Cellular Signalling 57 (2019) 65–75

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Multiple phosphorylation sites on the RegA phosphodiesterase regulate *Dictyostelium* development

Nick A. Kuburich[1], Nirakar Adhikari, Jeffrey A. Hadwiger*

Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, OK 74078-3020, United States

ARTICLE INFO

Keywords:
Phosphodiesterase
RegA
Dictyostelium
Development
PKA
cAMP

ABSTRACT

In *Dictyostelium*, the intracellular cAMP-specific phosphodiesterase RegA is a negative regulator of cAMP-dependent protein kinase (PKA), a key determinant in the timing of developmental morphogenesis and spore formation. To assess the role of protein kinases in the regulation of RegA function, this study identified phosphorylation sites on RegA and characterized the role of these modifications through the analysis of phosphomimetic and phospho-ablative mutations. Mutations affecting residue T676 of RegA, a presumed target of the atypical MAP kinase Erk2, altered the rate of development and impacted cell distribution in chimeric organisms suggesting that phosphorylation of this residue reduces RegA function and regulates cell localization during multicellular development. Mutations affecting the residue S142 of RegA also impacted the rate developmental morphogenesis but in a manner opposite of changes at T676 suggesting the phosphorylation of the S142 residue increases RegA function. Mutations affecting residue S413 residue altered aggregate sizes and delayed developmental progression suggesting that PKA operates in a negative feedback mechanism to increase RegA function. These results suggest that the phosphorylation of different residues on RegA can lead to increased or decreased RegA function and therefore in turn regulate developmental processes such as aggregate formation, cell distribution, and the kinetics of developmental morphogenesis.

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, etc.) [4]. Multiple signaling pathways use cAMP as an intermediate to regulate different cellular responses, even within a single cell, suggesting that cAMP signaling can be pathway specific. One mechanism proposed to explain pathway specificity is the formation of signaling complexes that localize cAMP signaling, even though cAMP can diffuse throughout 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 the ability to confer temporal and spatial regulation of cAMP signaling within a signaling complex and pathway. This idea is consistent with the diversity of cAMP phosphodiesterases that exist in many organisms. In mammals, eight families of cAMP phosphodiesterases have been identified and each family is composed of multiple isoforms that can have specificity with respect to tissue expression and cellular localization [10,11]. In addition to isoform diversity, phosphodiesterase function can also be regulated through protein kinase phosphorylation of specific phosphodiesterases. 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 MAP kinases (MAPKs), PKAs, and other protein kinases [12–17].

The soil amoeba *Dictyostelium discoideum* is an organism that uses

Abbreviations: cAMP, Cyclic adenosine monophosphate; PKA, cAMP-dependent protein kinase; MAPK, Mitogen activated protein kinase; ERK, Extracellular-signal regulated kinase; EPAC, Exchange protein directly activated by cAMP; AKAP, A-Kinase anchoring protein; G protein, Guanine nucleotide binding protein; GFP, Green fluorescent protein; ORF, Open reading frame.

* Corresponding author at: Department of Microbiology and Molecular Genetics, Oklahoma State University, 307 Life Sciences East, Stillwater, Oklahoma 74078-3020, United States.

E-mail address: jeff.hadwiger@okstate.edu (J.A. Hadwiger).

[1] Current address Department of Translational Molecular Pathology, University of Texas M.D. Anderson Cancer Center, Houston, Texas, 77030, USA

https://doi.org/10.1016/j.cellsig.2019.02.005
Received 30 November 2018; Received in revised form 13 February 2019; Accepted 17 February 2019
Available online 19 February 2019
0898-6568/

both intracellular and extracellular cAMP signaling during its developmental life cycle in which solitary cells form multicellular aggregates and then differentiate into spores and stalk cells [18–20]. Extracellular cAMP mediates the chemotactic movement of cells into multicellular aggregates and also contributes to the morphogenesis in later stages of development such as the formation of migratory slugs and eventually fruiting bodies, a mass of spores upon a stalk structure. During early development prespore and prestalk cells become localized to specific regions of the aggregate and then later, in a coordinated differentiation process, these cells become the spores and the stalk of the fruiting body [21]. Both cell movement and differentiation involve intracellular cAMP signaling as suggested by the phenotypes observed for cells lacking or overexpressing PKA function [22]. 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 PKA regulatory subunit or overexpression of the catalytic subunit) display defective aggregation and rapid cellular differentiation, including precocious spore formation [22–26]. The intracellular cAMP-specific phosphodiesterase, RegA, is expressed during growth and development where it plays an integral role in regulating intracellular cAMP signaling and the kinetics of development [27–29]. Loss of RegA function results in accelerated spore formation at about 16 h of development whereas wild-type cells generally form spores closer to 24 h into development [30]. In contrast, over-expression of RegA delays developmental progression consistent with intracellular cAMP signaling being an important factor for developmental kinetics. The loss of RegA or the PKA regulatory subunit result in similar developmental phenotypes such as precocious spore formation and this similarity is consistent with RegA being a primary regulator of intracellular cAMP signaling and PKA function. Some *in vitro* studies indicate that RegA can interact with PKA suggesting that RegA and PKA directly bind to each other or associate with a common complex but no AKAP homologs have not been identified in *Dictyostelium* [29,31].

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 folate and cAMP [32]. Extracellular cAMP and folate can stimulate cAMP receptors (Car1–4) and the folate receptor (Far1), respectively, leading to a burst of cAMP accumulation that peaks approximately 1 min after stimulation [33–36]. These stimuli also lead to the activation of the MAPK Erk2, a regulator of cAMP signaling [37–39]. A leaky $erk2^{RE}$ (reduced expression) allele that does not allow sufficient cAMP production to complete cell aggregation can be suppressed by the loss of RegA function suggesting Erk2 is a negative regulator of RegA [40,41]. A putative MAPK phosphorylation site, residue T676 in RegA, is required for this regulation implying that Erk2 might down regulate RegA function through direct phosphorylation of this residue [41]. RegA can also be positively regulated through the phosphorylation of the D212 residue in response to a histidine kinase-mediated pathway and this regulation ceases prior to spore formation [42,43].

Many mutant phosphodiesterases, particularly those with mutations that impact regulation, have been analyzed for catalytic activity but relatively little is known about how regulatory modifications of phosphodiesterases impact cell fate and differentiation. Given that *Dictyostelium* RegA is critical for multiple developmental processes we sought to further examine how the phosphorylation of this phosphodiesterase impacts developmental progression. Potential phosphorylation sites were identified through mass spectrometry and then genetic analysis of phospho-mimetic and phospho-ablative mutations was used to examine the roles of RegA phosphorylation in *Dictyostelium* development.

2. Materials and methods

2.1. Strains and cell culturing

All *Dictyostelium* strains were isogenic to the parental strain, KAx-3, and a derivative $thyA^-$ gene disruption (thymidine auxotroph) strain, JH10, except where noted [44]. Disruption of the *regA* gene in the KAx-3 strain has been previously described and this same gene disruption was also carried out in the JH10 strain [30,44,45]. The *regA* gene disruption was verified by PCR amplification of genomic DNA using PCR as previously described [45]. Cells were grown axenically in HL-5 medium (with or without thymidine supplement) or on bacterial lawns of *Klebsiella aerogenes* as previously described [46,47]. G418 and blasticidin drug selections were performed as described [48]. Electroporation of *Dictyostelium* was performed as previously described [49]. RegA expression in transformed cells was verified by immunoblot analysis using antiserum generated against a RegA peptide (PSSHRVSDFSDEY-SPC) located near the amino terminus or a RegA peptide (CKSKLPKI-DEEENR) located near the carboxyl terminus. The underlined cysteines (C) were added for peptide attachments. Immunoblots were performed as previously described [50].

2.2. Recombinant DNA constructs

The *regA* open reading frame (ORF) was PCR amplified from plasmid pDT12 and inserted as a *Bam*HI and *Xba*I fragment into multiple of *act15* promoter (relatively constitutive expression) vectors including the integrating pDXA-3H expression vector and the extrachromosomal FLAG-tagging vector pTX-FLAG [30,45,51]. Site-directed mutagenesis was used to construct the *regA* alleles $regA^{S142E}$, $regA^{S142A}$, $regA^{S413E}$, $regA^{S413A}$, $regA^{T676E}$. Oligonucleotides used for site-directed mutagenesis are listed in (Supplementary Fig. S1). Phosphomimetic alterations were constructed using codons for glutamatic acid rather than aspartic acid due to the reduced number of nucleotide changes. The $regA^{T676A}$ allele was a generous gift from G. Shaulsky and a fragment containing this allele was used to construct vectors like those used for the other *regA* alleles. All *regA* alleles were confirmed by DNA sequencing. Knock-in vectors for the *regA* locus were constructed by inserting a *Bam*HI/*Sal*I fragment of the *thyA* gene and *Bam*HI/*Xba*I fragments containing the *regA* allele ORFs into the vector pBluescriptSK- (Stratagene). The knock-in vectors were linearized at either a *Sal*I or *Eco*RV site near the amino terminal region of the ORF and then integrated into the knock-out *regA* locus of a $regA^-thyA^-$ double mutant through a single crossover mechanism. The knock-in vectors lacked promoter sequences for the *regA* ORFs and this limited the expression of the knock-in allele to the ORF integrating adjacent to the endogenous *regA* promoter. All knock-in clones were verified by PCR analysis and retained blasticidin resistance.

