maeda and Firtel, 1997; Nichols et al., 2019; Schwebs et al., 2018). *Dictyostelium* encodes only one other MAPK, Erk1 (also known as ErkA), and this protein is related to other more typical MAPKs found in most eukaryotes (Gaskins et al., 1994; Schwebs et al., 2018). Both Erk2 and Erk1 become activated through the phosphorylation of a conserved activation motif (TEY) in response to chemotactic stimulation, but the regulation and roles of these

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MAPKs are quite different (Schwebs and Hadwiger, 2015). Erk2 activation occurs initially and then a burst of Erk1 activation follows as a secondary response that is dependent on Erk2 function. Only Erk2 is essential for chemotaxis and only Erk1 is activated by the single conventional MAP2K in *Dictyostelium* (Schwebs et al., 2018).

Dictyostelium have a chemotaxis response to at least two chemoattractants, cAMP and folate (Gerisch, 1982). Chemotaxis to folate is a mechanism by which *Dictyostelium* can forage for bacterial food sources in the environment and resume vegetative growth as solitary cells. In contrast, chemotaxis to cAMP mediates the aggregation phase of the multicellular developmental life cycle by allowing cells to move toward each other as they relay cAMP signaling in a pulsatile manner. Responses to cAMP not only include chemotactic movement, but the regulation of many developmental genes, including developmental cell type-specific genes (Rosengarten et al., 2015). One of the key regulators of developmental genes is the GATA transcription factor GtaC (Cai et al., 2014; Keller and Thompson, 2008; Santhanam et al., 2015). Loss of GtaC impairs aggregation, and GtaC has been shown to bind many locations in the genome (Santhanam et al., 2015). Expression of GFP-tagged GtaC rescues the development of *gtaC*$^{-}$ strains and serves as a reporter for the translocation of this transcription factor from the nucleus to the cytoplasm when cells are stimulated with cAMP (Cai et al., 2014). The shuttling of this transcription factor between the nucleus and cytoplasm during the repeated cyclic pattern of cAMP stimulation has been suggested to function as a developmental timer in the assessment of cycle number and duration. In this report, we assessed the roles of both typical and atypical MAPKs in the translocation of this GATA transcription factor in response to multiple chemoattractant signals and also tested the specificity of chemoattractant receptors and associated G proteins in this process. The GFP–GtaC reporter was used to examine the roles of specific phosphorylation sites on GtaC translocation and the regulation of developmental processes. Our study suggests that multiple external signals can use atypical MAPK signaling to regulate the GtaC transcription factor and different cell fates.

RESULTS

The atypical MAPK Erk2 is required for GtaC translocation

The translocation of a GFP–GtaC reporter from the nucleus to the cytoplasm was previously observed in a strain containing a hypomorphic *erk2*$^{-}$ allele, but we re-examined the possible translocation of the reporter in an *erk2*-null strain (Cai et al., 2014). Transformants containing the GFP–GtaC reporter were only obtained with a low concentration drug selection (1.5 µg G418/ml) and none of the transformants displayed sufficient GFP fluorescence for analysis, suggesting that the reporter might be toxic to cells lacking Erk2. To reduce possible toxicity, a mutant version of the reporter, GFP–GtaC$^{c\text{-}s}$ (C500S/C503S), was used in which two of the required cysteine residues of the GtaC zinc finger domain were converted into serine residues (Cai et al., 2014). Many viable transformants were obtained using this reporter vector and GFP fluorescence was readily detectable at high drug selection concentrations (3–8 µg/ml) suggesting that the alteration of the zinc finger reduces toxicity of GFP–GtaC overexpression in *erk2*-null cells. The reporter was concentrated in the nucleus of the *erk2*-null cells during both growth and starvation, and the stimulation of cells with cAMP did not result in the translocation of the reporter to the cytoplasm (Fig. 1; Movie 1). Complementation of the *erk2*-null mutant with a wild-type *erk2* allele rescued the ability of cAMP stimulation to translocate the reporter to the cytoplasm confirming that Erk2 is essential for this process. The translocation of the reporter was also re-examined in an *erk1*-null strain and the reporter was found to translocate in the same manner as observed in wild-type cells, confirming that Erk1 function is not required for this process.

