REGULATION OF A PHOSPHODIESTERASE AND MAP KINASE DURING *DICTYOSTELIUM* SIGNALING AND MULTICELLULAR DEVELOPMENT

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REGULATION OF A PHOSPHODIESTERASE AND

MAP KINASE DURING *DICTYOSTELIUM* SIGNALING

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Abstract: Many eukaryotic signaling pathways use cAMP as a secondary messenger to evoke specific responses to different external stimuli. Here, localized levels of cAMP can be controlled by phosphodiesterases, which are sometimes regulated by phosphorylation. *Dictyostelium discoideum* offers an excellent model system to study signal transduction pathways such as the regulation of phosphodiesterases as it contains relatively few cAMP-specific phosphodiesterases compared to mammals. The cAMP-specific phosphodiesterase, RegA, regulates important steps in *Dictyostelium* development and is negatively regulated by the MAP kinase, Erk2. This inactivation occurs periodically by external cAMP pulse where a cell-signaling pathway activates Erk2. Mammalian studies have suggested that the cAMP-dependent protein kinase, PKA, can also regulate the phosphodiesterase activity. This putative regulation of PKA on the activity of RegA has not been fully investigated in *Dictyostelium*. Mass spectrometry was used to detect potential phosphorylation sites on RegA. Two sites of interest have been identified, including a PKA phosphorylation site. Phosphomimic and phosphoablative mutations for the three sites have been constructed. The phenotypes of cells carrying these mutations have been analyzed for their impact on development and cell fate. This study supports the hypothesis that RegA is regulated by multiple phosphorylation events to influence signaling during multicellular development. To study upstream aspects of this signaling pathway the *erk2* gene in *Dictyostelium* was disrupted The absence of Erk2 resulted in a complete loss of folate and cAMP chemotaxis suggesting that this MAPK plays an integral role in the signaling mechanisms involved with this cellular response. Multicellular development was also impacted as was the phosphorylation of Erk1 as a secondary response to folate stimulation. Loss of the only known *Dictyostelium* MAPK kinase, did not impact Erk2 phosphorylation in response to folate and cAMP. This lack of MAP2K phosphorylation of Erk2 and the sequence similarity of Erk2 to mammalian MAPK15 (Erk8) suggest that the *Dictyostelium* Erk2 belongs to a group of atypical MAPKs. These studies of RegA and Erk2 suggests that *Dictyostelium* represents a good model for the study of the regulation of phosphodiesterases and atypical MAPKs.

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CHAPTER I

Introduction to Signal Transduction, Phosphorylation, and Phosphodiesterases

1.1 Cyclic Adenosine Monophosphate as a Signaling Molecule

Cyclic AMP (cAMP) is a molecule that is constructed from adenosine triphosphate by the enzyme adenylyl cyclase. cAMP is widely used as a secondary messenger that transmits a signal inside of the cell in response to an extracellular signal for some signaling pathways. This message is transmitted by the levels of cAMP in the cell. During the beginning on a cAMP signaling pathway, adenylyl cyclase is activated where it binds and catalyzes the conversion of ATP to cAMP. Adenylyl cyclases are activated downstream of G-protein coupled receptors (GPCR) in different organisms and cell types to transmit a diverse array of signals. In humans for example, adenylyl cyclase is activated for signaling processes involved with the heart and reproduction by the binding of a hormone to a GPCR [1,2]. *Dictyostelium* utilize adenylyl cyclase activation in a similar GPCR mechanism in response to external cAMP and folate during development and foraging, respectively [3–5].]. This usage of cAMP *Dictyostelium discoideum* and other social amebae is unique as cAMP is used as both an intracellular signaling molecule to transmit a signal as well as an external signaling molecule to drive chemotaxis during starvation triggered development [6,7]. cAMP is released in oscillating pulses from starving *D. discoideum* cells to attract nearby cells to an aggregating center [8]. This usage of cAMP as an external chemoattractant allows for the controlled destruction of the signaling molecule through the action of a secreted phosphodiesterase, PdsA [9]. The control of PdsA in combination of regulation of secreting cAMP allows for the regulation of the timing of signaling events during early development [10]. The levels of intracellular cAMP is increased rapidly in a time scale of seconds

as a result of the activation of adenylyl cyclase [11]. One major target for cAMP signaling in the cell is the cyclic AMP dependent protein kinase, Protein Kinase A that is activated by high levels of cAMP and reversibly inactivated by decreased levels of cAMP [12]. The act of decreasing the levels of cAMP to basal levels at the end of a signal transduction event is also an important signaling event [6].

1.2 Phosphodiesterases as Regulators of cAMP

Phosphodiesterases function to inactivate cyclic nucleotides to regulate a signaling event and they are classified based on the type of cyclic nucleotide that they hydrolyze. These are categorized as cAMP-specific phosphodiesterases, cyclic GMP-specific phosphodiesterases, and phosphodiesterases that can hydrolyze both cAMP and cGMP. The cyclic nucleotides are actively degraded by phosphodiesterases by hydrolyzing the phosphodiester bond. For cAMP, this leads to the conversion of cAMP into the signal-inactive AMP, which is unable to bind to targets such as PKA. Phosphodiesterases are tightly controlled by post-translational modifications from a variety of sources. Kinases can regulate phosphodiesterases through phosphorylation, leading to inactivating or activating the catalytic site of the phosphodiesterase based on the context [13–15]. Other post-translational modifications regulate phosphodiesterase function, stability, or activity such as SUMOylation and ubiquitination [16,17]. The tight regulation of phosphodiesterases are needed to prevent antagonizing the increase of cyclic nucleotides during a signaling event, which could lead to aberrant signaling. Phosphodiesterases are also common therapeutic targets some diseases due to their important roles in cell signaling pathways. They are also good targets due to their high number of isoforms and tissue specific expression, which contributes to the specificity of therapeutic compounds allowing therapies to target tissues of interest. Phosphodiesterase inhibitors are suggested to help in the treatment of diseases such as Alzheimer's disease, pulmonary disease, cardiovascular disease, and erectile dysfunction [18–22].

1.3 Activation and Function of Protein Kinase A by cAMP

Protein Kinase A (PKA) is a major effector protein of cyclic AMP. It is a tetramer that is made up of four subunits: two PKA-regulatory subunits (PKA-R) and two PKA-catalytic subunits (PKA-C) [23]. However, *Dictyostelium discoideum* PKA forms as a dimer with one PKA-R and one PKA-C subunit [24]. PKA activity is dependent on the levels of cAMP in the localized subcellular compartment [25]. The PKA-R subunits function to bind and inactivate the PKA-C subunits at low levels of cAMP forming the inactive holoenzyme (Fig. 1.1). At high levels of cAMP, PKA-R binds cAMP resulting in a conformational change that leads to the release of active PKA-C [26]. PKA-C then functions to phosphorylate numerous downstream targets at while levels of cAMP are elevated depending on the context of cell type, substrate availability, and subcellular localization [27]. One mechanism for providing specificity of PKA-C activity is through the A Kinase Anchoring Proteins (AKAPs) which function to bind to the PKA-R subunit and localize PKA [28]. There are numerous types of AKAPs identified and they can function to localize PKA-C with specific signaling enzymes to increase signaling specificity and targeting [29]. The termination phase of the cAMP signaling event occurs as the level of cAMP is decreased in the cell. Phosphodiesterases hydrolyze cAMP in the cell to decrease the total concentration resulting in the PKA-R subunit to bind to the PKA-C subunit again, rendering the catalytic subunit inactivated [30,31].

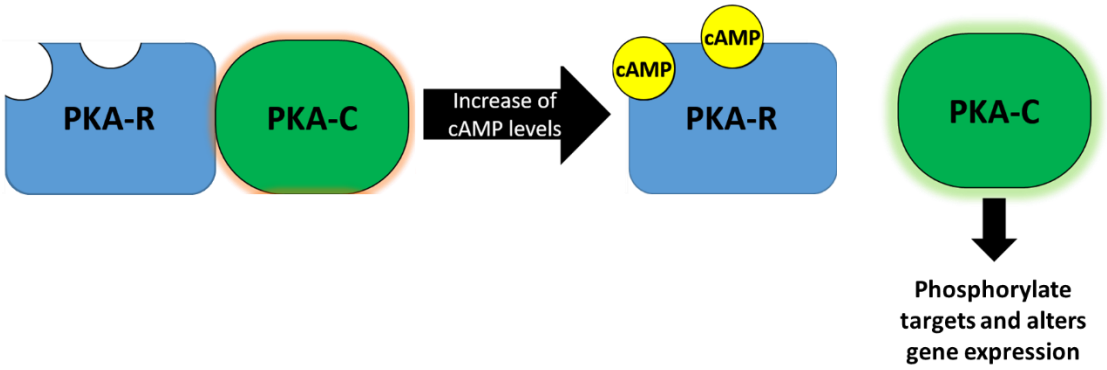

Figure 1.1. Activation of *Dictyostelium* PKA catalytic subunit by cAMP. When cAMP levels are low in the cell, the PKA regulatory subunit binds and inactivates the catalytic subunit. When the levels of the secondary messenger, cAMP, is increased in the cell during a signaling event, PKA-R binds cAMP, which results in a conformation change that releases the activated form of PKA-C.

1.4 Mitogen Activated Protein Kinases as Regulators of Other Proteins

Mitogen Activated Protein Kinases (MAPK) are signaling proteins that function to regulate other proteins through transferring a phosphoryl group through phosphorylation. The phosphate group can alter the function of a target protein due to the conformational change of the structure, the formation of new hydrogen bond interactions, and altering binding sites [32]. This post-translational modification is reversible through the process of dephosphorylation where the phosphate group is removed by a phosphatase [33]. MAPKs regulate a wide variety of substrates in cells performing different functions depending on the target and signaling event occurring. These substrates commonly contain the motif (P-X-S/T-P) where the serine/threonine is phosphorylated by the MAPK [34]. In mammals MAPKs function in cellular events such as embryogenesis, differentiation, cell growth, survival, and apoptosis [35]. While there are numerous pathways in which MAPKs are major components in, there are relatively few MAPKs encoded. *D. discoideum* for example only has two encoded MAPKs that are involved in multiple cellular processes such as cell growth chemotaxis during development and foraging [36,37].

Because of the large number of potential targets in different signaling pathways in a cell and the small number of MAPKs encoded, various mechanisms are utilized to provide specificity to transmit the signal to the appropriate substrate at the correct time [38–40]. Scaffolding proteins which function to bind, localize, and consolidate signaling proteins together to influence the transduction pathway [41]. Scaffolds help insulate and prevent aberrant signaling by localizing the kinases together, reducing crosstalk between different pathways [42]. MAP kinase phosphatases (MKPs) function to dephosphorylate MAPKs to limit their activity and duration of

their active state [43]. The termination of the MAPK signaling is as important as the activation of MAPKs as constitutive active MAPKs can be a contributor to diseases such as cancer and obesity [44,45].

1.5 *Dictyostelium discoideum* as a eukaryotic model organism

The amebae *Dictyostelium discoideum* has been used as a simple model organism to study eukaryotic cells for many years after it was originally discovered in 1935 [46]. As a model *Dictyostelium* has some advantages for looking at basic eukaryotic cellular processes such as cell growth, division, phagocytosis and chemotaxis. The chemotaxis of *D. discoideum* has been used as a model for neutrophils to look at conserved cellular processes [47–49]. However, the unique aspects of *Dictyostelium* defines its usefulness as a model organism, such as it being a haploid organism and its unique developmental life cycle. As a haploid organism, it is easier to observe knock-in and knock-out mutants relative to a diploid organism. It is also amenable to biochemical and genetic techniques due to the ability to collect isogenic cells in relatively large numbers at multiple time points during development [50]. Its developmental life cycle lead to it being termed a “social amebae” due to forming multicellular structures from previously solitary cells. Upon nutrient limitation cells will begin to aggregate together through cAMP chemotaxis. The aggregate of cells then begin to differentiate into either pre-stalk or pre-spore cell types that eventually form stalk and spore structures in the terminal fruiting body structures respectively [6].

1.6 Early development: The *Dictyostelium* internal cAMP and PKA signaling pathway

While using cyclic AMP as an intracellular signaling molecule is ubiquitous in eukaryotic organisms, the main cAMP signaling pathway during early development of *Dictyostelium* involves secreted external cAMP from cells for the purpose of chemotaxis-mediated aggregation. cAMP is secreted to attract cells into an aggregation center. The external cAMP released by cells binds and activates the cAMP receptor (cAR1) of nearby cells [51–53]. cAR1 is a G-protein

coupled receptor (GPCR) that associates with the *Dictyostelium* Gα2, Gβ, and Gγ for the cAMP signaling pathway [54]. The net goal of this signaling event is to alter gene expression and secrete more cAMP outside of the cell in an oscillating manner [55]. This continued signaling results in chemotaxis of cells towards an aggregating center. For this signaling event to occur, cAMP levels in the cell must increase rapidly by activating adenylate cyclase to produce more cAMP and inhibit the phosphodiesterase, RegA, to prevent excessive degradation of cAMP and then reduce levels of internal cAMP to terminate the signal (Fig. 1.2).

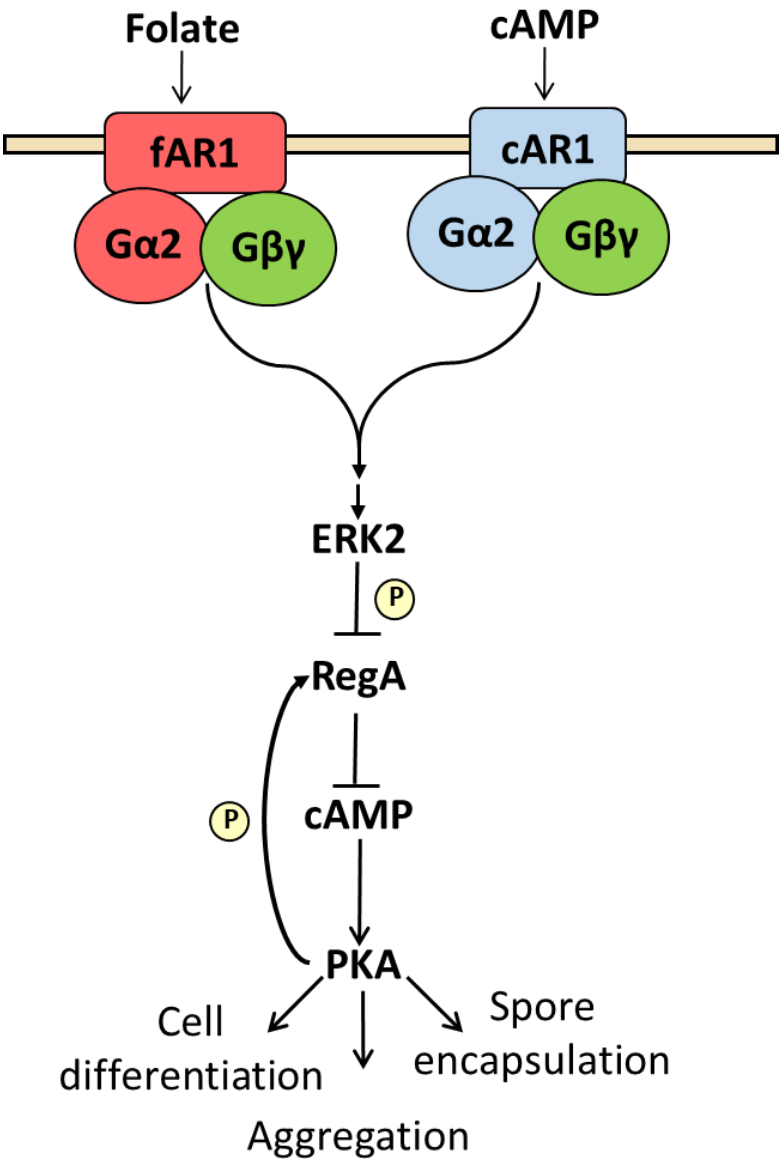

Figure 1.2. Model for RegA signaling pathway for cAMP and folate stimulation. The binding of folate or cAMP to their receptors results in the increase of cAMP levels in the cell by inhibiting the cAMP-specific phosphodiesterase RegA through Erk2 activation. The higher levels of cAMP activate PKA to regulate downstream targets involved in cell differentiation, spore development, and aggregation. PKA may then feedback to phosphorylate RegA at Serine 413 resulting in an increase of RegA activity.

The binding of cAMP to cAR1 results in the activation and disassociation of the heterotrimeric G-proteins that bind to the cAR1 receptor. While *Dictyostelium* only has one known Gβ subunit, there are multiple Gα subunits that provide pathway specificity [56,57]. In the cAMP pathway, cAR1 activates Gα2 with the downstream effect of rapidly activating adenylyl cyclase [58–60]. Adenylyl cyclase then synthesizes cAMP from ATP which is used both as an intracellular secondary messenger and is secreted outside of the cells to continue the intercellular signaling event [61–63].

The levels of cAMP are also increased by the downstream signaling from the cAR1 receptor in a parallel pathway through the inhibition of the major intracellular phosphodiesterase, RegA, by Erk2 [55]. The specifics of the signal relay from the cAMP receptor to activate Erk2 is not fully understood. Erk2 activation occurs rapidly within 30 seconds of stimulation with cAMP or folate [64]. However, Erk2 is activated by cAMP stimulation to at least 50% of the level of wild type cells when they are lacking either Gα2 or Gβ, which suggest that Erk2 can be activated through a G-protein independent mechanism or that there is redundancy in the G-protein function with other Gα subunits functioning in cAMP signaling [65,66]. Erk2 resembles an atypical MAPK as it has sequence similarities to known mammalian atypical MAPKs and it is not activated through the canonical MAPK cascade [37]. Erk2 activation is not impaired when the only known MAPK kinase, MekA. Erk2 is also regulated by a non-adaptive mechanism [67]. In an adaptive system, a signal will be terminated after a period of time even when there is a constant stimulation of the signaling molecule. This can be achieved through multiple

mechanisms such as a negative feedback loop, sequestering the receptor, and inactivating the receptor. However, the *Dictyostelium* Erk2 can be constantly activated when cells are stimulated with high levels of cAMP and dithiothreitol which inhibits the extracellular phosphodiesterase [67]. While this is a non-physiological condition due to the inactivation of the extracellular phosphodiesterase, it suggests that the duration of the signaling event is largely controlled by the extracellular phosphodiesterase that degrades cAMP outside of the cell, thus depleting the ligand rather than an adaptive system in the cell.

One target of activated Erk2 is RegA, as suggested by genetic data where disrupting *regA* in an *erk2* null strain rescued the aggregation deficient phenotype [55,68]. This inhibitory phosphorylation event is suggested to occur at residue T676 in RegA, as expressing *regA* with an alanine point mutation ($RegA^{T676A}$) in $regA^{-}$ cells resulted in a decrease of cAMP accumulation to 15% of wild type levels [55]. The result of this inhibition is to allow cAMP levels to increase without being antagonized by RegA, leading to the activation of PKA-C.

As cAMP levels increase in the cell the regulatory subunit of PKA (PKA-R) binds cAMP and changes conformation. This leads to the disassociation and resulting activation of PKA-C from PKA-R. PKA-C then functions to phosphorylate downstream targets to lead to a change of gene expression for at least 15 genes in response to cAMP pulsing, such as *aca* and *carA* [69]. This leads to a short period of gene expression of early developmental genes as a response to external cAMP stimulation [70].

However, the mechanism of restoration of the system to an adaptive state with low levels of intracellular cAMP has been debated. One way that cAMP levels are reduced is by the reduction of adenylyl cyclase activity, which prevents the production of new cAMP. cAR1 loses substrate affinity around two to three minutes after stimulation with cAMP due to the phosphorylation of multiple serine residues at the carboxyl terminus of cAR1 [71,72]. It has been

speculated that PKA phosphorylates these residues to indirectly inhibit the activation of adenylyl cyclase; however, this is debated as the residues do not contain the canonical PKA phosphorylation motif and the phosphorylation event is independent of cAMP levels [68,73]. The phosphorylation of cAR1 at these residues was determined to not be necessary for chemotaxis or regulating G-protein response [74]. It is important to note that these findings do not rule out the possibilities that PKA has an effect on cAR1 and adenylyl cyclase activity through other indirect mechanisms. PKA was suggested to inhibit adenylyl cyclase activity due to the prolonged activation of adenylyl cyclase A (ACA) in cells lacking *pkaC* [75]. Another mechanism for restoring the basal level of cAMP is for the reactivation of RegA to turnover cAMP. It was proposed that this occurred through the inactivation of Erk2 by PKA phosphorylation forming a negative feedback loop, which would in turn alleviate the inhibition of RegA [55]. However, Erk2 is activated normally in cells that could not produce intracellular cAMP (*aca*$^{-}$) where the catalytic subunit of PKA is underactive and in cells with constitutive PKA activity, (*pkaR*$^{-}$) [67]. This does not support the idea that PKA feeds back to regulate the activity of Erk2 and opens the possibility of another regulatory event to help in resetting the levels of cAMP to an adaptive state. One putative mechanism is by the regulation of RegA directly by phosphorylation to reactivate its phosphodiesterase activity. Mammalian phosphodiesterases are regulated by multiple mechanisms such as MAPKs and PKA to alter the levels of cAMP in the cell [13,15,76]. It is possible that RegA in *Dictyostelium* is regulated by similar mechanisms and this would provide a way for decreasing the levels of cAMP after the activation of PKA in concert with the reduction of adenylyl cyclase activity.

1.7 Differentiation of *Dictyostelium* during development

Cell differentiation and sorting occurs as early as the mound stage where cells begin to differentiate into pre-stalk and pre-spore cell types. The differentiation of cells into these cell types is driven by a position independent manner starting in the mounds before further separating

out into either pre-stalk or pre-spore cells [77]. While there are two main categories of cell types, the pre-stalk cell types are further divided based on gene expression and location in the slug with pre-stalk A cells being at the most anterior portion of the slug, prestalk O (pstO) cells being just posterior of prestalk A, prestalk B cells, prestalk AB (Fig 1.3) [78]. These subtypes of pre-stalk cells become different components of the stalk (the basal disc, stalk, and cup cells) to support the spore mass in the final fruiting body structure [79,80].

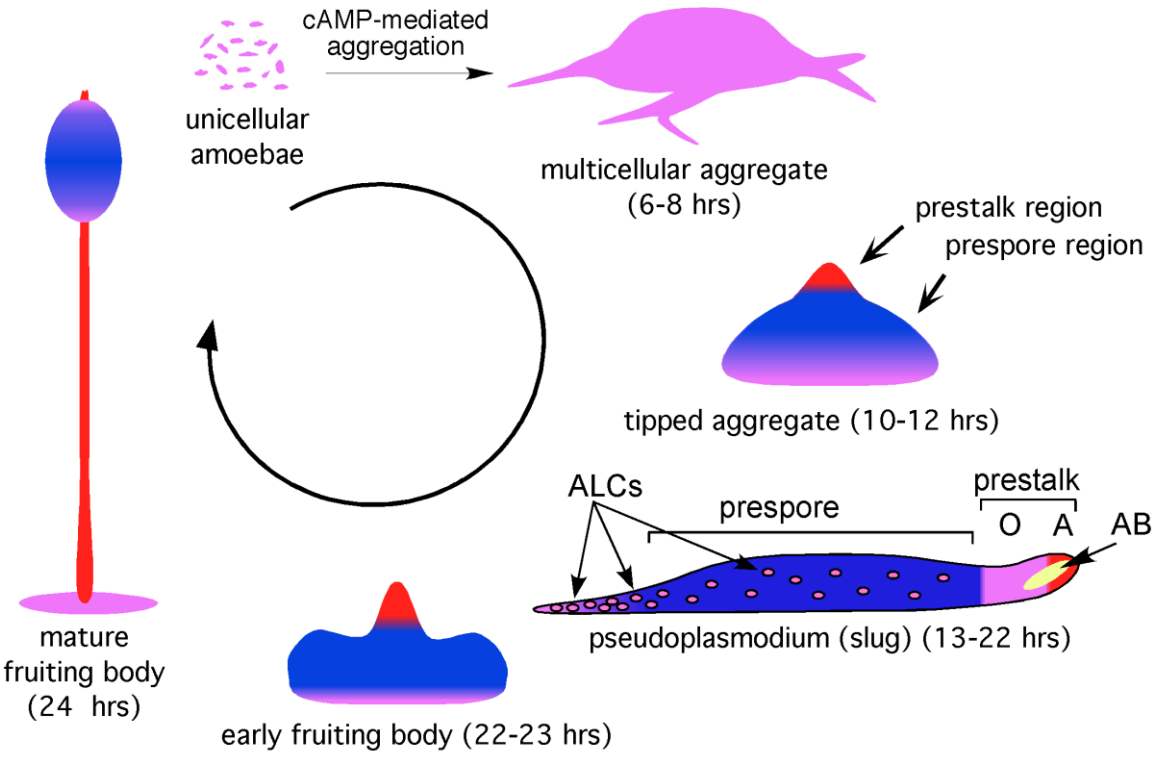

Figure 1.3. *Dictyostelium* developmental life cycle. *Dictyostelium* cells start as solitary cells and upon starvation, they aggregate by cAMP chemotaxis. In the aggregate they begin to differentiate into prespore and prestalk cell types before forming a slug. In the slug, various cell types further differentiate due to signaling events before culminating into a mature fruiting body when the spore and stalk cell types mature.

While the specific signaling that determines the decision between pre-stalk and pre-spore is unknown, PKA and cAMP are essential parts for both pre-stalk and pre-spore development [81,82]. Prespore differentiation is driven by an increase of cAMP levels in posterior region of

the slug due to the upregulation of the adenylate cyclase AcgA [83]. These prespore cells then start to produce a signaling molecule called DIF-1 which directs the differentiation of pstO cells that are anterior of the prespore cells [84,85]. RegA plays a large role for differentiation of these cell types due to the importance of cAMP signaling and it is regulated largely by a histidine kinase phosphorylation site in its N-terminal regulatory region. This site is phosphorylated by RdeA in a phosphorelay system resulting in an 8-fold increase of phosphodiesterase activity for RegA [86]. Spore maturation utilize this regulation of RegA through the release of the signaling peptide SDF-2 from prestalk cells, which binds and inactivates the histidine kinase activity of DhkA in prespore cells [87]. DhkA with SDF-2 bound acts as a protein phosphatase for RdeA to remove its phosphate group. This results in the removal the phosphorylation of from the aspartic acid residue 212 on RegA by RdeA, leading to the increase of cAMP levels, activation of PKA, and maturation of spores [6,88,89]. Stalk maturation is also regulated by RegA; however, RegA is activated rather than inhibited in this pathway. Prestalk cells in the anterior region of the slug sense ammonia which activates DhkC resulting in the phosphorylation of RdeA and in turn phosphorylation of RegA. Ammonia is produced from the breakdown of proteins in the cells of the slug and high concentrations of ammonia inhibits culmination [90]. However, ammonia concentration is lowered at the anterior tip of the slug resulting in the inhibition of RegA, activation of PKA, and stalk maturation required for culmination [82]. During culmination the vacuolated stalk cells elevate the spore mass and other prestalk cells differentiating into the lower and upper cup and basal disc that supports the entire structure.

1.8 Chemotactic Signaling

Dictyostelium utilize chemotaxis for both foraging for bacteria and development. While foraging *Dictyostelium* chemotax towards pterin-like molecules such as folic acid produced by bacteria [91]. Folic acid binds to the fAR1 GPCR in *Dictyostelium* which is essential for chemotaxis towards bacteria [92]. The stimulation of *Dictyostelium* results in the coupling of the

Gα4/Gβ/Gγ heterotrimer to the receptor resulting in the activation and dissociation of the subunits and the rapid activation of Erk2 [4,93]. fAR1, Gα4, and Erk2 are also all required for chemotaxis towards bacteria [4,92,93]. cAMP chemotaxis is utilized for the aggregation of cells during early development as it is released in oscillating waves. cAMP binds to the cAR1 GPCR leading to the activation of the Gα2/Gβ/Gγ heterotrimer and activation of Erk2 similar to the early signaling for folic acid [94]. cAMP levels are increased as a result of ligand binding to the receptor in both pathways resulting in the activation of PKA [4]. The Gβγ subunits function to upregulate the activation of phatidylinositol 3-kinase for the phosphorylation of PI(4,5)P2 to produce PI(3,4,5)P3 at the leading edge of the cell to direct actin polymerization [95–97].

1.9 Outline of dissertation

This research set out to investigate the regulation of signaling pathways that regulate early development and chemotaxis of *Dictyostelium* to apply information of its signaling pathways to higher eukaryotes as well as the pathogenic amoeba, *Acanthamoeba castellanii*. While attempting to transform *A. castellanii*, the author identified differences between the chemotactic responses of *A. castellanii* and *D. discoideum* to folate and bacteria. Specifically this included the observation that *A. castellanii* did not chemotaxis towards the chemoattractants as in the same robustness as *D. discoideum.* This observation prompted the research that is described in chapter 2 and was published in *Protist* [98]. The principle investigator conducted primary experiments to investigate the extent of the difference between the chemotactic reaction of the amoebas to potential chemoattractants such as molecules and bacteria. The author performed cell counting and calculated the chemotactic index to quantify chemotaxis for these assays. The genomes between the amoebas were compared by the principle investigator to identify the absence of key G-protein subunits in the current *A. castellanii* genomic sequence that were known to be essential for *D. discoideum* chemotaxis.

The research described in chapter 3 and 4 was performed to investigate the key signaling proteins Erk2 and RegA for early development of *Dictyostelium.* The intent was to investigate simple eukaryotic signaling pathways to aid in the understanding of complex signaling pathways present in other eukaryotes. The signaling interaction between RegA and Erk2 provided an interesting opportunity to study the regulation of a phosphodiesterase by an atypical MAPK in a model organism. The functionality of Erk2 was investigated in the research described in chapter 2. Previous studies involving Erk2 have been conducted using a mutant with significant reduction in *erk2* expression rather than a true disruption of the gene. The principle investigator constructed a disruption of the *erk2* gene through a disruption with the auxotrophic marker gene *thyA* as well as constructed a strain with both MAPK, *erk2* and *erk1,* genes disrupted. The lab group and collaborating authors then investigated the impact of *erk2* disruption on chemotactic response, bacterial engulfment, development, and growth. The author investigated the impact on Erk1 phosphorylation and activation as a response to folate stimulation due to the disruption of *erk2* because of the essential role of Erk2 in folate chemotaxis. This led to the finding that Erk1 phosphorylation as a result to folate stimulation was depended on Erk2. The study also concluded that Erk2 belongs to the group of atypical MAPKs due to sequence similarities to other atypical MAPKs and identified that Erk2 activation was not dependent on the only MAPK Kinase (MAP2K) in *Dictyostelium*. These studies are presented in chapter 3 and were published in *Cellular Signaling* [37].

The research described in chapter 4 set out to investigate the regulation of RegA during early development by phosphorylation. Prior genetic evidence supported the regulation of RegA by Erk2 phosphorylation; however, impacts on development and cellular localization have not been investigated [55]. Preliminary phosphorylation site screening identified potential PKA phosphorylation in the RegA amino acid sequence, an interaction that has been observed in the regulation of mammalian phosphodiesterases [99]. To identify phosphorylation events, the author immunoprecipitated FLAG-RegA from cells stimulated with cAMP during early development

and analyzed the sample with tandem mass spectroscopy. Three putative phosphorylation events were chosen for mutational analysis and both a phosphomimetic and phosphoablative mutation was constructed for each site by the author. These mutations were analyzed by the author for their impact on developmental kinetics, fruiting body morphology, sporulation, and cellular localization during development. These studies are presented in chapter 4 and are to be published.

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CHAPTER II

ACANTHAMOEBA AND *DICTYOSTELIUM* USE DIFFERENT FORAGING STRATIGES †

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Abstract

Amoeba often use cell movement as a mechanism to find food, such as bacteria, in their environment. The chemotactic movement of the soil amoeba *Dictyostelium* to folate or other pterin compounds released by bacteria is a well-documented foraging mechanism. *Acanthamoeba* can also feed on bacteria but relatively little is known about the mechanism(s) by which this amoeba locates bacteria. *Acanthamoeba* movement in the presence of folate or bacteria was analyzed in above agar assays and compared to that observed for *Dictyostelium*. The overall mobility of *Acanthamoeba* was robust like that of *Dictyostelium* but *Acanthamoeba* did not display a chemotactic response to folate. In the presence of bacteria, *Acanthamoeba* only showed a marginal bias in directed movement whereas *Dictyostelium* displayed a strong chemotactic response. A comparison of genomes revealed that *Acanthamoeba* and *Dictyostelium* share some similarities in G protein signaling components but that specific G proteins used in *Dictyostelium* chemotactic responses were not present in current *Acanthamoeba* genome sequence data. The

results of this study suggest that *Acanthamoeba* does not use chemotaxis as the primary mechanism to find bacterial food sources and that the chemotactic responses of *Dictyostelium* to bacteria may have co-evolved with chemotactic responses that facilitate multicellular development.

2.1 Introduction

Amoeboid organisms exist in various environments where they interact with many other microbial organisms. *Dictyostelium* and *Acanthamoeba* are two amoebae of general interest because *Dictyostelium* has been used as a model for eukaryotic cell movement and differentiation and *Acanthamoeba* has been documented as a human pathogen [1–8]. Both amoebae inhabit soil environments and feed on bacteria and other microbes [9–11]. Cell movement is a critical feature to allow these cells to find locations where bacteria exist. Mechanisms of cell movement have been extensively studied in *Dictyostelium discoideum* but relatively little is known about the movement of *Acanthamoeba castellanii* [12,13]. Since both organisms can inhabit similar environments, it is possible that they share similar foraging mechanisms. However, under extreme starvation conditions where food cannot be found the fates of these amoebae are very different. *Dictyostelium* is a social organism that can form multicellular aggregates when starved and these aggregates undergo an elaborate developmental program to produce a fruiting body with spores that can remain dormant in the absence of nutrients [14]. In contrast, starved *Acanthamoeba* form solitary cysts that allow survival in nutrient poor conditions [10,15]. The formation of dormant spores and cysts are developmental fates for these amoebae if the foraging efforts fail and therefore foraging and dormancy are likely to be tightly regulated with respect to each other.

Dictyostelium chemotactic responses have been regarded as an important model for understanding directed cell movement and the underlying signal transduction pathways [2]. Many

Dictyostelium studies have focused on the chemotactic responses to cAMP that occur during the aggregation phase of the developmental life cycle. After several hours of starvation, *Dictyostelium* increase the expression of cAMP surface receptors and become competent for cAMP chemotaxis [16–19]. The stimulation of cAMP receptors triggers a G protein-mediated signaling pathway that results in chemotactic movement [20–22]. The response also includes a release of extracellular cAMP so that cells can find each other during the aggregation process. Signaling through these cAMP receptors continues during the multicellular phases of development and contributes to cell sorting within the aggregate and the differentiation of cells into the stalk or spores of the fruiting body [23–25]. In contrast, chemotactic movement associated with foraging is present during vegetative growth and enhanced during the first few hours of starvation [26–29]. This chemotactic response requires receptors for pterin-like compounds such as folate. Foraging cells exhibit substantial meandering during chemotaxis and do not display the elongated morphology typical of aggregating cells. However, both cAMP and folate chemotaxis responses require G proteins that couple to cell surface receptors and many of the downstream cellular responses are quite similar including the transient accumulations of cAMP and cGMP, cytoplasmic influx of calcium, and the activation of regulatory proteins such as mitogen activated protein kinases (MAPKs) (ERK1 and ERK2) [21,27,30,31].