2.3. Dictyostelium developmental phenotype analysis

Cells were grown in shaking cultures to mid-log phase ($\sim 3 \times 10^6$ cells/ml) and then pelleted by centrifugation. Cells were washed in phosphate buffer (12 mM NaH_2PO_4 adjusted to pH 6.1 with KOH) and suspended at 1×10^8 cells/ml or 3×10^7 cells/ml before plating on non-nutrient agar (1.5% in phosphate buffer) plates for development. Images of developmental morphology were recorded at times indicated. For chimera experiments, cells were labeled with a GFP expression vector pTX-GFP and mixed with unlabeled control cells (wild-type *regA* knock-in strain) using a 1:10 ratio. Images were captured using fluorescence microscopy.

2.4. Immunoprecipitation of FLAG-RegA

The $regA^-$ cells transformed with the FLAG-RegA expression vector were starved in shaking cultures for 3 h and then pulsed with 100 μM

cAMP for 3 h to make cells competent for the aggregation phase of development as previously described [52]. The cell suspension was stimulated with a final treatment of 100 μM cAMP and after 0, 0.5, 1, 2, and 5 min cells were harvested in ice-cold phosphate buffer (10 ml), pelleted, and frozen at −80 °C. Cells were lysed by thawing in 0.5% *v*/v Triton X-100 immunoprecipitation lysis buffer composed of 50 mM Tris-HCl pH 8, 150 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF, 3 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM Sodium-orthovanadate, and 25 mM β-glycerophosphate. Samples from different time points were combined and incubated with OctA-probe antibody (Santa Cruz Biotechnology, sc-166,355) overnight at 4 °C on a test tube rotator. After incubation, 20 μl 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, twice in wash buffer (50 mM Tris-HCl pH 8, 150 mM NaCl), and then subjected to SDS-PAGE and coomassie staining.

2.5. Phosphoprotein mass spectroscopy analysis

The FLAG-RegA bands were prepared for mass spectrometry via the general methodologies developed by Shevchenko et al. [53], using chemicals purchased from SigmaAldrich. In detail, protein bands were excised from stained SDS-PAGE gels with a razor blade, cut into 1-mm cubes, and destained by rocking the gel cubes for 6 h in one-ml aliquots of 50% acetonitrile/50 mM ammonium bicarbonate pH 8.2, with changing of destain buffer at 2-h intervals. Destained gel cubes were dehydrated by incubating for 10 min in one ml of 100% acetonitrile, and dried for one hour under ambient temperature and pressure. The acetonitrile was withdrawn, and the dehydrated gel cubes were rehydrated with 0.5 ml of 10 mM tris(2-carboxyethyl)phosphine, 50 mM ammonium bicarbonate pH 8.2, and reduced for one hr at room temperature. Reducing buffer was withdrawn by pipeting, and replaced with 0.5 ml 55 mM iodoacetamide in 50 mM ammonium bicarbonate pH 8.2, and incubated for 1 h at room temperature in the dark. Then gel cubes were rinsed once with one ml 50 mM ammonium bicarbonate pH 8.2, dehydrated again with one ml acetonitrile, and rehydrated/infiltrated with protease solution containing either trypsin (Promega), elastase (Worthington), subtilisin (Sigma), or V8 protease (Worthington) at 8 μg protease per ml of 50 mM ammonium bicarbonate pH 8.2, using just enough protease solution to completely rehydrate and cover the cubes. After overnight digestion at 37 degrees C, the gel cubes were extracted thrice with 70 μl of 0.5% trifluoracetic acid, extracts containing the peptides were pooled, and the pooled peptide extracts were purified by solid phase extraction on 200-μl OMIX monolithic C18 pipet tips following the manufacturer's instructions (Agilent). The purified peptide eluates were then frozen at -80 °C, and lyophilized for 40 min in a centrifugal vacuum device. The samples were not further enriched for phosphopeptides. Dried peptides were redissolved in 45 μl of 0.1% formic acid/water, and 10 μl of each sample was injected for 10 min at 3 μl/min onto a 5-cm vented trap column [54]. The trap column was coupled via a liquid high-voltage junction (New Objective) to a 75 μm × 40 cm Picofrit nanocolumn (New Objective) packed with Magic AQ 3 μm resin (Bruker) fitted with an integral nanospray emitter eluting directly into the ion source [55,56] via 2000 V of ionizing potential applied to the liquid high-voltage junction. The trap-analytical column pair was heated to 55 °C using a column heater fabricated in house, and eluted using an Eskigent nano2D HPLC as previously described [57] to perform reversed phase column nano-chromatagraphy at a flow rate of 250 nL/min. The aqueous solvent (mobile phase A) consisted of 0.1% formic acid in water (Burdick & Jackson), while the organic solvent (mobile phase B) consisted of 0.1% formic acid in acetonitrile (Burdic and Jackson). Each sample was analyzed twice by LC-MS/MS, once using a 60-min HPLC gradient (0–30% mobile phase B) and once using 120-min gradient (0–30% mobile phase B).

Upon elution, peptides were analyzed using an OrbitrapXL mass spectrometer [58] fitted with an inline PV500 nanspray ion source (New Objective). The mass spectrometer was programmed to collect one full-range FT-MS scan (nominal resolution of 60,000 FWHM, *m/z* range 360 to 1400) using the Orbitrap mass analyzer, followed immediately by six data-dependent MS/MS scans [59] performed in the linear ion trap mass analyzer sector. MS/MS settings used a trigger threshold of 8000 ion counts, monoisotopic precursor selection (MIPS), and rejection of ions that had unassigned charge states, were previously identified as contaminants on blank gradient runs, or that had been previously selected for MS/MS (dynamic exclusion [60] with an exclusion count of 2 within 150% of the observed chromatographic peak width). In each scan cycle, each precursor ion selected for fragmentation was subjected to two different sequential MS/MS scans, with the second MS/MS scan programmed to include multistage activation of predicted phosphate neutral-loss species 107, 98, 64.4, 58, 49, 32.7, 24.5 [61].

Centroided ion masses were extracted using the extract_msn.exe utility from Bioworks 3.3.1 (ThermoFisher) and were used for database searching with the Mascot v2.2.04 protein mass spectrometry search application [62]; Matrix Science and X! Tandem v2007.01.01.1 ([63]; www.thegpm.org). Mascot was used to search a database of 12,318 *Dictyostelium discoideum* protein sequences dowloaded from the dictyBase resource (dictybase.org) on 5/17/2012. For the V8- and trypsin-digested samples, the Mascot searches were performed with statements of cleavage specificity matching the enzyme used. For the subtilisin- and elastase-digested samples, the Mascot searches were performed with statements of cleavage specificity "none," indicating non-specific informatic cleavage rules. Other Mascot settings included statements of fragment ion mass tolerance of 0.60 Da and parent ion tolerance of 6.0 PPM, corresponding to achievable mass accuracies in the ion trap and Orbitrap sectors, respectively, of the OrbitrapXL hybrid mass spectrometer [58]. Gln- > pyro-Glu of the N-terminus, oxidation of methionine, acetyl of the N-terminus, carbamidomethyl of cysteine, propionamide modification of cysteine as variable modifications in Mascot, using peptide modifications as curated at the Unimod database [62]. Phosphorylation was included as a variable modification in three separate discrete searches for phosphoserine, phosphothreonine, or phosphotyrosine, respectively.

Peptide and protein identifications were validated using the PeptideProphet algorithm within Scaffold v2.2.00 (Proteome Software) [64]. Protein identifications were accepted if their probabilities of correct identification were > 99%, based upon at least 2 peptides, with each peptide identified with 20% certainty. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Peptide-spectrum matches were diagrammed using resources within Scaffold.