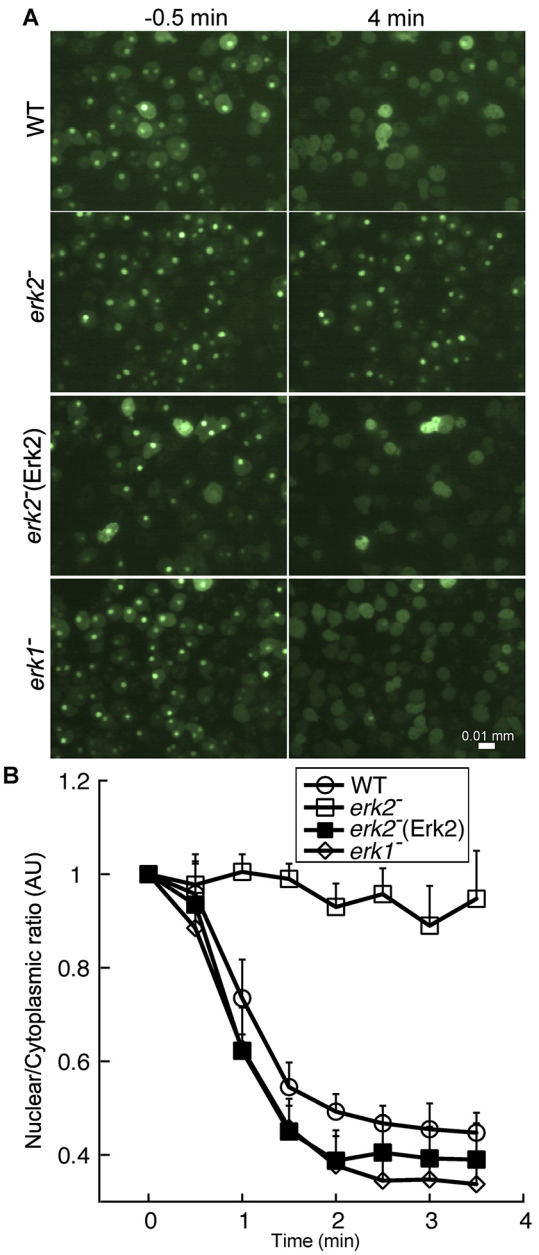

Fig. 1. Requirement of Erk2 function for the translocation of GtaC in response to cAMP stimulation. (A) Wild-type (WT), *erk2*$^{-}$, *erk2*$^{-}$ complemented with Erk2 expression vector [*erk2*$^{-}$(Erk2)] and *erk1*$^{-}$ strains expressing the GFP–GtaC$^{c\text{-}s}$ reporter were stimulated with 10 nM cAMP at time 0 min and time-lapse images were collected every 30 s. Images of –0.5 and 4 min of a representative assay of at least three independent assays are displayed. All images are the same magnification. (B) Graphical representation of mean nuclear/cytoplasmic ratios of GFP–GtaC$^{c\text{-}s}$ (AU, arbitrary units) of WT (n=35), *erk2*$^{-}$ (n=20), *erk2*$^{-}$(Erk2) (n=35), and *erk1*$^{-}$ (n=40) strains. Error bars represent s.d. (positive bar shown only).

Erk2 is required for phosphorylation of GtaC

The stimulation of wild-type cells with cAMP results in the rapid appearance of phosphorylated forms of the GFP–GtaC reporter, which migrate slower in electrophoretic gels, and an analysis of the GtaC sequence previously revealed many potential phosphorylation

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sites of several types of protein kinases (Cai et al., 2014). The slower migrating forms of GFP–GtaC were absent in *erk2*$^{-}$ cells stimulated with cAMP, suggesting that Erk2 is required for the phosphorylation of the reporter (Fig. 2). The slower migrating forms of the reporter were observed in *erk2*$^{-}$ cells complemented with the wild-type *erk2* gene and also in *erk1*$^{-}$ cells, suggesting a specific dependence on Erk2 function. The requirement of Erk2 function for GtaC phosphorylation does not exclude the possibility that other protein kinases, such as glycogen synthase protein kinase (Gsk3), might phosphorylate GtaC but only the loss of Erk2 prevents the mobility shift of the GFP–GtaC reporter (Cai et al., 2014). In an *in vitro* phosphorylation assay immunoprecipitated Erk2, but not Erk1, was capable of phosphorylating GFP–GtaC, resulting in a mobility shift in electrophoretic gels (Fig. 2B). In total, these data suggest that GtaC is specifically phosphorylated by Erk2, a mechanism consistent with the dependence of GtaC translocation on Erk2 function.

Erk2 regulates GtaC translocation in response to folate

Since Erk2 is also activated in response to folate, we postulated that folate stimulation could also result in GtaC translocation. Folate stimulation of wild-type cells resulted in the translocation of the GFP–GtaC$^{c\text{-}s}$ reporter from the nucleus to the cytoplasm with kinetics comparable to that occurring after cAMP stimulation (Fig. 3; Movie 2). This translocation was absent in *erk2*$^{-}$ cells, as observed for cAMP-stimulated *erk2*$^{-}$ cells. The translocation of the reporter was rescued by the expression of wild-type Erk2 indicating that Erk2 can regulate GtaC translocation in response to multiple chemotactic signals. As with cAMP responses, Erk1 was not required for the translocation of the reporter in response to folate, implying that only the atypical MAPK function regulates this process.

Folate signaling and GtaC function

Chemotaxis and other responses to folate require the folate receptor Far1 and the coupled G-protein subunit Gα4, and so the requirements of these signaling components were analyzed by assaying GFP–GtaC$^{c\text{-}s}$ reporter translocation in cells lacking either the receptor or the G protein. As expected, folate stimulation of the

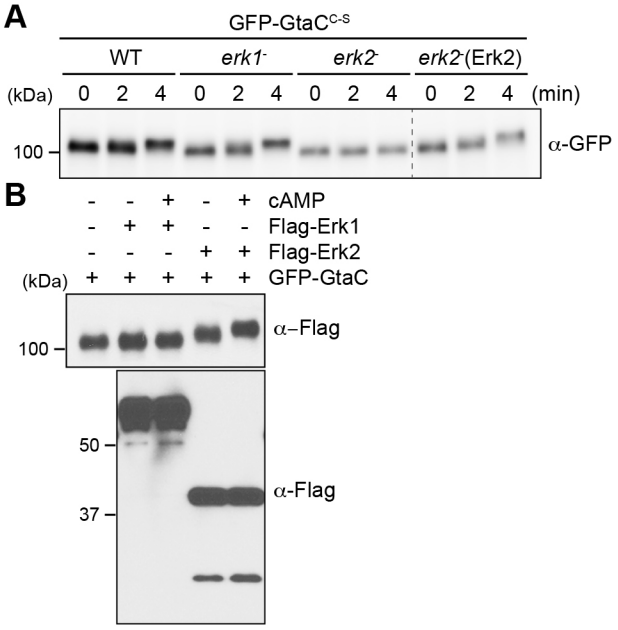

Fig. 2. Requirement of Erk2 function for GFP–GtaC mobility shifts. (A) Immunoblot of GFP–GtaC$^{c\text{-}s}$ from wild-type (WT), *erk1*$^{-}$, *erk2*$^{-}$, and *erk2*$^{-}$ complemented with Erk2 expression vector [*erk2*$^{-}$(Erk2)] strains stimulated with cAMP. Dashed line indicates removal of extra lanes in blot. (B) GFP–GtaC purified from wild-type cells and Flag–Erk1 and Flag–Erk2 purified from wild-type cells with or without cAMP treatment were assessed in an *in vitro* kinase assay. Immunoblot of GFP–GtaC$^{c\text{-}s}$ mobility shifts and immunoblots of Flag–Erk1 (~70 kDa) and Flag–Erk2 (~42 kDa) are shown. Blots shown are representative of three repeats.