The *Dictyostelium discoideum* genome encodes more than 60 G protein coupled receptors but relatively few of them have been genetically characterized [32]. Four cAMP receptors have been identified and two of these play a role in the cAMP chemotaxis involved with aggregated formation [16]. Several other receptors have been genetically analyzed, including some close paralogs of the cAMP receptors, and recently a receptor responsible for folate chemotaxis has been identified [33–36]. In regards to G proteins, folate responses require the G4 G protein subunit and cAMP responses require the G2 subunit [27,37,38]. Interestingly, the G4 subunit is also required for cellular localization and morphogenesis during multicellular development [39].

Both folate and cAMP chemotaxis responses require the single Gβ subunit encoded by the genome [40]. A single Gγ subunit that contributes to the heterotrimeric structure has also been identified [41]. These G protein mediated signal transduction pathways for *Dictyostelium* chemotaxis share many similarities to signaling pathways in chemotactic mammalian cells (e.g., neutrophils) suggesting that many chemotactic signaling components have been evolutionarily conserved in eukaryotes [2].

Compared to *Dictyostelium*, very few studies have been conducted on *Acanthamoeba* cell movement. These studies have assayed *Acanthamoeba* movement toward a variety of different compounds and to bacteria [12,13]. The results of these studies suggest that *Acanthamoeba* have variable responses to bacteria and compounds such as cAMP and formylated peptides. These studies did not compare *Acanthamoeba* cell movement to known chemotactic cells such as *Dictyostelium* or mammalian neutrophils. These studies were also conducted before the sequencing of any *Acanthamoeba* genomes and so comparisons of signaling proteins were not considered. The recent sequencing of the *Acanthamoeba castellanii* genome now provides an opportunity to compare genes that are potentially involved with chemotactic signaling in *Acanthamoeba* with those genes that have been characterized in chemotactic organisms [42]. A recent study has reported similarities in the cAMP-specific phosphodiesterase, RegA, found in both *Acanthamoeba* and *Dictyostelium*. RegA regulates the development of spore formation in *Dictyostelium* and the development of cysts in *Acanthamoeba* [43]. Like *Dictyostelium*, the *Acanthamoeba* genome also encodes many putative G protein coupled receptors and G protein subunits genes that could be potential contributors to their ability to find food sources and undergo cell differentiation [42,44].

In this study, we compared the ability of *Acanthamoeba* and *Dictyostelium* to forage for nutrient sources. Chemotactic assays to folate and bacteria were used to determine if these organisms possess similar mechanisms to find nutrients in similar environments. Analyses of

these two organisms under identical conditions suggest that these organisms have evolved different mechanisms to find bacteria. The genomes of these organisms were also compared for G proteins that potentially contribute to these responses. The difference in foraging strategies used by these amoebae is supported by the difference in G protein subunits encoded in the *Acanthamoeba* and *Dictyostelium* genomes.

2.2 Methods

2.2.1 Strains and Growth Conditions

The axenic *Dictyostelium discoideum* strain KAx3 and the *Acanthamoeba castellanii* strain ATCC 30010 were used in this study. Both amoebae were grown in HL5 medium [45]. *Klebsiella aerogenes* was grown on SM+/3 medium and *Escherichia coli* and *Pseudomonas aeruginosa* were grown on L broth [46]. Folate solutions were adjusted to pH 7 using 100 mM NaHCO3.

2.2.2 Chemotaxis assays

Above agar chemotaxis assays were performed as previously described [47]. Cells were grown in fresh HL5 medium 24 hrs prior to harvesting and washing in phosphate buffer (12 mM NaH2PO4 adjusted to pH 6.1 with KOH) and suspended at 1 × 108 cells/ml for *Dictyostelium* or 2 × 107 cells/ml for *Acanthamoeba* (*Acanthamoeba* are approximately 5 times the size of *Dictyostelium*) unless otherwise noted. Droplets (<1 μl) of cell suspensions were spotted on to non-nutrient agar plates (1.5% agar in phosphate buffer) and then 1 μl of chemoattractant was spotted approximately 2 mm from the cell droplet. Images of the cells were recorded immediately after the plating of the cells and chemoattractant and recorded again 2.5 h later. The agar surface near the cell droplet was scarred with a needle to allow the early and late images to be aligned so that the original cell droplet perimeter could be overlaid on the later image. Cell movement toward the chemoattractant source was determined by measuring the distance from the original

cell droplet perimeter to the leading edge of migrating cells. Chemotaxis index (A/B) was defined as the number of cells outside the original cell perimeter that moved toward the chemoattractant (A) divided by the number of cells outside the original cell perimeter that moved away from the chemoattractant (B). Chemotaxis to bacterial cells was performed as that described for folate except 1 µl droplets of bacterial cell suspensions were used as the chemotactic stimulus. Prior to being used in the chemotaxis assays, bacterial cultures were grown overnight in shaking cultures at 22 °C (*K. aerogenes*) or 37 °C (*E. coli* and *P. aeruginosa*). Chemotaxing cells were analyzed using a dissecting microscope (Nikon SMZ2). Videos were created using time-lapse photography with 20 s intervals between images for 33 min. ImageJ with MTrackJ plugin software was used to trace cell migration tracks and determine the migration distance for selected cells. Directionality values were determined using Chemotaxis and Migration Tool Version 1.01 plugin software.

2.2.3 G protein orthologs analysis

G protein sequences were identified using BLASTp searches using default parameters in the non-redundant protein sequences database (NCBI). Amoebae sequences were available primarily due to genome sequencing projects of *Dictyostelium discoideum*, *Acanthamoeba castellanii*, *Dictyostelium purpureum*, *Dictyostelium fasciculatum*, and *Polysphondylium pallidum* [42,48–50]. Initial searches were queried with *Dictyostelium discoideum* protein sequences but queries were also conducted using representative proteins from *Acanthamoeba castellanii*, mammals and yeast. Molecular phylogenetic analysis was conducted in MEGA7 using the Maximum Likelihood method based on the JTT matrix-based model [51,52]. The percentage of replicate trees in which the associated proteins clustered together in the bootstrap test (1000 replicates) are shown next to the branches [53]. Only branches corresponding to partitions reproduced in more than 50% of the bootstrap replicates are labeled. Each tree is drawn to scale, with branched lengths measured in the substitutions per site. Additional BLAST searches were conducted in the AmoebaDB database (AmoebaDB and MicrosporidiaDB:functional

genomic resources for Amoebozoa and Microsporidia species) using the *Acanthamoebae* data sets (Andrew Jackson, Liverpool, UK).

2.3 Results

2.3.1 Comparison of Amoeboid Chemotaxis to Folate

Acanthamoeba and *Dictyostelium* both feed on bacteria as a food source suggesting that *Acanthamoeba* might have chemotactic responses to folate as previously described for *Dictyostelium* [54]. To determine if *Dictyostelium discoideum* and *Acanthamoeba castellanii* have similar chemotactic responses to folate, both species were examined in an above agar chemotaxis assay. Both amoebae displayed robust cell movement as indicated by the maximum migration distance in the presence of folate (Fig. 2.1 A, B). The migration distance for *Dictyostelium* was greatly reduced in the absence of folate but *Acanthamoeba* displayed a similar migration distance in the presence or absence of folate suggesting *Acanthamoeba* cell movement was not dependent on the folate. This idea is further supported by the *Acanthamoeba chemotaxis* index of 1.1 compared to *Dictyostelium* chemotaxis index of 1.6 in the presence of 10μM folate (Fig. 2.1 C).

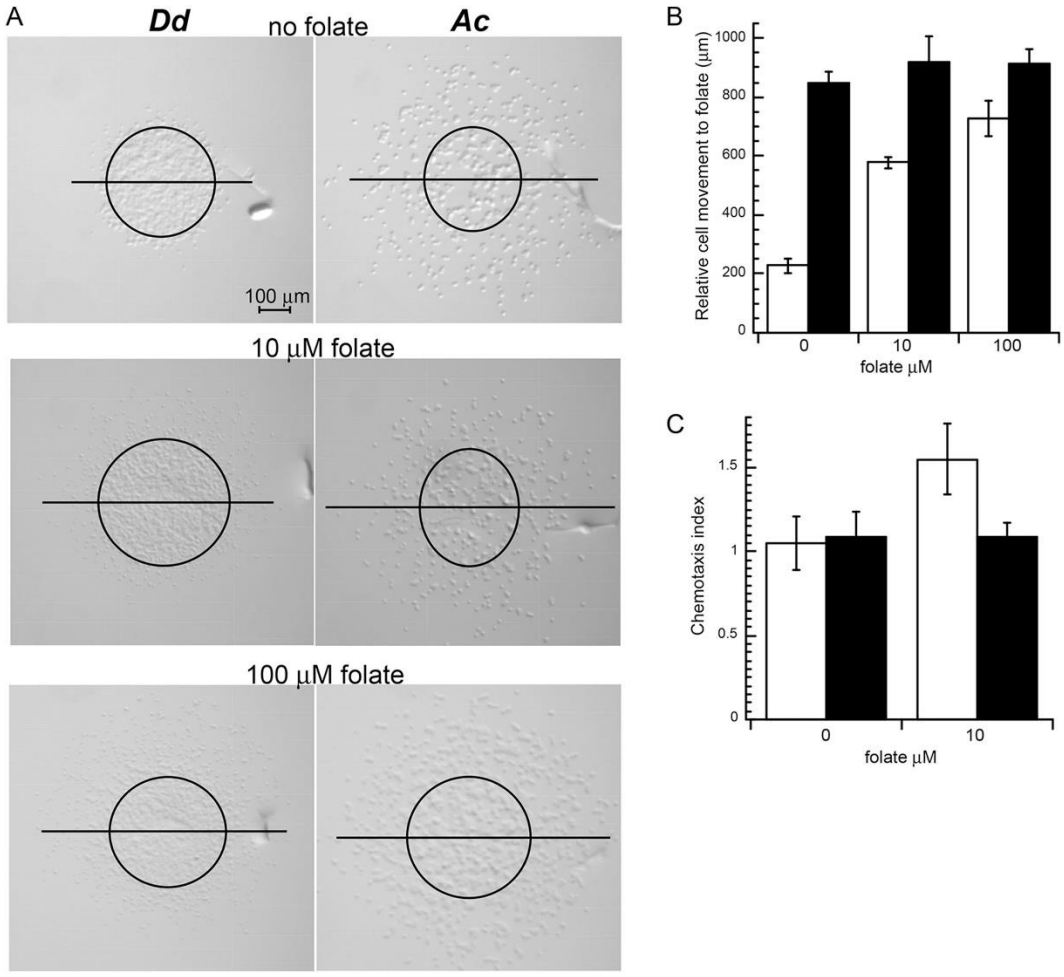

Figure 2.1. Chemotaxis of *Dictyostelium* and *Acanthamoeba* to folate. Chemotaxis assays were set up as described in the methods section. (A) Images of *Dictyostelium* (Dd) and *Acanthamoeba* (Ac) 2.5 h after plating with no folate, 10µM folate, or 100µM folate. Images are orientated with the folate chemoattractant diffusing from the upper side of the image. Representative images are shown. Each chemotaxis assay typically included the analysis of 6 droplets (minimum of 4) and each assay was repeated at least 2 times. Circles on the image represent the approximate cell droplet perimeter at the time of plating and the horizontal lines bisect the upper and lower halves of the circle. In cases where migrating cells moved beyond a single field of view multiple images were collected to account for all migrating cells. (B) Distance traveled of the leading edge of migrating *Dictyostelium* (open bars) and *Acanthamoeba* (black bars) toward the source of folate. Data are the mean distance measured for 6 droplets of cells. For each concentration of folate this chemotaxis assay was repeated 6 times and the data from one representative

assay is shown. Unpaired Student's t-test p values for assays with or without folate were determined (*Dictyostelium* assays p 0.001 and *Acanthamoeba* assays p > 0.03). (C) Chemotaxis index of *Dictyostelium* (open bars) and *Acanthamoeba* (black bars) assayed with folate (10μM) exposure or without (0μM). The chemotaxis index was determined by the number of cells outside the original droplet perimeter on the side facing the source of folate divided by the number of cells outside the perimeter facing away from the source of folate. Data is the mean chemotaxis index from 6 droplets of cells. This assay was repeated at least 3 times and data shown represents a typical assay. Error bars represent the standard deviation of the mean. Unpaired Student's t-test p values for chemotactic index assays with or without folate were determined (*Dictyostelium* p = 0.0006 and *Acanthamoeba* p = 1.0).

A chemotaxis index of one is expected for non-chemotactic (i.e., random migration) cells because the number of cells moving toward ansd away from the chemoattractant source is approximately equal. The greater than one chemotaxis index for *Dictyostelium* is consistent with previous studies that demonstrate this amoeba is chemotactic to folate [27,28,55]. *Dictyostelium* can inactivate folate as a chemoattractant through a deamination reaction and so as folate diffuses beyond the cell droplet a folate gradient can develop on all sides of the cell droplet [56]. This effect can explain the increased *Dictyostelium* movement in all directions as the assay proceeds. In comparison, *Acanthamoeba* moved robustly in all directions regardless of the presence of folate. No significant differences in migration were displayed by *Acanthamoeba* when exposed to different concentrations of folate, even up to 10 mM (data not shown), but *Dictyostelium* movement increased in distance and cell density at higher folate concentrations. *Acanthamoeba* also began moving beyond the original cell droplet perimeter much earlier than *Dictyostelium* consistent with *Acanthamoeba* movement not being dependent of the formation of a folate gradient.

Time-lapse photography of *Acanthamoeba* and *Dictyostelium* was also used to monitor cell movement in the presence of folate. Cell tracking software was used to map the movement of

individual cells over time (Fig. 2.2, Supplementary Material Figs S2.1, S2.2). Cells located on the edge facing the folate source were chosen because they had little or no contact with other cells.

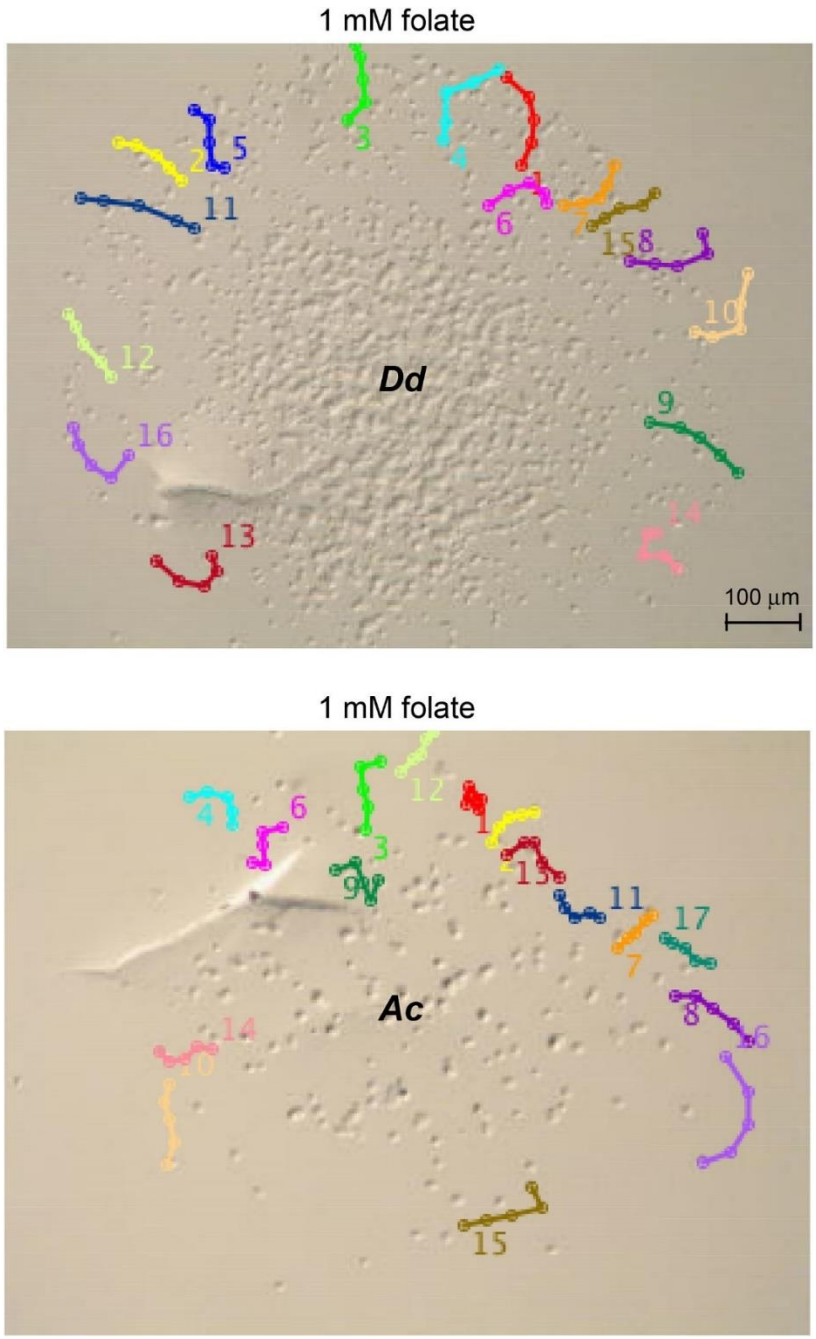

Figure 2.2. Migration maps of *Dictyostelium* and *Acanthamoeba* in folate chemotaxis assays. Time-lapse photography of *Dictyostelium* (Dd upper image) and *Acanthamoeba* (Ac lower image) in response to 1 mM folate (source diffusing from the upper side of each image) after approximately 2 h after plating.

Only the final image is shown but images were collected every 20 s for 33 min as described in the methods section. Tracks of individual cells were traced using 5 time points (approximately every 6 min) using MTrackplugin in ImageJ and overlaid on the final image. The number for each track is located near the tail of each track. Cells near the leading edge and with relatively few cell-cell interactions were chosen for track analysis. Movies containing all time-lapse images are included in the Supplementary Material Figures S1, S2.

The average distance traveled by *Dictyostelium* and *Acanthamoeba* during the 33 min period was comparable (Dd 35.6 ± 5.9 and Ac 29.3 ± 8.9). Many of the *Dictyostelium* and *Acanthamoeba* cells displayed movement with noticeable meandering and both populations contained some cells that did not move. This heterogeneity in cell movement is typical of *Dictyostelium* populations in response to folate [55]. Cell tracking was also used to assess directionality for *Dictyostelium* and *Acanthamoeba*. Migration directionality can be defined as the displacement (direct distance from start point to end point) divided by the total path length of the cell and so cells traveling in a relatively straight line (high persistence) have directionality values near one and cells that meander more have lower values [57]. The average directionality values for *Acanthamoeba* (0.84 ± 0.12) and *Dictyostelium* (0.83 ± 0.14) were very similar suggesting that both amoebae had similar levels of meandering. However, most *Dictyostelium* displayed a bias in cell movement toward the source of folate. In contrast, *Acanthamoeba* movement continued in all directions without being biased by the folate gradient. The *Acanthamoeba* movement was similar to that previously described for *Dictyostelium* in the absence of a chemoattractant and this movement has been defined as “random motion” because cells exhibit a stochastic combination of persistence and meandering [58].

2.3.2 Comparison of Amoeboid Chemotaxis to Bacteria

Although *Acanthamoeba* did not display a chemotactic response to folate it is possible that other molecules released from bacteria might serve as chemoattractants. Therefore

chemotaxis assays were conducted with a variety of bacterial species. Chemotaxis to *Klebsiella aerogenes* and *Escherichia coli* was analyzed because these species are often used in the laboratory setting as a food source for *Dictyostelium*. *Acanthamoeba* was also found to grow efficiently on lawns of these bacteria. Bacteria were harvested and washed with phosphate buffer to remove factors associated with the growth medium. As with the folate chemotaxis assays, *Dictyostelium* displayed a significant chemotaxis index but *Acanthamoeba* did not (Fig. 2.3). However, *Acanthamoeba* exhibited robust cell movement in all directions allowing some of the cells to reach the bacterial droplet. Both *Dictyostelium* and *Acanthamoeba* that reached bacterial droplets were capable of consuming the bacteria (data not shown).

A previous report has suggested that *Acanthamoeba* can chemotaxis to *Pseudomonas aeruginosa* with a chemotactic index of 1.6 in an under agar chemotaxis assay with substantially different parameters than our above agar assay [13]. Our above agar assay with *Acanthamoeba* in the presence of *P. aeruginosa* produced a comparable chemotaxis index of 1.5 (Fig. 2.3). This response was significantly less than the chemotactic response of *Dictyostelium* under the same conditions. Although the chemotaxis response of *Acanthamoeba* to *P. aeruginosa* was marginally statistically significant, the robust movement of *Acanthamoeba* in all directions suggests that any chemotactic contributions to cell movement were secondary to the mechanism underlying the process of cell dispersal. *Dictyostelium* displayed a chemotactic response to *P. aeruginosa* that was similar to its response to other bacteria suggesting that *Dictyostelium* might use a similar mechanism to detect all bacteria.

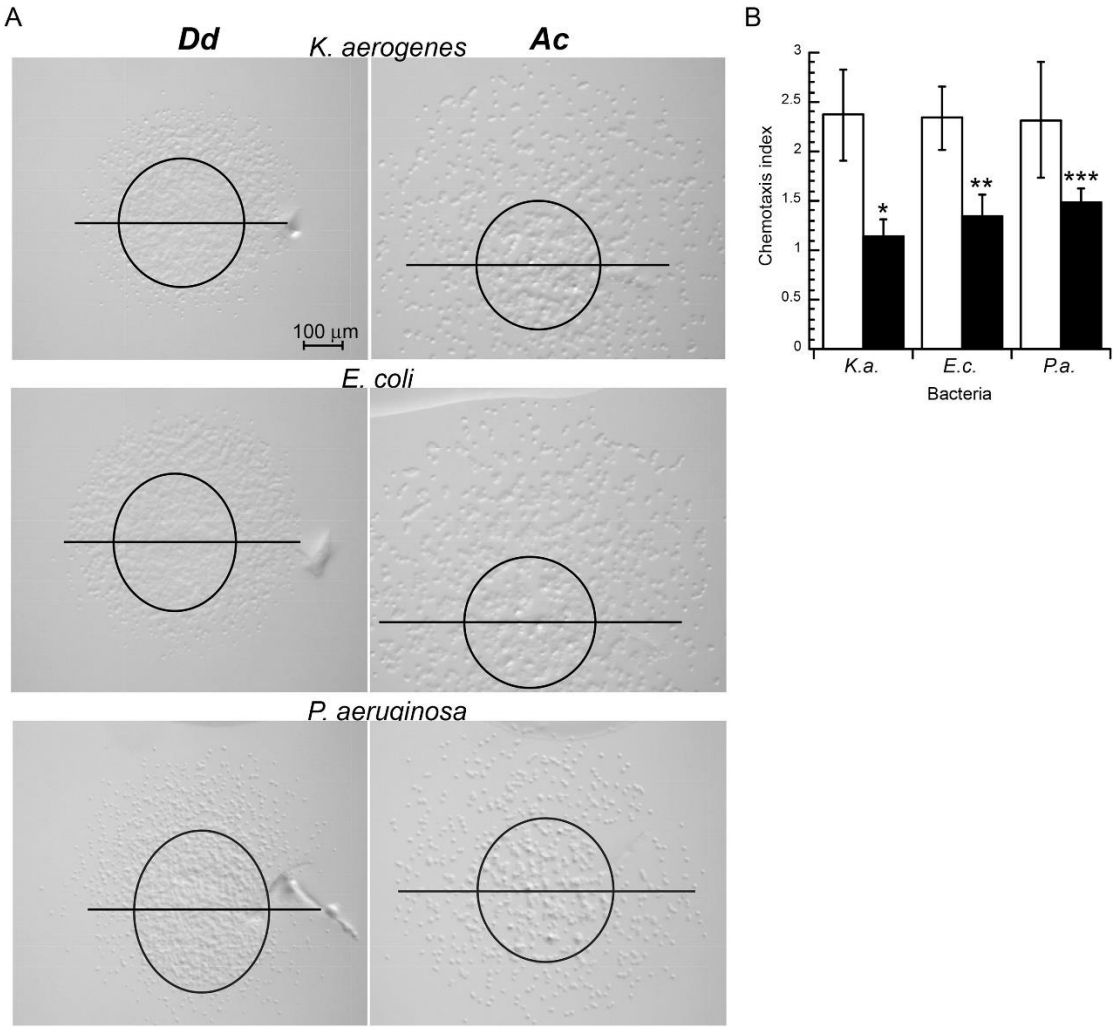

Figure 2.3. Chemotaxis of *Dictyostelium* and *Acanthamoeba* to bacteria. Chemotaxis assays were set up as described in the methods section. (**A**) Images of *Dictyostelium* (*Dd*) and *Acanthamoeba* (*Ac*) 2.5 h after plating with *Klebsiella aerogenes*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Images are orientated with the bacterial source orientated on the upper side of the image. Representative images are shown from an assay that included 6 droplet of either *Dictyostelium* (*Dd*) or *Acanthamoeba* (*Ac*). Each chemotaxis assay that included a minimum of 4 cell droplets was repeated at least 2 times. Original droplet perimeters and bisector lines were determined as described in Figure 2.1. In cases where migrating cells moved beyond a single field of view multiple images were collected to account for all migrating cells. (**B**) Chemotactic index of *Dictyostelium* (open bars) and *Acanthamoeba* (black bars) to bacterial droplets. Data is the mean chemotaxis index from 6 droplets of cells. This assay was repeated at least 3 times and data

shown represents a typical assay. Error bars represent the standard deviation of the mean. Unpaired Student's *t*-test *p* values for chemotactic index assays with or without bacteria was determined (all *Dictyostelium* assays $p < 0.001$ and *Acanthamoeba* assays $*p = 0.6$, $**p = 0.05$, $***p = 0.04$).

Time-lapse recordings of amoebae migration to bacteria (*K. aerogenes*) were very comparable to those observed for the amoeboid movement to folate (Fig. 2.4, Supplementary Material Figs S3, S4). Both amoebae displayed meandering movement and the average migration distances during the 33 min period were similar (*Dd* 31.1 + 7.1 and Ac 28.8 + 4.2, relative pixel units). The average directionality values for *Dictyostelium* (0.90 ± 0.10) and *Acanthamoeba* (0.92 ± 0.12) were also comparable to each other and similar to those observed in response to folate. In many cases, the *Dictyostelium* and *Acanthamoeba* movement significantly slowed when the cells reached the bacterial mound but some *Acanthamoeba* were capable of tunneling further into the bacterial mound (Supplementary Material Fig. S2.4). This tunneling behavior suggests that some cells might not immediately switch from foraging to feeding when making contact with bacteria. However, *Acanthamoeba* were never observed leaving the bacterial mound suggesting the foraging movement did not continue indefinitely in while in contact with the bacteria. The larger cell size of *Acanthamoeba* compared to *Dictyostelium* might possibly contribute to the ability of individual cells to physically penetrate further into the bacterial mound.

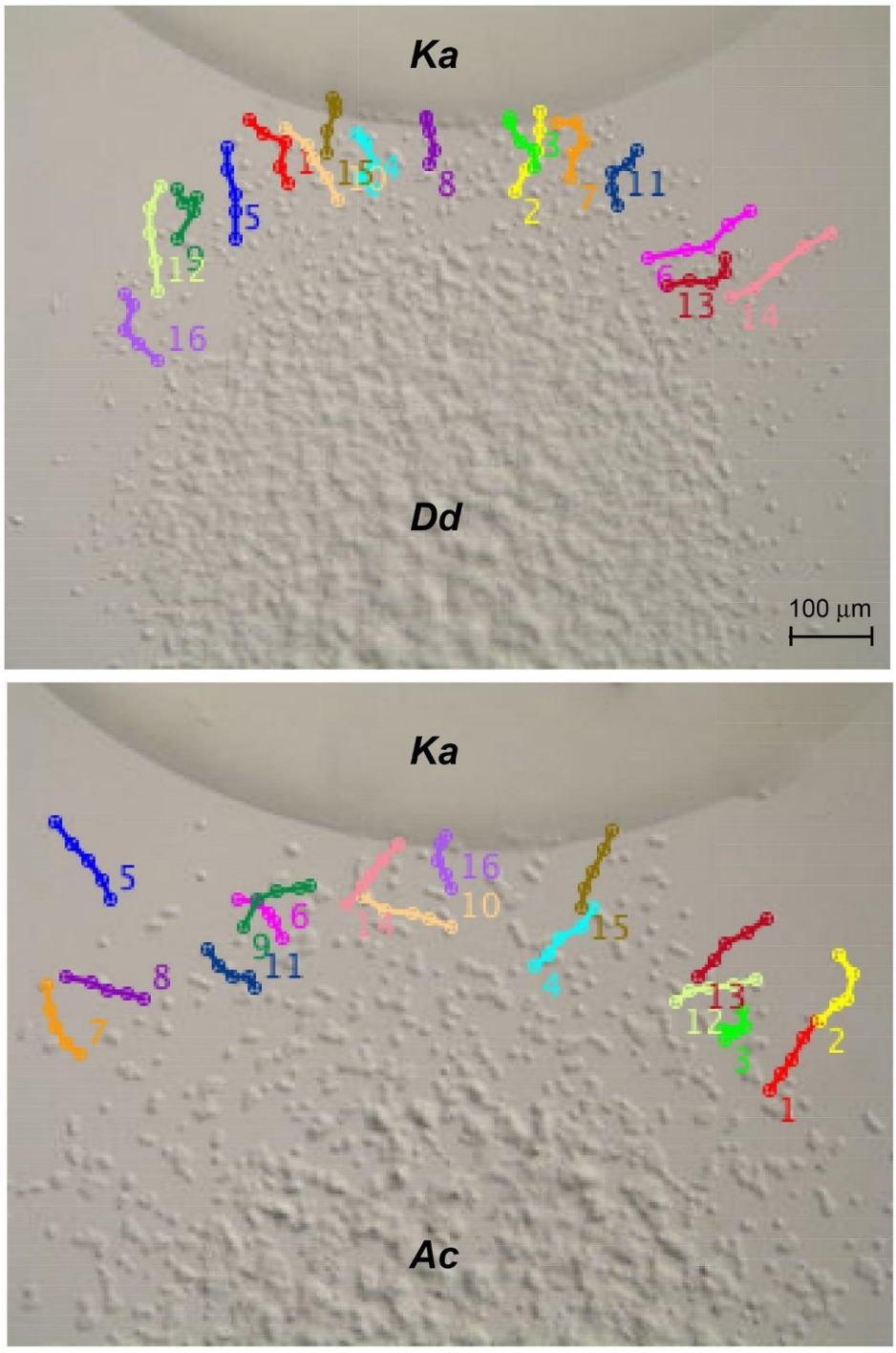

Figure 2.4. Migration maps of *Dictyostelium* and *Acanthamoeba* in chemotaxis to *Klebsiella aerogenes*. Time-lapse photography of *Dictyostelium* (*Dd* upper image) and *Acanthamoeba* (*Ac* lower image) in response to *K. aerogenes* droplets (positioned on the upper side of each image) after approximately 2 h after plating. Only the final image is shown but images were collected and tracks were traced as

described Figure 2.2. The number for each track is located near the tail of each track. Cells near the leading edge and with relatively few cell-cell interactions were chosen for track analysis. Movies containing all time-lapse images are included in the Supplementary Material Figures S3, S4.

2.3.3 Acanthamoeba Dispersal

The dispersal of *Acanthamoeba* might possibly be driven through a chemorepulsion mechanism that could potentially mask a response to a chemoattractant at high cell densities. Chemorepulsion has been observed for other amoebae including *Dictyostelium* and *Entamoeba* in response to molecules produced during vegetative growth. Although growth medium was washed away prior to the chemotaxis assays, the amoeba might still be capable of releasing chemorepellent molecules. To examine if chemorepulsion contributes to the movement of *Acanthamoeba*, chemotaxis assays were repeated using different densities of *Acanthamoeba* because chemorepulsion is expected to correlate with increased amoeba density. High (108 cells/ml) and low (5 × 106 cells/ml) density *Acanthamoeba* suspensions were used in chemotaxis assays to *K. aerogenes* (Fig. 2.5A). Chemotaxis indices were not determined for *Acanthamoeba* at the high cell density because of the difficulty of counting individual cells but at the lower cell density *Acanthamoeba* had a chemotaxis index of 1.2 ± 0.4, comparable to the higher cell density assays described in Figure 2.3.

Chemorepulsion, particularly at high cell densities, might be expected to disperse cells in a uniform radial pattern due the decreasing level of autocrine factors in all directions away from the cell droplet and also the potential repulsion between individual cells. However, *Acanthamoeba* displayed a disorderly pattern of dispersal (i.e., uneven distribution of migrating cells) at both high and low densities suggesting that dispersal is the result of random motion rather than chemorepulsion. Furthermore, *Acanthamoeba* at relatively low cell densities near droplets of bacteria were also capable of moving toward or away from the bacteria (Fig. 2.5 B, S). This observation suggests that random motion can occur in close proximity of bacterial cells

where potential chemoattractant and chemorepellent concentrations are expected to be relatively high and low, respectively. These results suggest that *Acanthamoeba* movement is not primarily determined by chemotaxis or chemorepulsion.