3. Results

3.1. RegA has multiple phosphorylations 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 could be regulated by multiple mechanisms. Earlier studies have verified the phosphorylation of RegA at residue D212 through a two-component histidine kinase response regulator mechanism prior to spore formation and another study has suggested that the MAPK Erk2 phosphorylates residue T676 in early development [30,41]. 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 aggregation competent cells (6 h of starvation in shaking culture). 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

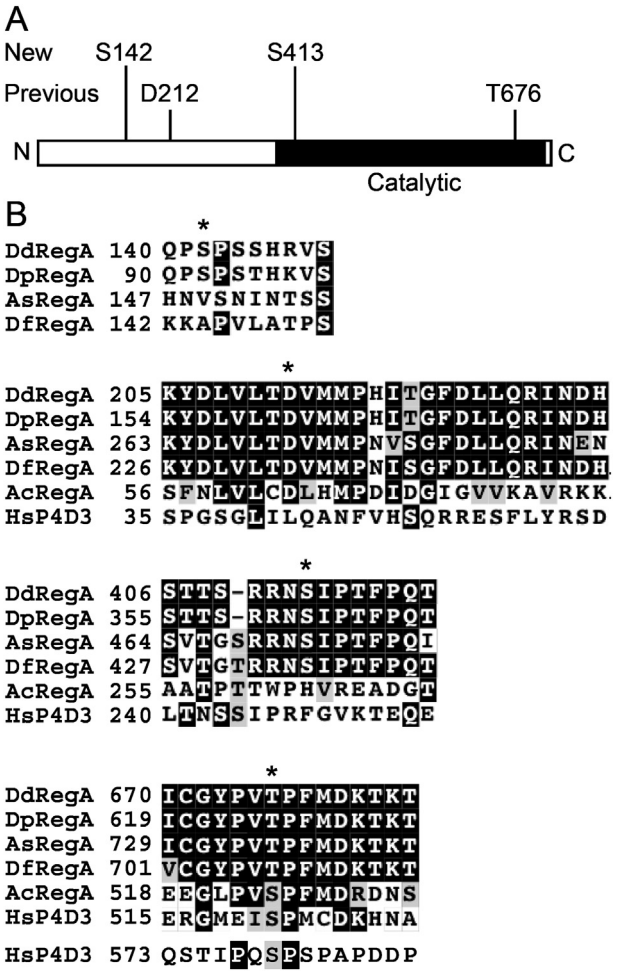

Fig. 1. Phosphorylation sites on RegA. (A) Map of phosphorylation sites on RegA detected through mass spectrometry (S142 and S413) and previously reported (D212 and T676). Location of phosphodiesterase catalytic domain (black bar). (B) Alignments of *Dictyostelium discoideum* RegA (DdRegA) phosphorylation sites and flanking residues with other Dictyostelids (Dp, *Dictyostelium purpureum*, As, *Acytostelium subglobosum*, Df, *Dictyostelium fasciculatum*), *Acanthamoeba castellanii* (Ac), and Human (HsP4D3 - PDE4D3 isoform) cAMP phosphodiesterases. Residues shaded in black are conserved in > 50% of the proteins and residues shaded in gray represent residues with similar structure or charge. The last alignment also includes a known phosphorylation site on HsP4D3 with flanking residues. Phosphorylated RegA residues are indicated by an asterisk (*).

proteolytic digestion strategies of the RegA protein did not alleviate the lack of peptide coverage in this region. Phosphopeptides in other regions of RegA suggest that residues S142 and S413 can be phosphorylated (Fig. 1A, Supplementary Fig. S2, and Table S1). The S413 residue is located near the amino terminal end of the predicted catalytic domain and the residue is part of a PKA phosphorylation site motif (RRXS) suggesting that PKA could be the kinase responsible for this phosphorylation event. PKA phosphorylation sites in mammalian phosphodiesterase PDE4E isoforms have been reported to serve as a mechanism for up regulating phosphodiesterase activity leading to reduced cAMP levels and PKA activity [17]. However, the PKA phosphorylation site of mammalian PDE4E is located near the amino terminus as opposed to the more centrally located PKA phosphorylation site in RegA [16]. The other detected RegA phosphorylated residue, S142, is located outside of the predicted catalytic domain and followed by a proline suggesting that it could be phosphorylated by a MAPK or a Cdc2-related kinase. Many MAPK phosphorylation sites also contain a proline at the −2 position but the absence of this proline does not preclude the possibility that MAPKs might phosphorylate the S142 residue.

Sequence alignments of RegA with related phosphodiesterases were used to determine if the phosphorylated regions of RegA are conserved in other organisms. Many regions within the catalytic domain of cAMP phosphodiesterases are highly conserved but the sequences outside of this domain are typically more divergent. The MAPK phosphorylation target motif (PXS/TP) that includes the RegA residue T676 is highly conserved in Dictyostelids and other protists (e.g., *Acanthamoeba*) (Fig. 1B, Supplementary Fig. S3). This motif is also conserved in mammalian PDE4D except for the optional proline at the −2 position. However, an earlier study indicates that MAPKs phosphorylate the S579 residue of PDE4D rather than the S521 residue but it is possible that modifications at either site might confer similar changes in phosphodiesterase function. The RegA residue S413, located within a PKA phosphorylation target motif, is conserved among Dictyostelids but not in other protists. Further away from the catalytic domain, the RegA S142 residue is conserved in some but not all Dictyostelids and the surrounding sequences are highly divergent with long runs of repeated residues. Only the S142 residue lies within a predicted disordered protein region (Supplementary Fig. S3). The varied conservation of these kinase phosphorylation target sites suggests that some phosphorylation events might be only conserved among closely-related species whereas other phosphorylation events are potentially widespread among eukaryotes.

3.2. Endogenous expression allows for equivalent expression and comparison mutant regA phenotypes

To further investigate the potential role of phosphorylated residues in RegA function, 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 cells to assess developmental phenotypes. This initial approach was complicated due to the overexpression of RegA, previously reported to delay developmental progression, and the challenge of identifying mutants with similar levels of RegA expression [30]. To reduce the overexpression of the mutant RegA proteins, the open reading frames of the *regA* alleles were inserted into vectors containing the *thyA* gene and integrated by single crossover into the disrupted *regA* locus of a *regA*$^-$*thyA*$^-$ strain (Fig. 2a). The single crossover recombination results in only a single copy of the wild-type or mutant *regA* alleles expressed from the endogenous *regA* promoter. Any additional integrated copies of the *regA* ORFs at the endogenous *regA* locus lack transcriptional regulatory and promoter sequences. These knock-in alleles of RegA were verified using PCR analysis (Supplementary Fig. S4) and relatively uniform RegA levels were detected through immunoblot analysis (Fig. 2b). The levels of RegA protein in the mutant and wild-type allele knock-in strains were at lower levels than that observed from the parental KAx3 strain and this reduced expression is likely to result from the absence of a designated transcriptional termination sequence. The wild-type knock-in allele developed with a similar morphology to the KAx3 strain and had only slightly accelerated development suggesting sufficient RegA protein is produced from the knock-in alleles (Fig. 2c). This strategy allowed the mutant alleles to be accurately compared to the wild-type allele without concerns of gene dosage or promoter variability and these knock-in mutants were used for all subsequent analyses.

3.3. Alteration of RegA residue T676 impacts developmental processes including cell localization

The RegA^{T676E} mutant was accelerated in morphological development compared to the RegA^{T676A} mutant and wild-type control supporting the previously proposed down regulation of RegA through the phosphorylation of the T676 residue (Fig. 3). The acceleration