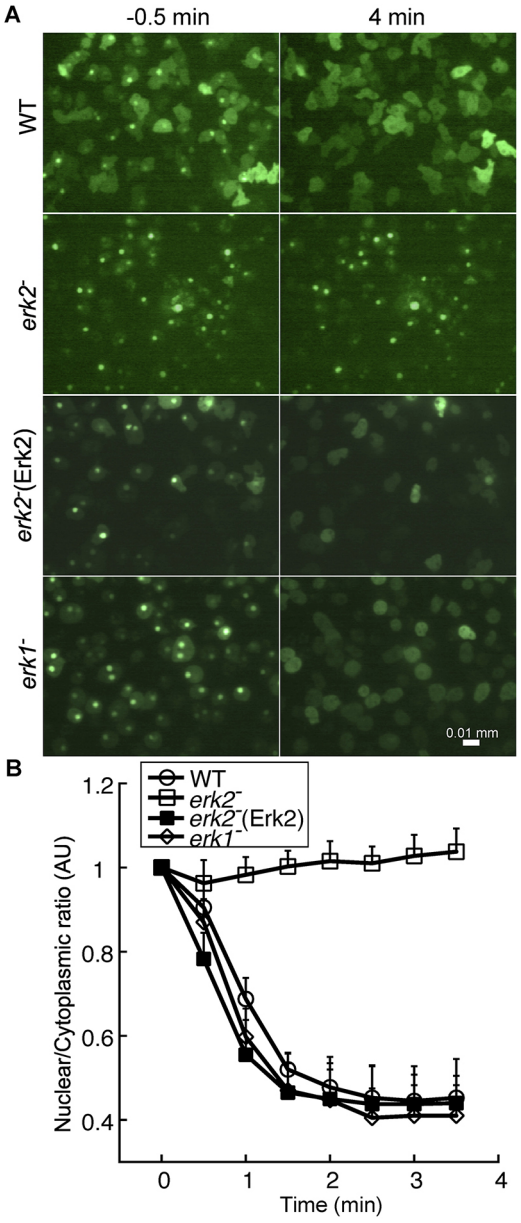

Fig. 3. Erk2 dependency of the GtaC translocation in response to folate. (A) Wild-type (WT), *erk2*$^{-}$, *erk2*$^{-}$ complemented with Erk2 expression vector [*erk2*$^{-}$(Erk2)], and *erk1*$^{-}$ strains expressing the GFP–GtaC$^{c\text{-}s}$ reporter were stimulated with 1 µM folate at time 0 min and time-lapse images were collected every 30 s as described in Fig. 1. Images of –0.5 and 4 min are displayed of representative assay of at least three independent assays. All images are the same magnification. (B) Graphical representation of mean nuclear/cytoplasmic ratios of GFP–GtaC$^{c\text{-}s}$ (AU, arbitrary units) of WT (n=23), *erk2*$^{-}$ (n=31), *erk2*$^{-}$(ERK2) (n=25), and *erk1*$^{-}$ (n=40) strains. Error bars represent s.d. (positive bar shown only).

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reporter translocation was absent in cells lacking either the folate receptor Far1 or the Gα4 subunit (Fig. 4; Movie 3). The noticeable dip in the nuclear-to-cytoplasmic ratio of reporter in *gα4*$^{-}$ cells stimulated with folate was observed in multiple assays and appeared to result from a transient change in the shape of some cells that temporarily moved the nucleus out of the focal plane. The loss of either Far1 or Gα4 did not prevent translocation of the reporter when cells were stimulated with cAMP, confirming that these signaling proteins mediate responses to folate and not to cAMP. The closest Gα4 subunit paralog in *Dictyostelium* is the Gα5 subunit and loss of the Gα5 subunit did not impede translocation of the reporter in response to either folate or cAMP. Previous studies have shown the Gα5 subunit is not required for chemotactic movement to folate but possibly acts in opposition to the Gα4 subunit in chemotactic movement and in regulation of developmental morphology (Natarajan et al., 2000). Other studies have indicated the requirement of the cAMP receptors Car1 and Car3, and the G protein subunit Gα2 for efficient translocation of the reporter in response to cAMP (Adhikari et al., 2021; Cai et al., 2014). To evaluate GtaC function in folate chemotaxis, a *gtaC*$^{-}$ strain was created through gene disruption and analyzed for chemotaxis to folate in the above agar assays. Cells lacking GtaC migrated further toward folate than the wild-type parental cells, consistent with a role for GtaC in the suppression of folate chemotaxis (Fig. 4C; Fig. S1). This result suggests that folate chemotaxis might be enhanced by Erk2-mediated translocation of GtaC, possibly through the loss of GtaC repression of the folate receptor gene (Santhanam et al., 2015).

Erk2-dependent translocation of GtaC can be mediated through multiple phosphorylation sites

The dependence of GtaC phosphorylation and translocation on Erk2 function suggests that Erk2 phosphorylates GtaC before it can be translocated to the cytoplasm. An earlier analysis of Erk2 phosphorylation sites *in vivo* and *in vitro* has indicated that preferred Erk2 phosphorylation sites include serine/threonine residues followed by a proline and a positively charged residue (S/T-P-K/R) (Nichols et al., 2019). The primary sequence of GtaC contains four of these sites (S357, S380, S386 and T492) and none of these sites exist in the GFP tag (Fig. 5A). Of these four sites, only the T492 residue is located within a region highly conserved among other Dictyostelid GtaC homologs, and the other sites are found just upstream of this conserved region (Fig. S2). Other GtaC orthologs also have putative Erk2 phosphorylation sites just upstream of the conserved region but the positions of these sites do not always align precisely with those in the *Dictyostelium discoideum* GtaC protein. An increase in the phosphorylation of the T492 site has been detected in a mass spectrometry analysis of phosphoproteins after folate stimulation (Nichols et al., 2019). In addition, GtaC contains 12 other sites (S/T-P) that are regarded potential targets for MAPKs or cyclin-dependent protein kinases. We examined the importance