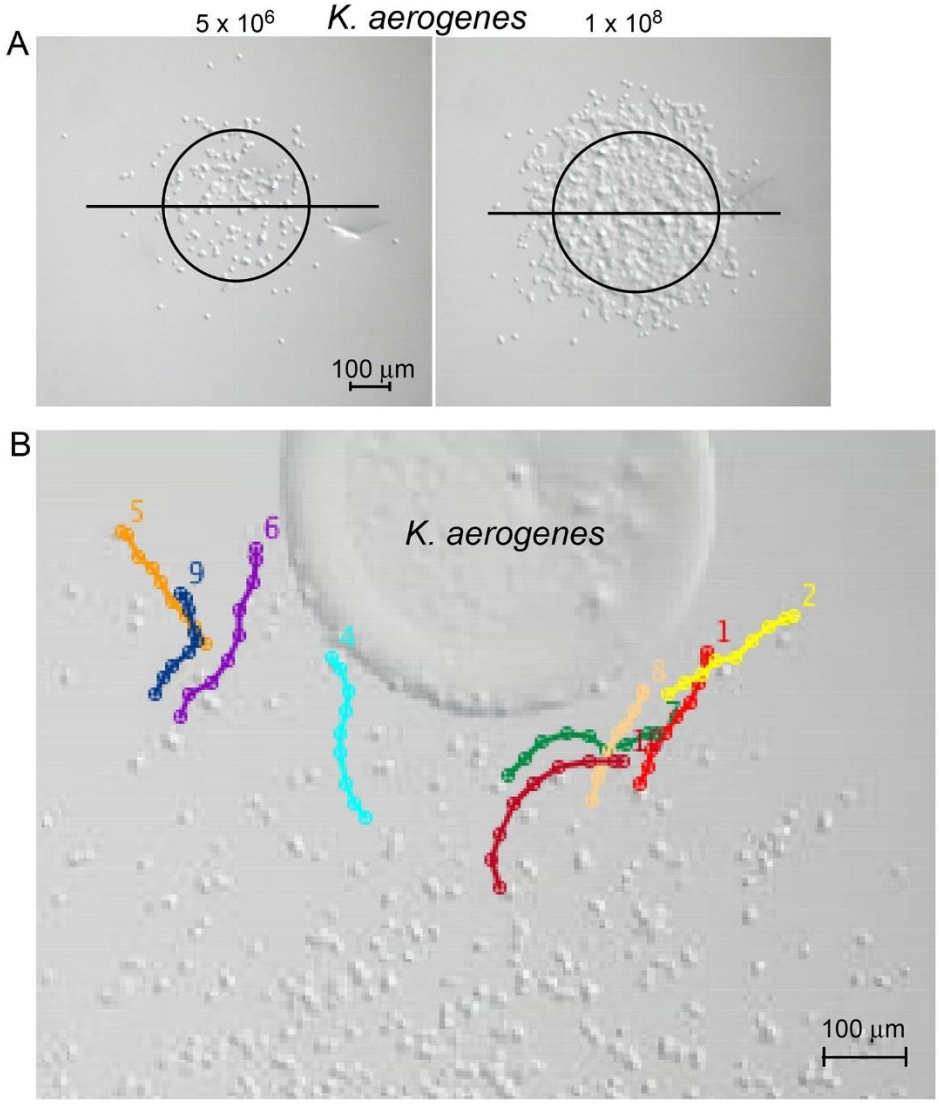

Figure 2.5. *Acanthamoeba* movement at different cell densities in the presence of bacteria. Chemotaxis assays were set up as described in the methods section. (**A**) Images of *Acanthamoeba* (*Ac*) movement 2 h after plating at different densities (5×10^6 or 1×10^8cells/ml suspensions) on agar plates near droplets of *Klebsiella aerogenes* (positioned on the upper side of each image). Representative images are shown from an assay that included 5 droplets of *Acanthamoeba* (*Ac*) for each cell density and each assay was

repeated 3 times. Original droplet perimeters and bisector lines were determined as described in Figure 1. (**B**) Time-lapse photography of *Acanthamoeba* in response to *K. aerogenes* droplet (positioned on the upper side of each image) after approximately 4.5 h after plating. Only the final image is shown but images were collected and tracks were traced as described Figure 2. The number for each track is located near the head of each track. Cells moving near but not directly toward the bacterial droplet were chosen for track analysis. Movies containing all time-lapse images are included in the Supplementary Material Figure S5.

2.3.4 Comparison of G Protein Gα Subunits

The chemotaxis assays to folate and bacterial sources suggest that the foraging strategy of *Acanthamoeba* is different than that of *Dictyostelium*. The basis of this difference could possibly be differences in the signaling components encoded by each organism's genome. Chemotaxis and other chemoresponsive processes (e.g., chemotrophic growth) in eukaryotes are typically associated with G protein-mediated signaling pathways [2]. Given that the *Dictyostelium discoideum* Gα4 subunit is required for chemotactic responses to folate and bacteria, the sequence of this subunit was used to search for homologous proteins in *Acanthamoeba* and other amoebae. *Dictyostelium discoideum* and related dictyostelid species (*Dictyostelium purpureum*, *Polysphondylium pallidum*, *Dictyostelium fasciculatum*, and *Actyostelium subglobosum*) all possessed a single closely related Gα4 ortholog with sequence identity greater than 90% (Table 2.1).

Table 2.1. Percent identity of amoeboid Gα subunits to the *Dd* Gα4 subunit.

Organism	Gα subunit	% identity
Dictyostelium purpureum	Gα4	99
Polysphondylium pallidum	Gα4	94
Dictyostelium fasciculatum	Gα4	91
Acytostelium subglobosum	Gα4	93
Acanthamoeba castellanii	Gα5	54
Polysphondylium pallidum	Gα5	53
Dictyostelium discoideum	Gα5	52
Dictyostelium discoideum	Gα2	44

A previous report has indicated that *Dictyostelium purpureum* and *Polysphondylium pallidum* have chemotactic responses to folate and while the other species have not been tested it is likely they also respond to folate given the conservation of the Gα4 subunit [28]. The most closely related *Acanthamoeba* Gα subunit to the *Dictyostelium* Gα4 subunit was a Gα5 ortholog but this subunit has a much lower sequence identity (54%) than the Gα4 orthologs in other amoebae. The *Dictyostelium discoideum* Gα5 and Gα2 subunits also have a much lower sequence identity (52% and 44%, respectively) with respect to the Gα4 subunit and this is indicative of their functional differences. The Gα2 subunit, like the Gα4 subunit, mediates a chemotactic response but this response is to cAMP. A phylogenetic analysis of the 12 *Dictyostelium* and 5 *Acanthamoeba* Gα subunits indicated that other than a similarity between Gα5 orthologs (60% identity), these amoebae do not share closely related Gα subunits (Fig. 2.6). Out of the other 4 *Acanthamoeba* Gα subunits, 3 of them shared greatest sequence similarity with each other rather than *Dictyostelium* Gα subunits. The remaining *Acanthamoeba* Gα subunit, Gα3, had sequence similarity with the *Dictyostelium* Gα7 subunit. As previously reported, most *Dictyostelium* Gα

subunits, except for Gα1-Gα2 pair and the Gα4- Gα5 pair share little sequence similarity with each other outside of the highly conserved sequences associated with guanine nucleotide binding. Even the most closely related pairs of *Dictyostelium* Gα subunits, Gα4-Gα5 and Gα1-Gα2, do not show functional redundancy in chemotactic or developmental phenotypes [21,59].

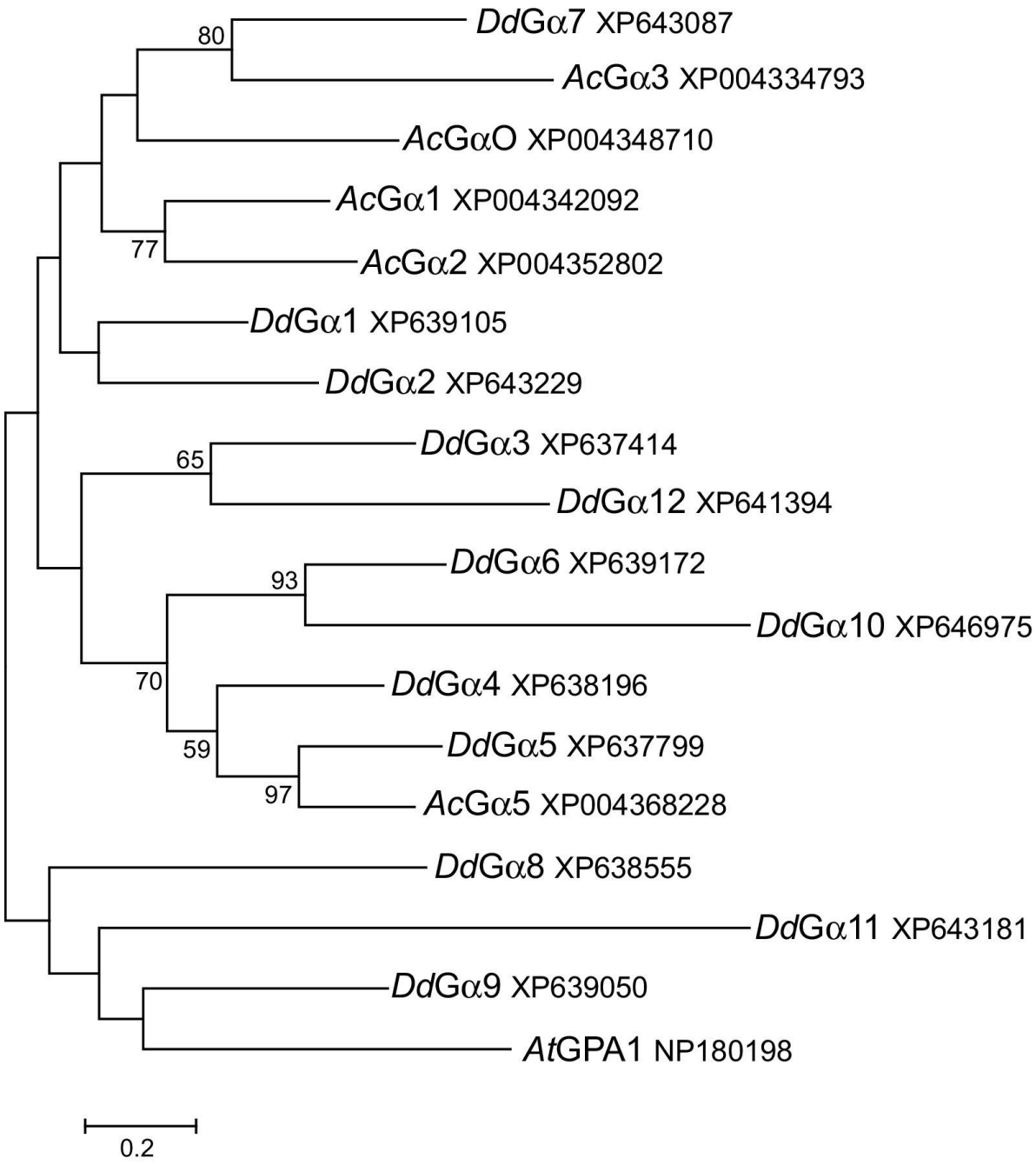

Figure 2.6 Phylogenetic tree of *Dictyostelium* and *Acanthamoeba* G protein Gα subunits. BLAST searches were used to identify Gα subunit homologs in *Dictyostelium discoideum* (*Dd*) and *Acanthamoeba castenalli* (*Ac*) genomes. A phylogenetic tree was generated as described in the methods section. The *Arabidopsis thaliana* Gα subunit (*At*GPA1) was used as an out-group. Each sequence has an accession reference number.

2.3.5 Comparison of Other G Protein Subunits

In *Dictyostelium*, the sole Gβ subunit is required for chemotaxis to both cAMP and folate indicating that both Gα2 and Gα4 complex with the Gβ in these chemotactic responses. The *Dictyostelium* Gβ subunit sequence was used to search for orthologs in *Acanthamoeba* but the search did not reveal any closely related proteins as suggested by the low range of sequence identities (20–29%). This contrasts *Dictyostelium* Gβ searches in other eukaryotes such as humans where Gβ orthologs were identified with much higher sequence identities (60–70%). Several of the *Acanthamoeba* proteins detected in the sequence similarity searches were identified as Gβ-like proteins because they contain WD-repeats like those found in Gβ subunits. One of these *Acanthamoeba* WD-repeat proteins has significant similarity to proteins commonly known as Rack homologs (Receptor associated with protein C kinase) that exist in a diverse range of eukaryotes. The *Acanthamoeba* and *Dictyostelium* Rack proteins share 59% identity but none of the other *Acanthamoeba* WD-repeat proteins shared more than 34% sequence identity with the *Dictyostelium* RACK protein. Phylogenetic analysis of Gβ related proteins from *Dictyostelium*, *Acanthamoeba*, and some other eukaryotes indicated that Gβ subunits and Rack proteins form distinct clades suggesting these WD repeat proteins represent two different classes of proteins (Fig. 2.7 A). The *Acanthamoeba* Rack protein shares comparable relatedness with higher eukaryotes as it does with other amoeboid species.

G proteins, which couple to cell surface receptors, are typically heterotrimeric proteins that contain a Gγ subunit. The Gγ subunit binds tightly to Gβ subunit and remains tightly

associated with this subunit upon activation of the Gα subunit. A *Dictyostelium* Gγ subunit has been identified and shown to couple with the Gβ subunit but its requirement in chemotactic movement has not been established [41]. The *Dictyostelium discoideum* Gγ subunit was used to search for related proteins in *Acanthamoeba* but the search did not identify any closely related proteins. Attempts with human or yeast Gγ to find related proteins in *Acanthamoeba* were also unsuccessful but the low sequence conservation and the small protein size make the searches for Gγ subunit homologs more challenging than homologs to other G protein subunits in distantly related organisms. However, Gγ subunits from other dictyostelids were detected. A phylogenetic analysis of Gγ sequences indicates that the dictyostelids species have closely related Gγ subunits compared to those found in other unrelated organisms (Fig. 2.7 B). The phylogenetic tree suggests higher sequence similarities between Gγ subunits within a given phylum.

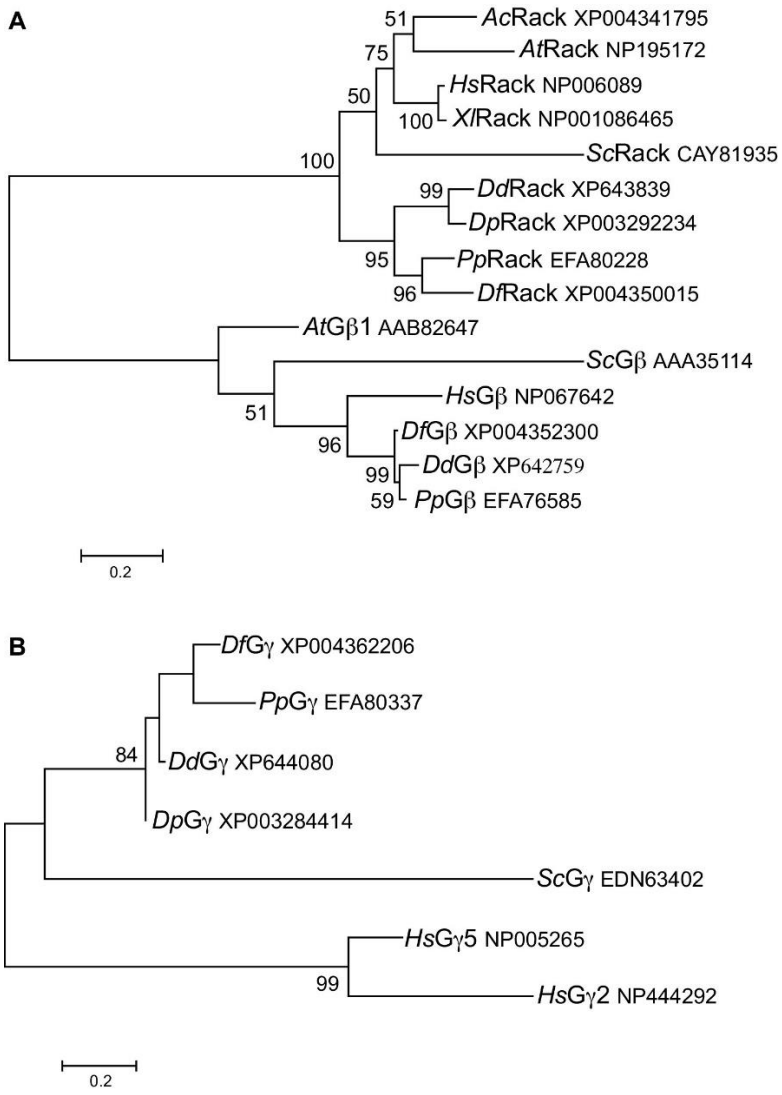

Figure 2.7 Phylogenetic trees of G protein Gβ subunits/Racks and Gγ subunits of some amoebozoan and other select eukaryotes. (A) Phylogenetic tree of Gβ and Rack subunit homologs identified through BLAST searches. Only one Gβ subunit of the multiple Gβ subunits from the human (*Hs*) genome was selected for comparison purposes. **(B)** Phylogenetic tree of Gγ subunit homologs identified through BLAST searches. Phylogenetic trees were generated as described in the methods section. Species represented include *Dictyostelium discoideum* (*Dd*), *Acanthamoeba castenalli* (*Ac*), *Homo sapien* (*Hs*), *Xenopus laevis* (*Xl*), *Arabidopsis thaliana* (*At*), *Dictyostelium purpureum* (*Dp*), *Polysphondylium pallidum* (*Pp*), *Dictyostelium fasciculatum* (*Df*), and *Saccharomyces cerevisiae* (*Sc*) genomes. Each sequence has an accession reference number.

2.4 Discussion

The results of this study suggest that *Acanthamoeba* forage for bacterial food sources using a mechanism distinct from *Dictyostelium*. *Dictyostelium* displayed directed movement to both folate and bacterial sources even though the migration paths included substantial meandering. *Acanthamoeba* displayed a slight bias in the movement toward some bacterial sources but the robust movement in all directions overshadowed any directed movement to the bacterial source suggesting that random motion rather than chemotaxis represents the primary foraging mechanism of *Acanthamoeba* (Fig. 2.8). Cell dispersal through random motion is likely to increase the chance that some members of an *Acanthamoeba* population find new food sources because the population can cover a larger area compared to a population directed by chemotaxis. Cell dispersal through random motion has also been described for *Dictyostelium* in the absence of a chemotactic signal [60].

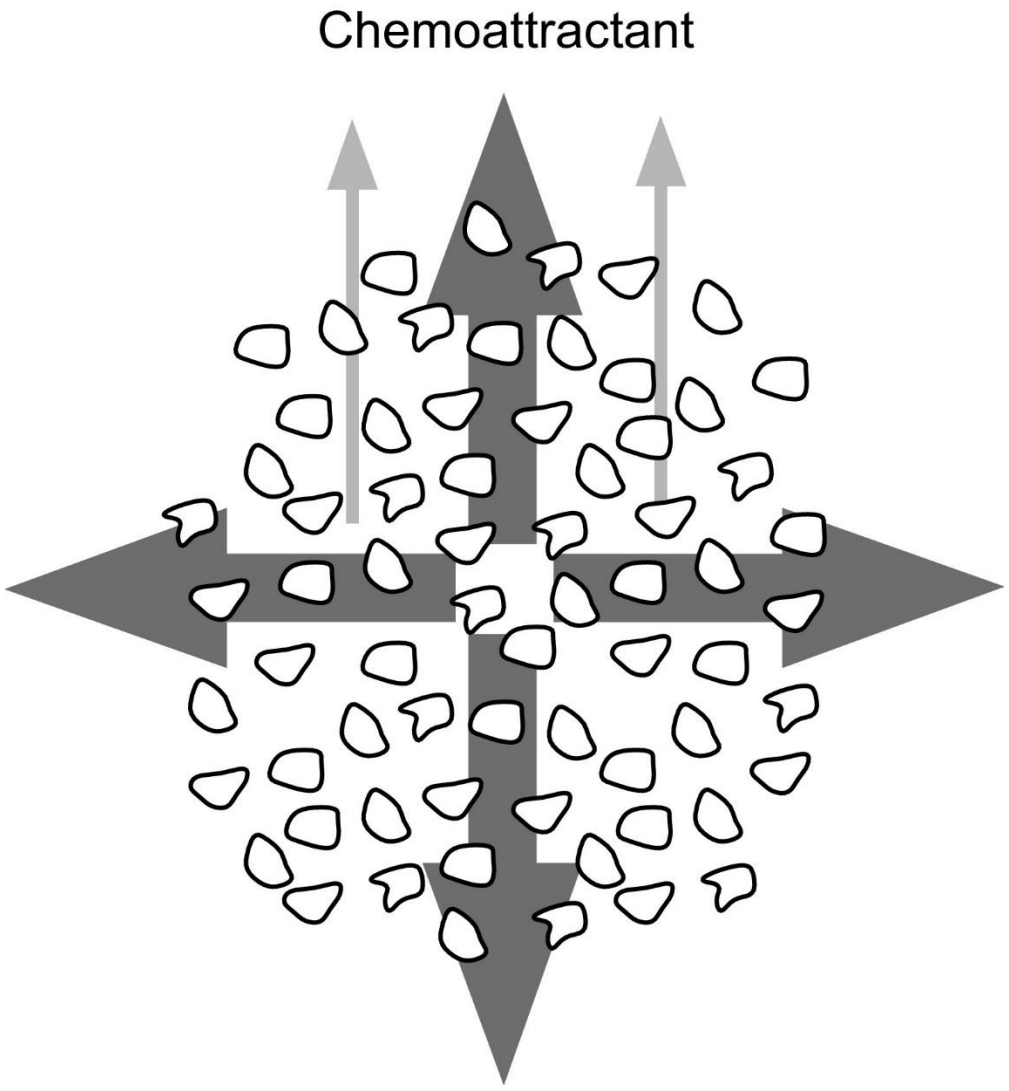

Figure 2.8. Model of *Acanthamoeba* movement. Nutrient deprivation primarily results in random motion allowing cells to disperse in all directions (thick dark gray arrows). Movement toward a chemoattractant (thin light gray arrows) is very weak compared to other amoebae such as *Dictyostelium*.

The efficient dispersal of *Acanthamoeba* at even low cell densities suggests that chemorepulsion is not an important contributor for *Acanthamoeba* dispersal in the conditions tested in this study but such a mechanism could possibly operate in populations of vegetatively growing cells. Under growing conditions *Dictyostelium* use an autocrine signal, AprA, as a chemorepellent and other amoebae, such as Entamoeba, use ethanol production as a

chemorepellent [61,62]. While the dispersal of cells reduces the competition for food in a localized area, it might also have important consequences with respect to cell fate. *Acanthamoeba* that are unsuccessful in foraging enter into a dormant state (encystation) as solitary cells, allowing survival until local food conditions change. In contrast, *Dictyostelium* enter into a multicellular state to form dormant cells (spores) if foraging fails and so mechanisms that promote cell dispersal would likely work in opposition to the cell aggregation process. Therefore, social amoebae such as *Dictyostelium* might limit cell dispersal mechanisms to allow for more efficient cell aggregation.

The lack of *Acanthamoeba* chemotaxis to folate is consistent with the absence of a Gα4 subunit ortholog in the genome given that this subunit is highly conserved among species that can chemotax to folate. While most of the reported folate-responsive amoebae belong to the Dictyostelid classification, a recent study indicates that *Vahlkampfia*, classified in a different subphylum, can also chemotax to folate [63,64]. It remains to be determined if the *Vahlkampfia* genome contains a Gα4 subunit homolog. Many amoebae, including *Acanthamoeba*, possess a Gα5 subunit homolog that is closely related to Gα4 subunits but phenotypic characterization of the *Dictyostelium gα5*$^{-}$ mutants indicates that this subunit is not required for folate chemotaxis [59]. Rather, the Gα5 subunit appears to act in opposition to the Gα4 subunit as suggested by the increased folate responsiveness of cells that lack the Gα5 subunit and the decreased folate responsiveness of cells that overexpress the Gα5 subunit [65]. The functional relationship of the Gα4 and Gα5 subunits is not fully understood but Gα subunit chimeric studies suggest that the functional differences are not solely due to receptor coupling but rather downstream signaling [66].

The absence of a Gβ subunit gene in the current *Acanthamoeba castellanii* genomic data is very surprising given that several putative Gα subunits are present. Biochemical and genetic characterization of Gβ subunits in other organisms suggest the Gβ subunits can have a variety of

interactions and roles in downstream signaling and in some cases they provide the primary role in signaling to downstream responses [67,68]. Not detecting a Gβ subunit gene could possibly result from incomplete coverage of the genome by the sequencing analysis. The *Acanthamoeba castellanii* genome project did not report the depth of sequencing coverage but did indicate 94% coverage of the transcriptome [42]. Searches for Gβ subunits were also conducted using recently deposited *Acanthamoeba* sequences in the AmoebaDB database (AmoebaDB and MicrosporidiaDB:functional genomic resources for Amoebozoa and Microsporidia species). In several *Acanthamoeba* species, including *Acanthamoeba castellanii*, these searches revealed partial gene sequences with 50–60% identity to known Gβ subunits suggesting that Gβ homologs are present in the *Acanthamoeba* genus. It is also possible that other WD-repeat proteins might provide Gβ subunit function. The presence of the Gβ-like Rack protein in *Acanthamoeba* offers the possibility that this protein could function in G protein signaling pathways. Studies in the yeast *S. cerevisiae* indicate that the Gpa2 Gα subunit does not couple with the single Gβ or Gγ subunit in this organism [69]. Instead the yeast Rack homolog, Asc1, has been reported to serve in place of a Gβ subunit for the Gpa2 signaling pathway [70]. The Gpa2/Asc1 G protein pathway mediates the sensing of glucose to control cell growth and division in yeast but thus far this pathway has not been implicated in cell polarity or chemotrophic growth, unlike the Gpa1/Gβ pathway [71]. While Rack proteins may mediate G protein signaling it is possible that these proteins might not be capable of contributing to chemotactic or chemotrophic responses like Gβ subunits. The Rack ortholog in *Dictyostelium* cannot compensate for the chemotactic deficiencies that occur in the absence of the Gβ subunit but that does not exclude the possibility that the Rack protein might function in signaling pathways that do not require Gβ proteins [72].

While *Acanthamoeba* and *Dictyostelium* might exist in similar environments and consume common bacteria, these amoebae use very different approaches to finding their food sources based on their cell movement. It is interesting that both amoebae share many families of

proteins that participate in G protein-mediated signal transductions but yet very little overlap exists with specific signaling proteins such as specific Gα subunits. Perhaps some of the differences in signaling proteins are the result of social or solitary strategies for surviving starvation. Chemotactic responses of *Dictyostelium* to folate and bacteria might have co-evolved with chemotactic responses to cAMP because many similarities exist between these responses. These similarities include the activation of MAPKs and other kinases and transient changes in the level of cyclic nucleotides that are important for regulating cell morphology and gene expression [21,31,73]. The use of similar signaling proteins downstream of G protein function for both foraging and cell aggregation could save time and critical energy reserves in the switch between foraging and the cell aggregation process. In addition, the relatively close proximity of cells with each other during chemotactic foraging, a process somewhat analogous to herding, can also help expedite the aggregation process if needed. A potential drawback to "herding" is that it increases the competition between cells for food sources that might be found. However, if food is not found then the close proximity of cells is beneficial because it reduces the distance cells need to migrate to form a multicellular aggregate. In contrast to social amoeba, *Acanthamoeba* does not require cell aggregation to form cysts and so an investment in chemotactic signaling mechanisms might not be warranted. These differences in developmental fates among social and non-social amoebae might provide some of the basis for the different foraging strategies.

2.5 Supplemental Materials:

https://ars.els-cdn.com/content/image/1-s2.0-S1434461016300487-mmc1.mp4

Figure S2.1 Movie of *Dictyostelium* movement in the presence of folate. Time-lapse recording of *Dictyostelium* movement over 33 min period with images collected every 20 s. The folate source is oriented at the upper side of the image.

https://ars.els-cdn.com/content/image/1-s2.0-S1434461016300487-mmc2.mp4

Figure S2.2. Movie of *Acanthamoeba* movement in the presence of folate. Time-lapse recording of *Acanthamoeba* movement over 33 min period with images collected every 20 s. The folate source is oriented at the upper side of the image.

https://ars.els-cdn.com/content/image/1-s2.0-S1434461016300487-mmc3.mp4

Figure S2.3. Movie of *Dictyostelium* movement in the presence of *K. aerogenes*. Time-lapse recording of *Dictyostelium* movement over 33 min period with images collected every 20 s. The bacterial source is oriented at the upper side of the image.

https://ars.els-cdn.com/content/image/1-s2.0-S1434461016300487-mmc4.mp4

Figure S2.4. Movie of *Acanthamoeba* movement in the presence of *K. aerogenes*. Time-lapse recording of *Acanthamoeba* movement over 33 min period with images collected every 20 s. The bacterial source is oriented at the upper side of the image.

https://ars.els-cdn.com/content/image/1-s2.0-S1434461016300487-mmc5.mp4

Figure S2.5. Movie of *Acanthamoeba* movement near a droplet of *K. aerogenes*. Time-lapse recording of *Acanthamoeba* movement over 33 min period with images collected every 20 s. The bacterial source is oriented at the upper side of the image and the original droplet of *Acanthamoeba* was positioned at the lower side of the image.

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CHAPTER III

DICTYOSTELIUM ERK2 IS AN ATYPICAL MAPK REQUIRED FOR CHEMOTAXIS †

†This chapter is reproduced with slight modifications from the following publication:

Schwebs, D.J., Pan, M., Adhikari, N., Kuburich, N. A., Jin, T., & Hadwiger, J. A. (2018). *Dictyostelium* Erk2 is an atypical MAPK required for chemotaxis. *Cellular signalling, 46,* 154-165. Reprinted with permission.

Abstract

The *Dictyostelium* genome encodes only two MAPKs, Erk1 and Erk2, and both are expressed during growth and development. Reduced levels of Erk2 expression have been shown previously to restrict cAMP production during development but still allow for chemotactic movement. In this study the *erk2* gene was disrupted to eliminate Erk2 function. The absence of Erk2 resulted in a complete loss of folate and cAMP chemotaxis suggesting that this MAPK plays an integral role in the signaling mechanisms involved with this cellular response. However, folate stimulation of early chemotactic responses, such as Ras and PI3K activation and rapid actin filament formation, were not affected by the loss of Erk2 function. The *erk2*$^{-}$ cells had a severe defect in growth on bacterial lawns but assays of bacterial cell engulfment displayed only subtle changes in the rate of bacterial engulfment. Only cells with

no MAPK function, *erk1*$^{-}$*erk2*$^{-}$ double mutants, displayed a severe proliferation defect in axenic medium. Loss of Erk2 impaired the phosphorylation of Erk1 in secondary responses to folate stimulation indicating that Erk2 has a role in the regulation of Erk1 activation during chemotaxis. Loss of the only known *Dictyostelium* MAPK kinase, MekA, prevented the phosphorylation of Erk1 but not Erk2 in response to folate and cAMP confirming that Erk2 is not regulated by a conventional MAP2K. This lack of MAP2K phosphorylation of Erk2 and the sequence similarity of Erk2 to mammalian MAPK15 (Erk8) suggest that the *Dictyostelium* Erk2 belongs to a group of atypical MAPKs. MAPK activation has been observed in chemotactic responses in a wide range of organisms but this study demonstrates an essential role for MAPK function in chemotactic movement. This study also confirms that MAPKs provide critical contributions to cell proliferation.

3.1 Introduction

Mitogen activated protein kinases (MAPKs) are components of many eukaryotic signal transduction pathways [1–4]. These proteins generally function downstream of protein kinase cascades that include MAPK kinases (MAP2Ks) and MAPK kinase kinases (MAP3Ks). Once activated, MAPKs phosphorylateand regulate a wide variety of proteins throughout the cell. Mitogens, chemoattractants, and other extracellular signals can stimulate MAPK pathways and lead to changes in cell growth, movement, gene expression, and differentiation [3,5]. While the interactions and functions of some MAPKs have been well documented, many members of this regulatory protein family remain uncharacterized [6]. Sequence similarities and functional roles have provided the basis to organize the family of mammalian MAPKs into subfamilies such as the ERKs (extracellular signal regulated kinases), p38 MAPKs, pJNKs (c-Jun N-terminal kinases), and other smaller groups but not all of these groups are present in other eukaryotes [5]. Some MAPKs are known to have redundant functions (e.g., mammalian Erk1/Erk2) and others can have common activators and substrates but promote different cellular

responses (e.g., yeast Fus3 and Kss1 in regulating mating responses and filamentous growth, respectively) [4,7,8]. The signaling pathways that use MAPKs can be quite varied but the activation mechanism of most characterized MAPKs includes a dual phosphorylation of residues in a highly conserved motif (TXY) within a catalytic domain [5]. This activation is typically mediated by MAPK kinases (MAP2Ks; also known as Meks) that are capable of phosphorylating both serine/threonineand tyrosine residues [9]. However, a group of atypical MAPKs does not appear to be phosphorylated by conventional MAP2Ks [5,10,11]. MAPKs have been found in all eukaryotic kingdoms and appear to be present in all free-living eukaryotes suggesting these proteins regulate basic cellular processes in eukaryotes [12,13].

Simple eukaryotic organisms have been particularly useful for the characterization of MAPK function and specificity. These eukaryotes are typically amenable to both genetic and biochemical analysis and they tend to have relatively few MAPKs. The yeast *Saccharomyces cerevisiae* has 5 MAPKs and the soil amoeba *Dictyostelium discoideum* has only 2 MAPKs compared to the 13 MAPKs found in mammals [3,4]. MAPKs in yeast have been associated with responses to mating pheromones, starvation, osmotic stress, and cell wall stress [4]. The two MAPKs in *Dictyostelium*, designated as Erk1 and Erk2 (also referred to as ErkA and ErkB, respectively), play important roles in the developmental life cycle that is initiated by the loss of nutrients [3]. Starved *Dictyostelium* aggregate using a relayed intercellular cAMP signal and the multicellular mounds undergo morphogenesis to form a slug and then a fruiting body consisting of a spore mass on top of a stalk [14,15]. Cells lacking Erk1 aggregate into small mounds that have accelerated morphogenesis and the overexpression of Erk1 results in large aggregates that have delayed morphogenesis indicating that Erk1 function can inhibit developmental progression [16–18]. Genetic analysis of Erk2 function has been extensive but limited to the characterization of a leaky erk2 allele in which Erk2 expression is reduced but not eliminated [17,19–23]. A reduction of Erk2 expression results in cells with insufficient external cAMP signaling to allow

cell aggregation in clonal populations but cells retain the ability to chemotax to cAMP [19,23,24]. In the presence of wild-type cells, the reduced Erk2 expression mutant can co-aggregate because of the cAMP provided by wild-type cells [19,22,23]. The deficiency in cAMP signaling of this mutant can also be suppressed by the loss of the cAMP-specific phosphodiesterase, RegA, allowing the double mutant to undergo and complete multicellular development [20].

Stimulation of *Dictyostelium* with the chemoattractants folate or cAMP results in a rapid phosphorylation of Erk2 that is followed by the phosphorylation of Erk1 as the level of phosphorylated Erk2 decreases [17,25]. Folate stimulation of Erk2 phosphorylation requires the folate receptor, Far1, and its coupled G protein, Gα4 [21,26]. However, cAMP stimulation of Erk2 phosphorylation only requires a cAMP receptor, cAR1 or cAR3, and appears to be independent of G protein function, at least the function of Gα2 and Gβ subunits [27,28]. The basis for this distinction remains to be determined and the proteins that transduce the signals from the receptor to the MAPK are not well characterized [29]. The *Dictyostelium* genome encodes only a single MAP2K, MekA (also known as Mek1), based on sequence similarity to characterized orthologs [16]. Cells lacking MekA form small aggregates with accelerated development, similar to the phenotype observed for *erk1*$^{-}$ cells. Previous studies have also suggested that Erk1 but not Erk2 kinase activity is dependent on MekA function [16,27,30].