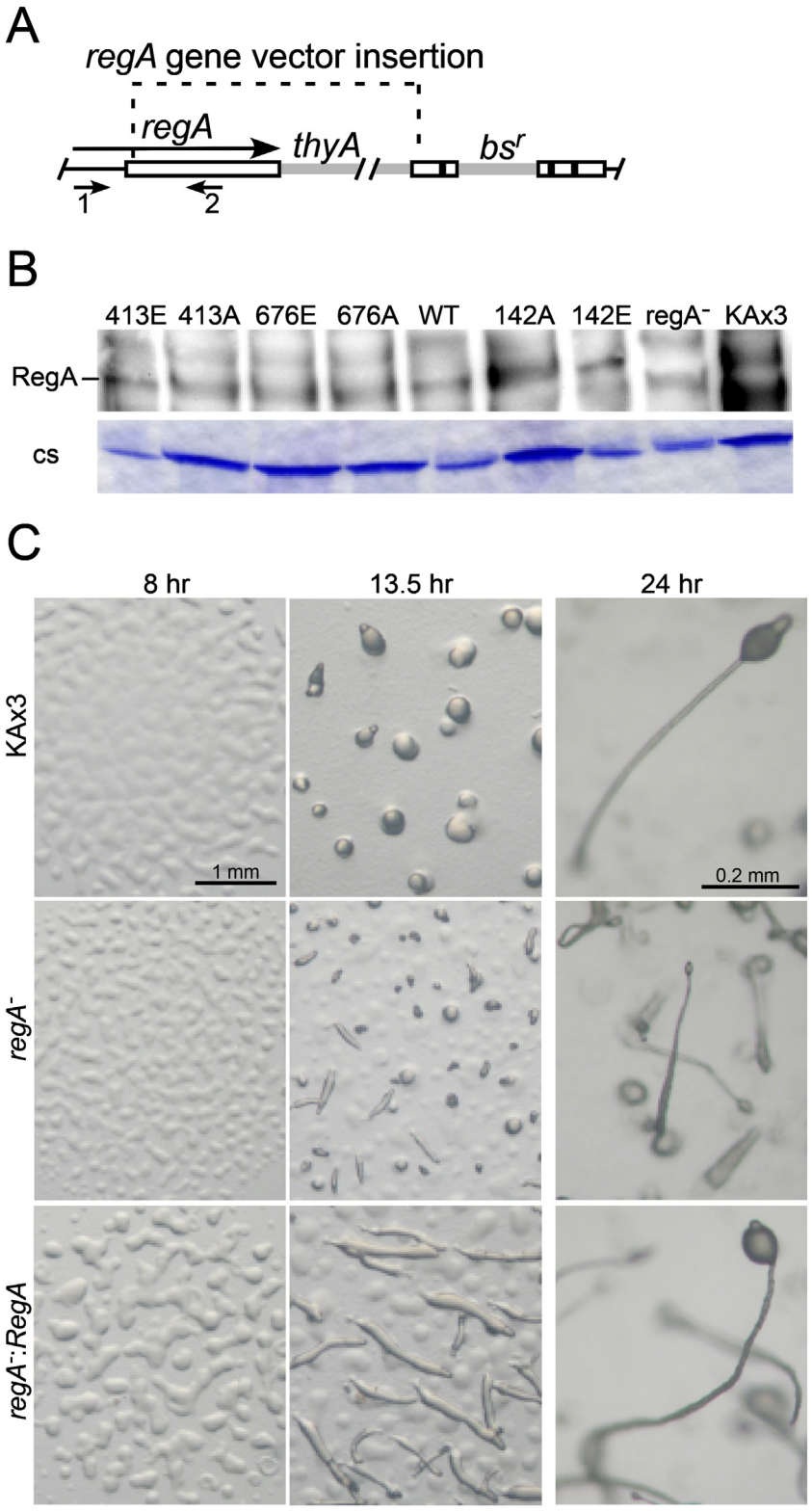

Fig. 2. Integration and expression of *regA* alleles. (A) Map of *regA* allele single crossover integration into disrupted *regA* locus. Open bar represents *regA* open reading frame and black bars within represents intron sequences upstream and downstream of the replacement of *regA* sequences with the blasticidin resistance gene (bs^r - gray bar). Other gray bar represents the *thyA* gene and vector backbone of integration constructs (slashes −//− indicate that approximately 4 kb of sequences have not been represented in the map). Dotted line indicates the borders of the *regA* gene integration vector. Black line represents intergenic genomic sequences and arrow indicates *regA* transcription. (B) Immunoblot of RegA protein in WT and RegA mutants. Parental KAx3, *regA*$^-$, or cells expressing the integrated wild-type allele (WT) or mutant alleles were harvested after 2 h starvation, lysed, and subjected to immunoblot analysis using antiserum generated against a peptide sequence in the amino terminal region of RegA. Each lane was loaded with lysates from 1×10^6 cells. Coomassie staining (cs) of the blotted gel (lower panel) used as a loading control. (C) Developmental morphology of parental KAx3, *regA*$^-$, and *regA*$^-$ cells with wild-type *regA* allele knock-in (*regA*$^-$:*regA*). Cells were plated from 5×10^7 cells/ml suspensions and images were recorded at the times indicated.

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 typically aberrant with many spores at the base of the stalk rather than at the top, a phenotype similar to *regA*$^-$ strains. In contrast, the $RegA^{T676A}$ cells formed large aggregates that developed into fruiting bodies with wild-type morphology. The $RegA^{T676E}$ strain produced many spores by 18 h of starvation, resembling the *regA*$^-$ phenotype, and the $RegA^{T676A}$ strain did not initiate spore formation until after the wild-type control strain, implying a delay in prespore cell differentiation (Fig. 4). The distinctive phospho-mimetic and phospho-ablative morphological phenotypes suggest that the phosphorylation of this residue reduces RegA function and disrupts the coordination of prespore cell differentiation with the culmination of development.

Chimera analysis of $RegA^{T676}$ mutants and wild-type strains was used to assess RegA mutant cell distribution in multicellular aggregates composed primarily of wild-type cells. Cells were labeled with a GFP

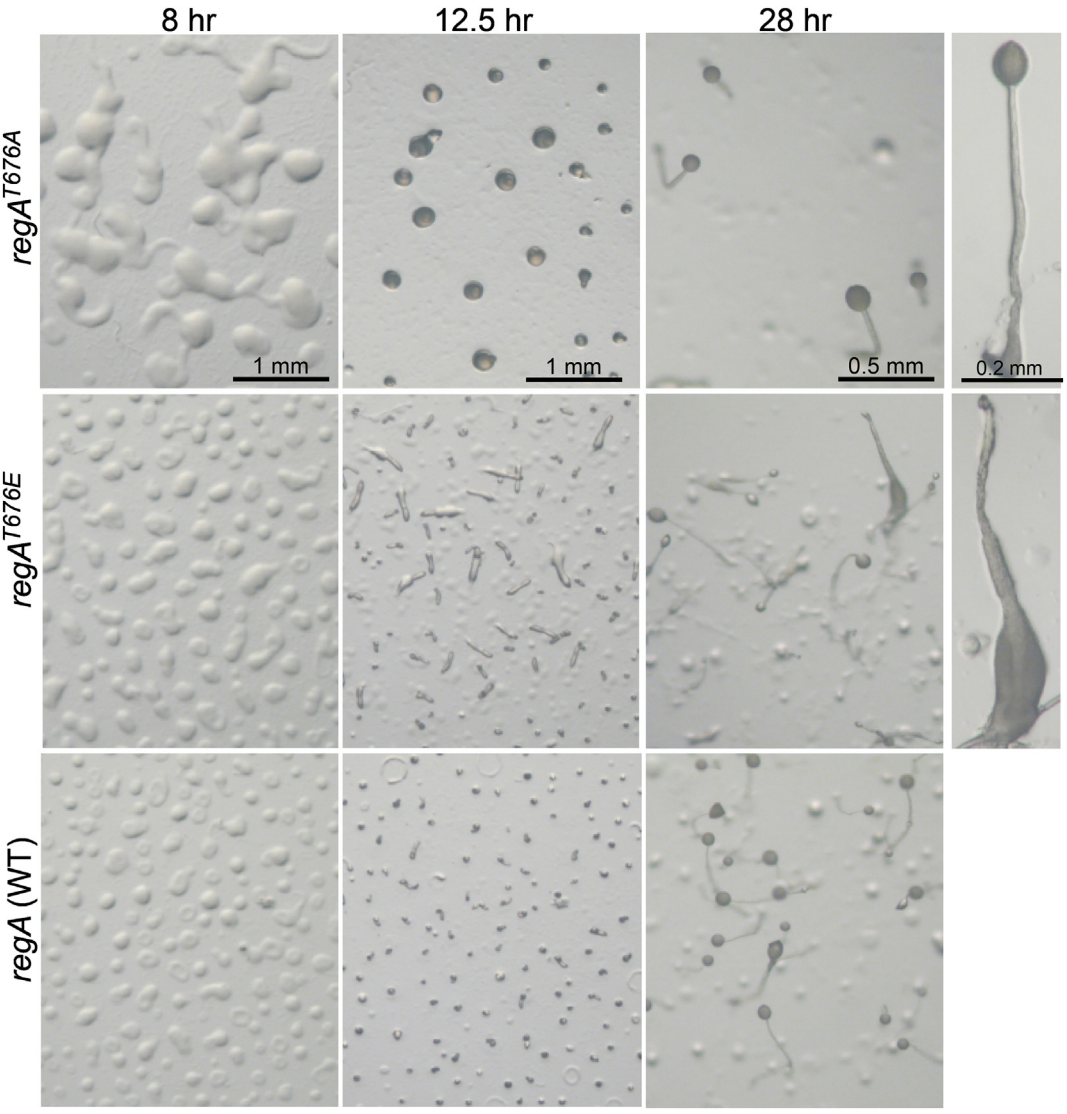

Fig. 3. Developmental morphology of RegA^{T676} mutants and wild-type (WT) control cells. Cells were plated from 1×10^7 cells/ml suspensions on nonnutrient agar and images from above of developmental morphology were recorded at the times indicated. In each column the cells were developed for the same period of time and the images have the same magnification (scale). Images are representative of multiple assays. Representative fruiting body structures (last column).

expression vector to allow positional tracking in the developing aggregates. Cells expressing RegA^{T676A} displayed a strong bias for localization near the anterior region of the chimeric aggregates, a region typically occupied by cells designated as prestalk A cells (Fig. 5). Many of these RegA^{T676A} mutant cells remained at this anterior tip throughout fruiting body development. Conversely, RegA^{T676E} mutants exhibited a weaker bias toward the posterior-central regions of chimeric aggregates, a region occupied by anterior-like cells and prespore cells. This posterior region of the chimeric aggregates was often left behind as the anterior portion underwent fruiting body development. The RegA^{T676E} cells that participated in fruiting body development were present in both spore and stalk populations. These observations suggest the phosphorylation of the residue T676 of RegA can impact cell distribution during multicellular development.