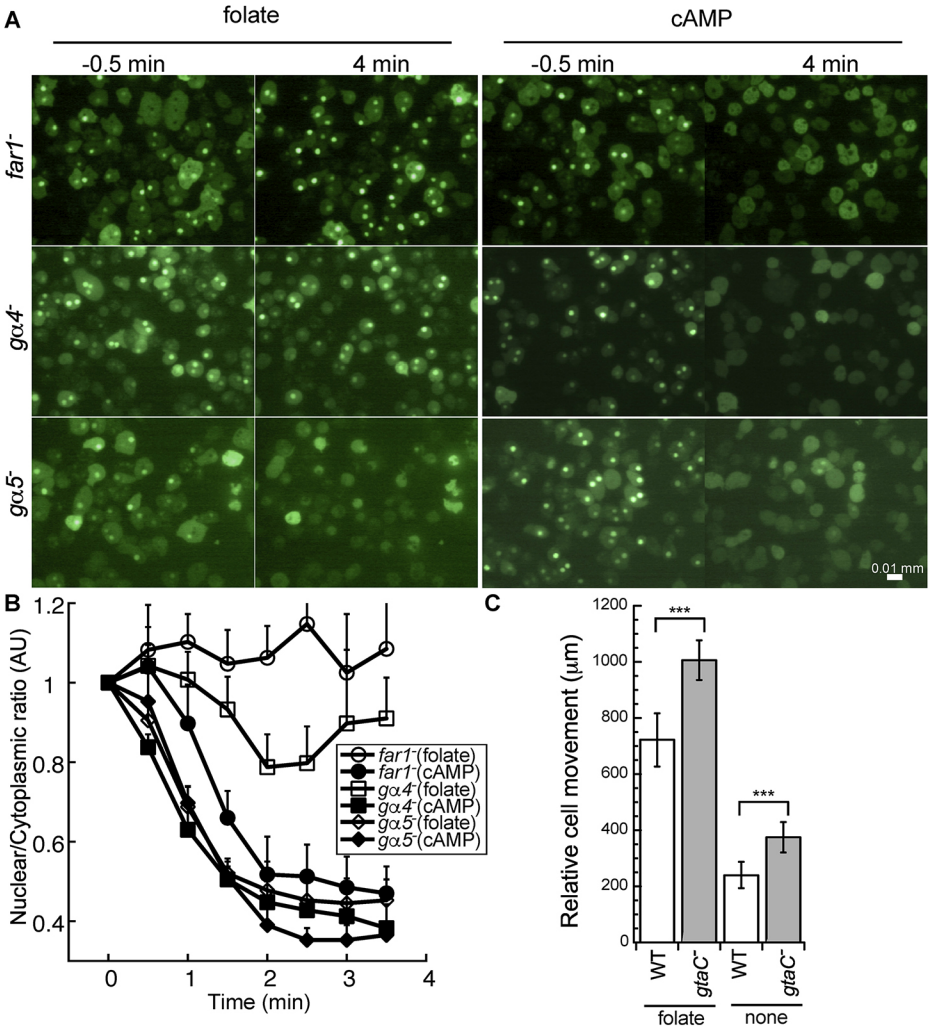

Fig. 4. Receptor and G protein requirements for GtaC translocation in response to folate. (A) *far1*$^{-}$, *gα4*$^{-}$ and *gα5*$^{-}$ strains expressing the GFP–GtaC$^{c\text{-}s}$ reporter were stimulated with 1 µM folate or 10 nM cAMP at time 0 min and time-lapse images were collected every 30 s as described in Fig. 1. Images of −0.5 and 4 min are displayed of representative assay of at least three independent assays. All images are the same magnification. (B) Graphical representation of mean nuclear/cytoplasmic ratios of GFP–GtaC$^{c\text{-}s}$ (AU, arbitrary units) of *far1*$^{-}$ (folate *n*=28, cAMP *n*=30), *gα4*$^{-}$ (folate *n*=30, cAMP *n*=40), *gα5*$^{-}$ (folate *n*=23, cAMP *n*=25) strains. Error bars represent s.d. (positive bar shown only). (C) Above agar chemotaxis assays of wild-type (WT) and *gtaC*$^{-}$ cells to folate. Data shown is representative of at least four assays. Error bars represent standard deviation of means. ***P<0.001 (unpaired two-tailed Student's *t*-test).

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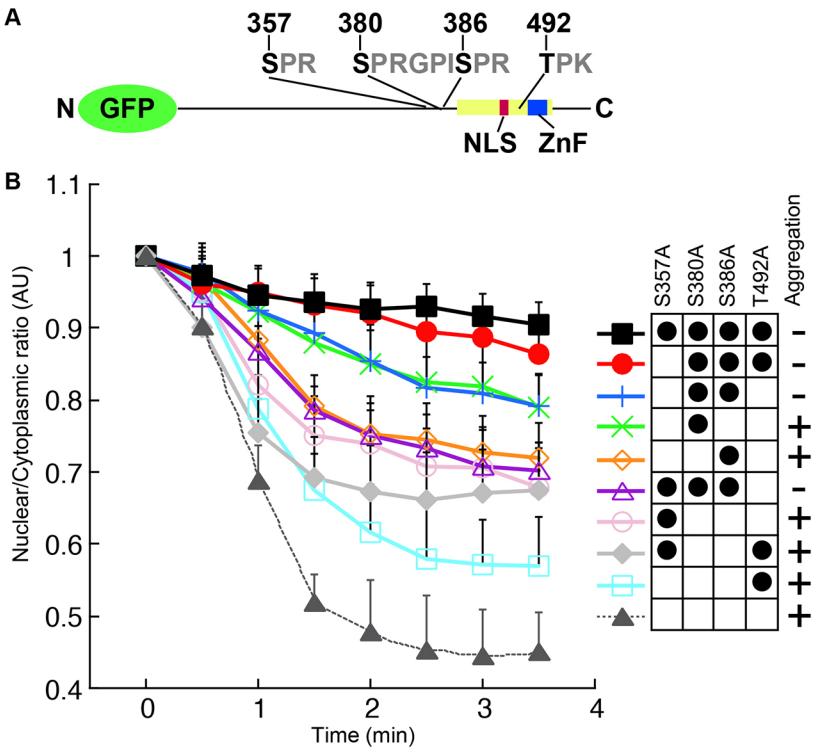