3.2 Methods

3.2.1 Strains and Development

All of the *Dictyostelium* strains used in this study were derived from the parent axenic strain KAx-3 except for the noted loci. Axenic strains have been derived from wild-type strains through mutations, including those at the *NF-1* locus [31,32]. The JH10 thymidine auxotrophic strain, disrupted at the *thyA* locus (also designated *thy1*), has been previously described [33]. The *erk1*$^{-}$, *erk1*$^{-}$*thyA*$^{-}$, and *mekA*$^{-}$ strains have been previously described [17,18]. Phenotypic

comparisons of the MAPK mutants were done with KAx-3 cells due to the auxotrophic requirements of the JH10 strain. *Dictyostelium* were grown in HL-5 axenic medium (with or without thymidine supplement) or on lawns of *Klebsiella aerogenes* on SM+/3 agar plates [34]. For the analysis of plaque growth, rate cells were mixed with bacteria and plated at a low density on SM+/3 plates to allow the formation of plaques from single cells. DNA constructs and vectors were inserted into *Dictyostelium* using electroporation as previously described [35]. For developmental analysis, cells were harvested from axenic medium by centrifugation and washed in phosphate buffer (12 mM NaH_2PO_4 adjusted to pH 6.1 with KOH). Cells were plated on non-nutrient plates (1.5% agar in phosphate buffer) from suspensions of 5×10^7 cells/ml or less. For chimeric development, clonal populations were mixed at indicated ratios prior to plating cells on non-nutrient plates. Fluorescent images were detected and recorded using fluorescence microscopy.

3.2.2 Recombinant DNA Constructs and Amplifications

A genomic fragment containing the *thyA* gene excised with *Bam*HI was inserted into the unique *Bgl*II sites of an *erk2* cDNA to disrupt the *erk2* open reading frame.
The *erk2::thyA* construct was excised at flanking sites with *Xho*I and *Xba*I and electroporated into *thyA*$^-$ or *erk1*$^-$*thyA*$^-$ cells to disrupt the *erk2* locus. Erk1 and Erk2 expression vectors utilizing the *act15* promoter were created using the pTX-GFP2 vector (replacing the GFP with MAPK sequence) as previously described [22]. The pTX-GFP2 vector was also used to label strains with GFP as previously described [36]. An Erk2 expression vector conferring blasticidin resistance was created by transferring a p*act15:GFP2:erk2 Spe*I fragment into a pBluescript SK- vector containing a blasticidin resistance gene at the *Pst*I site [37]. This vector was linearized at a unique *Sph*I site near the amino-terminal coding region of *erk2* and integrated into the *erk2::thyA* locus of *erk2*$^-$ cells through a single homologous recombination event.
This knock-in construct regenerated a complete *erk2* open reading frame downstream of the

endogenous *erk2* promoter. Verification of integration events into the *erk2* locus was conducted using PCR amplification. PCR primer sequences and binding locations are described in Supplementary figures (Fig. S1).

3.2.3 Chemotaxis Assays

Above agar chemotaxis assays were performed as previously described [22]. Cells were grown in fresh axenic medium 24 h prior to harvesting, washing and suspension in phosphate buffer at 2×10^7 cells/ml for *Dictyostelium*. Droplets (<1 μl) of cell suspensions were spotted on nonnutrient agar plates and then 1 μl of chemoattractant was spotted approximately 2 mm from the cell droplet. Images of the cells were recorded immediately after the plating of the cells and chemoattractant and recorded again 2.5–3 h later. The agar surface near the cell droplet was scarred with a needle to allow the early and late images to be aligned so that the original cell droplet perimeter could be overlaid on the later image. Cell movement toward the chemoattractant source was determined by measuring the distance from the original cell droplet perimeter to the leading edge of migrating cells. Chemotaxis was analyzed using a dissecting microscope (Nikon SMZ2). Videos were created using time-lapse photography with 20s intervals between images for 33 min. ImageJ with MTrackJ plugin software was used to trace cell migration tracks and determine the migration distance for selected cells.

3.2.4 Analysis of Bacterial Cell Engulfment

Dictyostelium engulfment of bacteria in suspension cultures was conducted as previously described [26]. Axenic *Dictyostelium* cells were washed and resuspended at 1×10^6 cells/ml in phosphate buffer. Live *K. aerogenes* bacteria were labeled with pHrodo Red (Thermo Scientific) and incubated with *Dictyostelium* in phosphate buffer at a 100:1 ratio at 22 °C in shaking cultures (150 rpm). At indicated times, *Dictyostelium* cells were centrifuged and suspended in buffer containing 50 mM Tris pH 8.8 and 150 mM NaCl to quench the fluorescence of non-engulfed

bacteria. *Dictyostelium* and bacteria were distinguished through forward and side scatter (FSC and SSC) and the engulfment of bacteria was indicated by the level of fluorescence detected using FACSort flow cytometer (BD Bioscience) with Cell Quest software (v. 3.3). Data analysis was conducted using FlowJo (v. 10.0.8; Tree Star). Quantification of engulfed bacteria number per *Dictyostelium* cell was analyzed using confocal microscopy. *Dictyostelium* were plated in four well chambers (Lab-Tek) and incubated with pHrodo labeled *K. aerogenes* in phosphate buffer. After 15 min, phosphate buffer was replaced with basic buffer to stop engulfment and quench extracellular bacteria fluorescence for imaging.

3.2.5 Reporter Protein Translocation

Reporter protein translocation was measured as previously described [26]. Cells expressing PH_{CRAC}-GFP, RBD-GFP or LimEΔcoil-GFP were harvested, washed with phosphate buffer prior to plating in four well chambers (Lab-Tek). A Zeiss Laser Scanning Microscope 880 with a 60x, 1.3 NA Plan-Neofluar objective lens was used to acquire time-lapse images every 2 s. Cells were exposed to a final concentration of 100 μM folic acid to induce PH_{CRAC}-GFP, RBD-GFP or LimEΔcoil-GFP translocation from cytosol to plasma membrane. Confocal images were used to determine the temporal-spatial intensity changes of PH_{CRAC}-GFP or LimEΔcoil-GFP. Fluorescence intensity at the plasma membrane was measured over time and normalized to the first frame when folate was added. At least ten cells were quantified for each strain.

3.2.6 Immunoblot Analysis of MAPKs

For analysis of MAPK abundance, cells were harvested from axenic medium, washed in phosphate buffer and lysed by mixing with SDS-PAGE loading buffer on ice. Immunoblot analysis of Erk2 protein was conducted using an affinity-purified Erk2 antiserum as the primary antibody. This antiserum was generated in rabbits using the ERK2 peptide ERKKQTNPTKPD (containing a cysteineresidue at the amino terminus for

attachment procedures) as an antigen and the peptide was also used for the affinity purification (Genscript). The analysis of MAPK phosphorylation was conducted as previously described [17]. Cells were grown in shaking cultures to mid log phase (~3×10^6 cells/ml) and then harvested by centrifugation. Cells were washed once in phosphate buffer and suspended at 1×10^8 cells/ml. Starved cells were shaken in a conical tube for 3–5 h with pulses of 100 nM cAMP every 15 min except for the 15 min prior to an assay. For analysis of cAMP stimulation, cells were stimulated with 100 nM cAMP and cell samples were collected and lysed at the indicated time by mixing with SDS-PAGE loading buffer on ice. Cell extracts were subjected to immunoblot analysis using a rabbit monoclonal antibody phospho-p42/44 MAPK (#4370, Cell Signaling Technology). For folate stimulation of MAPK phosphorylation, cell suspensions were shaken for 1 h in phosphate buffer prior to stimulation with 50 μM folate. A secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP) and bioluminescence reaction was used for detection of the primary antibodies. In some blots the biotinylated mitochondrial3-methylcrotonyl-CoA carboxylase α (MCCC1) was used as a gel loading control and this protein was detected using HRP-Streptavidin as previously described [38].

3.2.7 MAPK Ortholog Analysis

MAPK sequences were identified in sequence databases using UniProt and BLASTp searches using default parameters in the non-redundant protein sequences database (NCBI). Molecular phylogenetic analysis was conducted in MEGA7 using the Maximum Likelihood method based on the JTT matrix-based model [39,40]. The percentage of replicate trees in which the associated proteins clustered together in the bootstrap test (1000 replicates) are shown next to the branches [41]. Only branches corresponding to partitions reproduced in >50% of the bootstrap replicates are labeled. Each tree is drawn to scale, with branched lengths measured in the substitutions per site.

3.3 Results

3.3.1 Disruption of the erk2 Gene

Previous analyses of *Dictyostelium* Erk2 function have used *erk2* mutants that contain an insertion mutation near the *erk2* open reading frame [17,19–23]. This allele was originally created through restriction enzyme-mediated insertion (REMI) mutagenesis and the allele has been recapitulated in axenic strains through homologous recombination. This allele has a significant reduction of *erk2* expression that leads to inadequate cAMP production for the aggregation stage of development but only subtle changes in chemotactic responses to either cAMP or folate [24]. Although this allele results in developmental defects, activated Erk2 can still be detected [17]. For clarity this allele will now be referred to as $erk2^{RE}$ (reduced expression) to distinguish it from an $erk2^{-}$ allele described in this report that does not produce functional Erk2 protein. Given the apparent importance of Erk2 in *Dictyostelium* development, we created and verified an $erk2^{-}$ strain that contains the *erk2* open reading frame disrupted with the auxotrophic marker gene *thyA* (Fig. 1A and Fig. S1A). *Dictyostelium discoideum* has only two MAPKs, Erk1 and Erk2, and so we also created a strain with no MAPKs by disrupting the *erk2* gene in a strain already containing a disrupted *erk1* gene. Both $erk2^{-}$ and $erk1^{-}erk2^{-}$ strains were transformed with Erk2 expression vectors for complementation and the $erk1^{-}erk2^{-}$ strain was also transformed with an Erk1 expression vector. Levels of Erk2 protein in mutants and complemented strains were verified by immunoblot analysis using antiserum generated against an Erk2 peptide (Fig. 1C). Wild-type and complemented $erk2^{-}$ mutants displayed a band at approximately 42 kDa (predicted size of Erk2) and this band was absent in $erk2^{-}$ and $erk1^{-}erk2^{-}$strains.

3.3.2 erk2⁻ Cells have Growth Defects on Bacterial Lawns

When grown on bacterial lawns, cells with the *erk2* gene disruption displayed a slow plaque growth rate and no multicellular development compared to wild-type cells (Fig. 2A). Transformation of the *erk2*$^{-}$ cells with Erk2 expression vectors (both extrachromosomal and integrating) using the relatively constitutive *act15* promoter resulted in a rescue of the plaque growth rate and, in some transformants, a rescue of multicellular development was also observed.

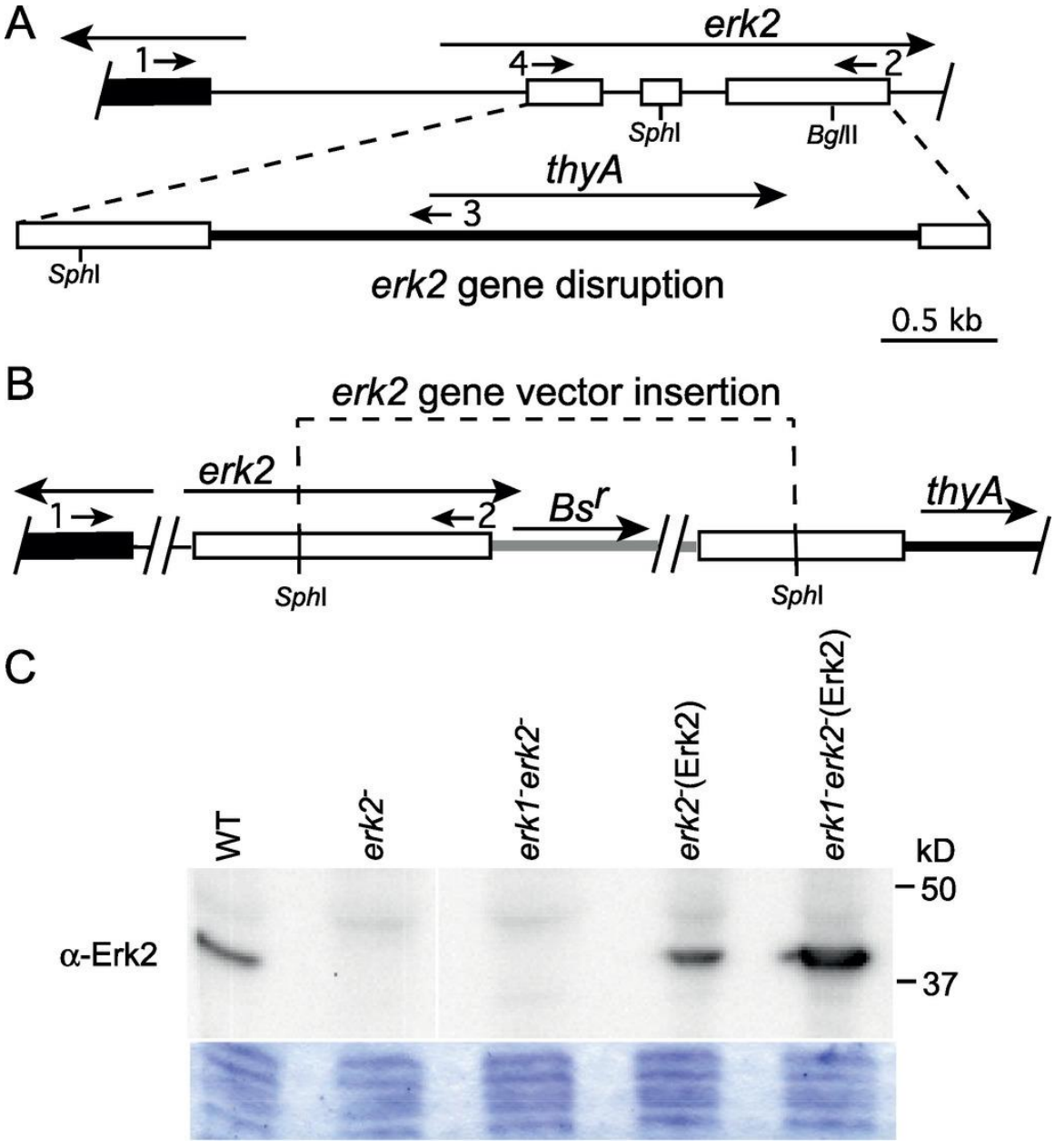
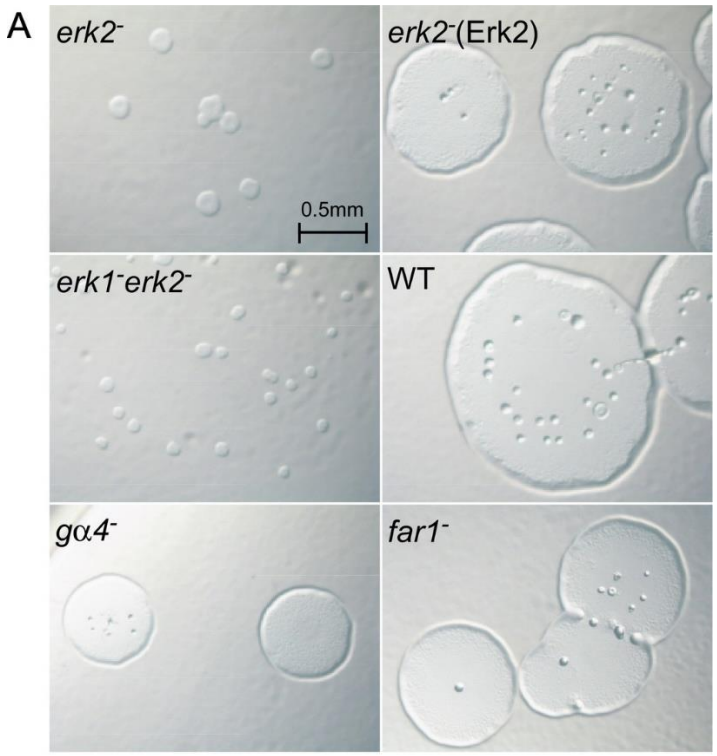


Figure 3.1 Disruption and knock-in complementation of the *erk2* locus. A) Homologous recombination of the *erk2::thyA* fragment (see Materials and Methods for construction) with the *erk2* locus. The location of primer binding sites (arrows) used for PCR verification of recombination are shown. Open rectangles represent the *erk2* open reading frame, the closed rectangle represents the open reading from of an adjacent gene and the thick black line represents the *thyA* genomic fragment. B) Knock-in of an Erk2 expression vector with blasticidin resistance into the disrupted *erk2::thyA* locus at the *Sph*I site. Hashed lines represent sequences not shown to reduce the size of image. Description of PCR products and primer sequences are described in Fig. S3.1. C) Immunoblot of Erk2 protein in wild-type(WT), *erk2*$^{-}$, and *erk1*$^{-}$*erk2*$^{-}$ strains and in mutant strains complemented with Erk2 expression vector (Erk2). Lysates of cells grown in axenic medium were analyzed for Erk2 protein by immunoblot analysis. Coomassie staining of the gel was used as a lane loading control.

The lack of multicellular development in some transformants might be due to the heterologous overexpression of Erk2 because these vectors can also result in aggregation deficient phenotypes in wild-type cells. To express Erk2 from its endogenous promoter an Erk2 expression vector conferring blasticidin resistance was linearized within the *erk2* open reading frame and integrated into the *erk2::thyA* locus upstream of the gene disruption site (Fig. 3.1B, Fig. S3.1). This knock-in integration of an *erk2* vector resulted in cells with a single copy of a complete *erk2* open reading frame downstream of the endogenous *erk2*promoter. Erk2 expression from the endogenous promoter provided a more efficient rescue of both plaque growth rate and multicellular development. Cells with both MAPK gene disruptions, *erk1*$^{-}$*erk2*$^{-}$, also had a slow plaque growth phenotype but this phenotype was more extreme than that of cells with only the disruption of the *erk2* gene. Reduced plaque growth rates have also been observed with other *Dictyostelium* mutants, particularly those defective in responding to folate. Two mutants with defects in folate responses, *far1*$^{-}$ and *gα4*$^{-}$ strains, displayed plaque growth rates slower than wild-type cells but faster than *erk2*$^{-}$ cells suggesting that Erk2 likely functions in cellular processes other than folate responses. To assess whether the slow plaque growth phenotypes

of *erk2*$^{-}$ and *erk1*$^{-}$*erk2*$^{-}$ are due to general growth defects the mutants were analyzed for cell proliferation in shaking cultures of axenic medium. Interestingly, the *erk2*$^{-}$ mutants had proliferation characteristics similar to that of complemented cells and wild-type cells (Fig. 3.2B).



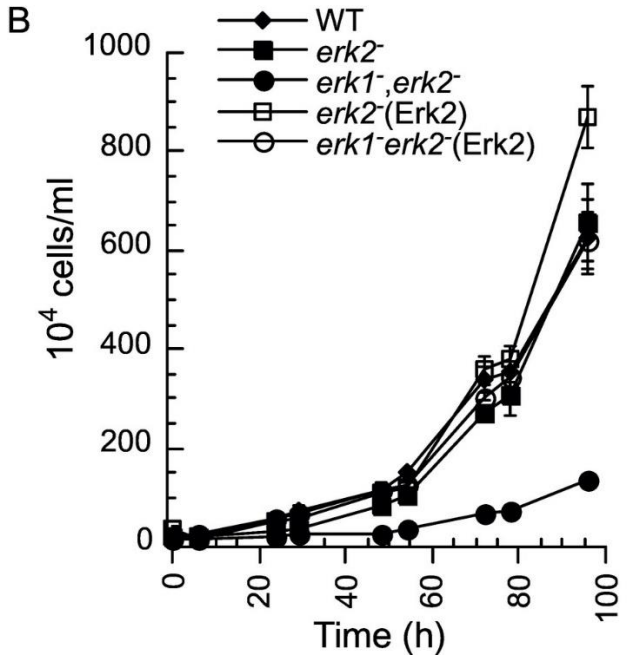


Figure 3.2 *Dictyostelium* growth. A) Growth of MAPK mutants and wild-type cells on bacterial lawns. Individual strains were mixed with bacteria and plated on SM+/3 plates as described in the Materials and Methods section. Images of plaques were captured 5 days later. All images are the same magnification. B) Growth of MAPK mutants and wild-type cells in shaking cultures of axenic medium. Wild-type (WT), *erk2*$^{-}$, *erk1*$^{-}$*erk2*$^{-}$, and the *erk2* mutant strains complemented Erk2 expression vectors (Erk2) were inoculated into shaking cultures of HL-5 axenic medium and cells concentrations were determined using a hemacytometer at the indicated times. Each data point represents 4 counts of at least 100 cells. Error bars represent standard deviation in multiple counts.

However, *erk1*$^{-}$*erk2*$^{-}$cell proliferation was much slower under these conditions but complementation of the *erk2*$^{-}$ allele in this strain rescued this defect suggesting that only a loss of both MAPKs has an impact on proliferation in axenic medium. The *erk1*$^{-}$*erk2*$^{-}$cell proliferation defect was not the result of unusual cytokinesis mechanisms because the distribution of single and multinucleated cell particles was similar to the other strains (Fig. S3.2).

3.3.3 Loss of Erk2 Impairs Bacterial Engulfment

Engulfment of bacteria by *Dictyostelium* is mediated in part by the folate receptor Far1 and stimulation of this receptor also activates Erk2 [26]. To determine the potential role of Erk2 in bacterial phagocytosis, we used a flow cytometry analysis to quantitatively compare the engulfment of pHrodo-labeled *Klebsiella aerogenes* by wild-type, *erk2*$^{-}$, and other mutant strains. Cells lacking Erk2 or Far1 displayed a similar delay in the initial engulfment in the phagocytosis of the fluorescently-labeled bacteria in suspension cultures compared to wild-type cells. The phenotypic similarity between *erk2*$^{-}$ and *far1*$^{-}$cells suggests that Erk2 plays a role in Far1-mediated bacterial phagocytosis (Fig. 3.3A and B). This delayed uptake of bacteria was also consistently observed when cells were monitored by confocal microscopy (Fig. 3.3C and D). The number of engulfed bacteria in *erk2*$^{-}$ cells was less than wild-type cells. This defect was rescued by complementation with the Erk2 expression vector. After the initial delay, the rate

of *erk2*$^{-}$ bacterial engulfment was similar to that of wild-type cells implying that other mechanisms might contribute to the slow plaque growth rate on bacterial cell lawns. The lower fluorescence intensity of pHrodo-bacteria in *erk2*$^{-}$ cells suggests a potential defect in phagosomal maturation.

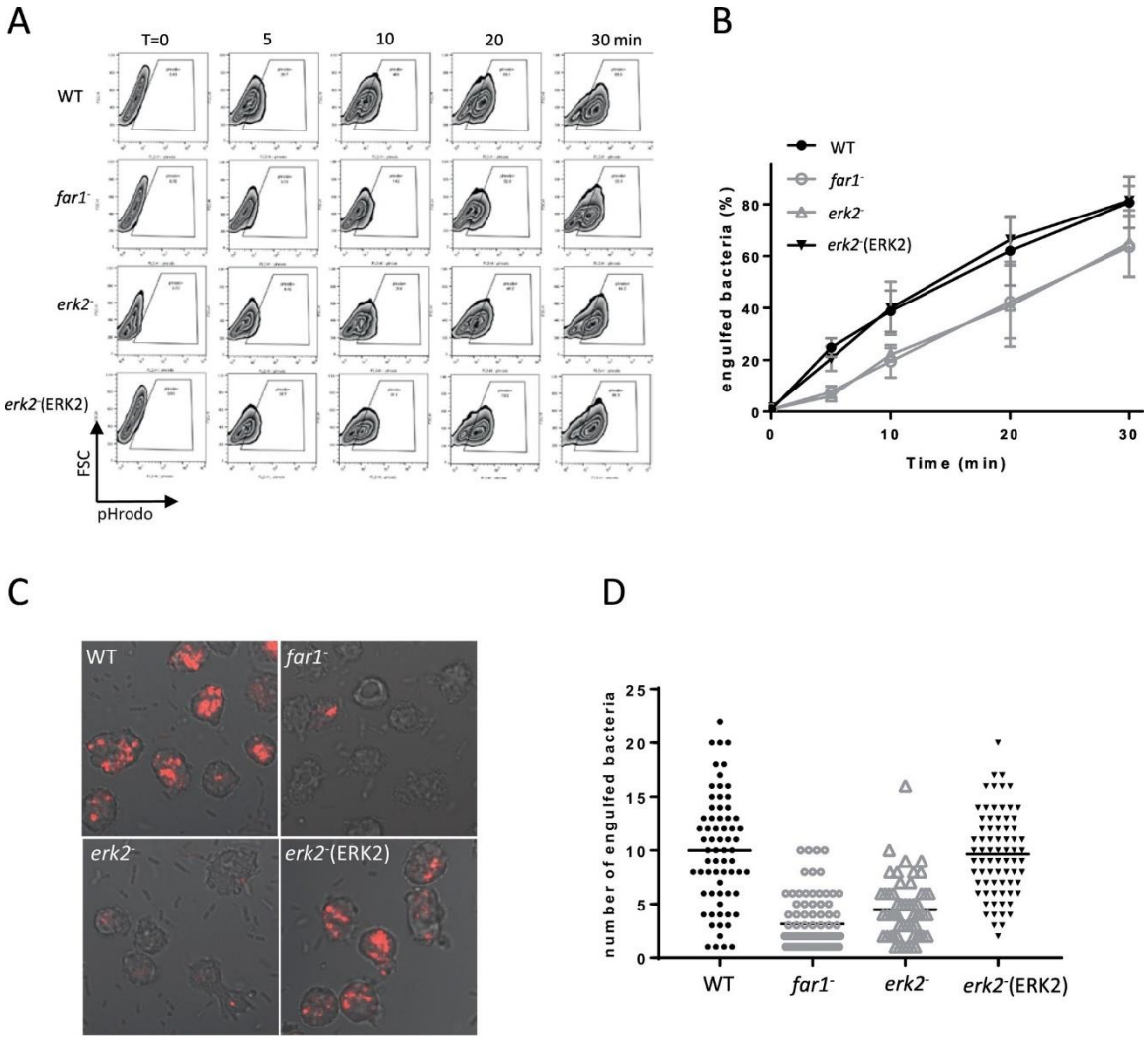


Figure 3.3. Engulfment of bacteria. A) Wild-type (WT), *far1*$^{-}$, and *erk2*$^{-}$ strains and *erk2*$^{-}$mutant complemented with Erk2 expression vector (Erk2) were mixed with pHrodo-labeled live bacteria and analyzed at indicated times for the percentage of pHrodo-positive cells. B) Graphical representation of data from (A). C) Images of engulfed bacteria in cells after 15 min. D) Quantitation of bacterial cell uptake into cells. The engulfed bacterial number in each cell was measured and plotted.

3.3.4 Erk2 is Required for Folate Chemotaxis

Reduced plaque growth rates can potentially result from defects in the ability of cells to properly forage for bacteria at the perimeter of the plaque. *Dictyostelium* forage for bacteria primarily using the folate receptor and downstream G proteins as a mechanism to facilitate chemotactic movement [42,43]. The *erk2*$^{-}$cells displayed a defect in folate chemotaxis similar to that of *far1*$^{-}$ and *gα4*$^{-}$mutants when analyzed in an above agar assay (Fig. 3.4A and Fig. S3.3). This defect is rescued by the presence of the *erk2* gene. In the absence of folate stimulation *erk2*$^{-}$ cells did not migrate as far as wild-type and the complemented *erk2*$^{-}$ cells suggesting Erk2 function contributes to cell motility, directionality, and/or other mechanisms (e.g., cell repulsion) associated with cell dispersal. The *erk1*$^{-}$*erk2*$^{-}$ cells also lacked chemotaxis to folate but these cells did not disperse from the initial cell droplet as much as the *erk2*$^{-}$ cells suggesting that the loss of both MAPKs has a detrimental impact on cell dispersal. Time-lapsed videos of *erk2*$^{-}$ cells showed migratory paths typical of random movement compared to the more directed movement paths observed for wild-type cells or complemented mutants in the presence of folate (Fig. 3.4 B, C and Fig. S3.4–S3.8). The average path lengths for *erk2*$^{-}$ or *erk1*$^{-}$*erk2*$^{-}$ cells were comparable but substantially less than that of wild-type or complemented cells. This difference in individual cell path lengths suggests that chemotactic cell movement is compromised in the MAPK mutants. The reduced cell dispersal of the double MAPK mutant compared to the *erk2*$^{-}$ mutant was surprising given that *erk1*$^{-}$cells do not have a defect in folate chemotaxis (Fig. S3.9). The basis of this cell dispersal defect is not known but the defect was also observed in cAMP chemotaxis assays (Fig. 3.6C) and during drug selection of transformants (Fig. S3.10). It is possible that both MAPKs might have overlapping contributions to cell movement. The requirement of Erk2 for folate chemotaxis indicates that this MAPK plays an important role in the foraging of *Dictyostelium* to bacteria.

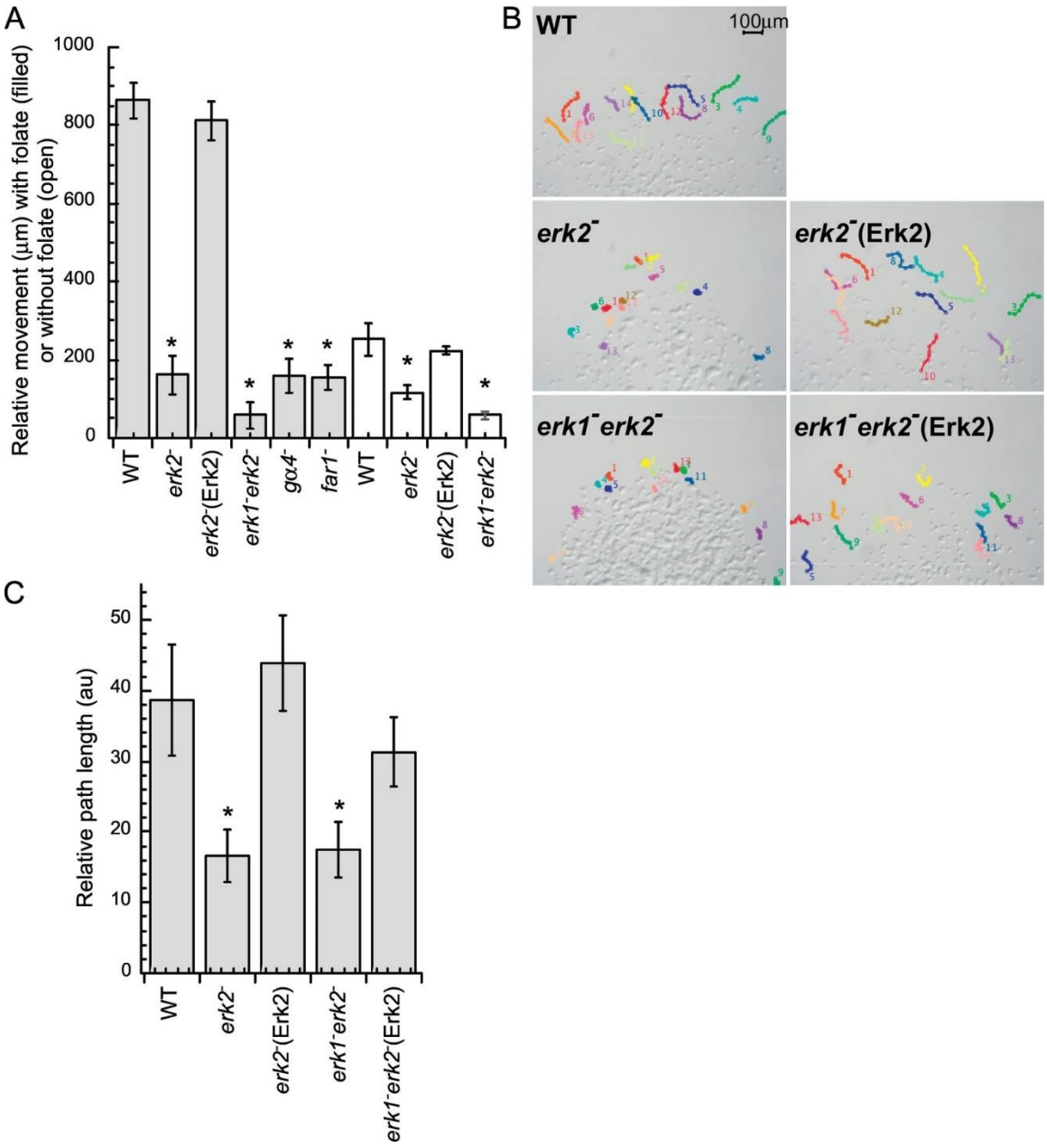


Figure 3.4 Chemotaxis of MAPK mutants to folate. A) Above-agar chemotaxis assay images for wild-type (WT), *erk2*$^{-}$, and *erk1*$^{-}$*erk2*$^{-}$ strains and *erk2*$^{-}$ mutants complemented with Erk2 vector (Erk2) after 2.5 h exposure to droplets of 100 µM folate. A) Relative movement of wild-type (WT), *erk2*$^{-}$, *erk1*$^{-}$*erk2*$^{-}$, *gα4*$^{-}$ and *far1*$^{-}$ strains and MAPK mutants complemented with Erk2 expression vector (Erk2) toward folate (filled bars) and relative movement in the absence of folate (open bars). Values indicate maximum distance of cell migration toward the source of folate or migration in any direction in the absence of folate. Error bars represent the standard deviation of the error. B) Cell migration paths of select

cells were mapped over a 30 min period using time-lapse photography as described in the Materials and Methods section. All images are the same magnification. C) Graphical representation of the average path lengths in arbitrary units (a.u.) from (B). Error bars represent standard deviation. Student's unpaired *t*-test comparing to WT, $P < 0.0001$ (*).

3.3.5 Loss of Erk2 does not Affect Folate Detection and Early Signaling Events

Chemoattractant sensing in *Dictyostelium* is mediated in part by the rapid activation of Ras proteins, phosphoinositide 3-kinases (PI3Ks), and actinpolymerization [44,45]. Fluorescent reporters that bind to activated Ras (RBD-GFP), phosphorylated inositol lipids (PH_{CRAC}-GFP), and actin filaments(LimEΔcoil-GFP) can assess these cellular responses through the translocation of the reporter to the plasma membrane [46–48]. All of these responses typically begin within a few seconds of chemoattractant stimulation and prior to the activation of Erk2 suggesting these responses occur independently of Erk2 function. When expressed in *erk2*$^{-}$ cells, these reporters translocated to the membrane with kinetics and amplitudes similar to that observed for wild-type or complemented *erk2*$^{-}$ cells (Fig. 3.5A–C). This observation is also consistent with previous studies that suggest MAPK activation might occur in a parallel signaling pathway [49,50]. Therefore, the loss of Erk2 function does not significantly impact early chemotactic responses to folate.