3.4. Alteration of the RegA residue S413 impacts aggregate size and developmental kinetics

When starved, cells expressing RegA^{S413A} formed large aggregates that were delayed in the transition into slugs compared to wild-type cells (Fig. 6). In contrast, the expression of RegA^{S413E} resulted in aggregates that were typically smaller than that of the wild-type cells but these aggregates were also delayed in the transition to the slug stage. The distinction in aggregate size suggests that the phosphorylation of the RegA S413 residue can impact the aggregation territory. Both mutant strains displayed normal fruiting body morphology suggesting that both alterations to this residue result in sufficient RegA function to provide proper culmination. This morphological phenotype was consistent with both mutant strains displaying delayed spore formation compared to the wild-type strain (Fig. 4). Neither mutant displayed any bias in their distribution in chimeric aggregates when developed with wild-type cells (Supplementary Fig. S5). The phosphorylation of the RegA residue S413 was also examined in cAMP stimulated cells at early or late stages of aggregation using antibodies that recognize phosphorylated PKA substrate motifs (#9624 Cell Signaling Tech.). Immunoblots of whole cell extracts detected many bands but a RegA specific band was not revealed suggesting that a PKA phosphorylated RegA band might be masked by other PKA phosphorylated proteins.

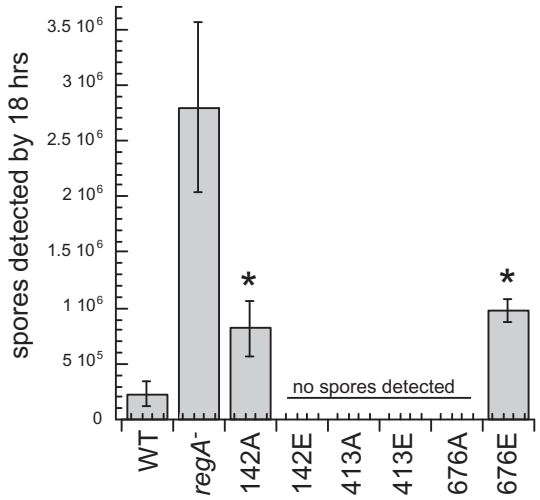

Fig. 4. Precocious spore formation assay of *regA*$^{-}$ strains expressing the wild-type or mutant RegA proteins (RegA^{S142A}, RegA^{S142E}, RegA^{S413A}, RegA^{S413E}, RegA^{T676A}, and RegA ^{T676E}). Equal numbers of cells from each strain were starved on filters and developed for 18 h. Cells were collected, labeled with Bright fluorescent dye (primary binds to cellulose in spore coat), and spores were counted using a hemacytometer in triplicate with at least 50 cells examined in each count. Average spore yields are displayed and error bars represent standard deviations. Note that this assay only assesses precocious spore formation and that all strains displayed spore formation by 26 h of starvation. Student's unpaired *t*-test comparing to WT and *regA*$^{-}$, $P < .05$ (*).

3.5. Alteration of the RegA residue S142 impacts the kinetics of developmental morphology

Cells expressing RegA^{S142E} exhibited a delayed aggregate formation and a slow transition from mound to the slug stage (Fig. 7). These cells also produced fruiting bodies with wild-type morphology. In contrast cells expressing RegA^{S142A} displayed accelerated development for the mount to slug stage transition and produced fruiting body morphology resembling that of the *regA*$^{-}$ strain. These developmental morphologies were consistent with RegA^{S142A} cells having precocious spore formation and the RegA^{S142E} cells having delayed spore formation (Fig. 4). Neither S142 residue mutant exhibited a bias in cell localization when developed with wild-type cells in chimeras suggesting that this phosphorylation site does not have a role in cell distribution within a developing aggregate (Supplementary Fig. S5). Overall, the phenotypes observed for the RegA^{S142E} and RegA^{S142A} cells suggest that the phosphorylation of the S142 residue increases RegA function during development.

4. Discussion

In mammals, the diversity of cAMP-specific phosphodiesterase isoforms can contribute to signaling pathway specificity through their association with different tissues or even intracellular domains [7,65]. The phosphorylation of some of these isoforms by multiple protein kinases allows for the modulation of phosphodiesterase function by either changes in catalytic activity or possibly changes to phosphodiesterase interactions within signaling complexes [14–16]. In *Dictyostelium*, the regulation of intracellular cAMP signaling and PKA function during development appears to be primarily achieved through a single phosphodiesterase, RegA [26,30,42]. The results of this study suggest that the phosphorylation of RegA, through the actions of multiple protein kinases, might serve to both regulate phosphodiesterase function and pathway specificity (Fig. 8). These modifications of RegA can impact a variety of processes such as aggregate formation, developmental progression and morphology, and cell distribution in multicellular aggregates. Some, if not all of these processes, are likely to be regulated

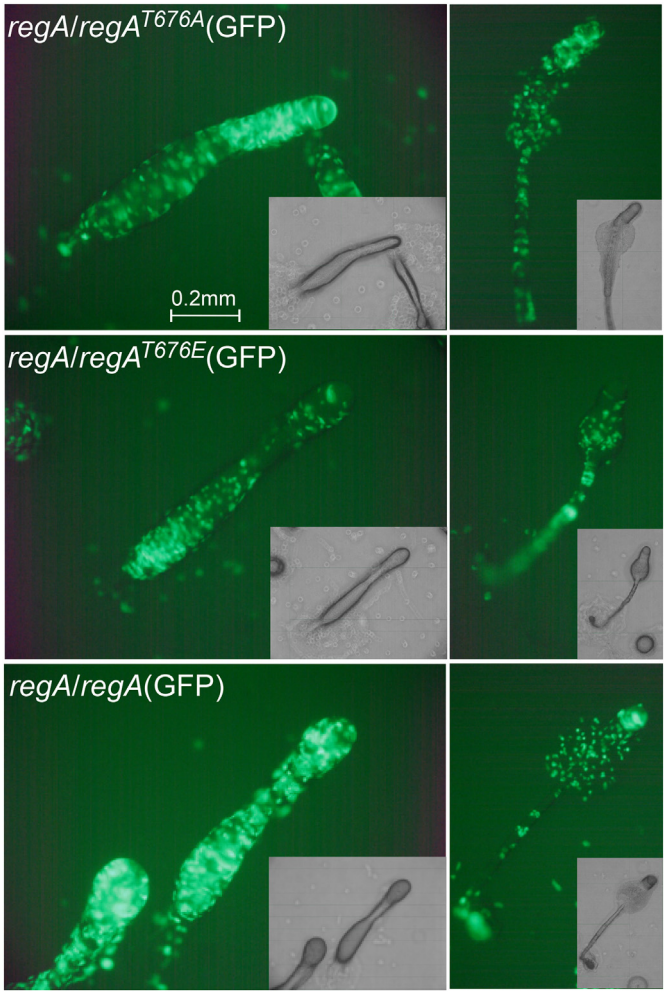

Fig. 5. Distribution of wild-type and RegA^{T676} mutants in chimeras. Cells expressing the wild-type, RegA^{T676A}, or RegA^{T676E} were labeled with a GFP expression vector and then mixed with unlabeled wild-type cells at a 1:10 ratio and then plated for development on nonnutrient agar. Images of fluorescence cell distribution in slugs were recorded at 14 h of development.

through changes in cAMP concentration and PKA activity and consistent with this idea PKA function has been previously linked to aggregation, developmental kinetics and cell distribution in the multicellular state [23–25,66,67]. The phosphorylation state of RegA could also impact the association of this phosphodiesterase with signaling complexes and therefore the binding of RegA to other signaling proteins could be an important contribution to signaling pathway specificity.

As might be expected from the phenotypes of *regA*$^{-}$ or RegA overexpression mutants, modifications of RegA that decrease or increase function can have a profound impact on the rate of development. Based on the phenotypes of phospho-mimetic or phospho-ablative mutants, the phosphorylation of the S142 residue and T676 residue result in increased and decreased RegA function, respectively, and so these modifications impact the rate of development in opposing ways. This versatility in the regulation of RegA supports the idea that one or more protein kinases act on RegA in response to different upstream signaling. Erk2 is a good candidate protein kinase for the phosphorylation of the RegA T676 residue based on the suppression of a leaky *erk2*$^{-}$ phenotype by the loss of RegA [41]. The presence of a MAPK phosphorylation motif around this residue also supports the idea that a MAPK is the modifying protein kinase. The identity of the kinase that phosphorylates S142 is unknown but the presence of the adjacent proline (P143) suggests the serine residue could be phosphorylated by a MAPK or a cyclin-dependent kinase (CDK) [68]. The Erk2 MAPK is not