Fig. 5. Erk2 phosphorylation requirements for GtaC translocation in response to folate. (A) Map of GFP–GtaC reporter construct with locations of Erk2-preferred phosphorylation motifs. Location of nuclear localization signal (NLS; red bar) and zinc finger domain (ZnF; blue bar) are indicated and occur within highly conserved region (yellow bar, see also Fig. S2) of Dictyostelid homologs. (B) Graphical representation of nuclear/cytoplasmic ratios of GFP–GtaC$^{c\text{-}s}$ mutants expressed in wild-type cells after stimulation with 1 µM folate. Time-lapse images were collected and analyzed as described in Fig. 1 and the Materials and Methods. Wild-type cells with the parental GFP–GtaC$^{c\text{-}s}$ reporter construct data from Fig. 1 is included for comparison. Key indicates the combination of Erk2 phosphorylation site alterations, graph line color, and vector number. Multiple representative assays of wild-type cells with pJH911 (n=56), pJH913 (n=54), pJH925 (n=74), pJH926 (n=59), pJH929 (n=97), pJH931 (n=81), pJH932 (n=77), pJH935 (n=75), pJH937 (n=55), and pHC329 (n=23) were used to generate the mean of nuclear/cytoplasmic ratios of the reporter (AU, arbitrary units) and error bars indicate s.d. (positive bar shown only). (C) Aggregation capability of *gtaC*$^{-}$ cells expressing Erk2 phosphorylation mutant vectors with functional zinc finger domains (targeted sequence reversion) on bacterial lawns (images displayed in Fig. 6).

of the four putative Erk2-preferred phosphorylation sites that could mediate translocation of the GFP–GtaC$^{c\text{-}s}$ reporter in response to folate stimulation by converting one or more of the target residues (serine or threonine) into alanine. Individual residue changes did not prevent the translocation of the reporter in wild-type cells but in some cases the extent of translocation was reduced (Fig. 5B; Movie 4). These observations indicate that no single site is essential and that redundancy might exist in the ability of these sites to allow translocation of the reporter. Converting all of the sites or all but the S357 site significantly interfered with the reporter translocation, and in general the reporter translocation decreased as more sites were altered. Conversion of S380 and/or S386 appeared to have more impact on reducing reporter translocation than other single or double conversions suggesting these residues might be primary targets of Erk2 phosphorylation. However, the relationship of specific conversions with the impact on reporter translocation appears to be complex, and we cannot rule out the possibility that a conversion of one site might indirectly impact the importance of other sites.

Erk2 phosphorylation sites are necessary for GtaC function in early development

The GFP–GtaC$^{c\text{-}s}$ reporter offers a real-time analysis of Erk2-mediated GtaC translocation, but the translocation analysis might not accurately reflect the importance of Erk2 phosphorylation sites on GtaC function, particularly since this reporter lacks a functional zinc finger domain. To examine the impact of the Erk2 phosphorylation site alterations on GtaC function, the zinc finger domain in these mutant alleles was restored by converting the zinc finger domain sequence back to the wild-type sequence (S500C/S503C). When introduced into a *gtaC*$^{-}$ strain all of the single phosphorylation site mutants and one of the double site mutants (S357A/T492A) were capable of rescuing the aggregation phase of development when cells were grown on bacterial lawns (Fig. 6). Interestingly, the S380A allele rescued aggregation to form tight mounds, but most mounds did not undergo further developmental morphogenesis. None of the mutants possessing alterations at both S380 and S386 were capable of restoring sufficient aggregation to form tight mounds of cells. These results indicate that Erk2 phosphorylation sites on GtaC are not just important for translocation but that the sites play a regulatory role in GtaC function, consistent with a previous study demonstrating a correlation between translocation and developmental gene expression (Cai et al., 2014).

DISCUSSION

The dependence of GtaC translocation on the presence of the atypical MAPK Erk2 and on the Erk2-preferred phosphorylation sites within this transcription factor provide strong support that Erk2 directly regulates the GtaC function in response to chemoattractants (Fig. 7). The interaction between Erk2 and GtaC is further supported by the phosphorylation of GtaC in combined immunoprecipitates of GtaC and Erk2, as indicated by mobility shifts of GFP–GtaC in electrophoretic gels. These results reveal a previously unrealized role for atypical MAPKs in the regulation of transcription factor translocation, suggesting that this class of MAPKs can directly modify key regulators of gene expression. This study demonstrates that Erk2 can phosphorylate and regulate the translocation of the GtaC transcription factor in response to multiple chemoattractants, suggesting that the regulation of GtaC might have roles in foraging responses in addition to its role in multicellular development. The regulation of transcription factors in response to external signals has been observed for other classes of MAPKs. Mammalian ERK1 and ERK2 (also known as MAPK3 and MAPK1) regulates the activation and nuclear accumulation of the transcription factor Elk-1 in response to growth factors and p38 MAPKs regulates the translocation of TEAD transcription factors from the nucleus to the cytoplasm in response to stress (Lavaur et al., 2007; Lin et al., 2017; Slone et al., 2016; Yang et al., 1999). Heterologous overexpression of the atypical MAPK, MAPK15 (also known as Erk8), in