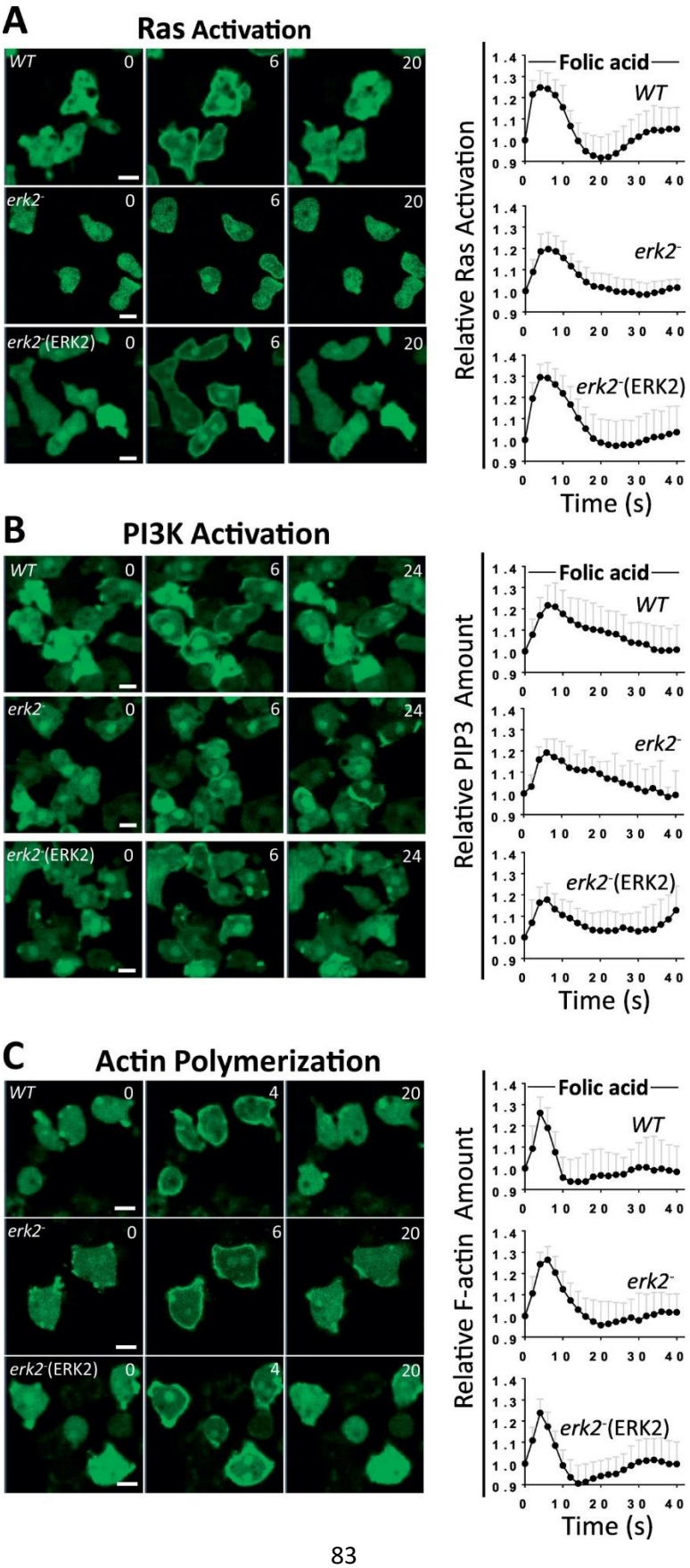

A
Ras Activation
WT
erk2-
erk2-(ERK2)
0
6
20
Folic acid
Relative Ras Activation
Time (s)
B
PI3K Activation
24
Relative PIP3 Amount
C
Actin Polymerization
4
Relative F-actin Amount

Figure 3.5 Early chemotactic signaling in response to folate. Translocation of Ras, PI3K, and actin filament reporters in wild-type (WT), *erk2*$^{-}$ cells (*erk2*$^{-}$), and complemented *erk2*$^{-}$cells (Erk2) in response to folate stimulation was assayed as described in the Materials and Methods section. A) Translocation of the Ras activation reporter RBD-GFP to the membrane. B) Translocation of the PI3K activation reporter PH_{CRAC}-GFP to the membrane. C) Translocation of the actin filament reporter LimEΔcoil-GFP to the membrane. Graphs indicate relative intensity of fluorescence at the membrane and 1 represents the intensity at the start of the response. Error bars represent standard deviation. All images are the same magnification and scale bar represents 5 μm.

3.3.6 Erk2 is Required for Development and cAMP Chemotaxis

The *erk2*$^{-}$ and *erk1*$^{-}$*erk2*$^{-}$ mutants failed to aggregate when synchronously starved on nonnutrient agar but the expression of Erk2 in these mutants restored multicellular development similar to that of wild-type and *erk1*$^{-}$ cells, respectively, including the small aggregate and accelerated development characteristic of *erk1*$^{-}$ development (Fig. 3.6A). Earlier studies have shown that the aggregation defect of mutants with reduced Erk2 expression can be rescued by the presence of wild-type cells in a chimeric population because the *erk2*^{RE}mutants retain the ability to chemotaxis to cAMP [22,23]. In contrast, the *erk2*$^{-}$or *erk1*$^{-}$*erk2*$^{-}$ cells do not co-aggregate with wild-type cells as indicated by lack of cell elongation and the absence of these cells in aggregation streams (Fig. 3.6B). This observation suggests that the mutants do not respond to wild-type cAMP signaling or produce an inhibitory mechanism to the cAMP-mediated aggregation of wild-type cells. The *erk2*$^{-}$ cells were also analyzed in above-agar cAMP chemotaxis assays. Cells lacking Erk2 were not capable of chemotaxing to cAMP but chemotaxis could be restored by complementation with the Erk2 expression vector (Fig. 3.6C). Both the lack of cAMP chemotaxis and the inability to co-aggregate with wild-type cells suggest that the failure of *erk2*$^{-}$ mutants to undergo multicellular development is due to a chemotaxis defect and not just a defect in cAMP production.

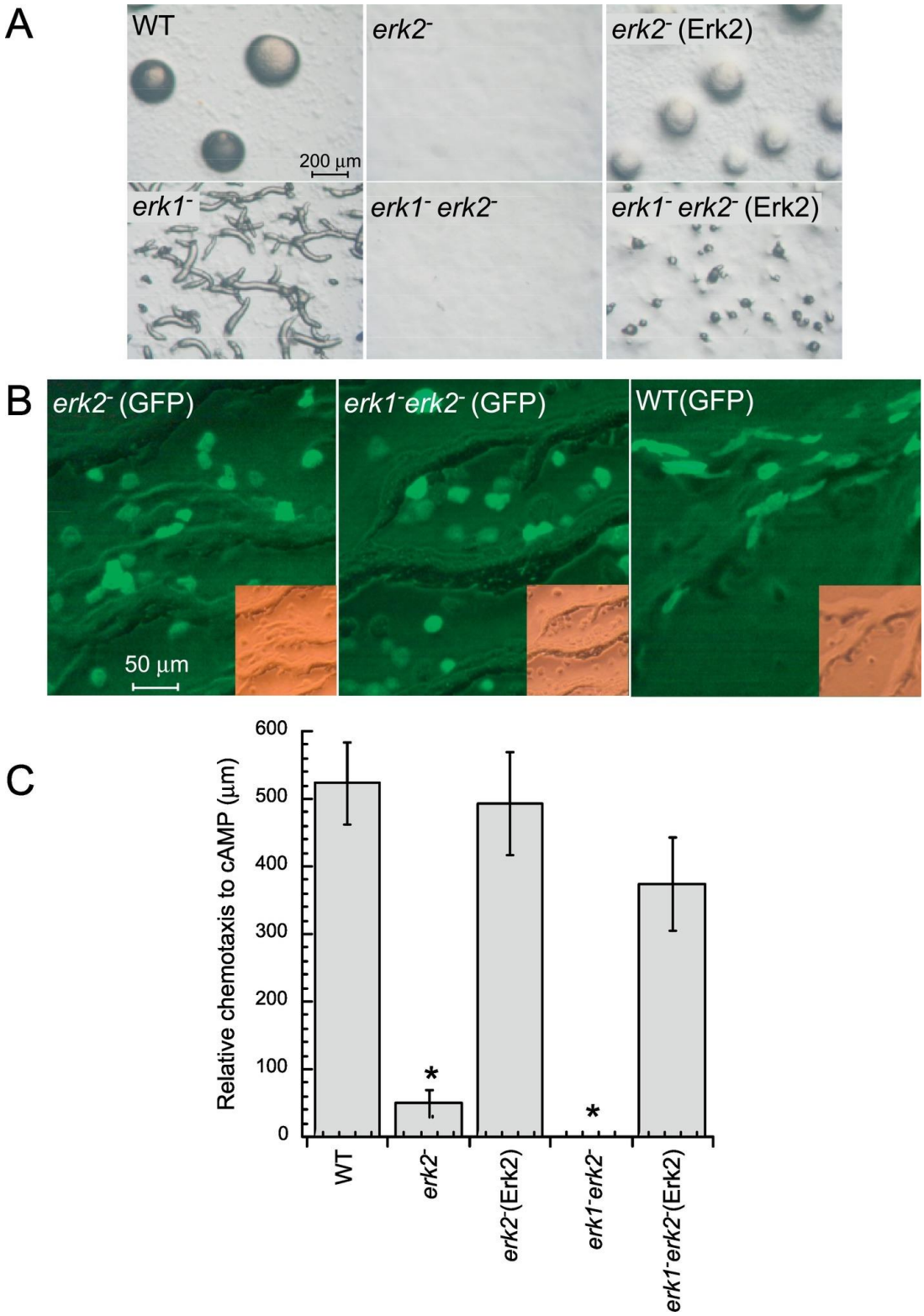


Figure 3.6 Development and cAMP chemotaxis. A) Wild-type (WT), *erk2*$^{-}$, and *erk1*$^{-}$*erk2*$^{-}$mutants and mutants complemented with Erk2 expression vector (Erk2) developed on nonnutrient plates for 12 h. All

images are the same magnification. B) A GFP vector was used to label *erk2*$^{-}$, *erk1*$^{-}$*erk2*$^{-}$, and wild-type (WT) cells. Labeled cells (GFP) were mixed in a 1:9 ratio with unlabeled wild-type cells and and cell droplets (1 × 107 cells/ml) plated for development on nonnutrient agar plates. Images of aggregation streams were taken at 12 h. All images are the same magnification. C) Above-agar cAMP chemotaxis assay. After 4 h of starvation in shaking phosphate buffer cells were plated on nonnutrient plates near droplets of 100 μM cAMP. Images of cells were taken at 0 h and 2.5 h and distance was measured of the leading edge of cells toward the source of cAMP. Migration distances under 100 μm are typical for random movement in the absence of exogenous cAMP. Error bars represent the standard deviation of the error. Student's unpaired *t*-test comparing to WT, $P < 0.0001$ (*).

3.3.7 Loss of Erk2 Impairs Erk1 Activation in Folate Chemotactic Response

We had previously shown that reduced levels of Erk2 in *erk2*^{RE} cells did not impact the phosphorylation of Erk1 in the secondary response to folate stimulation [17]. Given the importance of Erk2 in chemotaxis, the phosphorylation of Erk1 in response to folate was examined in the *erk2*$^{-}$ cells and found to be absent, indicating a dependence on Erk2 function (Fig. 3.7A). However, a low level of phosphorylated Erk1 could be detected in *erk2*$^{-}$ cells suggesting that Erk2 function is only required for the burst of phosphorylated Erk1 as a secondary response to chemotactic stimulation. Erk1 activity has been previously shown to be dependent on MekA, the only known MAP2K in *Dictyostelium*, but a requirement of MekA for the phosphorylation of Erk1 had not been demonstrated [16]. The stimulation of *mekA*$^{-}$ cells with folate or cAMP resulted in the phosphorylation of Erk2 but not Erk1 indicating that MekA only regulates Erk1 and not Erk2 (Fig. 3.7B). This result implies that the phosphorylation of Erk2 must be facilitated through a mechanism that does not require a conventional MAP2K.

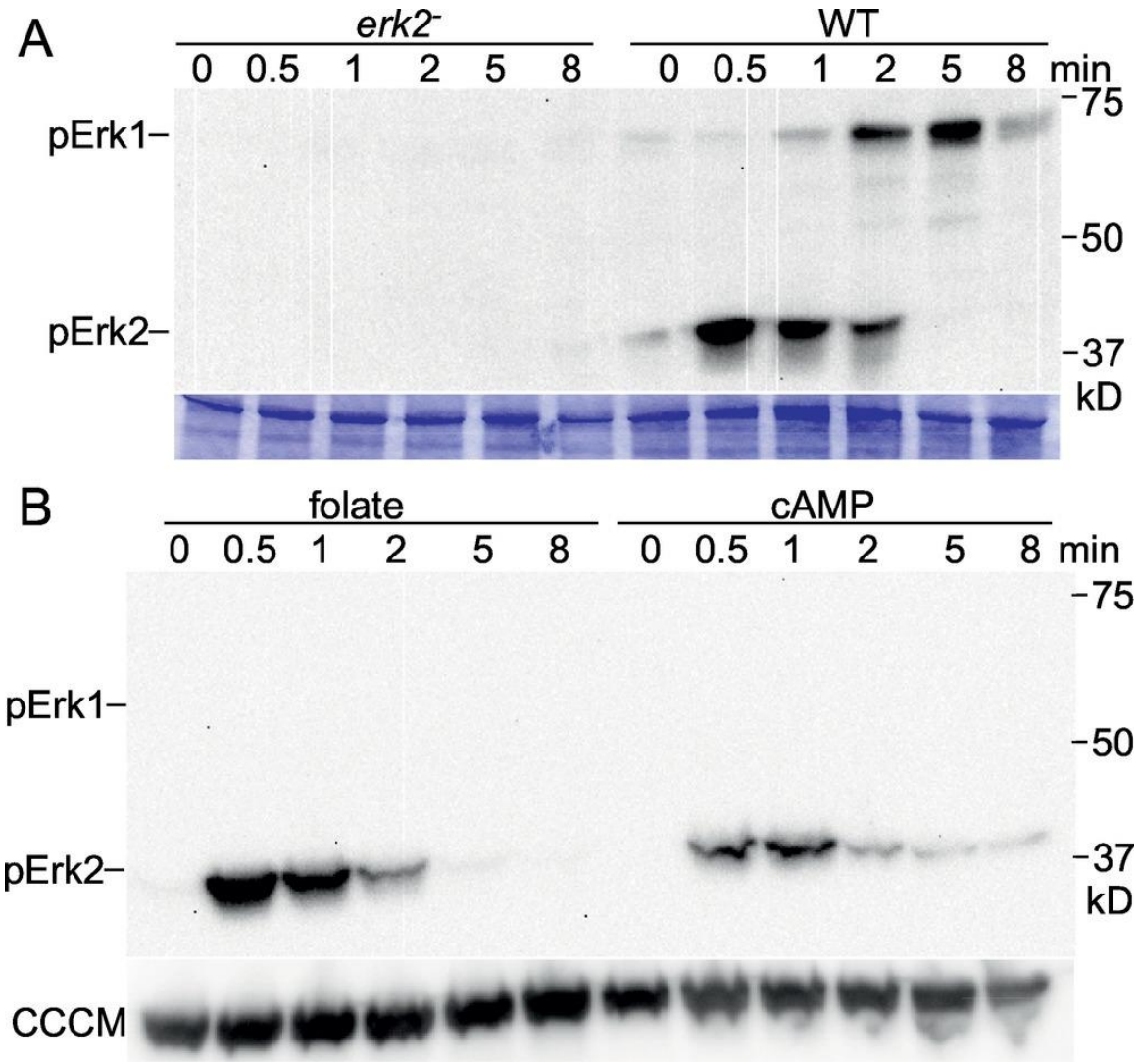


Figure 3.7 Phosphorylation of MAPKs. A) After 50 μM folate stimulation *erk2*⁻ and wild-type(WT) were lysed at times indicated and analyzed for the phosphorylation of MAPKs by immunoblots using phospho-MAPK specific antibodies (upper panel). Coomassie blue stained gel as loading control (lower panel). B) Phosphorylation of MAPKs in *mekA*⁻ cells in response to folate or cAMP. Cells were stimulated with either 50 μM folate or 100 nM cAMP and then analyzed for phosphorylation of the MAPKs as described in (A) (upper panel). Detection of CCCM using HRP-streptavidin as a loading control (lower panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3.8 Erk2 Sequence is Related to Atypical MAPKs

A phylogenetic analysis of the *Dictyostelium* MAPKs with other eukaryotic MAPKs suggests that the *Dictyostelium* Erk1 belongs to a group of MAPKs that is found in a wide variety of eukaryotes (Fig. 3.8). This group contains prototypical MAPKs in yeast (e.g., Fus2) and mammals (e.g., ERK1/2) that have been characterized extensively. In contrast, the *Dictyostelium* Erk2 shares more sequence similarity to a group of MAPKs that includes the mammalian MAPK15 (also referred to as Erk8). This group of kinases has been previously referred to as atypical MAPKs because typical MAP2Ks have not been identified as the activators of these MAPKs [5,10,11]. This atypical regulation is consistent with the *Dictyostelium* Erk2 belonging to this group of MAPKs. Orthologs of the *Dictyostelium* Erk2 exists in other amoebae and in animals where cell movement plays important roles but not in fungi where cell movement is absent. The fungal MAPKs (e.g., *Aspergillus* nidulans, *An*MAPK) that are most closely related to the *Dictyostelium* Erk2 belong to other MAPK groups suggesting the evolution of organisms without cell movement did not require this group of atypical MAPKs.

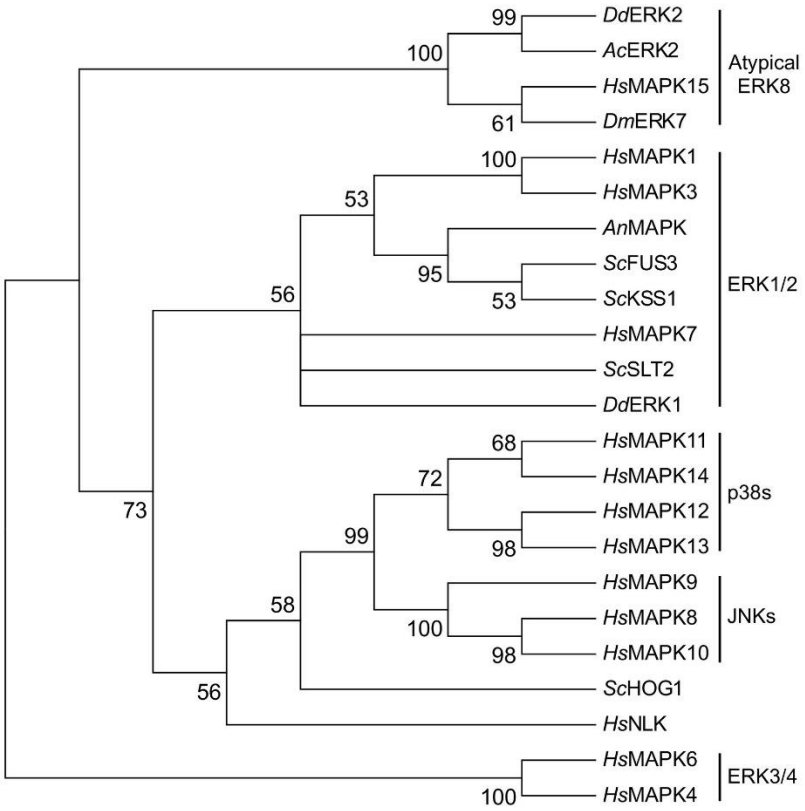


Figure 3.8 Phylogenetic analysis of MAPKs. All known MAPKs in human (*Hs*), yeast/*Saccharomyces cerevisiae* (Sc), and *Dictyostelium discoideum* (*Dd*) were used to construct the phylogenetic tree using MEGA7 as described in the Materials and Methods. Selected MAPKs with similarity to atypical human MAPK15 (Erk8) from *Drosophila*melanogaster (*Dm*) and *Acanthamoeba castellanii* (*Ac*) were also included in the tree. A BLAST search of fungal genomes using the *Dictyostelium* Erk2 protein as the query yielded only MAPKs with similarities to the human Erk1/2 group such as the one representative MAPK included from *Aspergillus* nidulans (*An*).

3.4 Discussion

This study of *erk2* gene disruption mutants has revealed the essential role of the *Dictyostelium* Erk2 in chemotaxis to folate and cAMP whereas previous studies of $erk2^{RE}$ mutants had implied only a subtle role in chemotaxis (Fig. 3.9). While required for chemotaxis, Erk2 function does not impact early chemotactic responses such as Ras and PI3K activation and early actin filament assembly. Previous studies have shown levels of Erk2 activation in $rasC^{-}$ mutants to be the same as wild-type cells in response to cAMP and only reduced by half in response to folate suggesting Erk2 regulation occurs through a parallel signaling pathway [49,50]. The requirement of Erk2 function for two different chemotactic responses suggests that this MAPK plays an integral role in general chemotactic responses and could possibly be important for other cell fates that involve chemotactic movement. The rapid activation of Erk2 in response to chemoattractants argues that Erk2 function is necessary for cell movement in response to chemoattractants rather than being a general requirement for all cell movement. However, $erk1^{-}erk2^{-}$ mutants show a strong defect in cell dispersal suggesting both MAPKs have overlapping contributions to cell movement in the absence of an exogenous stimulus. The role of Erk2 function in chemotactic responses is clearly different than that of Erk1 function. In an earlier study, $erk1^{-}$ cells have been described as impaired with respect to cAMP responses but these cells can aggregate and complete all other

phases of development in clonal populations [17,18]. However, *erk1*$^{-}$ cells typically form smaller aggregates with precocious development suggesting that developmental signaling is aberrant. Folate chemotaxis and foraging capabilities of *erk1*$^{-}$ cells are comparable to that of wild-type cells (Fig. S3.9). The phenotypic differences between *erk1*$^{-}$ and *erk2*$^{-}$ cells in foraging and multicellular development suggest that the two *Dictyostelium* MAPKs regulate different cellular processes even though they appear to have overlapping contributions to cell movement.

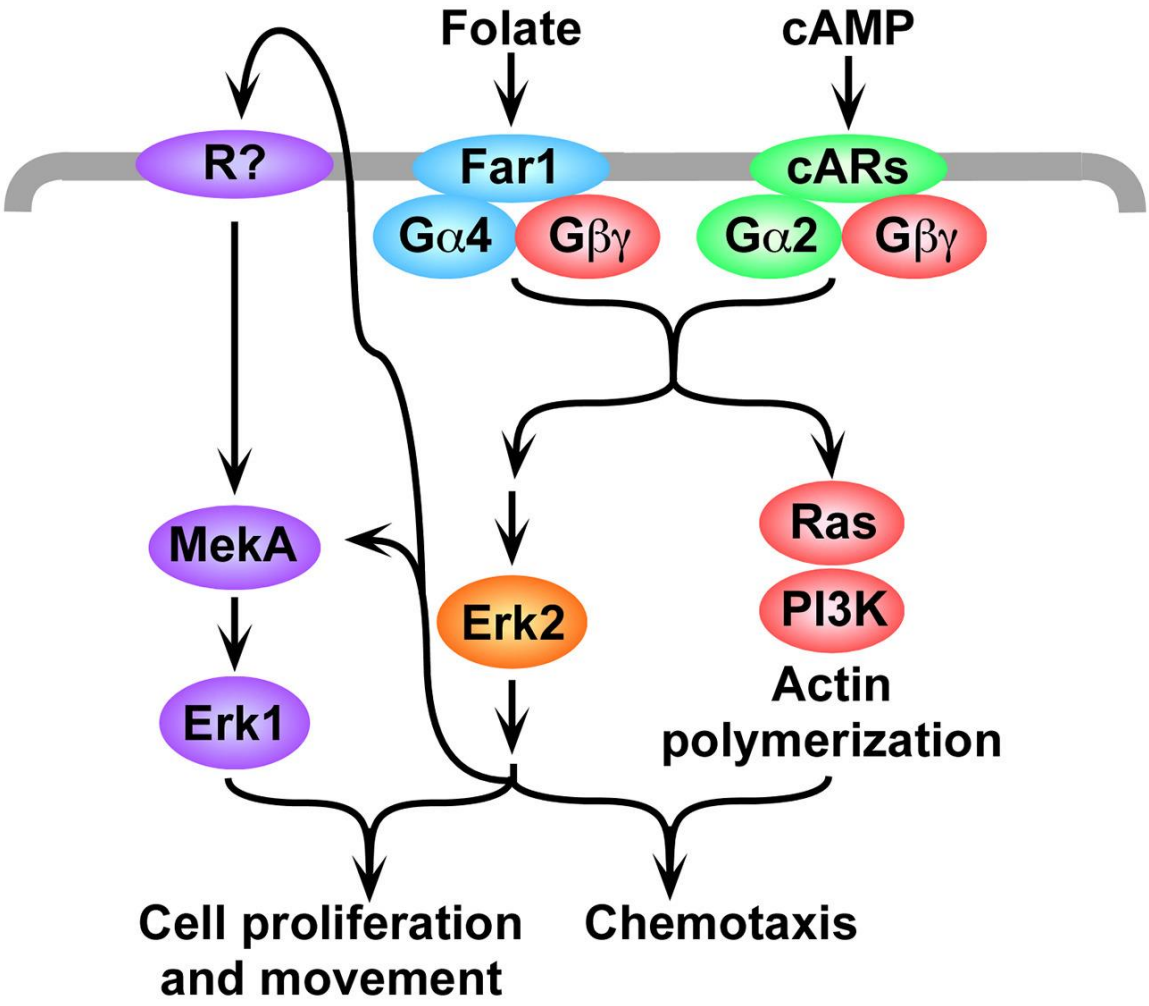


Figure 3.9 Model of Erk2 mediated signaling pathways. Multiple chemoattractant stimulated pathways lead to the activation of Erk2 and downstream cellular responses such as chemotaxis and Erk1 activation. Early chemotactic responses such as Ras and PI3Kactivation and actin filament formation are not dependent on Erk2 function. Like mammalian atypical MAPKs, the activation of Erk2 does not require the

only known MAP2K in *Dictyostelium*. Folate but not cAMP responses require G protein function for Erk2 activation.

How Erk2 mediates chemotaxis independent of early chemotactic responses remains a major question because little is known about the regulators and targets of atypical MAPKs. Genetic evidence suggests Erk2 is a negative regulator of the cAMP-specific phosphodiesterase, RegA, and therefore Erk2 function could indirectly lead to increased cAMP-dependent protein kinase (PKA) activity [20]. However the loss or over-expression of RegA does not eliminate chemotaxis, suggesting other downstream regulatory proteins exist. Epp2, a protein phosphorylated in an Erk2-dependent manner, is important but not essential for cAMP chemotaxis and cAMP production [51]. The primary structure Epp2 has so far not provided clues as to the function of this protein. Typical MAPKs are known to phosphorylate and regulate other protein kinasesand transcription factors but such downstream regulatory proteins have not yet been reported in *Dictyostelium* [5]. Erk2 function could potentially regulate the expression of genes that facilitate chemotaxis but such function would not address the role of Erk2 activation during chemotaxis.

MAPKs in other organisms have been associated with the regulation of cell proliferation and so MAPK pathways have been a focus for understanding and treating cancerous growth [1,52–54]. The association of MAPK function with cell growth and proliferation in mammalian systems has been largely based on the activation of MAPKs downstream of receptor tyrosine kinases and Ras proteinsthat drive these processes [5,52,55–59]. The compromised proliferation of the *Dictyostelium* double MAPK mutant indicates that the MAPKs are important but not essential for proliferation. Some synergy may exist between the *erk1*$^{-}$ and *erk2*$^{-}$ gene disruptions because no proliferation defects have been noted for strains carrying one or the other mutant alleles implying Erk1 and Erk2 signaling pathways might have some overlap in the regulation of cell proliferation. While the basis of the *erk1*$^{-}$*erk2*$^{-}$ proliferation defect remains to

be determined, this proliferation phenotype supports early assertions that MAPK signal transduction might be a good target for inhibiting cell proliferation. *Dictyostelium* growth and proliferation are typically intertwined with finding nutrient sources but the cell proliferation defect of *erk1*$^{-}$*erk2*$^{-}$ mutants in axenic suspension cultures occurs in the absence of cell migration. However it is possible that nutrient uptake in *Dictyostelium* suspensions could include cellular processes related to those important for cell migration.

The roles of Erk2 and Erk1 function in *Dictyostelium* development are quite different and possibly oppositional. The loss of Erk2 function blocks development at the aggregation stage and the loss of Erk1 function can accelerate developmental progression [17]. Therefore it is interesting that both MAPKs become activated in response to cAMP and folate stimulation. The rapid phosphorylation of Erk2 and then later phosphorylation of Erk1, as Erk2 becomes dephosphorylated, indicates a temporal distinction in the regulation of these MAPKs. The timing of Erk1 phosphorylation correlates with the adaptation to the stimulus and therefore Erk1 activation could be associated with a mechanism to down regulate the initial chemotactic signal. The mechanism by which Erk2 function regulates the phosphorylation of Erk1 is unclear but it requires the activation of MekA and possibly intercellular signaling, as suggested by a previous study [17]. Interestingly, the timing of Erk1 phosphorylation in response to chemotactic signals in *Dictyostelium* is similar to that of mammalian Erk1/Erk2 phosphorylation in mammalian neutrophils after chemotactic stimulation with fMLP, in that the phosphorylated form persists for over 5 min [60–62]. If *Dictyostelium* and mammalian MAPK orthologs play analogous roles in chemotaxis then it is possible that mammalian Erk1/Erk2 could be involved with an adaptive secondary response to chemoattractants and the mammalian MAPK15 might have a role in mediating the initial chemotactic signaling. Studies of the mammalian MAPK15 regulation have often focused on relatively slow or long term responses (10 min to hours after stimulation) rather than rapid responses (within a couple minutes), like the rapid phosphorylation

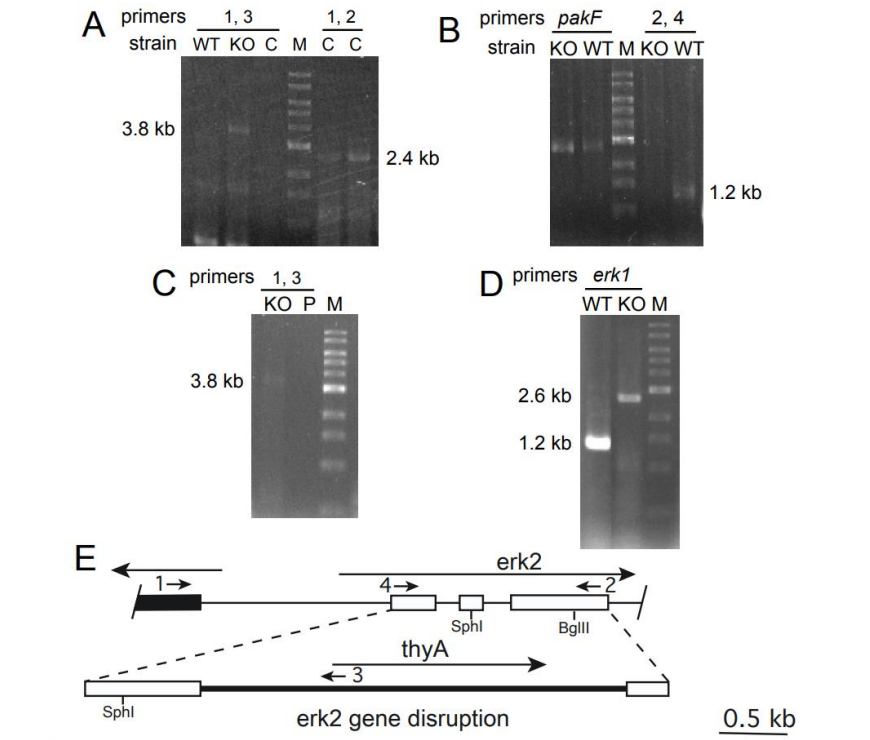
of Erk2 in *Dictyostelium*, and so possible chemotactic regulation of MAPK15 activity might have been overlooked [63–65]. *Dictyostelium* and mammals share many similarities in chemotactic responses including G protein-mediated signaling and a rapid rise in cAMP suggesting similarities could possibly extend to MAPK function and regulation [45,66,67].

The sequence similarity of the *Dictyostelium* Erk2 with the mammalian MAPK15 and inability of these MAPKs to be activated by typical MAP2Ks suggest that these MAPKs might share related functions and regulation [5,10,11]. Thus far no gene disruptions have been created in the animal orthologs but recently mutations within the kinase domain of a nematode (*Caenorhabditis elegans*) ortholog have been shown to interfere with the formation of motile ciliumformation [68]. RNA interference and kinase inhibitor analyses suggest that orthologs in trypanosomes (*Trypanosoma brucei*) are important for proliferation [69,70]. The human MAPK15 has been found widespread in tissue distribution and throughout development and studies using RNA interference suggest this MAPK can regulate proliferation in a variety of cell types [11,71–73]. The corresponding ortholog in flies, Erk7, regulates insulin-like peptide secretion and perhaps this production of a secreted hormone has some analogy with the intercellular signaling associated with Erk1 activation in *Dictyostelium* [74]. Understanding the activation kinetics of mammalian MAPK15 and other orthologs has been hampered due to the limited characterization of possible endogenous extracellular signals that activate these pathways [64,65]. Therefore defining the regulation and function of the *Dictyostelium* Erk2 MAPK in response to known endogenous signals is likely to provide a useful model for characterizing the regulation and function of this group of atypical MAPKs.

The *Dictyostelium* Erk2 MAPK is required for chemotactic movement to both folate and cAMP suggesting Erk2 plays a critical role in the general cellular response to chemoattractants. While not necessary for early chemoattractant detection, the signaling pathway mediated by Erk2 function is responsible for the secondary response of Erk1 phosphorylation. Both Erk2 and Erk1

have overlapping roles in both cell growth and cell movement. In contrast to Erk1, Erk2 is an ortholog of atypical MAPKs and has an activation mechanism independent of conventional MAP2Ks. Therefore, Erk2 represents a good model for the study of atypical MAPKs.

3.5 Supplemental Materials



G

Primer	Nucleotide sequence (5' to 3')
1	GGGTGATCGATATTGCGTGAATATC
2	GGATGAGCCAATGCCTCCTCTGC
3	GGGAAAAACACATACACCAAATGTTCC
4	GGAGCCTATGGTATTGTTTGGGAAGC

Figure S3.1. Verification of genomic insertions. A) PCR amplification of genomic DNA from JH10 (WT), *erk2*$^{-}$ (KO), and *erk2*$^{-}$ complemented with Erk2 vector knock-in (KI). PCR with primers 1 and 3 generates a 3.8 kb band in only the *erk2*$^{-}$ strain. PCR with primers 1 and 2 generatres a 2.4 kb band only in the complemented *erk2*$^{-}$ strains. B) PCR amplification of genomic DNA from *erk2*$^{-}$ (KO) and JH10 (WT) genomic DNA using control primers to another locus (*PakF*) generates fragments in both strains but primers 2 and 4 only generate a fragments in only the JH10 strain and not the *erk2*$^{-}$ strain. C) PCR amplification of genomic DNA from *erk1*$^{-}$*erk2*$^{-}$ (KO) and parental strain *erk1*$^{-}$*thyA*$^{-}$ (P) using primers 1 and 3 produces a 3.8 kb band in only the *erk1*$^{-}$*erk2*$^{-}$ strain. D) PCR amplification of genomic DNA from JH10 (WT) and *erk1*$^{-}$*erk2*$^{-}$ (KO) genomic DNA using *erk1* specific primers produces a fragment 1.4 kb greater in size for the *erk1*$^{-}$*erk2*$^{-}$ strain confirming the disruption of the *erk1* locus with the blasticidin resistance marker. E) Model for expected *erk2* gene disruption in the *erk2* loci in the *Dictyostelium* genomic DNA. F) Model for the expected outcome of the *erk2* disruption. G) List of primers used for PCR verification.