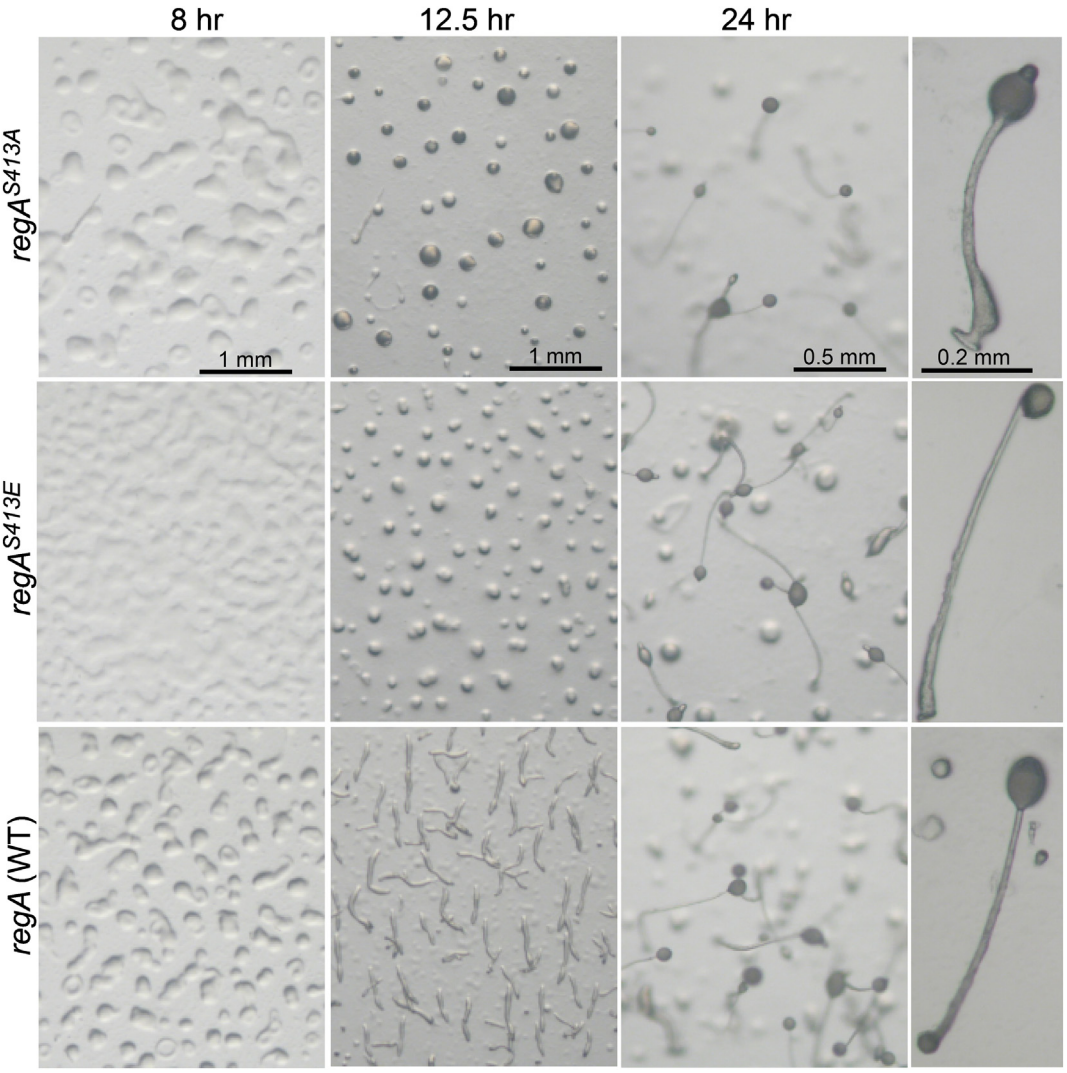

Fig. 6. Developmental morphology of RegA^{S413} mutants and wild-type control cells. Cells were prepared, developed, and imaged as described in Fig. 3 at indicated time points. In each column the cells were developed for the same period of time and the images have the same magnification (scale). Images are representative of multiple assays. Representative fruiting body structures (last column).

likely a modifier of the S142 residue because Erk2 generally acts to reduce RegA function and increase cAMP signaling. *Dictyostelium* encodes only one other MAPK, Erk1, and the loss of Erk1 also accelerates developmental progression but with a different terminal fruiting body morphology. Therefore Erk1 represents a potential modifier of this RegA residue but Erk1 is also likely to function in RegA-independent signaling pathways because of differences in *erk1*$^-$ and *regA*$^-$ developmental phenotypes. *Dictyostelium* encodes multiple CDKs but none have been associated with an accelerated development phenotype [69]. Attempts to detect the phosphorylation of S142 or T676 through MAPK substrate specific (pT/pSP) or phospho-threonine/serine specific (pT/pS) antiserum were unsuccessful in identifying a RegA specific band among background bands. The suspected modifier of the S413 residue is PKA based on the surrounding motif but it is not clear how both the phospho-mimetic and phospho-ablative mutations at S413 can delay development. Perhaps a cycling between phosphorylated forms is necessary for efficient development or that each form is necessary to facilitate a different developmental process. The difference in the aggregate size phenotypes associated with these mutations suggests these two RegA alleles impact signaling during aggregate formation in distinct ways, but ultimately both result in developmental delays. PKA phosphorylation of the mammalian PDE4 cAMP phosphodiesterase ablates an inhibitory phosphorylation by MAPKs but it remains to be determined if PKA phosphorylation of RegA impacts other phosphorylation events [70].

Based on this and previous studies, the phosphorylation of the RegA D212, S142 and S413 residues all increase RegA function suggesting that multiple mechanisms can be used to up regulate RegA function and down regulate PKA activity [42]. The developmental phenotypes associated with the alteration of each site are different implying that the each phosphorylation event might regulate a different signaling pathway or stage of development. Mimicking the phosphorylation of residue S142 decreases the rate of development, mimicking the phosphorylation of residue S413 decreases the size of aggregates and rate of development, and the phosphorylation of D212 has been previously proposed to regulate the time of spore encapsulation [43]. All of these regulatory mechanisms are presumed to down regulate cAMP signaling but whether this regulation alters the amplitude or the duration of signaling remains to be determined. Interestingly, all of these phosphorylation sites are located on the amino terminal side of the catalytic domain whereas the single phosphorylation modification on the carboxyl side of the catalytic domain down regulates RegA function (Fig. 8). Whether or not this apparent spatial separation of regulatory sites is related to intrinsic protein conformation and catalytic activity or interactions with other signaling proteins remains to be determined.

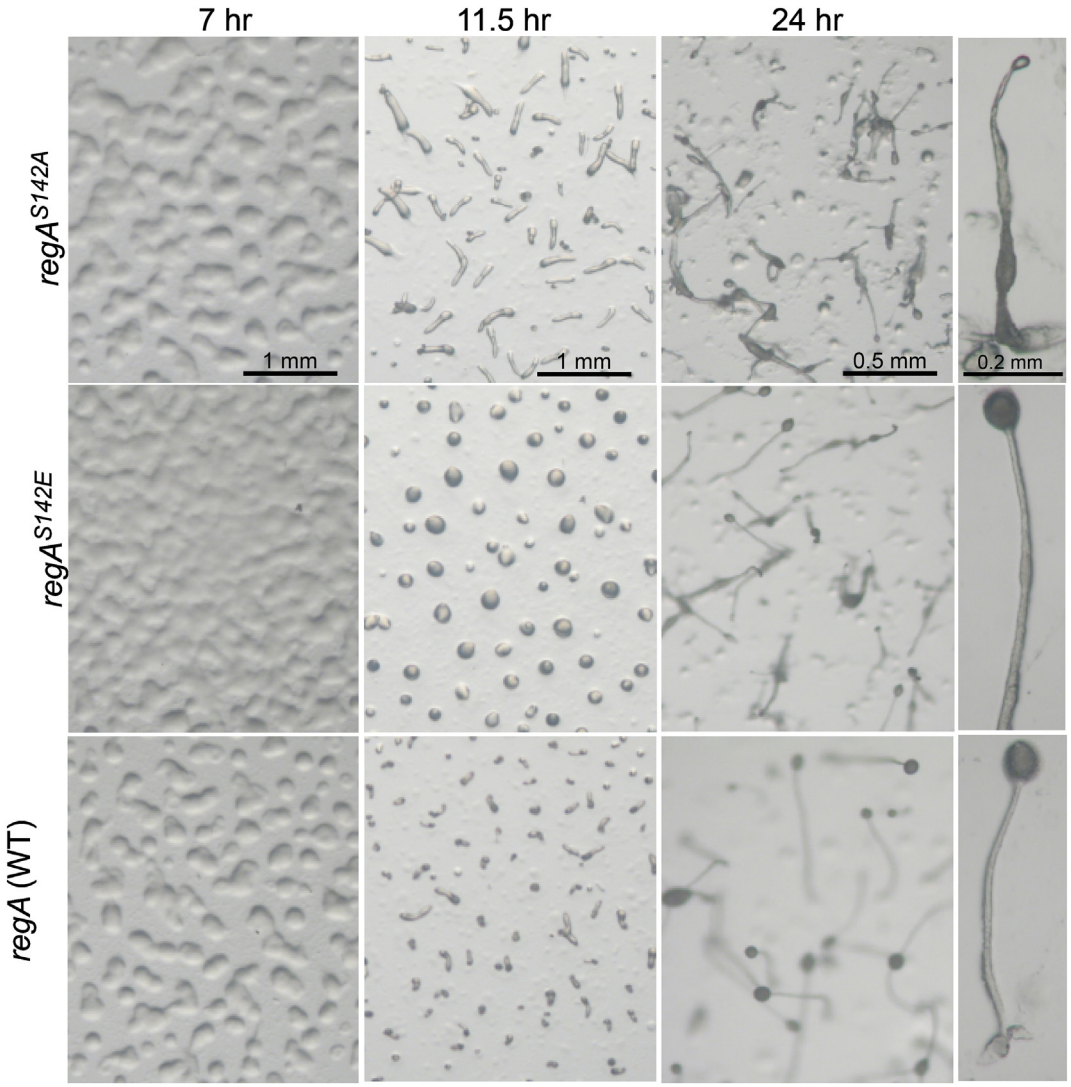

Fig. 7. Developmental morphology of RegA^{S142} mutants and wild-type control cells. Cells were prepared, developed, and imaged as described in Fig. 3 at indicated time points. In each column the cells were developed for the same period of time and the images have the same magnification (scale). Images are representative of multiple assays. Representative fruiting body structures (last column).