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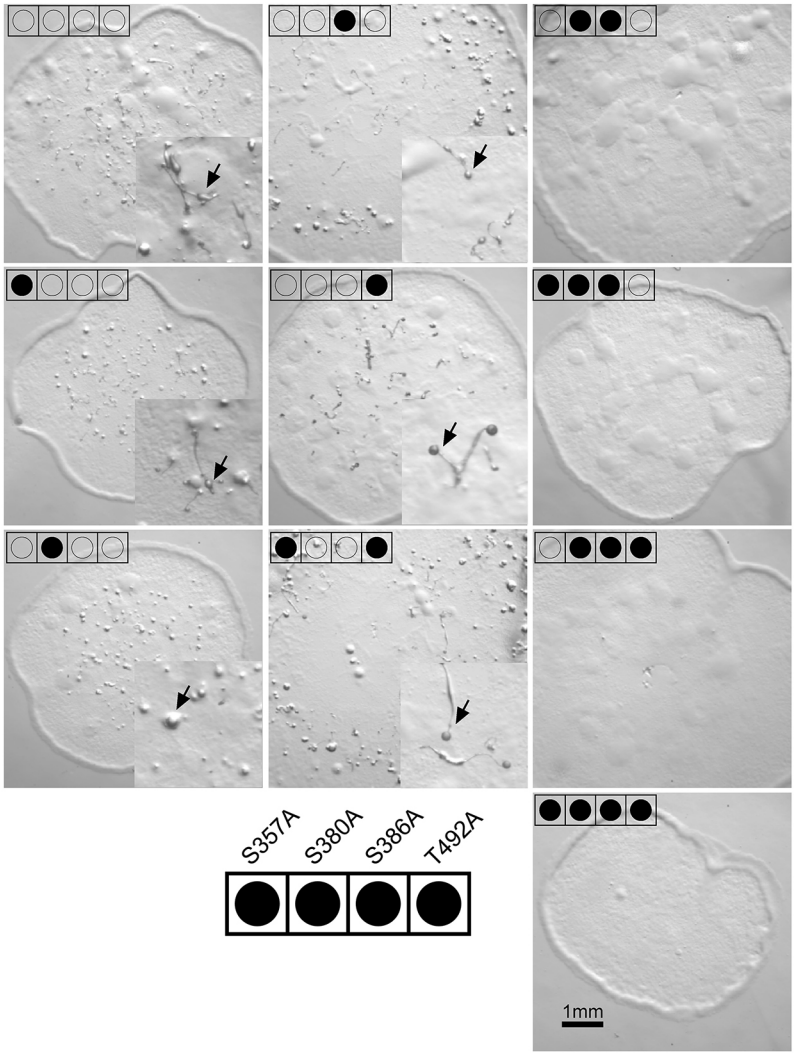

Fig. 6. Erk2 phosphorylation site-mediated regulation of GtaC function in early development. GFP–GtaC constructs with altered Erk2 phosphorylation sites were expressed in *gtaC*$^{-}$ cells. Strains expressing each construct were plated on to bacterial lawns and allowed to grow and develop for 2–3 days. All images are the same magnification except for insets (3× magnification). Representative aggregates that have formed tight mounds are indicated by arrows. The key indicates which sites have been altered (filled in circles) for each construct.

mammalian cells has been reported to regulate CRM1-dependent ERRα (also known as ESRRA) localization to the cytoplasm through protein–protein interactions but the phosphorylation state of ERRα was not investigated (Rossi et al., 2011). Our analysis showing Erk2-mediated regulation of GtaC in *Dictyostelium* is the first report of transcription factor phosphorylation and translocation by an atypical MAPK. Translocation of GtaC is rapid and reversible, and correlates with the activation of Erk2 in response to chemoattractants. If other atypical MAPKs operate in a manner similar to Erk2, then atypical MAPK signaling pathways in other organisms might be expected to regulate transcription factors and possibly other MAPKs in response to chemoattractants or environmental stresses.

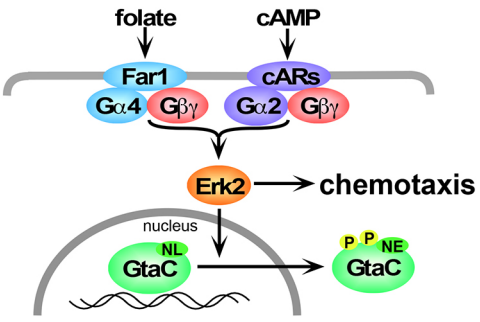

Fig. 7. Model of Erk2 regulation of GtaC translocation. Activation of cAMP (cARs) or folate (Far1) G protein-coupled receptors leads to the activation of the atypical MAPK Erk2 and the phosphorylation of the GtaC transcription factor. Translocation of GtaC from the nucleus to the cytoplasm is possibly mediated through masking the nuclear localization signal (NL) or exposing a nuclear export signal (NE).

Erk2 function is essential for folate chemotaxis, but the loss of GtaC increases the movement of cells to folate, suggesting that GtaC has a negative impact on this response. An earlier analysis of GtaC function on gene regulation has indicated that the loss of GtaC function increases the expression of the gene encoding the folate receptor Far1, consistent with increased chemotactic movement of *gtaC*$^{-}$ cells to folate (Santhanam et al., 2015). GtaC might possibly repress *far1* expression and then release this repression when translocated to the cytoplasm. However, the presence of GtaC positively impacts the expression of cAMP receptor genes suggesting that a different Erk2-regulated GtaC mechanism might exist for these genes. Erk2 is likely to have other cellular targets that might regulate chemotactic movement through mechanisms other than gene regulation. The Erk2-mediated regulation of GtaC translocation in response to multiple chemoattractants that promote different cell fates (i.e. foraging versus multicellular development) suggests that GtaC-mediated regulation of gene expression might be

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different in each response or that other signaling mechanisms might contribute to cell fate. External cAMP regulation of GtaC translocation has been previously described as a developmental timer mechanism to coordinate changes in gene expression with the repeated cycles of cAMP stimulation that occur during aggregation, but how GtaC might regulate gene regulation in response to folate remains to be assessed (Cai et al., 2014). Differential regulation of GtaC binding to DNA or other transcription factors could possibly result in the regulation of different target genes in response to folate compared to cAMP, similar to the different Erk2 phosphorylation targets associated with each chemotactic response (Nichols et al., 2019).