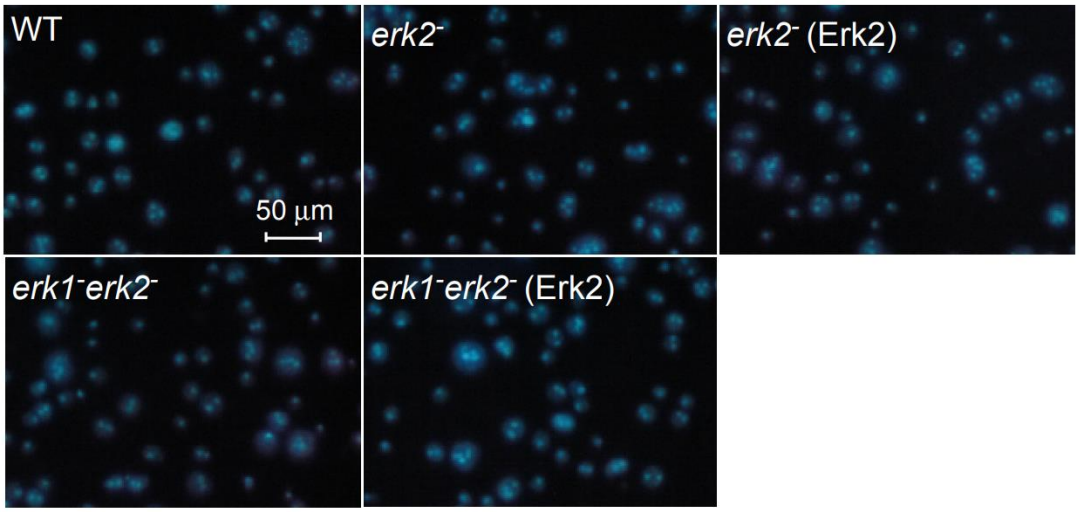


Figure S3.2. Nuclei staining of axenic shaking cultures. Wild-type (WT), *erk2*$^{-}$, *erk1*$^{-}$*erk2*$^{-}$, and the *erk2*$^{-}$ mutant strains complemented Erk2 expression vectors (Erk2) were inoculated into shaking cultures of HL-5 axenic medium for 3 days and then fixed and stained with Hoechst dye to detect nuclei. All images are the same magnification.

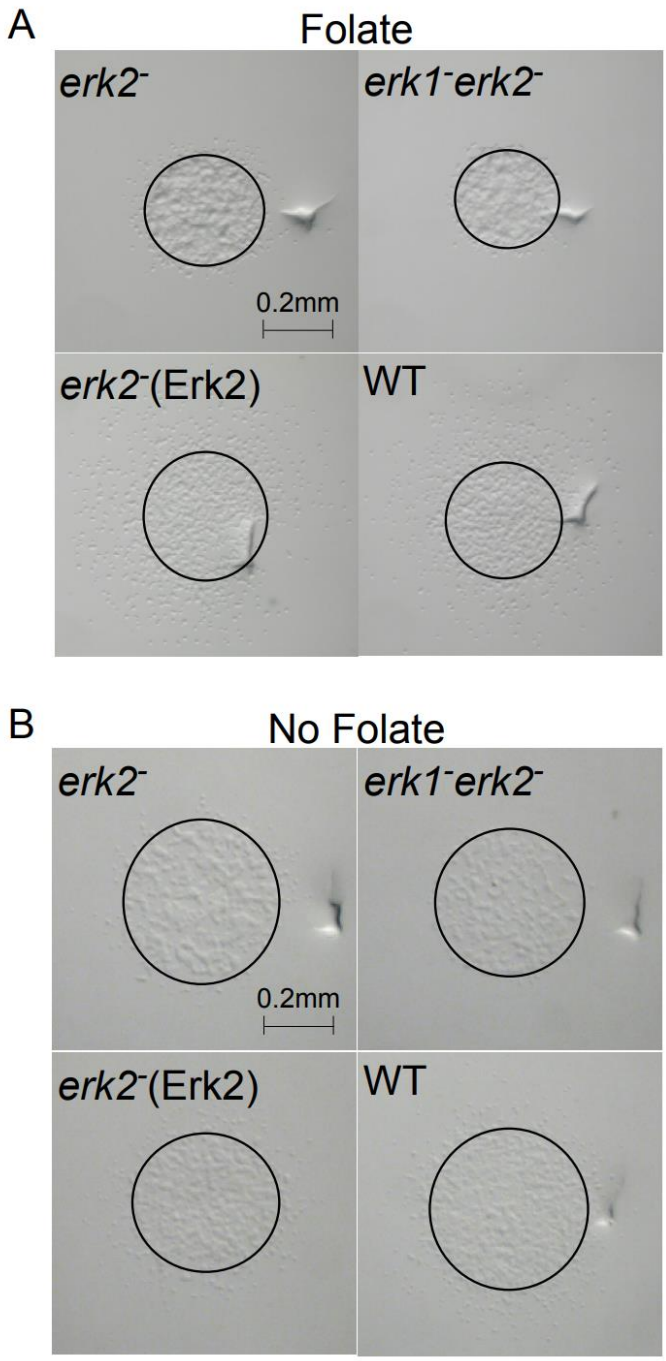


Figure S3.3. Chemotaxis of MAPK mutants to folate. A) Above-agar chemotaxis assay images for wild-type (WT), *erk2*$^-$, and *erk1*$^-$*erk2*$^-$ strains and *erk2*$^-$ mutants complemented with Erk2 vector (Erk2) after 2.5 h exposure to droplets of 100 μM folate. Circle indicates original perimeter of cell droplet and the source of folate is located on the top of each image. B) Same assay as (A) except in the absence of folate exposure. All images are the same magnification.

https://ars.els-cdn.com/content/image/1-s2.0-S0898656818300706-mmc4.mp4

Figure S3.4. Movie of wild-type cell movement in the presence of folate. Time-lapse recording of *Dictyostelium* movement over 33 min period with images collected every 20 s. The folate source is oriented at the upper side of the image.

https://ars.els-cdn.com/content/image/1-s2.0-S0898656818300706-mmc5.mp4

Figure S3.5. Movie of *erk2*$^{-}$ cell movement in the presence of folate. Time-lapse recording of *Dictyostelium* movement over 33 min period with images collected every 20 s. The folate source is oriented at the upper side of the image.

https://ars.els-cdn.com/content/image/1-s2.0-S0898656818300706-mmc6.mp4

Figure S3.6. Movie of *erk2*$^{-}$ mutant complemented with Erk2 expression vector cell movement in the presence of folate. Time-lapse recording of *Dictyostelium* movement over 33 min period with images collected every 20 s. The folate source is oriented at the upper side of the image.

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Figure S3.7. Movie of *erk1*$^{-}$*erk2*$^{-}$ cell movement in the presence of folate. Time-lapse recording of *Dictyostelium* movement over 33 min period with images collected every 20 s. The folate source is oriented at the upper side of the image.

https://ars.els-cdn.com/content/image/1-s2.0-S0898656818300706-mmc8.mp4

Figure S3.8. Movie of *erk1*$^{-}$*erk2*$^{-}$ mutant complemented with Erk2 expression vector cell movement in the presence of folate. Time-lapse recording of *Dictyostelium* movement over 33 min period with images collected every 20 s. The folate source is oriented at the upper side of the image.

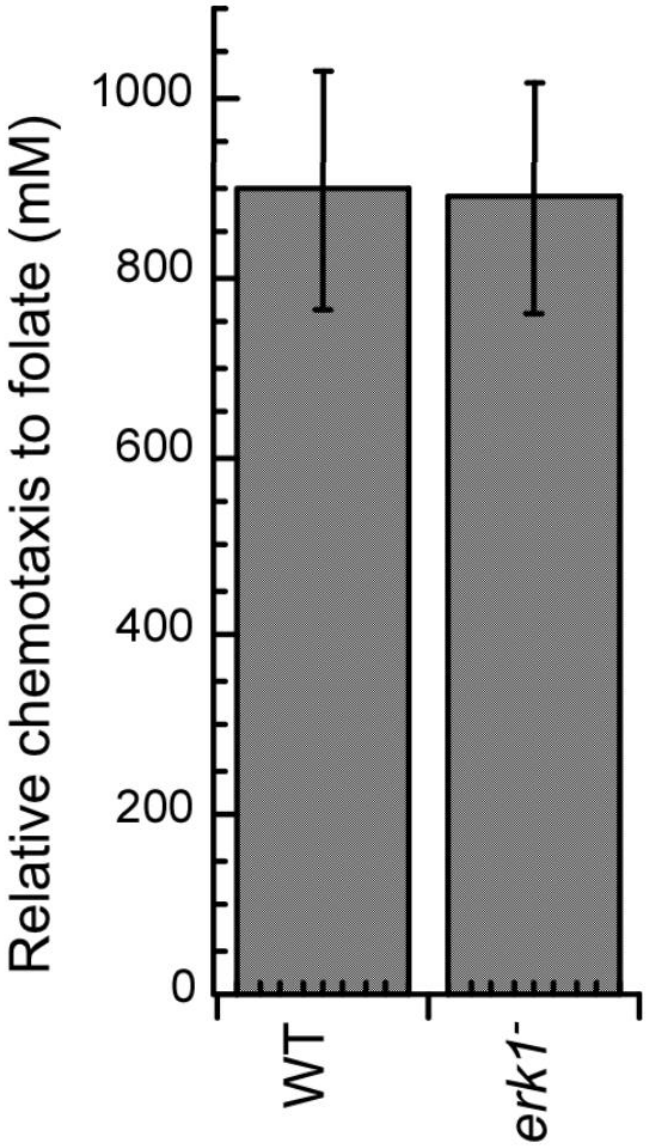

Figure S9. Wild-type (WT) and *erk1*$^{-}$ cell chemotaxis to folate. Folate chemotaxis assays were performed as described in the Methods. Quantitation of wild-type and *erk1*$^{-}$ cell chemotaxis was measured as described for other strains in Fig. 3.4.

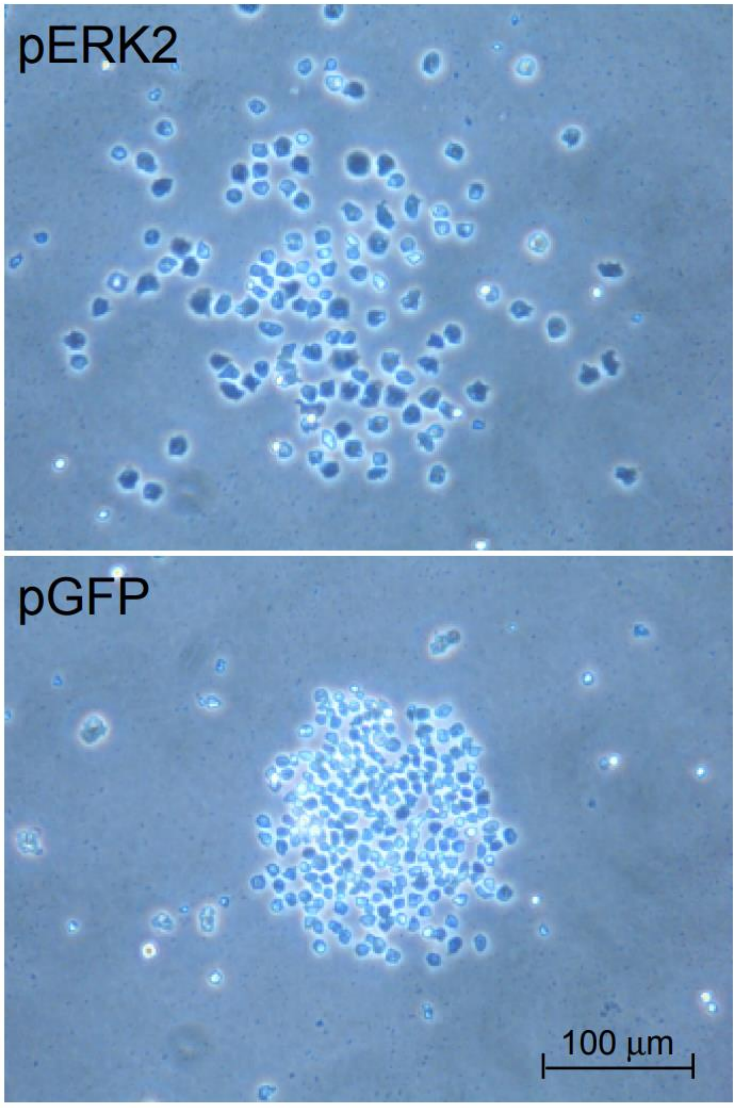

Figure S10. Images of typical *erk1⁻erk2⁻* colonies. The *erk1⁻erk2⁻* cells were electroporated with different expression vectors conferring G418 drug resistance and grown in axenic medium. After several days images were taken of the drug resistant colonies expressing Erk2 or GFP.

3.6 Chapter 4 References

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CHAPTER IV

PHOSPHORYLATION OF THE *DICTYOSTELIUM* RegA PLAYS AN IMPORTANT ROLE IN THE REGULATION OF DEVELOPMENT

Abstract

The association of phosphodiesterases with cAMP-dependent protein kinases in signaling complexes suggests that the regulation of phosphodiesterase function within signaling complexes can be a key determinant of PKA function by modulating local levels of cAMP. In Dictyostelium, the cAMP-specific phosphodiesterase RegA is an important regulator of PKA function with respect to the rate of development and spore encapsulation. To assess the role of protein kinases in the regulation of RegA function phosphorylation sites were identified and the roles of these sites were characterized through the analysis of phosphomimetic and phosphoablative mutations. Mutations of a suspected target of MAP kinase phosphorylation at T676 altered the rate of development suggesting that phosphorylation of this residue reduces RegA function. The phosphoablative mutation also altered the distribution of cells in chimeras implying the phosphorylation of this residue impairs prestalk cell development. Mutations of S412 and S413 also impacted developmental kinetics suggesting the phosphorylation of these residues increases RegA function. Mutations in the S413 residue also altered aggregate formation during development. These results suggest that phosphorylation events in the amino-terminal half of RegA increase function in developmental processes whereas the phosphorylation event near

the carboxyl terminus decreases RegA function

4.1 Introduction

The role of cAMP as an intracellular signal is widespread among many organisms and the modulation of cAMP signaling can have important consequences with respect to cell function and fate [1–3]. The synthesis and turnover of cAMP are regulated through the activities of adenylyl cyclases and phosphodiesterases, respectively, and the target of cAMP signaling in eukaryotes is often cAMP-dependent protein kinases (PKAs) but can include other proteins (e.g., EPACs, ion channels, and popeye domain containing proteins) [4]. Different external stimuli can trigger cAMP signaling to elicit specific cellular responses even within a single cell suggesting that cAMP signaling can be pathway specific. How different pathways can utilize a common diffusible intermediate without activating responses associated with other pathways has been an intriguing question in the area of signal transduction. One mechanism that can account for signaling specificity is the formation of specific signaling complexes that localize cAMP signaling to a particular region within the cell [5–8]. PKA anchoring proteins (AKAPs) can form complexes with PKA and phosphodiesterases allowing the phosphodiesterases to regulate local cAMP levels and PKA activity [9]. Such a mechanism provides upstream regulators of the phosphodiesterase to confer temporal and spatial regulation of cAMP signaling within a signaling complex and pathway. This idea has been supported by the plethora of cAMP phosphodiesterases that exist in some organisms. In mammals, 8 families of cAMP phosphodiesterases have been identified and each family is composed of multiple isoforms that might provide specificity with respect to cellular and tissue distribution [10,11].

The soil amoeba *Dictyostelium discoideum* is an organism that uses both intracellular and extracellular cAMP signaling during its developmental cycle in which solitary cells undergo a multicellular developmental cycle in response to starvation [12–14]. Extracellular cAMP serves

as an intercellular signal that allows for chemotactic movement and the formation of multicellular aggregates. Aggregates undergo a variety of morphological stages including a migratory slug stage before culminating development as fruiting bodies composed of a mass of spores upon a stalk structure. During this developmental life cycle, the individual cells undergo sorting and differentiation to form prespore or prestalk cells which eventually contribute to the fruiting body [15]. Both cellular differentiation and cell movement include intracellular cAMP signaling as suggested by the phenotypes observed for cells lacking or overexpression PKA function [16]. Mutants lacking the PKA catalytic subunit do not aggregate or differentiate into spores or stalk cells and cells with excessive PKA activity (e.g., due to the loss of regulatory subunit or overexpression of the catalytic subunit) display defective aggregation and rapid cellular differentiation into spores [16–20]. The intracellular cAMP-specific phosphodiesterase, RegA, is expressed during growth and development where it plays an integral role in regulating the kinetics of development [21–23]. Loss of RegA results in small aggregate formation and spore formation at 16-18 hrs of development whereas wild-type cells form spores closer to 20-24 hrs of development [24]. Overexpression of RegA delays developmental progression consistent with internal cAMP signaling being an important factor in regulating developmental kinetics. The loss of RegA or PKAR (PKA regulatory subunit) have similar developmental phenotypes (aggregation defects and precocious spore encapsulation) and presumably, these phenotypes result from increase PKAC function. Some studies suggest that RegA can also associate with PKA, at least in vitro [23]. This process functions to aide in the termination phase of PKA signaling where RegA rapidly clears intracellular cAMP [25].

Levels of cAMP in *Dictyostelium* are rapidly increased in response to the stimulation of G protein-coupled receptors by external signals such as the chemoattractants [26]. Extracellular cAMP and folate can both stimulate receptors, cARs and Far1, respectively leading to a burst of cAMP accumulation that peaks around 1 min after stimulation [27–30]. These stimuli also lead to

the activation of the MAPK Erk2 that is important for cAMP signaling [31–33]. A leaky *erk2*^{RE} (reduced expression) allele does not allow sufficient cAMP production to allow aggregation but disrupting *regA* function can restore aggregation and development suggesting Erk2 is a negative regulator of RegA [34,35]. A putative MAPK phosphorylation site at residue T676 in RegA is required for this regulation implying that Erk2 down regulates RegA function through direct phosphorylation [35]. RegA is also regulated through an aspartic acid phosphorylation mediated through a histidine kinase pathway. This regulation occurs late in development and is important for sporulation [36,37].

Studies in mammalian cells suggest that cAMP-specific phosphodiesterases such as members of the PDE4 family can be regulated by multiple mechanisms that include regulators such as MAPKs, PKA, and potentially other kinases [38–43]. Most of these studies have been conducted in cell lines that might have altered signaling mechanisms and most studies have described molecular interactions rather than changes in physiology or cell function. Given that regulation of RegA is critical for multiple developmental processes, we sought to further examine the role of phosphorylation on phosphodiesterase function during development. Potential phosphorylation sites were assessed through mass spectrometry and genetic analysis was used to examine the role of potential phosphorylation events in *Dictyostelium* development.

4.2 Methods

4.2.1 Strains and Cell Culturing

All *Dictyostelium* strains were isogenic to the wild-type strain, KAx-3, except where noted. Cells were grown axenically in HL5 medium or on bacterial lawns of *Klebsiella aerogenes*. *regA* was disrupted in both KAx-3 and JH10, a stain that is auxotrophic for thymidine, using a *regA* gene disruption construct as previously described [24,44,45]. Strains with disrupted *thy1* gene were maintained in HL5 with supplemental thymidine. The *regA* gene disruption was

verified by PCR amplification of genomic DNA using PCR as previously described [45]. Electroporation of *Dictyostelium* was performed as previously described [46]. Transformed cells were selected for and maintained in HL5 containing 2-5 μg/ml of the drug G418 or in HL5 in the absence of exogenous thymidine. RegA expression in transformed cells was verified by immunoblot analysis using antiserum generated against a RegA peptide located near the amino terminus.

4.2.2 Recombinant DNA Constructs

The *regA* open reading frame was PCR amplified from plasmid pDT12 using oligonucleotides to introduce flanking BamHI, XhoI, and XbaI restriction sites. The *regA* amplicon was inserted with BamHI and XbaI into the integrating cloning vector pDXA-3H, which contains a constitutive actin promotor for overexpression of *regA* [47]. Site-directed mutagenesis was used to construct the *regA* mutants *regA*^{S142E}, *regA*^{S142A}, *regA*^{S413E}, *regA*S413A, *regA*^{T676E}. Primers used for site directed mutagenesis are listed in (Supplemental Materials Table S4.1). The *regA*^{T676A} allele was a generous gift from G. Shaulsky and fragment containing this allele was inserted into a RegA construct like the other RegA mutations [35]. These mutations were chosen to either prevent the phosphorylation at the residue of interest (alanine substitution) or to constitutively mimic a phosphorylation at the residue of interest (glutamic acid substitution) (Supplemental Materials Fig. S4.1). This functions Site directed mutations were screened for undesired mutations and confirmed by DNA sequencing. A knock-in vector was constructed by inserting a 3.2 kB BamHI/SalI fragment containing the *Dictyostelium THY1* gene and then inserting the *regA* amplicon with BamHI and XbaI into pBluescript- (Stratagene). These knock-in vector were constructed for the wildtype *regA* amplicon as well as the phosphorylation mutations made with site directed mutagenesis. The wildtype *regA* amplicon was also inserted into the *Dictyostelium* extrachromosomal vector, pTX-FLAG, using XhoI and XbaI [48].

4.2.3 Dictyostelium Developmental Phenotype Analysis

Cells were grown overnight in shaking cultures to mid-log phase (~3×10^6 cells/ml) and then pelleted by centrifugation [49]. Cells were washed in phosphate buffer (12 mM NaH_2PO_4 pH 6.1) and resuspended in 1×10^8 cells/ml. Cells were plated on non-nutrient agar plates for development at 1×10^8 cells/ml or diluted 1:1 with phosphate buffer (0.5×10^8 cells/ml). Pictures were taken at multiple time points during development as indicated. To investigate the effect of the mutated putative phosphorylation sites on cell differentiation, all phosphorylation mutant clones, *regA* null, and the *regA* wildtype rescue clones was transformed with pTX-GFP, a GFP expression vector [48]. The labeled cell lines were mixed with unlabeled *regA* wildtype rescue clones (1:10 ratio) prior to plating on non-nutrient plates to construct chimeras. Fluorescent images were captured using fluorescence microscopy.

4.2.4 Immunoprecipitation of FLAG-RegA

regA null cells transformed with pTX FLAG-*regA* vector were starved and stimulated with cAMP for 6 hours. Samples were pulsed with a final 100 µM cAMP stimulation and then time points were taken at 0, 0.5, 1, 2, and 5 minutes. When harvesting cells for an assay, cells were added to excess ice-cold phosphate buffer (10mL), pelleted, and frozen at -80°C. Cells were lysed by thawing in 0.5% v/v Triton X-100 immunoprecipitation lysis buffer composed of 50mM Tris-HCl pH 8, 150 mM NaCl, protease inhibitors (10µg/ml aprotinin, 10µg/ml leupeptin, and 1mM PMSF), and phosphatase inhibitors (3 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM Sodium-orthovanadate, and 25 mM β-glycerophosphate). These time points were combined and incubated with OctA-probe antibody (Santa Cruz Biotechnology, sc-166355) overnight at 4°C on a test tube rotator. After incubation, 20µL 50% Protein A Agarose slurry (9863S, Cell Signaling) was added and incubated for 2 h on a test tube rotator. The immunoprecipitate was washed 3 times with lysis buffer lacking PMSF and then twice in wash

buffer (50mM Tris-HCl pH 8, 150 mM NaCl). SDS-PAGE loading buffer was added to the immunoprecipitate and SDS-PAGE was performed.

4.2.5 Phosphoprotein Mass Spectroscopy Analysis

The FLAG-RegA band was excised, destained, reduced, and alkylated prior to an in-gel digestion. Mass Spectral analysis of RegA phosphorylation was performed twice using two sets of proteases to construct different peptide fragments for analysis. First, a triple protease approach was used with trypsin, elastase, and subtilisin. To further expand the RegA coverage map, a second protease approach of trypsin and V8 was used. Phosphopeptides were purified and then analyzed by nano-scale reverse phase chromatography before analysis with a hybrid LQ-Orbitrap mass spectrometer. MS/MS scans employed multi-stage activation in the Orbitraps LTQ sector because phosphate groups are typically lost prior to fragmentation of the peptide backbone. Phosphopeptides were identified by database searching using proteomics software applications and scored for statistical significance (following the "Paris" guidelines). Spectra was inspected manually to confirm neutral loss fragments. Confirmatory LC-MS/MS was performed on each candidate peptide using the Orbitrap's ultra-accurate FT sector to provide unambiguous confirmation of peptide identifications.

4.3 Results

4.3.1 RegA has Multiple Phosphorylation Sites

The important role of cAMP in the developmental life cycle of *Dictyostelium* and the impact of RegA function on developmental progression suggests that RegA might serve in multiple signaling pathways and therefore be regulated by multiple mechanisms. Phosphodiesterase function in other organisms has been reported to be regulated by a variety of protein kinases and therefore the *Dictyostelium* RegA could potentially be regulated by other protein kinases than the currently known interactions. Earlier studies have verified the

phosphorylation of RegA at D212 through a two component histidine kinase response regulator mechanism late in developmental spore formation and another study has suggested that the MAPK Erk2 phosphorylates T676 in early development [24,35]. To search for RegA phosphorylation sites during early development, a mass spectrometry analysis of phosphopeptides was conducted on FLAG-tagged RegA immunoprecipitated from cAMP stimulated cells. The expected phosphorylation of the T676 residue was not observed in this analysis because the peptides spanning this region were not observed in the 77% coverage of the RegA protein. Additional analyses with different proteolytic digestion strategies of the RegA protein did not alleviate the lack of peptide coverage in this region. However, phosphopeptides in other regions of RegA suggest that residues S142 and S413 can be phosphorylated (Fig. 4.1, Supplemental Materials Fig. S4.2). The S413 residue is part of a PKA phosphorylation site motif (RRXS) suggesting that PKA might phosphorylated this residue. PKA phosphorylation sites in mammalian phosphodiesterase PDE4E have been reported to serve as a mechanism for up regulating phosphodiesterase activity leading to reduced cAMP levels and a negative feedback mechanism to down regulate of PKA activity. However, the PKA phosphorylation site of mammalian PDE4E is a residue near the be amino terminal side of the regulatory domain and the RegA S413 residue lies at the carboxyl side of the regulatory domain near the center of the RegA protein. The other phosphorylated residue S142 is located with the regulatory domain and is followed by a proline residue suggesting this site might be phosphorylated by MAPKs or Cdc2-related kinases. This N-terminal site does not contain the minus 2 proline often observed in MAPK target motifs (PXS/TP). The phosphorylation of the D212 residue was not detected in the mass spectrometry analysis even though peptides were identified in that region of the RegA protein. This absence of D212 phosphorylation is perhaps consistent with this modification being used to promote spore encapsulation later in development.

Figure 4.1 Mass Spectroscopy phosphoprotein analysis of RegA. FLAG-tagged RegA was analyzed for phosphorylation sites by mass spectroscopy. Two previously unidentified phosphorylation sites were detected (S142 and S413). Gray shaded amino acid residues denotes the peptide coverage that was produced from the protease digestion and detected by the mass spectral analysis. Yellow shaded amino acids indicates detected phosphorylated peptides by the mass spectral analysis. Green shaded amino acid residues indicates detected phosphorylated residues from the analysis. Red outlined amino acid residues indicates putative phosphorylation sites, either from this analysis or from prior studies.

In general, sequences in the C-terminal catalytic domain of cAMP phosphodiesterases are highly conserved but the regulatory domain sequences are more divergent when comparing Dictyostelids to each other and to mammals. The putative MAPK phosphorylation site T676 and surrounding sequences in the *Dictyostelium discoideum* RegA, is highly conserved in Dictyostelids and other protists (e.g., *Acanthamoeba*) (Fig. 4.2A). This conservation includes the proline at the -2 position (PXS/TP) motif which is typical of many MAPK target sequences. The mammalian PDE4D protein also has a serine (S521) at this alignment, but studies in cell lines suggest that a MAPK phosphorylates another position for the regulation of the phosphodiesterase PDE4D3 (S579) [41]. The S413 residue and upstream positively charged arginine residues (RRXS) are conserved among Dictyostelids but not in other protists (Fig. 4.2B). In contrast, the S142 residue is conserved in some but not all Dictyostelids (Fig. 4.2C). However, the N-terminal region of phosphodiesterases is highly divergent and can contain long runs of repeated residues in Dictyostelids. The differences in the conservation of these kinase recognition sites suggests that

phosphorylation of cAMP phosphodiesterases can be species specific or potentially widespread among diverse eukaryotes.

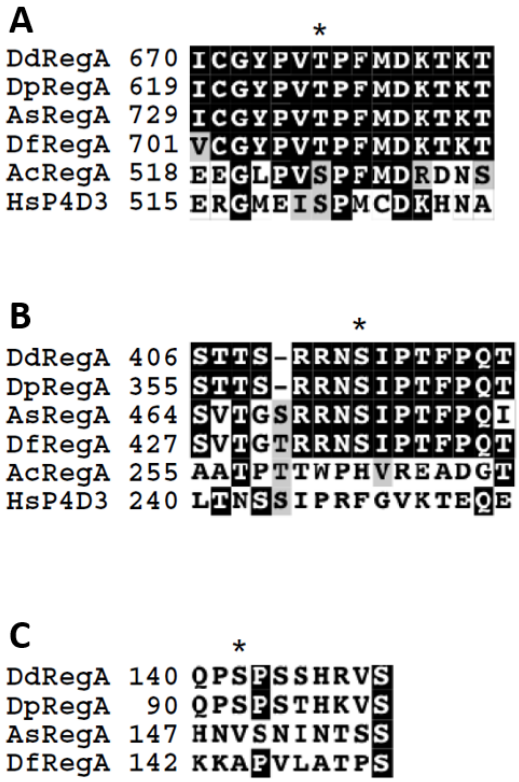


Figure 4.2 Multiple sequence alignment of phosphodiesterases around putative *D. discoideum* RegA phosphorylation sites. Dictyostelids (*Dd, Dp, As,* and *Df*) and *Acanthamoeba castellanii* (Ac) RegA amino acid sequences were analyzed for conservation around *D. discoideum* phosphorylation sites of interest. The Human (*Hs*) phosphodiesterase PDE4D3 was also included in the alignments for the *D. discoideum* RegA T676 and S413 residues. A) Multiple sequence alignment of both amoeba and human phosphodiesterases around the threonine 676 phosphorylation site of the *D. discoideum* RegA. B) Multiple sequence alignment of both amoeba and human phosphodiesterases around the serine 413 phosphorylation site of the *D. discoideum* RegA. C) Multiple sequence alignment of amoeba phosphodiesterases around the serine 142 phosphorylation site of the *D. discoideum* RegA. Black shaded amino acid residues indicate residues that are identical in the alignments. Gray shaded amino acid residues indicate similar aligned amino acids. Non-

shaded residues denotes amino acid residues that are not identical or similar. Asterisks indicates the phosphorylation residue of interest from *D. discoideum* RegA.

4.3.2 Genetic Analysis of Phospho-mimetic and Phospho-ablative Mutants

To further investigate the potential role of phosphorylated residues in regulating the function of RegA, site-specific mutations were created in the *regA* gene to create phospho-mimetic and phospho-ablative mutants. These mutant *regA* alleles were inserted into *Dictyostelium* expression vectors driven by the constitutive Act15 promoter and the vectors were transformed into *regA* null mutants to assess developmental phenotypes. This initial approach was complicated by a gross overexpression of RegA that can delay developmental progression as previously reported and the challenge of identifying mutants with similar levels of RegA expression [24]. To reduce the variability and overexpression of the mutant *regA*, the open reading frame sequences of the *regA* alleles were inserted into vectors containing the *thy1* gene and integrated by single crossover into the disrupted *regA* locus of a *regA*$^-$*thy1*$^-$ strain (Supplementary Material S4.3A and B). The single recombination results in only a single copy of the wild type or mutant allele to be expressed from the endogenous *regA* promoter. Any additional integrated copies of the *regA* ORFs at the *regA* endogenous locus lack transcriptional regulatory sequences. These knock-in alleles of RegA were verified using PCR analysis (Supplementary Material S4.3C and D) and relatively uniform RegA levels were detected through immunoblot analysis (Fig. 4.3).

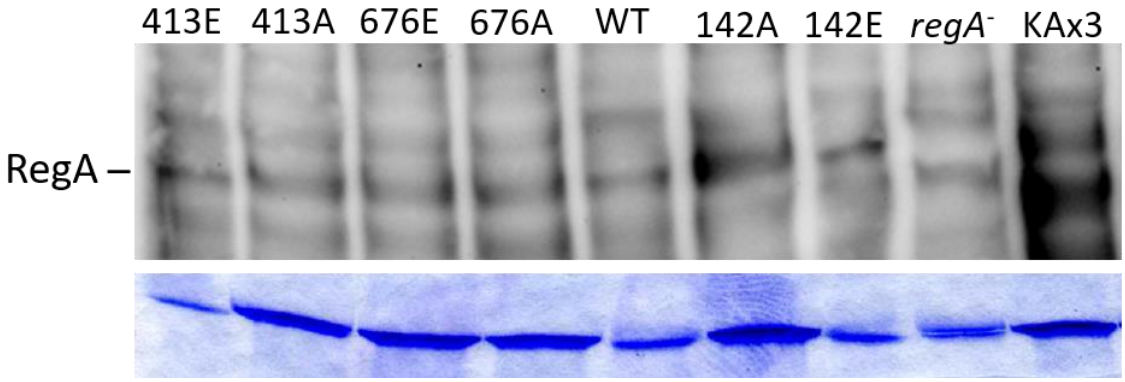


Figure 4.3 Detection of RegA levels. Cell extract was prepared from cells starved for two hours in phosphate buffer. RegA was detected using antibody raised to a peptide fragment at the amino terminal of RegA (GenScript, peptide: PSSHRVSDFSDEYSPC). The RegA band is detected in all of the knock-in strains at relatively uniform levels and slightly higher in KAx3 cells. The RegA band was not detected in the *regA*$^{-}$ cell line. Commassie blue stained gel was used as a loading control. All knock-ins were also verified by PCR amplification as detailed in the Supplementary Material.