5. Conclusion

The genetic analysis of protein kinase regulation of RegA function offers new perspectives on the complexity of phosphodiesterase regulation and how this regulation can impact developmental processes such as developmental progression, aggregation size, and cell distribution. The phenotypes of phospho-ablative mutations substantiate the requirements for the phosphorylation of RegA but interpretations of the phospho-mimetic mutation phenotypes are more challenging. This interpretive challenge stems from the assumption that the phosphorylation events are expected to be transient rather than constitutive. Therefore the phospho-mimetic mutations represent constitutive modifications that might potentially produce phenotypes that do not represent physiological parameters of RegA function. In this study, only individual changes in phosphorylation status were assessed through *regA* mutations but it is possible that some phosphorylation events coexist on RegA and potentially introduce additional levels of complexity to the regulation. The analysis of mutant phosphodiesterase function has the potential to reveal insights on signaling pathway specificity that may not be apparent from measuring cellular or tissue levels of cAMP because specific alterations in phosphodiesterase function might impact some, but not all, cAMP signaling pathways. In this regard, increased cAMP concentrations at the cellular or tissue level might only be descriptive of signaling pathways with down regulated phosphodiesterase function. However, the development and use of single cell reporters of cAMP signaling in relatively simple organisms such *Dictyostelium* might provide the capability to address some of these parameters in the future [6]. Many parameters of phosphodiesterase function such as the prevalence and duration of the phosphorylated states, the impact of these states on catalytic activity and protein interactions, and the cell-type specificity of the regulation remain to be determined but this study reveals some of the potential complexity of phosphodiesterase regulation during development.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.02.005.

Author contributions

NAK isolated the phosphorylated RegA, created and analyzed mutants, NA provided RegA antiserum analysis and JAH and NAK wrote the manuscript. All authors contributed comments to the manuscript.

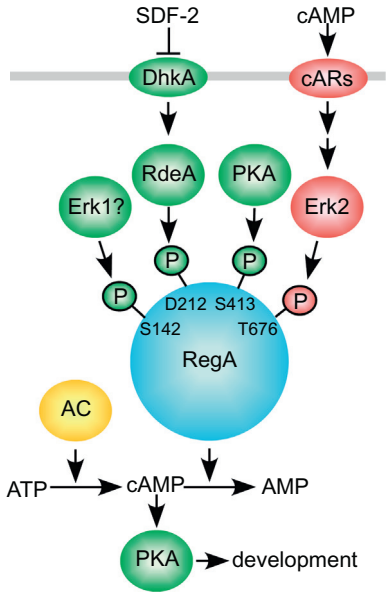

Fig. 8. Model of kinase regulation of RegA function. External signals SDF-2 (spore differentiation factor) and cAMP stimulate pathways that impact the phosphorylation state of RegA. The circled P represents phosphorylations that increase (green) or decrease (red) RegA function. Regulation of adenylyl cyclase (AC) and RegA function modulates cAMP levels and the function of PKA in developmental processes such as aggregation, cell distribution and spore formation. DhkA and RdeA are a histidine kinase and a phosphorelay intermediate protein, respectively. The MAPK Erk2 is activated in response to extracellular cAMP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Acknowledgment

This work was supported by grants NIGMS R15 GM097717-01 and OCAST HR13-36 to JAH. The authors thank Steven Hartson and the OSU Core facility for performing the mass spectrometry and Joshua Moore for technical assistance.

Funding

This work was supported by grants National Institute of General Medical SciencesR15 GM097717-01 and Oklahoma Center for the Advancement of Science and TechnologyHR13-36 to JAH. Mass spectrometry analyses were performed in the Genomics and Proteomics Center at Oklahoma State University, using resources supported by the NSF MRI and EPSCoR programs (award DBI/0722494).