The analysis of the Erk2-preferred phosphorylation sites on GtaC revealed that almost any combination of these sites would allow translocation of the transcription factor to the cytoplasm suggesting that the translocation process might be mediated by general changes in GtaC conformation. The precise mechanism of nuclear export is not known, but sequences near the nuclear localization signal have been reported to resemble features of nuclear export signals in other systems (Cai et al., 2014). It is possible that exposure of a nuclear export signal or the masking of the nuclear localization signal might allow for translocation but associations with other proteins might also contribute to cytoplasmic localization. As previously reported, GtaC has sites that might be phosphorylated by other classes of protein kinases, such as glycogen synthase kinase (GSK) or cAMP-dependent protein kinase (PKA), but thus far only alterations in the Erk2-preferred phosphorylation sites have been shown to severely impact the translocation process. Gsk3 function in *Dictyostelium* has been previously shown to affect the efficiency of GFP–GtaC translocation in response to cAMP, and GSKs typically recognize substrates already phosphorylated by other protein kinases, suggesting that phosphorylation of GtaC by Gsk3 might be a secondary modification (Cai et al., 2014). The ability of Erk2-phosphorylated GtaC to serve as a substrate for other protein kinases remains to be determined, but the multiple shifts in electrophoretic gel migration suggest GFP–GtaC undergoes multiple phosphorylations or other modifications after chemoattractant stimulation (Cai et al., 2014). The distribution of the phosphorylation sites preferred by Erk2 or other protein kinases in the C-terminal half of GtaC does not account for the requirement of an N-terminal region (residues 180–222) for cytoplasmic translocation. Perhaps this region contains binding sites necessary for protein kinase interactions or this region interacts with the C-terminus to regulate the exposure of translocation signals.

In mammalian cells, MAPK15 has been shown to be associated with general cellular stress responses through protein-binding analysis, heterologous expression or RNA silencing experiments, but thus far no specific signals or signaling pathways have been identified that cause rapid activation of the MAPK like that observed for chemoattractant activation of Erk2 in *Dictyostelium* (Chia et al., 2014; Colecchia et al., 2018; Groehler and Lannigan, 2010; Hasygar and Hietakangas, 2014; Iavarone et al., 2006; Klevernic et al., 2009; Liwak-Muir et al., 2016; Rossi et al., 2011; Saelzler et al., 2006; Zacharogianni et al., 2011). If MAPK15 has roles in cell movement and development similar to that of Erk2 in *Dictyostelium*, then disrupting the MAPK15 gene could potentially impact early embryonic development making the genetic analysis of MAPK15 in mammals challenging. Many important phenotypes associated with the loss of Erk2 in *Dictyostelium*, including defects in chemotaxis and transcription factor translocation, were not revealed with hypomorphic *erk2*$^{-}$ alleles, suggesting that reduced levels of atypical MAPK expression might not be sufficient to uncover all MAPK-associated phenotypes. However, developing a kinase translocation reporter for atypical MAPKs in mammals could provide a powerful tool for investigating the signals that activate MAPK15. In *Dictyostelium* the GFP-tagged GtaC protein serves as the first known kinase translocation reporter specific for an atypical MAPK. This reporter will likely benefit future studies in the identification of other signals, perhaps other chemoattractants or morphogens that activate atypical MAPK function.

MATERIALS AND METHODS

Strains and culturing

The *erk2*$^{-}$, *erk1*$^{-}$, *erk1*$^{-}$*erk2*$^{-}$, *gα4*$^{-}$ and *gα5*$^{-}$ and strains were created from the parental axenic strain KAx3 and a thymidine auxotrophic derivative JH10 as previously described (Hadwiger and Firtel, 1992; Hadwiger et al., 1996; Schwebs and Hadwiger, 2015; Schwebs et al., 2018). The *far1*$^{-}$ strain was created from KAx3 cells as previously described (Pan et al., 2016). The *gtaC*$^{-}$ strain was created from JH10 cells through a disruption of the *gtaC* locus with an insertion of the *thyA* gene resulting in developmental phenotypes as previously described for *gtaC*$^{-}$ cells (Cai et al., 2014). Expression of wild-type gene expression vectors, pFar1-Y (Far1) and pHC326 (GtaC) were capable of rescuing chemotaxis and developmental phenotypes associated with the gene disruptions. *Dictyostelium* strains were grown in axenic HL-5 medium (with thymidine supplement for JH10 strain) or on lawns of *Klebsiella aerogenes* as previously described (Dynes and Firtel, 1989; Watts and Ashworth, 1970). Vectors were transformed into *Dictyostelium* strains using electroporation parameters as previously described (Dynes and Firtel, 1989). Multiple clones from all transformations were assessed for developmental morphogenesis or GFP–GtaC reporter translocation.