The levels of RegA protein in the mutant and wild-type allele knock-in strains were lower than that observed from the strain KAx3 from which the *thyA*$^{-}$*regA*$^{-}$ strain was derived. This reduced expression is likely to result from the absence of a signature transcriptional termination sequence and possible genetic background differences. However, the wild-type knock-in allele developed with a similar morphology and only slightly accelerated kinetics compared to the KAx3 strain suggesting sufficient RegA protein is produced in these strains and that the mutant alleles can be accurately compared to the wild-type allele without the complexity of transcriptional expression variability (Fig 4.4).

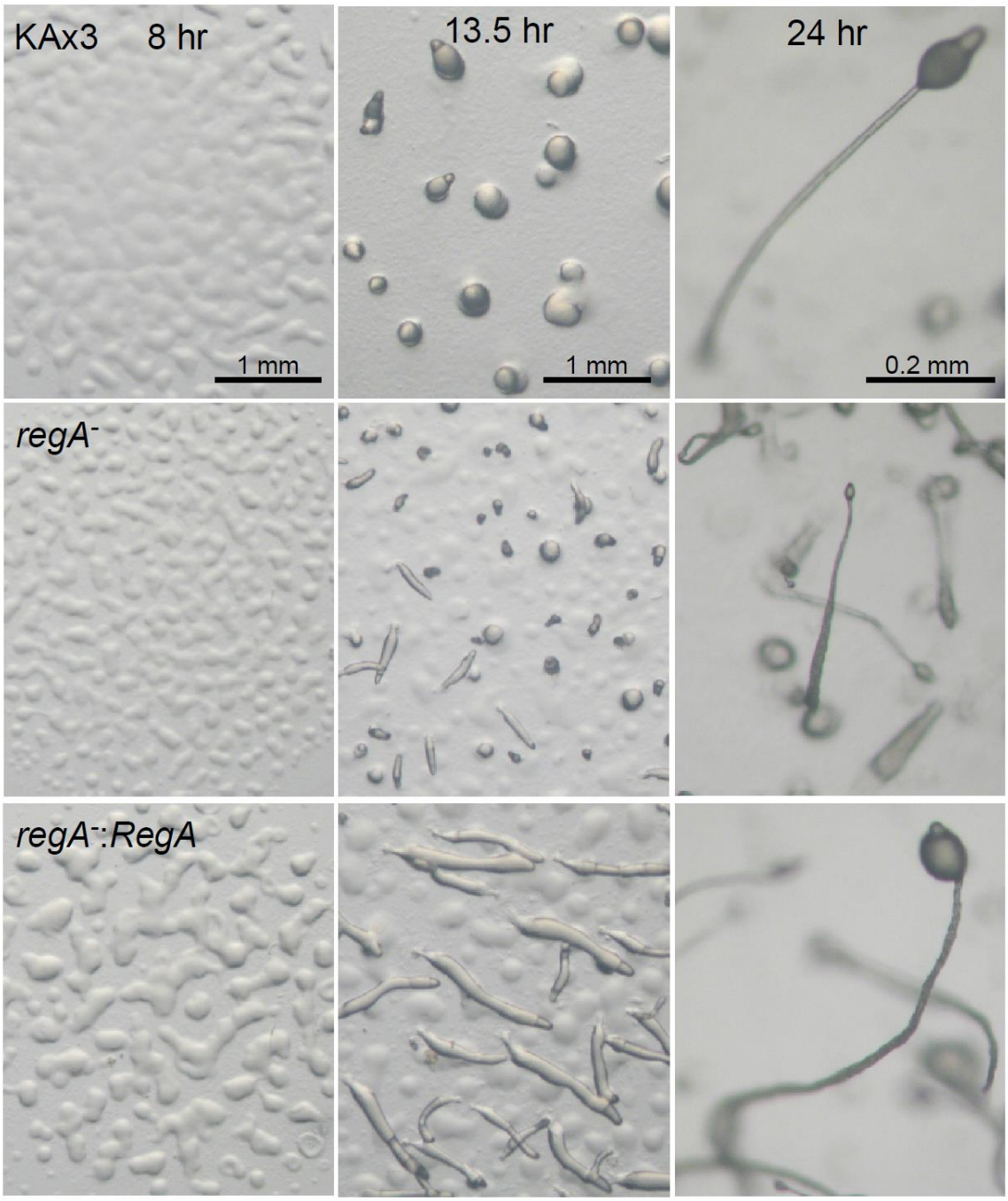


Figure 4.4 Morphological development of KAx3, *regA*$^{-}$, and wild-type allele knock-in. All strains were plated on non-nutrient phosphate plate as described in the methods section (4.2.3). Pictures were taken at the indicated hours after starvation. All images in a column were photographed at the same magnification and a scale bar indicates accurate distance.

4.3.3 Alteration of T676 Residue Impacts RegA Function and Prestalk Differentiation

A previous study suggested that Erk2 phosphorylates the RegA T676 residue to down regulate RegA phosphodiesterase activity. Supporting this idea, the *regA*^{T676E} phospho-mimetic mutant displayed an acceleration in morphological development compared to the phospho-ablative (*regA*^{T676A}) mutant and wild-type control (Fig. 4.5A). The acceleration primarily affected the transition of the aggregates into migratory slugs and then fruiting body structures. The fruiting body morphology of the *regA*^{T676E} strain was aberrant with a small spore head and a collection of spores at the base of the stalk, similar to *regA*$^{-}$ strains (Fig. 4.5B). In addition, the *regA*^{T676E} strain produced spores around 18 hours of starvation and this phenotype closely resembled the *regA*$^{-}$ precocious spore production around 16 hours of starvation. These late-developmental stage phenotypes suggest that the phosphorylation of this residue could impact the culmination process. The phospho-ablative *regA*^{T676A} mutant showed a contrasting phenotype with a delay in development affecting the transition from aggregates to slugs and the fruiting body structures resembled wild-type morphology. The *regA*^{T676A} mutant produced spores at a delayed rate relative to the wild-type control. These phenotypes resemble those of strains overexpressing *regA* and suggest the phospho-ablative mutant has excessive RegA function due to the lack of down regulation associated with the phosphorylation of the T676 residue. These observations are consistent with the idea that phosphorylation of the T676 residue as a mechanisms to down regulate RegA function during aggregation and early stages of multicellular development.

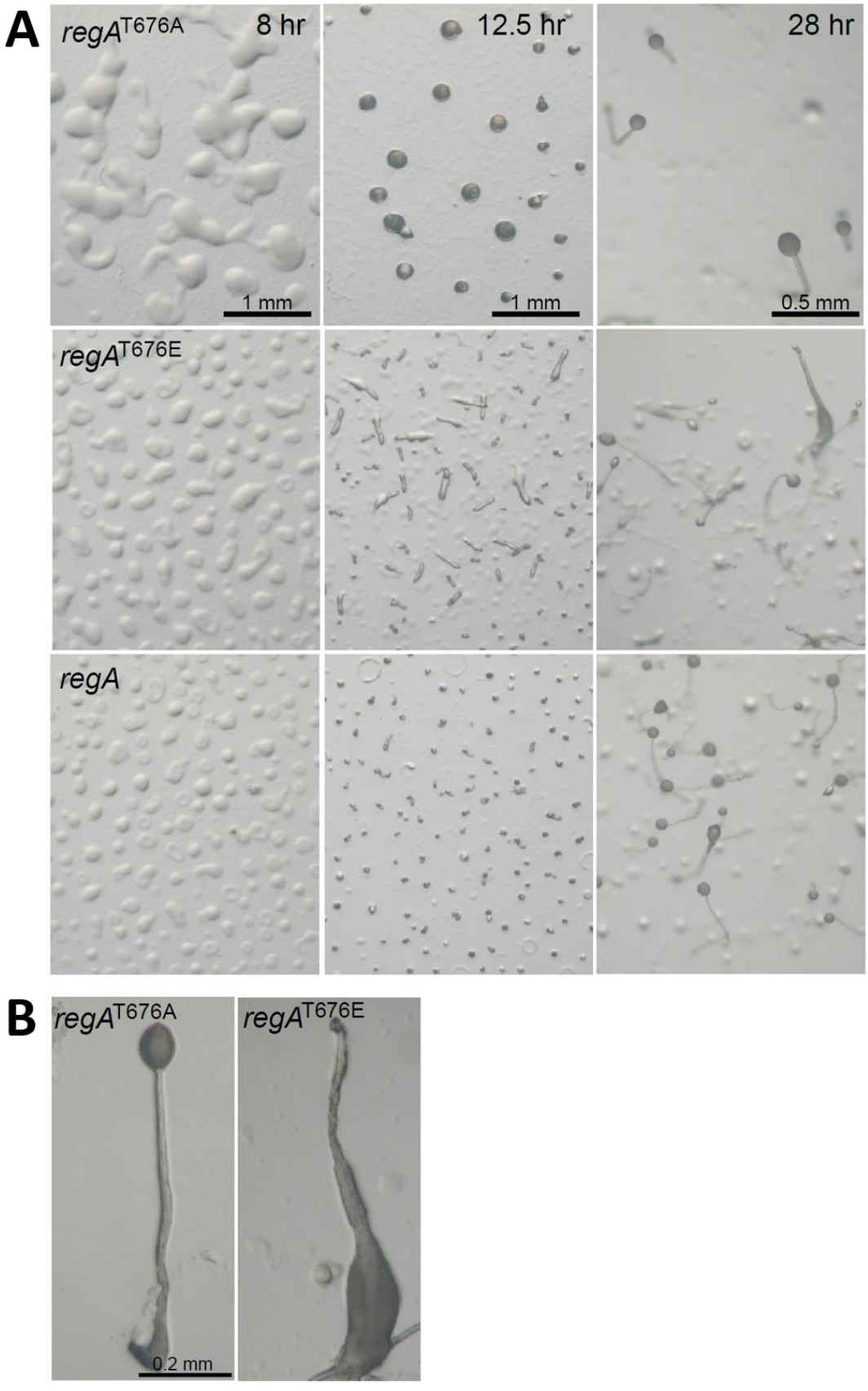

A
regA^T676A
8 hr
12.5 hr
28 hr
1 mm
1 mm
0.5 mm
regA^T676E
regA
B
regA^T676A
regA^T676E
0.2 mm

Figure 4.5 Morphological development of *regA*^{T676A}, *regA*^{T676E}, and wild-type allele knock-in. A) All strains were plated on non-nutrient phosphate plate as described in the methods section (4.2.3). Pictures were taken at the indicated hours after starvation. All images in a column were photographed at the same magnification and a scale bar indicates accurate distance. B) Fruiting bodies for *regA*^{T676A} and *regA*^{T676E} strains were photographed at 6x magnification to show detail of fruiting body morphology.

Labeling T676 mutant and wild-type strains with a GFP expression vector was used to track biasness in spatial distributions during multicellular development in a chimera because changes in cAMP signaling might impact cell distribution or differentiation when developed with non-labeled wild-type cells. Additionally, the disrupting *regA* results in a strong bias for prestalk B cell development and gene expression when developed in a chimera with wild-type cells [50]. Interestingly, the *regA*^{T676A} mutant, which was observed to result in increased RegA activity, displayed a strong bias for localization near the anterior region of the chimeric multicellular aggregate (Fig. 4.6). Cells in this anterior region are typically prestalk A cells that eventually contribute to the development of the stalk of fruiting bodies. The ability of *regA*^{T676A} mutants to develop with normal morphology in clonal populations without the increase of biasness toward prestalk development suggests a mechanism to compensate biasness in cell movement or cell differentiation [50].

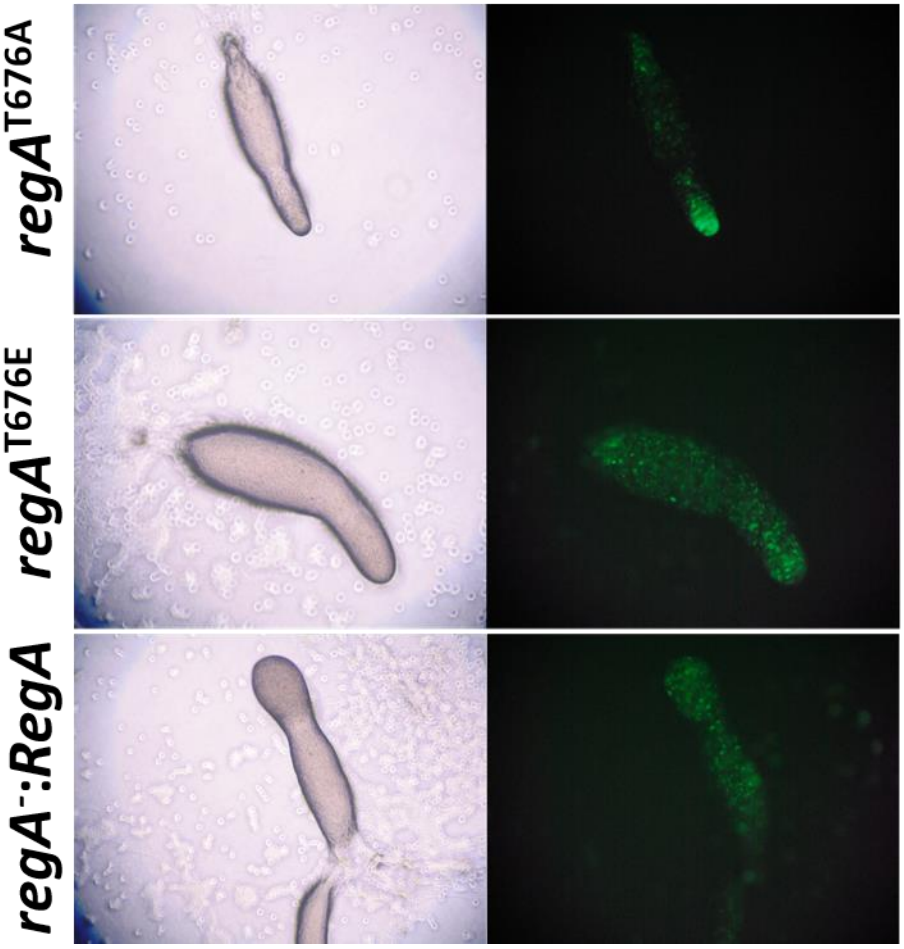


Figure 4.6 Chimeras of knock-in T676 mutations expressing a GFP vector and wild-type cells without GFP vector. GFP expressing knock-in cells were mixed 1:10 with non-labeled wild-type allele knock-in. Bias in localization is observed by preferential sorting in areas of slug during development. Pictures taken at 14 hours into development.

4.3.4 Phosphorylation of the RegA S413 Residue Impacts Aggregate Size and Developmental Kinetics

Development phenotypes of the phospho-ablative and phospho-mimetic mutations were observed to investigate a potential function phosphorylation at S413. Cells expressing the phospho-mimetic mutant (*regA*^{S413E}) are delayed in the aggregate to slug transition relative to wild-type controls (Fig. 4.7A). The S413A phospho-ablative mutation resulted in large aggregates

that showed similar developmental kinetics to wild-type control cells rather than an expected acceleration in developmental rate. Both mutant strains produce normal fruiting body structures suggesting that modifying this residue does not affect the culmination process (Fig. 4.7B). These observations suggest that the phosphorylation of this residue is important for establishing aggregate size and that dephosphorylation of this residue is necessary for appropriate transition from the mound to the slug. Spore production was delayed in the *regA*^{S413E} further suggesting that this mutation resulted in an increase of RegA activity. The delay in the phospho-mimetic phenotype suggests that phosphorylation of the S413 residue increases RegA function and subsequently delays developmental transitions by lowering cAMP levels, assuming reduced cAMP levels delay development at this stage. Neither S413 mutant displayed any biasness in cell localization in chimeric aggregates when developed with wild-type cells (Supplemental Material, Fig. S4.4) This residue is likely phosphorylated by PKA based on the surrounding sequence (RRNS) matching the conserved PKA substrate phosphorylation site (RRXS/T) suggesting a possible negative feedback mechanism to limit PKA function. While the phosphorylation of this residue was detected by mass spectroscopy during early development, attempts to identify the phosphorylation of this residue in immunoblots using antibodies that recognize PKA phosphorylated proteins were unsuccessful (data not shown, 9624, Cell Signaling). These attempts were conducted on early and late stages of aggregation. This lack of detection suggests that either this region is not recognized efficiently with the antibody or the amount of RegA phosphorylated at this site is very limited.

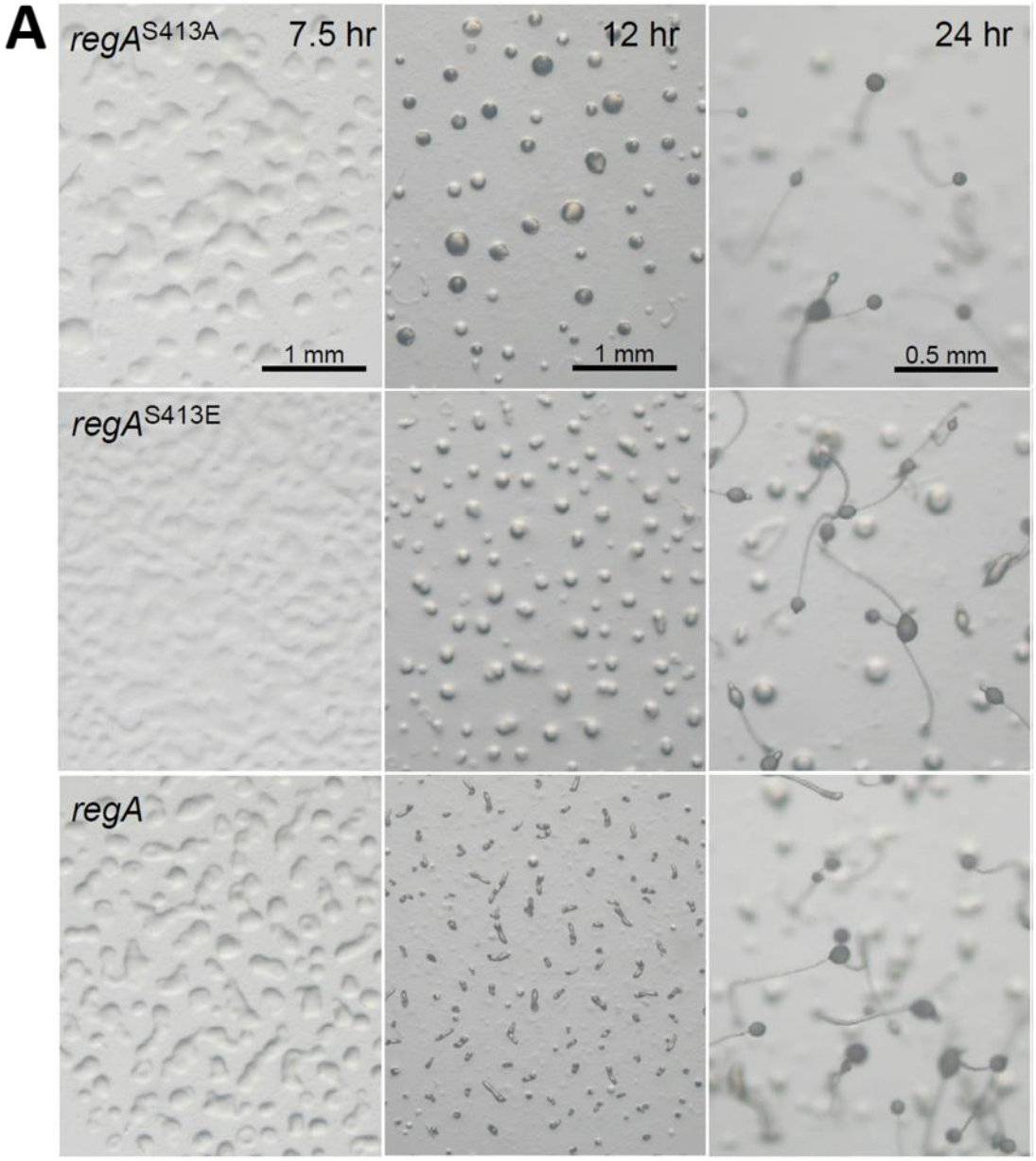

A
regA^S413A
7.5 hr
12 hr
24 hr
1 mm
1 mm
0.5 mm
regA^S413E
regA
B
regA^S413A
regA^S413E
0.2 mm

Figure 4.7 Morphological development of *regA*^{S413}, *regA*^{S413}, and wild-type allele knock-in. A) All strains were plated on non-nutrient phosphate plate as described in the methods section (4.2.3). Pictures were taken at the indicated hours after starvation. All images in a column were photographed at the same magnification and a scale bar indicates accurate distance. B) Fruiting bodies for *regA*^{S413A} and *regA*^{S413E} strains were photographed at 6x magnification to show detail of fruiting body morphology.

4.3.5 Phosphorylation of the RegA S142 Residue Impacts the Kinetics of Developmental Morphology

The function of the S142 phosphorylation event in the upstream regulatory region of RegA was investigated using the phosphorylation mutations. The expression of the *regA*^{S142E} mutant results in a delay in aggregate formation during early development and the transition from mound to the slug stage (Fig. 4.8A). The phospho-ablative *regA*^{S142A} mutant accelerates development for the mount to slug stage transition and the fruiting body structure is similar to that of *regA*$^{-}$ aggregates (Fig. 4.8B). The impact in fruiting body structure in the *regA*^{S142A} mutant suggest that modifying this residue affects the culmination process. The *regA*^{S142E} mutant also results in a delay in spore production relative to wild type and while the *regA*^{S142A} mutant precociously produces spores similar to that of *regA*$^{-}$ cells. These observations suggest that phosphorylation of the S142 residue increases RegA function during development. Neither S142 GFP expressing mutant showed any bias in cell localization when developed as a chimera with wild-type cells suggesting that this phosphorylation site does not have a role in cell localization in development (Supplemental Materials, Fig. S4.5).

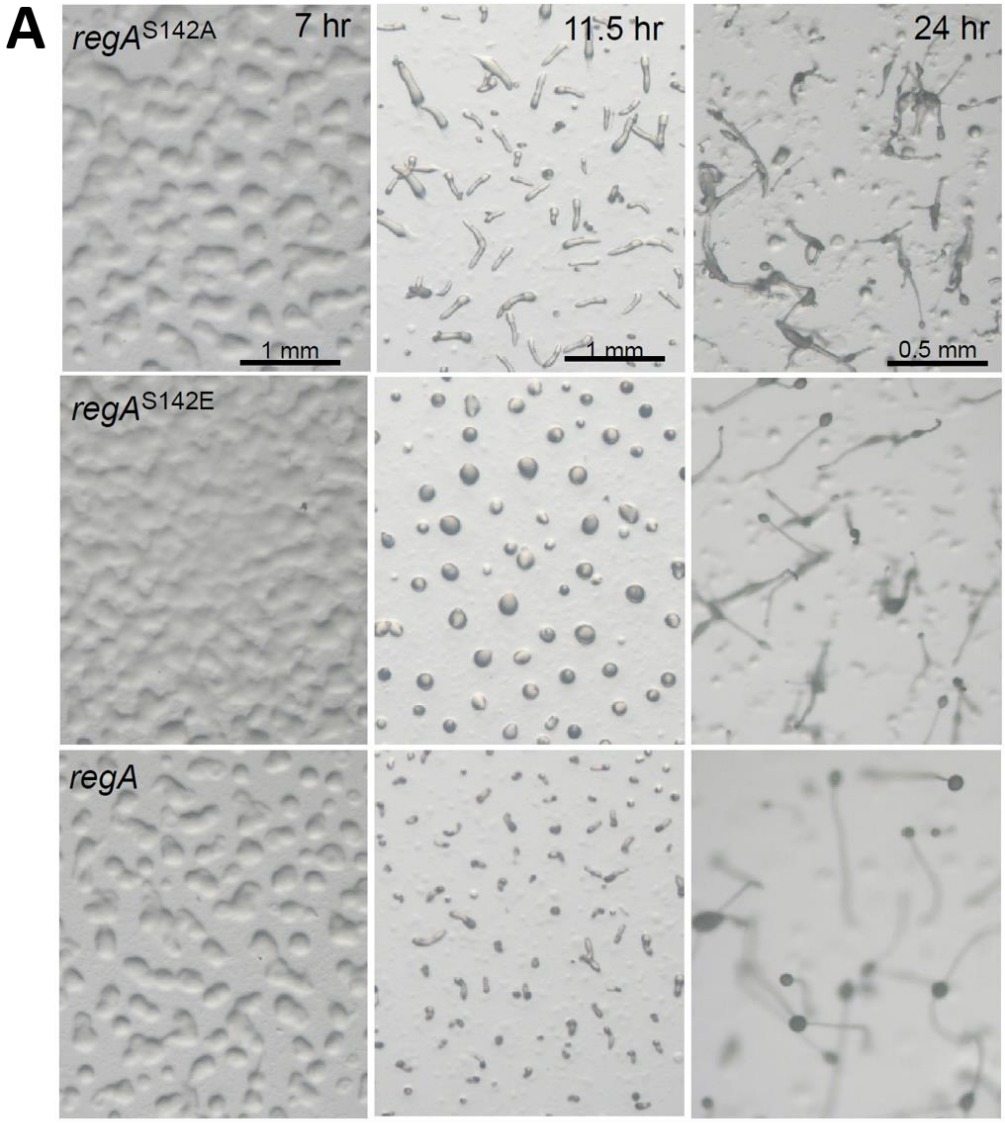

A
regA^S142A
7 hr
11.5 hr
24 hr
1 mm
1 mm
0,5 mm
regA^S142E
regA
B
regA^S142E
regA^S142A
0.2 mm

Figure 4.8 Morphological development of *regA*^{S142A}, *regA*^{S142E}, and wild-type allele knock-in. A) All strains were plated on non-nutrient phosphate plate as described in the methods section (4.2.3). Pictures were taken at the indicated hours after starvation. All images in a column were photographed at the same magnification and a scale bar indicates accurate distance. B) Fruiting bodies for *regA*^{S142A} and regA^{S142E} strains were photographed at 6x magnification to show detail of fruiting body morphology.

4.4 Discussion

The delayed developmental transition of *regA*^{T676A} results in delayed developmental kinetics similar to that observed for r*egA*^{S413E} and *regA*^{S142E} suggesting that the effect of the mutated phosphorylation sites are similar by altering the overall activity of RegA since levels of RegA has an impact on developmental kinetics. However, these sites are suggested to have different roles for regulating RegA function, as mimicking the phosphorylation event through glutamic acid substitutions resulted in an increase of development relative to wild-type for the *regA*^{T676E} mutant and a delay of developmental kinetics for the r*egA*^{S413E} and *regA*^{S142E} mutations. Additionally, the *regA*^{T676A} mutant showed biased cellular localization towards the anterior end of the slug while the *regA*^{S142E} and r*egA*^{S413E} mutants did not. The S142 and S413 sites are supported to function in activating RegA when phosphorylated by an unknown kinase and PKA respectively. In contrast, the T676 site is supported to result in the inhibition of RegA when phosphorylated by Erk2 (Fig 4.9). The *regA*^{S142A} and *regA*^{T676E} mutants showed accelerated developmental kinetics in addition to some properties of *regA* null cells such as precocious sporulation and aberrant fruiting body formation [23]. This is suggested to be caused by the increase of PKA-C signaling because of higher levels of intracellular cAMP, as overexpressing PKA-C or disrupting the PKA regulatory subunit (PKA-R) also results in precocious sporulation [18,51]. This suggests that the *regA*^{S142A} and *regA*^{T676E} mutants results in a decrease in RegA activity to the extent where it impaired its ability to downregulate PKA-C activation. Interestingly the *regA*^{S413A} mutant did not lead to a similar acceleration of development as the *regA*^{S142A} and

regA^{T676E} mutations even though the *regA*^{S413E} mutant did delay developmental kinetics. This suggests that the *regA*^{S413A} mutation does not directly impact on the phosphodiesterase activity of RegA. Rather, we propose that this mutation aids in alleviating the inhibitory effects of the T676 phosphorylation event by Erk2, thus restoring phosphodiesterase activity. In this hypothesis, *regA*^{S413E} would be resistant to Erk2 inhibition, thus leading to low levels of cAMP and the observed delay in aggregate to slug transition. Interestingly, Erk2 is most active during the aggregation stage of development [49].

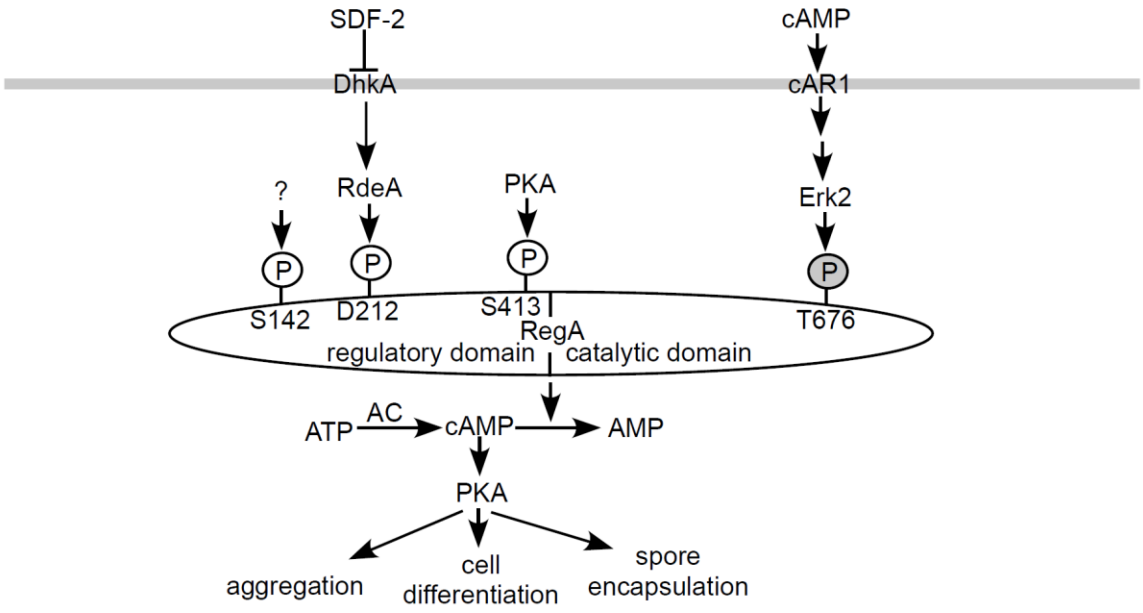


Figure 4.9 Model of RegA phosphorylation events. RegA is inhibited through the phosphorylation of Erk2 during cAMP signaling at T676 allowing for cAMP to accumulate in the cell. This results in an increase of cAMP and the activation of PKA. PKA is hypothesized to phosphorylate S413 to activate RegA and forming a negative feedback loop leading to decreased cAMP accumulation and inhibition of PKA. The S142 site was detected during early development and results in the activation of RegA when phosphorylated. However, the kinase and other time points during development this site is phosphorylated

is unknown. The D212 site is phosphorylated in the histidine two component phosphorelay system in later development where it functions in the development of pre-stalk and pre-spore cells.

Although the T676 site was not detected during the Mass Spectrum analysis, mutating the site with either alanine or glutamic acid led significant changes during development. The *regA*^{T676A} clone had a delayed development suggesting this mutant has a lower level of cAMP signaling and PKA activity compared to cells expressing the wild type or *reg*^{T676E}. Based on previous studies a likely explanation for this phenotype is the inability of Erk2 to down regulate RegA function. The phospho-mimetic RegA^{T676E} mutation gave a contrasting developmental phenotype resulting in a quicker progression to slug and fruiting body stages. The *regA*^{T676A} mutation also led to an interesting phenotype in a chimera where the mutated cells were localized in the pre-stalk A region at the tip of the slug. This indicates that MAPK regulation of cAMP through RegA likely plays a role in cell differentiation. The T676 residue of RegA has shown interesting phenotypes suggesting consistent with Erk2 down regulation of RegA. Erk2 is activated within 30 sec of cell stimulation with cAMP and therefore could potentially aid in the reduction of cAMP observed after the peak accumulation around 1 min post stimulation. Attempts to monitor this phosphorylation event were unsuccessful using phospho-threonine/serine antiserum or serum recognizing MAPK specific phosphorylation sites suggesting that this phosphorylation could be short lived or only occur in a small population of the total RegA protein in the cell. However, the lack of detection through antibody approaches could also be due the region not being recognized efficiently with the antibody.

Alteration of the RegA S413 residue had an impact on aggregation size but not on final fruiting body morphology suggesting the regulation of RegA through this residue is likely to occur primarily during early development. The slow transition of *regA*^{S413E} mutant mounds to slugs suggests that phosphorylation of S413 might have a positive impact on RegA function and therefore reduce cAMP levels. Such a mechanism would be similar to the upregulation of PDE

activity in response to PKA phosphorylation in mammalian cells [42,43]. The phosphorylation of PDE4D3 by PKA in mammals occurs near the amino terminal of the regulatory domain, but RegA S413 is near the carboxyl terminal of the regulatory domain in the middle of the protein. However, it is possible that both lead to up regulation of catalytic function by reducing negative interactions by the regulatory domain. Attempts to monitor the phosphorylation of S413 of RegA using PKA substrate specific antiserum were unsuccessful suggesting that this state of RegA is transient or present in only low levels. During aggregation, the repetitive oscillation of cAMP levels occurs about every 6 minutes and this might allow oscillating PKA activity to provide negatively feedback to cAMP concentrations to increase RegA function and decrease cAMP levels. Whether this type of regulation has a direct impact on Erk2 down regulation of RegA remains to be determined but PKA phosphorylation has been proposed to negate the down regulation of PDE4 long isoform by MAPKs [52]. Computational models incorporating PKA forming a negative feedback loop to reduce its own activity have resulted in simulations that faithfully account for the periodic signaling observed during development [35]. However, this computational model calculates for PKA inhibiting Erk2 directly to inhibit it. Later studies do not support the ability for PKA to feedback and phosphorylate Erk2 as cells with altered PKA activity where the catalytic activity of PKA is either upregulated or inhibited do not alter the duration of Erk2 activation after cAMP stimulation [53]. If the negative feedback loop of PKA occurred due to PKA phosphorylation of RegA resulting in increased RegA activity, then the model is likely to still accurately simulate the periodic signaling during early *Dictyostelium* development.

The phosphorylation in the regulatory region of RegA at S142 leads to an increase of RegA function based the delayed development of the *regA*^{S142E} mutant and the alter fruiting body morphology of the *regA*^{S142A} mutant. While the kinase(s) that phosphorylates this site is unknown, the MAPK ERK1 could be a potential candidate because Erk1 overexpression can delay the

transition from mound to slug in development. ERK1 and RegA also show similar phenotypes when they are disrupted resulting in accelerated development [49,54]. ERK1 is activated as a secondary response to chemotaxis signaling and therefore Erk1 activation overlaps with period in which phosphorylation of RegA S142 is detected [49]. However, these correlations do not exclude other kinases from regulating S142 on RegA as a response of cAMP signaling.

The existence of multiple phosphorylation sites on individual phosphodiesterases allows for different mechanisms of phosphodiesterase regulation in response to different cell signals. Such regulation also opens the possibility of a combinatorial system in which multiple phosphorylation events on the same protein could be synergistic or antagonistic with respect to each other. While the regulation of catalytic activity could be the goal of phosphorylation, the association or dissociation of the phosphodiesterase from a signaling complex could also be altered through phosphorylation. Therefore, the phosphorylation of a phosphodiesterase could have many outcomes and these might be difficult to define without additional knowledge of the other components in cAMP signaling pathways. Unfortunately, the analysis of phosphodiesterases in vitro or heterologous environments can provide only limited insight into the regulation and measuring overall cAMP levels in complex tissues might be insufficient to reveal critical parameters of specific signaling pathways. This study of RegA mutants in vivo provides some insight into potential developmental roles for some phosphorylation events but also contains some limitations. One of these limitations is that phosphorylation events are likely to be transient modifications, perhaps limited to specific cell types or signaling pathways for short periods, whereas the mutant RegA proteins mimic the phosphorylated or unphosphorylated states throughout development. This study demonstrates that not all phosphorylated residues that promote RegA function serve the same roles in the different stages of development. It is interesting to note that with both RegA and the mammalian PDE4D3 phosphorylation events in the amino-terminal regulatory domain tend to increase phosphodiesterase function whereas the

MAPK phosphorylation of residues near the carboxyl terminus in the catalytic domain tend to result in a reduction of phosphodiesterase function. This similarity is consistent with previous studies that suggest the regulatory domain of mammalian PDE4D3 can interact and limit the activity of the catalytic domain and suggests a possible conservation of phosphodiesterase function among eukaryotes.

4.5 Supplemental Figures

Table S4.1 Site directed mutagenesis primers used to construct phosphorylation mutations

Primer	Nucleotide sequence (5' to 3')
S142A Forward	CCA GCT CCG AGC TCT CAT AGA GTA TCG GAT TTT TCT G
S142A Reverse	CTA TGA GAG CTC GGA GCT GGT TGG TCG TTC AAT TTG TC
S142E Forward	CCA GAG CCG AGC TCT CAT AGA GTA TCG GAT TTT TCT G
S142E Reverse	CTA TGA GAG CTC GGC TCT GGT TGG TCG TTC AAT TTG TC
S413A Forward	GTC GAC GAA ATG CAA TAC CAA CTT TCC CTC AAA C
S413A Reverse	GCA GTT GAT GTT CAG CTG CTT TAC GTT ATG GTT G
S413E Forward	GTC GAC GAA ATG AAA TAC CAA CTT TCC CTC AAA C
S413E Reverse	GCA GTT GAT GTT CAG CTG CTT TAC TTT ATG GTT G
T676E Forward	GTG GAT ATC CAG TTG AAC CAT TTA TGG ATA AAA CC
T676E Reverse	GGT TCA ACT GGA TAT CCA CAA ATA GTT TCA TAA TG

Figure S4.2 Spectra from Mass Spectroscopy analysis of RegA. A) Spectra data of the S142 phosphorylation site that displayed a -98 neutral loss fragment diagnostic. B) Multiple spectra data of the S413 phosphorylation site. Each spectra represent different proteolytic phosphorylated peptide fragments.

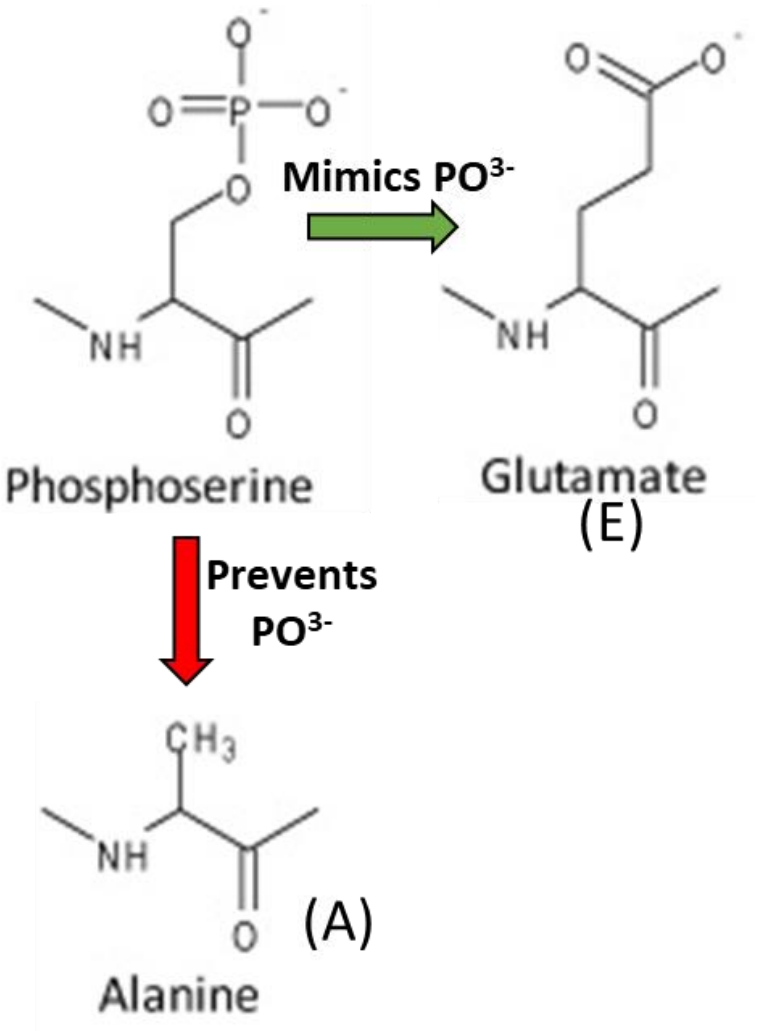


Figure S4.1 Model of phosphomimetic and phosphoablative mutations through substitutions of residues of interest. Glutamate substitution is used to constitutively mimic a phosphorylation event due to the added negative charge and increased size of the residue that is comparable to phosphoserine. The alanine substitution results in removing the hydroxyl group from serine and threonine, thus preventing the potential for phosphorylation at this site.

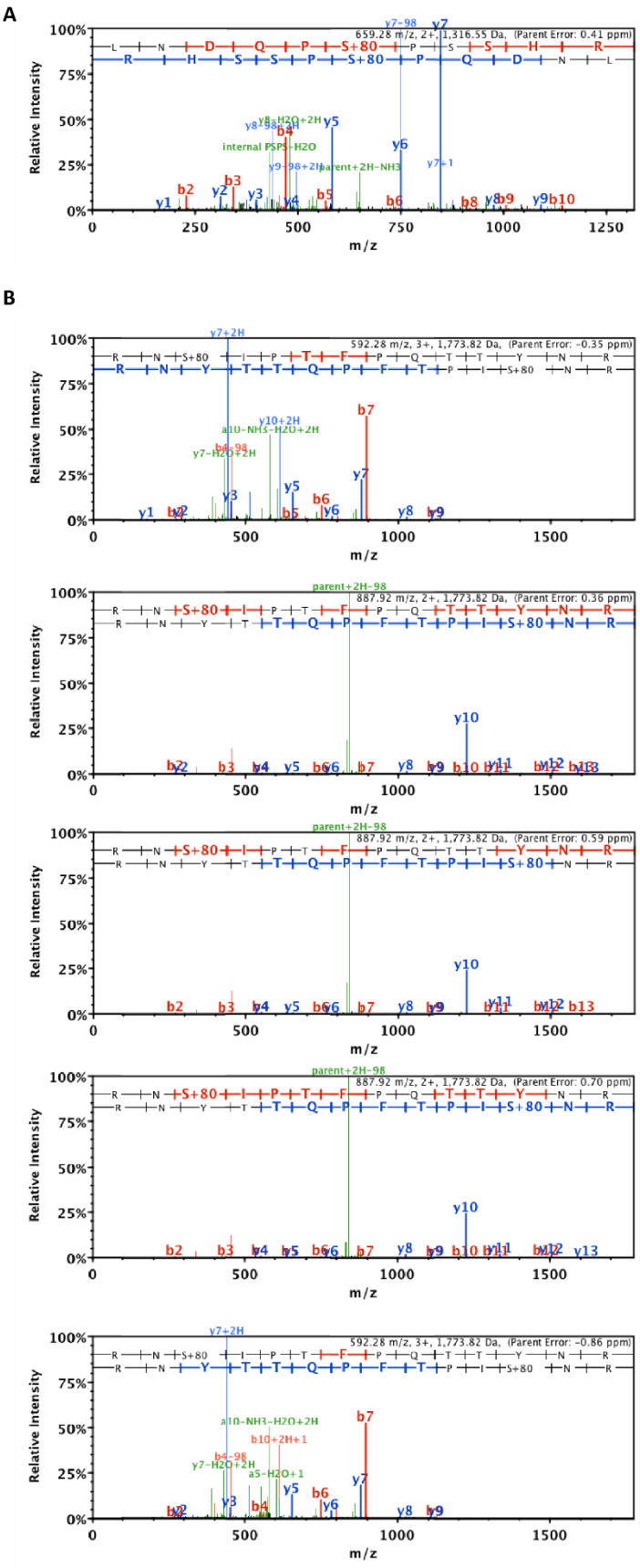
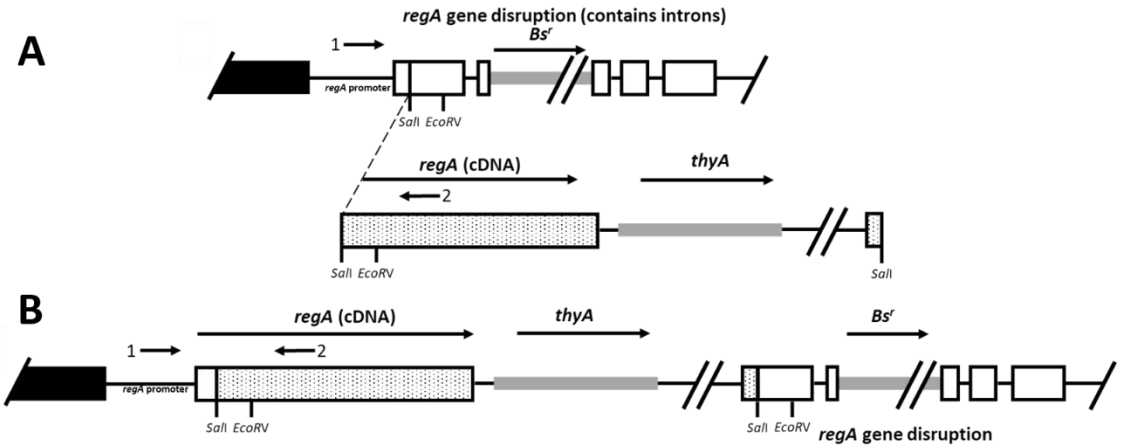
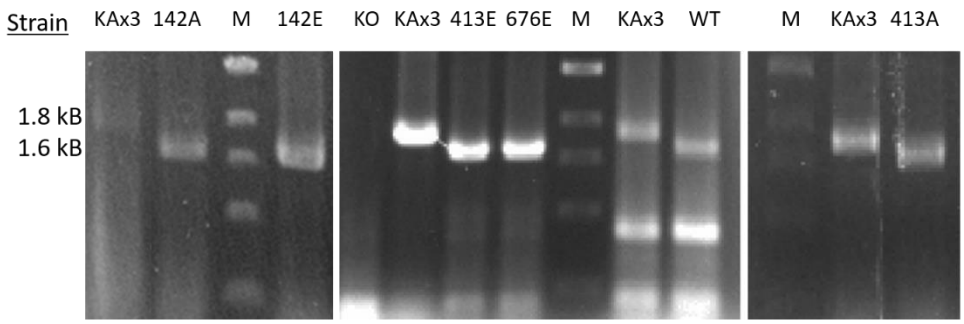

A
659.28 m/z, 2+, 1,316.55 Da, (Parent Error: 0.41 ppm)
B
592.28 m/z, 3+, 1,773.82 Da, (Parent Error: -0.35 ppm)
887.92 m/z, 2+, 1,773.82 Da, (Parent Error: 0.36 ppm)
887.92 m/z, 2+, 1,773.82 Da, (Parent Error: 0.59 ppm)
887.92 m/z, 2+, 1,773.82 Da, (Parent Error: 0.70 ppm)
592.28 m/z, 3+, 1,773.82 Da, (Parent Error: -0.86 ppm)
Relative Intensity
m/z

Figure S4.2 Spectra from Mass Spectroscopy analysis of RegA. A) Spectra data of the S142 phosphorylation site that displayed a -98 neutral loss fragment diagnostic. B) Multiple spectra data of the S413 phosphorylation site. Each spectra represent different proteolytic phosphorylated peptide fragments.



C



D

Primer	Nucleotide sequence (5' to 3')
1	CCGAGCTCACTCAAATACCTTTTAAGGGAAAAAACC
2	CTTGTGSCTGATACCAATGGTGATTC

Figure S4.3 Verification of genomic insertions. A) Model for expected *regA* gene knock-in in the *regA* disruption at the endogenous *regA* locus in the *Dictyostelium* genomic DNA. B) Model for the expected outcome of the *regA* knock-in C) PCR amplification of genomic DNA from KAx3, *regA*$^{-}$ (KO), *regA*$^{-}$:*regA* (WT), *regA*^{S142A} (142A), *regA*^{S142E}(142E), *regA*^{S413E}(413E), *regA*^{T676A}(T676A), and *regA*^{T676E}(T676E), and

regA^{S413A}(413A). All amplifications used primers 1 and 2 during PCR. These primers generate a 1.8 kB band in strains that amplify the RegA gene with all of its introns (KAx3 only). For knock-in strains, cDNA was used, thus the primers generate a 1.6 kB band due to the lack of introns. *regA*$^{-}$ strains lack the second primer binding site, thus does not produce a band. D) List of primers used for PCR verification.

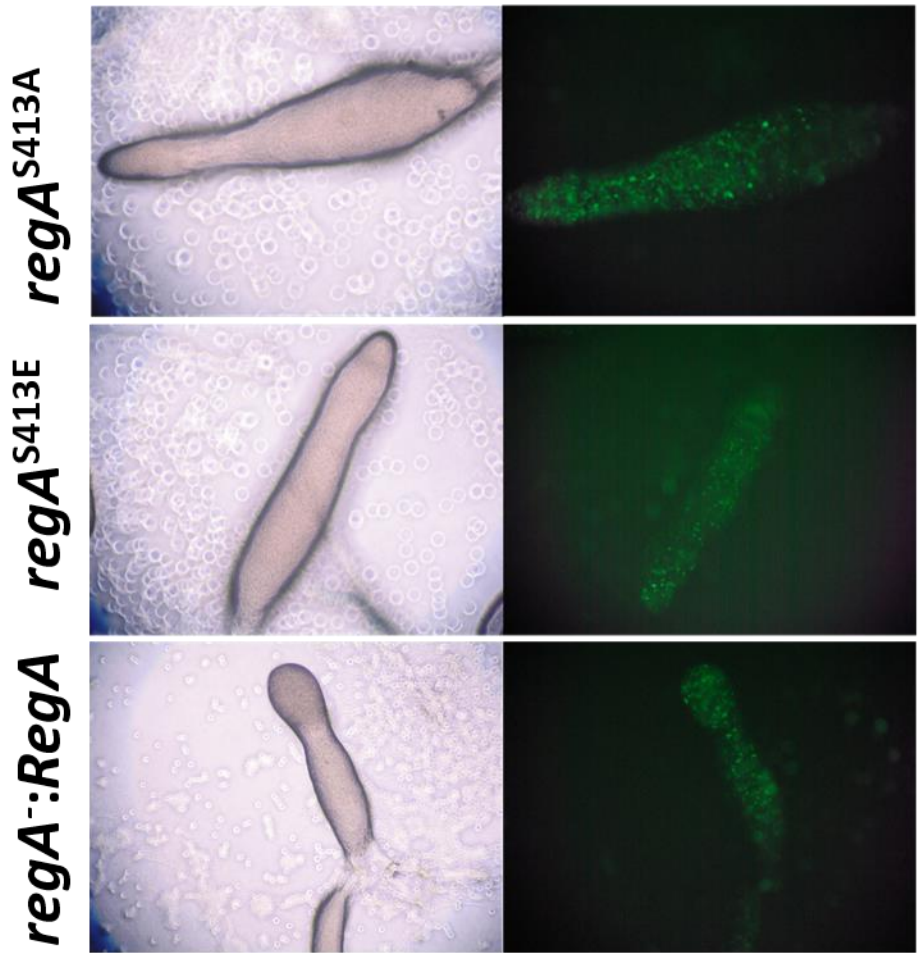


Figure S4.4 Chimeras of knock-in S413 mutations expressing a GFP vector and wild-type cells with without GFP vector. GFP expressing knock-in cells were mixed 1:10 with non-labeled wild-type allele knock-in. Bias in localization is observed by preferential sorting in areas of slug during development. Pictures taken at 14 hours into development.

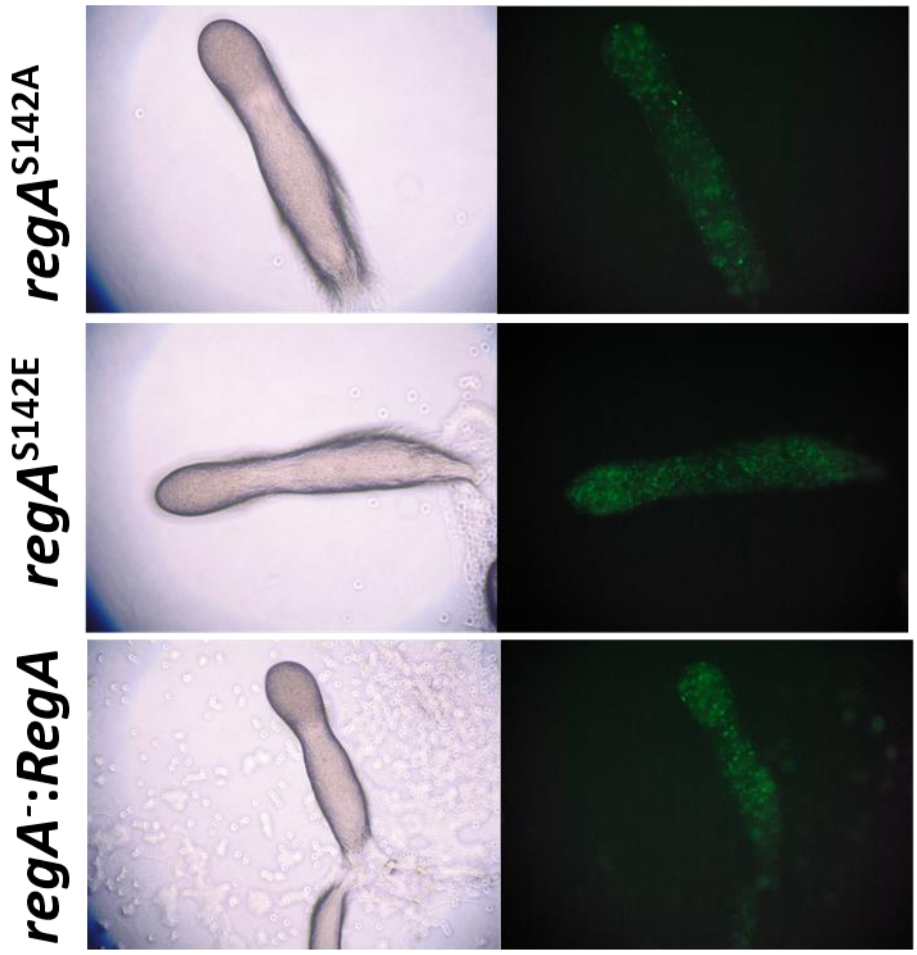


Figure S4.5 Chimeras of knock-in S142 mutations expressing a GFP vector and wild-type cells with without GFP vector. GFP expressing knock-in cells were mixed 1:10 with non-labeled wild-type allele knock-in. Bias in localization is observed by preferential sorting in areas of slug during development. Pictures taken at 14 hours into development.

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CHAPTER V

CONCLUSIONS

The focus of this research was to investigate the chemotaxis and signaling of *Dictyostelium* as a model organism to uncover insights for other systems. The primary focus described in chapters III and IV was to investigate cellular signaling pathways involving an atypical MAPK and a cAMP specific phosphodiesterase. The other main area research was to into *Dictyostelium* chemotaxis. *Dictyostelium* chemotaxis has been studied extensively due to its function in development and when foraging for bacteria. *Dictyostelium* chemotaxis has also been applied as a model for neutrophil chemotaxis in mammalian systems as the overall pathways that regulate chemotaxis are similar [1]. In our studies described in chapter 2, we compared the chemotaxis of *Dictyostelium* and the pathogenic *Acanthamoeba* to investigate foraging differences. *Dictyostelium* demonstrated extensive chemotaxis to both chemoattractants such as folate and bacteria, and with little motility in the lack of a chemoattractant. However, *Acanthamoeba* was observed to show robust motility with or without a potential chemoattractant and minor chemotaxis in the presence of bacteria. This observation lead to the conclusion that *Acanthamoeba* does not use chemotaxis as a primary mechanism when foraging for bacteria, rather it employs a robust motility and cell dispersal. This distinction between the foraging mechanisms of the two amoebas may be involved with their evolved responses to nutrient limitation. *Dictyostelium* uses robust cAMP chemotaxis in order to aggregate with other nearby

cells to form multicellular structures to produces spores. However, *Acanthamoeba* employs a solitary life cycle of encystation.

While *Acanthamoeba* did not appear to contain Gβ subunits orthologs based on the genomic sequencing data, this does not rule out the possibility that a Gβ was missed in the sequencing efforts. Looking for the presence of a Gβ using PCR with primers that bind to highly conserved regions of Gβ subunits may lead to identification of a putative gene. This could also be performed to look for a Gα4 subunit ortholog. The *Acanthamoeba* putative WD-repeat containing proteins, such as Rack could provide functionality in the motility or the chemorepulsion of *Acanthamoeba,* thus disrupting this putative gene would be useful in investigating its function. However, the genetic tools to work with *Acanthamoeba* is not nearly as extensive as it is with *Dictyostelium*, thus this could prove to be challenging.

The small number of two MAPKs encoded in the *Dictyostelium* genome offers a great model for studying the function of MAPK in signaling pathways. Studying signaling pathways in higher eukaryotes can be challenging due to the number of signaling proteins in different families and isoforms for these proteins. The two encoded MAPKs in the *Dictyostelium* genome also offer an interesting look at a typical and atypical MAPK in regards to Erk1 and Erk2, respectively. As an atypical MAPK Erk2 was identified to have roles in chemotaxis, bacterial engulfment, growth, development, and Erk1 activation. While the described research identified functional roles of Erk2 in *Dictyostelium*, there are still unknown aspects of Erk2 that might be interesting in regards to atypical MAPKs.

The activation of atypical MAPKs is thought to be due to auto-phosphorylation based on studies of heterologous expression of mammalian atypical MAPK, Erk7, in bacteria resulting in phosphorylated Erk7 [2]. However, the mechanism of atypical MAPK activation has not been studied in a physiological system. The transformation of a kinase dead mutation of the

Dictyostelium Erk2 into an *erk2*$^{-}$ cell line and analyzing for impaired Erk2 activation could be used to test the hypothesis that atypical MAPKs phosphorylate themselves. Identification of Erk2 interaction partners through mass spectroscopy during non-stimulated and prior to Erk2 activation (<30 second after stimulation of cAMP) may lead also to potential proteins that have roles in Erk2 activation. Disrupting these target genes and analyzing for impaired activation of Erk2 could also be an approach to study the activation of atypical MAPKs.

Our studies in chapter 4 investigated the regulation of the phosphodiesterase RegA by phosphorylation using mutations at putative phosphorylation sites. Our purpose was to further investigate the role of phosphorylation on phosphodiesterase function during the developmental process and observe changes in physiological or cell function. Two Phosphorylation sites were detected in cells that were stimulated with cAMP during early stages of development (6 hours). One identified site was a putative PKA phosphorylation site at S413. This site was initially detected through screening for conserved phosphorylation motifs in the primary amino acid sequence of RegA. The other identified site at S142 is followed by a proline residue, which may indicate the site may be phosphorylated by a MAPK or a Cdc2-related kinase. Two known phosphorylation sites were not detected through this experiment. One was the T676 site which is suggested to be phosphorylated by Erk2 and the D212 site that is phosphorylated by RdeA in a two component histidine kinase mechanism [3–5]. The former site was included in the peptide coverage in the analysis, even after a second attempt with different proteases. The latter phosphorylation event was not expected during early development as it is suggested to be involved in spore maturation later in development.

Our approach was to determine the effect of these mutations in regards to changes in physiology and cellular function. Disrupting *regA* results in an increase of developmental kinetics, precocious sporulation, altered distribution of cells when developed as a chimera with wild type cells, and altered fruiting body morphology with spores accumulating at the base of the

stock [6,7]. Based on this approach, the T676 residue was determined to be function due to the altered developmental phenotypes observed during development. Previous studies suggested that phosphorylation at T676 functioned in the inhibition of RegA, since *regA*$^{-}$ cells expressing an alanine substitution at this residue (*regA*^{T676A}) resulted in a significant decrease in cAMP accumulation when compared to *regA*$^{-}$ cells expressing wild type *regA* [5]. This suggests that the T676A mutation resulted in increased phosphodiesterase activity of RegA. This leads to the hypothesis that phosphorylation at this site normally functions to inhibit RegA. Our observations of the development *regA*^{T676A} cells supports this hypothesis due to a delay in developmental kinetics, which is similar to the observation of overexpressing wild type RegA. The phenotypes of the glutamic acid phosphomimetic mutation (*regA*^{T676E}) resembled the development of *regA*$^{-}$ cells in terms of increased developmental kinetics, precocious sporulation, and altered fruiting body morphology. This supports our hypothesis that the phosphorylation event functions to inhibit RegA.

Interestingly, the mutations at the T676 site were the only mutations in our studies that altered the distribution of the mutant cells during development when developed as a chimera with wild type cells. The *regA*^{T676A} mutation resulted in a strong bias in localizing in the anterior region of the slug where pre-stalk A (pstA) cells localize. Interestingly the *regA*^{T676E} mutants showed localization just posterior to this pstA region. This resembles the bias of *regA*$^{-}$ cells which show a strong bias for prestalk B (pstB) cell development, an area that is located posterior to the pstA cells in the slug [7]. The contributions of cell migration and localization by RegA as well as the potential role for the phosphorylation site at T676 for this bias is not fully known. RegA is involved in late developmental process such as stalk maturation in prestalk cells during late development and this function is suggested to be regulated by the histidine kinase two component signaling pathway that controls the phosphorylation state of RegA D212 [8,9]. Since these mutations were observed to have bias in localizing to the pre-stalk region, the regulation at

the T676 residue may have a role in the development or differentiation of pre-stalk cells. More studies to investigate the bias in localization with these mutants are required. One study would be to analyze changes in pstA specific gene expression in the *regA*^{T676A} mutant and pstB specific gene expression in the *regA*^{T676E} mutant relative to the gene expression of a wild type control chimera. This approach was utilized when analyzing the bias of *regA*$^{-}$ cells to localize int the pstB region of the slug when developed as chimeras with wild-type cells [10].

It is possible that the phosphorylation event at T676 has roles in late development that results in the bias in cell sorting, as well as its supported roles during early development for cAMP signaling. Determining the phosphorylation activity of this site at different times of development would help identified this potential function of this site during later development. An experiment to investigate this would be to develop wild type cells on filters and then harvest cells at different time points during development. Next, an immunoprecipitation of RegA would be performed on these cell extracts. Detecting the phosphorylation state of the residue could be performed through either antibody detection on a western blot or phosphoprotein mass spectroscopy. In my studies, I was unsuccessful in detecting this phosphorylation site with two different antibodies, phospho-threonine-proline mouse antibody (Cell Signaling Tech #9391) and phospho-threonine antibody (Cell Signaling Tech #9381). However, if an antibody either constructed or identified to detect this site then it could be used to determine the phosphorylated state of this residue throughout development by western blot. Detecting the phosphorylation event by mass spectroscopy could be challenging as well based on the poor peptide fragment coverage at this site. When identifying potential phosphorylation sites on RegA, we were unsuccessful in obtaining coverage of this site even after using different proteases to generate other peptide fragments. The initial approach used trypsin, elastase, and subtilisin, to generate peptide fragments and a second approach utilized trypsin and V8. Troubleshooting with different proteases could lead to increased coverage and the detection of the phosphorylation event at the T676 residue.

The phosphorylation site in the upstream regulatory region at serine 142 was concluded to have a functional effect on RegA activity based on the altered developmental phenotypes. Phosphorylation at this site is suggested to activate RegA rather than inhibit it like the T676 site. This is supported by the resulting delay of development when the phosphorylation site was mimicked the serine residue was substituted with a glutamic acid (*regA*^{S142E}). In addition, the phenotype of the phosphoablative mutation for this site (*regA*^{S142A}) resembled the phenotype of *regA*$^{-}$ cells in regards to accelerated developmental kinetics, precocious sporulation, and altered fruiting body structures. The kinase that phosphorylates this residue is unknown.

The MAPK Erk1 is a plausible kinase for the phosphorylation of the serine 142 residue. The surrounding sequence of the S142 site resembles a MAPK or a Cdc2-kinase phosphorylation motif. Cells with *erk1* disrupted resemble *regA*$^{-}$ cells in terms of both cells resulting in accelerated developmental rate, which suggests the two proteins could be in the same pathway [11]. Additionally, Erk1 is activated as a secondary response to chemotaxis signaling at the same time point that the phosphorylation of S142 was detected [12]. However, this does not rule out other kinases that could phosphorylate this site and more studies will need to be performed. This could be approached by determining if disrupting *erk1* is sufficient to prevent the phosphorylation of the S142 residue. RegA would be immunoprecipitated during early development from *erk1*$^{-}$ and WT cells after stimulating with cAMP. The sample would then be analyzed for the presence of the phosphorylated S142 residue in the immunoprecipitated samples using mass spectroscopy. Alternatively, an antibody that is able to detect this phosphorylation event could be used. However, I was unable to identify an antibody that could detect this phosphorylation event or sequence with high specificity during my studies. A custom antibody raised to the phosphorylated peptide and is able detect this phosphorylated residue could be used to determine if disrupting *erk1* is sufficient to prevent the phosphorylation event.

The serine 413 phosphorylation site was supported to be a functional phosphorylation site for the regulation of RegA based on the delay in developmental kinetics observed in the phosphomimetic mutation (*regA*^{S413E}) suggesting that the phosphorylation event at this site results in increased RegA activity. However, the phosphoablative phenotypes did not produce contrasting phenotypes when compared to RegA on developmental kinetics as observed with the T676 and S142 mutations. This suggests that the *regA*^{S413A} mutation does not directly impact on the phosphodiesterase activity of RegA. We propose that this mutation functions to alleviate the inhibition of Erk2's phosphorylation at T676 to restore phosphodiesterase activity. In this hypothesis, *regA*^{S413E} would be resistant to Erk2 inhibition, thus leading to low levels of cAMP and the observed delay in aggregate to slug transition. Computational models incorporating PKA forming a negative feedback loop to reduce its own activity have resulted in simulations that faithfully account for the periodic signaling observed during development [13]. However, this computational model has PKA inhibiting Erk2 directly to inhibit it. Later studies do not support the ability for PKA to feedback and phosphorylate Erk2 as cells that do not produce adenylyl cyclase (*aca*$^{-}$), thus have a low level of intracellular cAMP and reduced PKA catalytic activity, do not show increased duration of Erk2 phosphorylation after cAMP stimulation [14]. Additionally, *pkaR*$^{-}$ cells that have over active PKA catalytic activity due to not expressing the regulatory subunit of PKA do not show a decrease in the time that Erk2 is phosphorylated after stimulating with cAMP [14]. If the negative feedback loop of PKA through PKA phosphorylation of RegA to result in increased RegA activity, then this model is likely to still accurately simulate the periodic signaling during early *Dictyostelium* development.

The timing of RegA phosphorylation after stimulating with cAMP is currently unknown and multiple computational models have been proposed. The consensus of these models is that after stimulating with cAMP, the intracellular levels of cAMP increase in the cell as RegA is inhibited by Erk2. However, the mechanism that RegA is reactivated is not fully understood. It was

initially proposed that PKA phosphorylated Erk2 to inactivate it, resulting in the alleviation of RegA inhibition [5]. However, Erk2 phosphorylation was not altered when the catalytic subunit of PKA was overactive (by either overexpressing the catalytic subunit or disrupting the regulatory subunit of PKA) [15]. Another model supported by the research described in chapter 4, hypothesizes that PKA phosphorylates RegA directly resulting in the restoration of phosphodiesterase activity after Erk2 inhibits RegA by phosphorylation. However, additional studies could be performed to further test this model such as determining the timing of RegA phosphorylation. The hypothesis would be supported if after stimulating with cAMP, RegA is initially phosphorylated at T676 and then phosphorylated at S413. This could be investigated through antibodies that detects these phosphorylated sequence or through detecting the phosphorylation events using phosphoprotein mass spectroscopy. Current attempts to detect either the T676 or the S413 phosphorylation sites with phospho-threonine-proline mouse antibody (Cell Signaling Tech #9391) or phospho-PKA substrate (Cell Signaling Tech #9624) were unsuccessful in detecting the respective sites. Thus constructing custom antibodies for these sites could be one approach.

While investigating cAMP levels could lead to issues due to inaccuracies in measuring, looking at the phosphodiesterase activity of the RegA phosphorylation mutations could provide supporting evidence that backs up the current observations described in chapter 4. It is expected that T676 and S142 mutations will result in altering the phosphodiesterase activity directly as the *regA*^{T676E} and *regA*^{S142A} showed phenotypes that were similar to *regA* null. However, it is not expected that the *regA*^{S413} mutations would result in altered phosphodiesterase activity. This is because the *regA*^{S413A} mutation did not result in an increase in the developmental kinetics, even though the *regA*^{S413E} mutation did result in a delay in developmental kinetics. For this assay, cAMP levels for all six phosphorylation site mutants, *regA* null, and a wild type control will be

measured using a cAMP assay as described [16]. This data would be useful in furthering the understanding of the functions of these phosphorylation site.

The importance of the studies in chapter 4 could be applied to both mammalian systems and towards potential applications for pathogenic amoebas. Many studies in mammalian cell lines have been conducted in cell lines that might have altered signaling mechanisms due to expressing exogenous proteins and most studies have described molecular interactions rather than changes in physiology or cell function [17-22]. Studying the regulation of phosphodiesterases in *Dictyostelium* provides an advantage in both of these areas. The regulation of RegA may also have importance in looking at therapeutic approaches for pathogenic amoebas. RegA is highly conserved in both social and pathogenic amoebas and is involved with sporulation and/or encystation [16]. Inhibiting RegA in *Acanthamoeba castellanii* resulted in triggering encystation, thus targeting RegA and other parts of the cAMP signaling pathway may be a potential therapeutic strategy for pathogenic amoeba infections [16].

5.1 Chapter 5 References

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