GFP–GtaC translocation assay

Strains expressing the GFP–GtaC$^{c\text{-}s}$ or derivatives were grown in fresh axenic medium for several hours prior to harvesting and plating on coverslips attached to the bottom side of 10 mm holes drilled into the 60 mm diameter Petri dishes. After cells were allowed to attach to the coverslip for 10 min, unattached cells were removed by two or three washes with developmental buffer (DB) composed of phosphate buffer (12 mM NaH_2PO_4 adjusted to pH 6.1 with KOH) with the addition of 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$. A final solution of 100 µl solution of DB was placed over the attached cells. Within 6 min of the final DB wash, the coverslip Petri dishes were mounted on 60× oil-immersion objective of an Olympus XI89 spinning disc confocal microscope. The software Cellsens was used to program time lapse exposures at 30 s intervals for a duration of 4–8 min. Chemoattractant solutions (cAMP and folate; USB Chemicals, cat. #10470, cat. 15880, respectively) were added to the cells during the second time-lapse interval to a final concentration of 10 nM cAMP or 1 µM folate, unless otherwise noted. ImageJ software was used to create videos of the time lapse and to quantify the translocation of the reporter from the nucleus to the cytoplasm. The mean pixel intensity of the nucleus was divided by the mean pixel intensity of the cytoplasm. Measurements of the nuclear-to-cytoplasmic ratio in the stimulated cells were compared to an average of the ratios obtained from the first two time-lapse images representing unstimulated cells. The middle 50% values were used to generate the plotted data due to transient variability of values in some cells undergoing movement and/or shape changes that temporarily cause the nucleus to leave the focal plane.

GFP–GtaC$^{c\text{-}s}$ mobility shift assay

Wild-type, *erk1*$^{-}$, *erk2*$^{-}$ and *erk2*$^{-}$(Erk2) (i.e. *erk2*$^{-}$ cells expressing an Erk2 rescue construct) cells expressing GFP–GtaC$^{c\text{-}s}$ were starved in DB for 1 h. Cells were then washed with cold DB, resuspended in DB to a density of 2×10^{7} cells/ml, and kept on ice before assay. Cells were stimulated with 1 µM of cAMP at room temperature, lysed in SDS sample buffer at various time points before or after stimulation, and boiled for 5 min. Immunoblotting was performed as described before using an anti-GFP antibody (1:400, Roche, cat. #11814460001) or an anti-Flag antibody (1:4000, Sigma, cat. #A8592) (Kamimura et al., 2009).

In vitro phosphorylation assay

To purify GtaC as the substrate, GFP–GtaC/*gtaC*$^{-}$ cells were washed with cold DB, and lysed at a density of 10^7 cells/ml by adding equal volume of 2× lysis buffer [50 mM Tris-HCl pH 7.5, 200 mM NaCl, 1% NP-40, 100 mM NaF, 50 mM sodium pyrophosphate, 4 mM Na_3VO_4, 2× complete EDTA-free protease inhibitor mixture (Roche)], and incubated on ice for 5 min. Cleared lysate was incubated with GFP-Trap (ChromoTek) beads at 4°C for 1.5 h. Beads were washed with lysis buffer and elution buffer (25 mM Tris-HCl pH 7.5, 100 mM NaCl and 0.1% NP-40) and kept on ice before the assay. To purify Erk1 and Erk2, Flag–Erk1/*erk1*$^{-}$ and Flag–Erk2/*erk2*$^{-}$ cells were collected before and after cAMP stimulation (1 µM of cAMP for 1 min), washed with DB, and lysed at a density of 2×10^7 cells/ml by adding equal volume of 2× lysis buffer. Cleared lysate was incubated with anti-Flag M2 affinity resin (Sigma, cat. #F2426) at 4°C for 2 h. Beads were washed with lysis buffer and elution buffer. Proteins were eluted by the addition of elution buffer containing 3× Flag peptide (Sigma, cat. #F4799) at 4°C for 30 min. For the kinase reaction, 10 µl of GFP–GtaC-containing beads were mixed with 25 µl of Flag–Erk1 or Flag–Erk2-containing eluate. The reaction was initiated by the addition of ATP mix to a final concentration of 10 mM $MgCl_2$, 5 mM DTT and 0.3 mM ATP. Reaction was allowed to proceed for 15 min at room temperature and stopped by the addition of protein sample buffer.

Vector construction

Expression vectors GFP–GtaC (pHC326) and GFP–GtaC$^{c\text{-}s}$ (C500S/C503S) (pHC329) have been previously described (Cai et al., 2014). Erk2 phosphorylation site alterations at residues S357, S380, S386 and T492 were created through PCR mutagenesis of an intron-less version of pHC329 using the oligonucleotides (#1-14) and HiFi assembly (NEB) (Table S1). All of the phosphorylation site mutant vectors were further modified to restore the wild-type sequence in the zinc finger domain (S500C/S503C) using oligonucleotides (#15 and 16) (Table S1). All modified vectors were sequenced to verify changes in the sequence. The *gtaC::thyA* gene disruption was created through the insertion of a *thyA* gene fragment, amplified with oligonucleotides (#17 and 18), and cloned into a unique *Eco*RI site in the *gtaC* open reading frame and a *Bgl*II fragment containing the disruption site and flanking sequences was excised from a vector and electroporated into JH10 cells.

Chemotaxis and developmental assays

The 'above agar' chemotaxis assays to folate were conducted as previously described by placing droplets of cell suspensions on non-nutrient agar plates followed by the placement of droplets of 100 µM folate (approximately 2 mm away from the cell droplets) (Nguyen et al., 2010). Images of cells acquired after plating and 3 h later were compared to measure the maximum distance traveled by the leading edge of cells. The developmental morphogenesis of *gtaC*$^{-}$ cells, with or without GFP–GtaC vectors, was analyzed by transferring cells from clonal colonies in axenic culture onto a bacterial lawn. Images of *Dictyostelium* plaques on the lawn were acquired 2–3 days after the inoculation. Multiple transformants were analyzed for each strain.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.A.H., H.C.; Methodology: J.A.H., H.C.; Validation: J.A.H., H.C.; Formal analysis: J.A.H., H.C., R.G.A., S.F.; Investigation: J.A.H., H.C.; Data curation: J.A.H., H.C., R.G.A., S.F.; Writing - original draft: J.A.H., H.C.; Writing - review & editing: J.A.H., H.C., R.G.A., S.F.; Supervision: J.A.H.; Project administration: J.A.H., H.C.; Funding acquisition: J.A.H., H.C.

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