References

[1] M. Kamenetsky, S. Middelhaufe, E.M. Bank, L.R. Levin, J. Buck, C. Steegborn, Molecular details of cAMP generation in mammalian cells: a tale of two systems, J. Mol. Biol. 362 (4) (2006) 623–639.
[2] P. Schaap, Evolution of developmental cyclic adenosine monophosphate signaling in the Dictyostelia from an amoebozoan stress response, Develop. Growth Differ. 53 (4) (2011) 452–462.
[3] K. Soberg, L.V. Moen, B.S. Skalhegg, J.K. Laerdahl, Evolution of the cAMP-dependent protein kinase (PKA) catalytic subunit isoforms, PLoS ONE 12 (7) (2017) e0181091.
[4] T. Brand, R. Schindler, New kids on the block: the Popeye domain containing (POPDC) protein family acting as a novel class of cAMP effector proteins in striated muscle, Cell. Signal. 40 (2017) 156–165.
[5] G.S. Baillie, Compartmentalized signalling: spatial regulation of cAMP by the action of compartmentalized phosphodiesterases, FEBS J. 276 (7) (2009) 1790–1799.
[6] H.V. Edwards, F. Christian, G.S. Baillie, cAMP: novel concepts in compartmentalised signalling, Semin. Cell Dev. Biol. 23 (2) (2012) 181–190.
[7] L. Wills, M. Ehsan, E.L. Whiteley, G.S. Baillie, Location, location, location: PDE4D5 function is directed by its unique N-terminal region, Cell. Signal. 28 (7) (2016) 701–705.
[8] B.A. Fertig, G.S. Baillie, PDE4-mediated cAMP signalling, J. Cardiovasc. Dev. Dis. 5 (1) (2018).
[9] O. Torres-Quesada, J.E. Mayrhofer, E. Stefan, The many faces of compartmentalized PKA signalosomes, Cell. Signal. 37 (2017) 1–11.
[10] M.D. Houslay, Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown, Trends Biochem. Sci. 35 (2) (2010) 91–100.
[11] C. Lugnier, Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents, Pharmacol. Ther. 109 (3) (2006) 366–398.
[12] G.S. Baillie, S.J. MacKenzie, I. McPhee, M.D. Houslay, Sub-family selective actions in the ability of Erk2 MAP kinase to phosphorylate and regulate the activity of PDE4 cyclic AMP-specific phosphodiesterases, Br. J. Pharmacol. 131 (4) (2000) 811–819.
[13] E.V. Hill, M.D. Houslay, G.S. Baillie, Investigation of extracellular signal-regulated kinase 2 mitogen-activated protein kinase phosphorylation and regulation of activity of PDE4 cyclic adenosine monophosphate-specific phosphodiesterases, Methods Mol. Biol. 307 (2005) 225–237.
[14] K.F. MacKenzie, D.A. Wallace, E.V. Hill, D.F. Anthony, D.J. Henderson, D.M. Houslay, J.S. Arthur, G.S. Baillie, M.D. Houslay, Phosphorylation of cAMP-specific PDE4A5 (phosphodiesterase-4A5) by MK2 (MAPKAPK2) attenuates its activation through protein kinase a phosphorylation, Biochem. J. 435 (3) (2011) 755–769.
[15] S.J. MacKenzie, G.S. Baillie, I. McPhee, G.B. Bolger, M.D. Houslay, ERK2 mitogen-activated protein kinase binding, phosphorylation, and regulation of the PDE4D cAMP-specific phosphodiesterases. The involvement of COOH-terminal docking sites and NH2-terminal UCR regions, J. Biol. Chem. 275 (22) (2000) 16609–16617.
[16] S.J. MacKenzie, G.S. Baillie, I. McPhee, C. MacKenzie, R. Seamons, T. McSorley, J. Millen, M.B. Beard, G. van Heeke, M.D. Houslay, Long PDE4 cAMP specific phosphodiesterases are activated by protein kinase A-mediated phosphorylation of a single serine residue in upstream conserved region 1 (UCR1), Br. J. Pharmacol. 136 (3) (2002) 421–433.
[17] C. Sette, M. Conti, Phosphorylation and activation of a cAMP-specific phosphodiesterase by the cAMP-dependent protein kinase. Involvement of serine 54 in the enzyme activation, J. Biol. Chem. 271 (28) (1996) 16526–16534.
[18] M. Brenner, Cyclic AMP levels and turnover during development of the cellular slime mold *Dictyostelium discoideum*, Dev. Biol. 64 (2) (1978) 210–223.
[19] W.F. Loomis, The Development of *Dictyostelium discoideum*, Academic Press, New York, 1982.
[20] W.F. Loomis, Role of PKA in the timing of developmental events in *Dictyostelium* cells, Microbiol. Mol. Biol. Rev. 62 (3) (1998) 684–694.
[21] J.G. Williams, Transcriptional regulation of *Dictyostelium* pattern formation, EMBO Rep. 7 (7) (2006) 694–698.
[22] H. Zhang, P.J. Heid, D. Wessels, K.J. Daniels, T. Pham, W.F. Loomis, D.R. Soll, Constitutively active protein kinase a disrupts motility and chemotaxis in *Dictyostelium discoideum*, Eukaryot. Cell 2 (1) (2003) 62–75.
[23] S.K. Mann, R.A. Firtel, cAMP-dependent protein kinase differentially regulates prestalk and prespore differentiation during *Dictyostelium* development, Development 119 (1) (1993) 135–146.
[24] S.K. Mann, D.L. Richardson, S. Lee, A.R. Kimmel, R.A. Firtel, Expression of cAMP-dependent protein kinase in prespore cells is sufficient to induce spore cell differentiation in *Dictyostelium*, Proc. Natl. Acad. Sci. U. S. A. 91 (22) (1994) 10561–10565.
[25] S.K. Mann, R.A. Firtel, A developmentally regulated, putative serine/threonine protein kinase is essential for development in *Dictyostelium*, Mech. Dev. 35 (2) (1991) 89–101.
[26] C. Anjard, S. Pinaud, R.R. Kay, C.D. Reymond, Overexpression of Dd PK2 protein kinase causes rapid development and affects the intracellular cAMP pathway of *Dictyostelium discoideum*, Development 115 (3) (1992) 785–790.
[27] C. Anjard, C. Zeng, W.F. Loomis, W. Nellen, Signal transduction pathways leading to spore differentiation in *Dictyostelium discoideum*, Dev. Biol. 193 (2) (1998) 146–155.
[28] G. Shaulsky, R. Escalante, W.F. Loomis, Developmental signal transduction pathways uncovered by genetic suppressors, Proc. Natl. Acad. Sci. U. S. A. 93 (26) (1996) 15260–15265.
[29] G. Shaulsky, D. Fuller, W.F. Loomis, A cAMP-phosphodiesterase controls PKA-dependent differentiation, Development 125 (4) (1998) 691–699.
[30] P.A. Thomason, D. Traynor, G. Cavet, W.T. Chang, A.J. Harwood, R.R. Kay, An intersection of the cAMP/PKA and two-component signal transduction systems in *Dictyostelium*, EMBO J. 17 (10) (1998) 2838–2845.
[31] S. Krishnamurthy, N.K. Tulsian, A. Chandramohan, G.S. Anand, Parallel Allostery by cAMP and PDE coordinates activation and termination phases in cAMP Signaling, Biophys. J. 109 (6) (2015) 1251–1263.
[32] R.J. De Wit, R. Bulgakov, T.F. Rinke de Wit, T.M. Konijn, Developmental regulation of the pathways of folate-receptor-mediated stimulation of cAMP and cGMP synthesis in *Dictyostelium discoideum*, Differentiation 32 (3) (1986) 192–199.
[33] J.A. Hadwiger, S. Lee, R.A. Firtel, The G alpha subunit G alpha 4 couples to pterin receptors and identifies a signaling pathway that is essential for multicellular development in *Dictyostelium*, Proc. Natl. Acad. Sci. U. S. A. 91 (22) (1994) 10566–10570.
[34] A. Kumagai, J.A. Hadwiger, M. Pupillo, R.A. Firtel, Molecular genetic analysis of two G alpha protein subunits in *Dictyostelium*, J. Biol. Chem. 266 (2) (1991) 1220–1228.
[35] P. Pan, E.M. Hall, J.T. Bonner, Folic acid as second chemotactic substance in the cellular slime moulds, Nature (London) New Biol. 237 (1972) 181–182.
[36] R.H. Insall, R.D. Soede, P. Schaap, P.N. Devreotes, Two cAMP receptors activate common signaling pathways in *Dictyostelium*, Mol. Biol. Cell 5 (6) (1994) 703–711.
[37] H.N. Nguyen, J.A. Hadwiger, The Galpha4 G protein subunit interacts with the MAP kinase ERK2 using a D-motif that regulates developmental morphogenesis in

Dictyostelium, Dev. Biol. 335 (2) (2009) 385–395.
[38] M. Maeda, L. Aubry, R. Insall, C. Gaskins, P.N. Devreotes, R.A. Firtel, Seven helix chemoattractant receptors transiently stimulate mitogen-activated protein kinase in *Dictyostelium* - role of heterotrimeric G proteins, J. Biol. Chem. 271 (1996) 3351–3354.
[39] M. Maeda, R.A. Firtel, Activation of the mitogen-activated protein kinase ERK2 by the chemoattractant folic acid in *Dictyostelium*, J. Biol. Chem. 272 (38) (1997) 23690–23695.
[40] J.E. Segall, A. Kuspa, G. Shaulsky, M. Ecke, M. Maeda, C. Gaskins, R.A. Firtel, W.F. Loomis, A MAP kinase necessary for receptor-mediated activation of adenylyl cyclase in *Dictyostelium*, J. Cell Biol. 128 (3) (1995) 405–413.
[41] M. Maeda, S. Lu, G. Shaulsky, Y. Miyazaki, H. Kuwayama, Y. Tanaka, A. Kuspa, W.F. Loomis, Periodic signaling controlled by an oscillatory circuit that includes protein kinases ERK2 and PKA, Science 304 (5672) (2004) 875–878.
[42] P.A. Thomason, D. Traynor, J.B. Stock, R.R. Kay, The RdeA-RegA system, a eukaryotic phospho-relay controlling cAMP breakdown, J. Biol. Chem. 274 (39) (1999) 27379–27384.
[43] C. Anjard, W.F. Loomis, Peptide signaling during terminal differentiation of *Dictyostelium*, Proc. Natl. Acad. Sci. U. S. A. 102 (21) (2005) 7607–7611.
[44] J.A. Hadwiger, R.A. Firtel, Analysis of Ga4, a G-protein subunit required for multicellular development in *Dictyostelium*, Genes Dev. 6 (1) (1992) 38–49.
[45] D.J. Schwebs, H.N. Nguyen, J.A. Miller, J.A. Hadwiger, Loss of cAMP-specific phosphodiesterase rescues spore development in G protein mutant in *Dictyostelium*, Cell. Signal. 26 (2) (2014) 409–418.
[46] J.A. Hadwiger, K. Natarajan, R.A. Firtel, Mutations in the *Dictyostelium* heterotrimeric G protein a subunit Ga5 alter the kinetics of tip morphogenesis, Development 122 (1996) 1215–1224.
[47] D.J. Watts, J.M. Ashworth, Growth of myxameobae of the cellular slime mould *Dictyostelium* discoideum in axenic culture, Biochem. J. 119 (2) (1970) 171–174.
[48] H.N. Nguyen, B. Raisley, J.A. Hadwiger, MAP kinases have different functions in *Dictyostelium* G protein-mediated signaling, Cell. Signal. 22 (5) (2010) 836–847.
[49] J.A. Hadwiger, Developmental morphology and chemotactic responses are dependent on G alpha subunit specificity in *Dictyostelium*, Dev. Biol. 312 (1) (2007) 1–12.
[50] D.J. Schwebs, M. Pan, N. Adhikari, N.A. Kuburich, T. Jin, J.A. Hadwiger, *Dictyostelium* Erk2 is an atypical MAPK required for chemotaxis, Cell. Signal. 46 (2018) 154–165.
[51] D.J. Manstein, H.P. Schuster, P. Morandini, D.M. Hunt, Cloning vectors for the production of proteins in *Dictyostelium discoideum*, Gene 162 (1) (1995) 129–134.
[52] D.J. Schwebs, J.A. Hadwiger, The *Dictyostelium* MAPK ERK1 is phosphorylated in a secondary response to early developmental signaling, Cell. Signal. 27 (1) (2015) 147–155.
[53] A. Shevchenko, H. Tomas, J. Havlis, J.V. Olsen, M. Mann, In-gel digestion for mass spectrometric characterization of proteins and proteomes, Nat. Protoc. 1 (6) (2006) 2856–2860.
[54] L.J. Licklider, C.C. Thoreen, J. Peng, S.P. Gygi, Automation of nanoscale microcapillary liquid chromatography-tandem mass spectrometry with a vented column, Anal. Chem. 74 (13) (2002) 3076–3083.
[55] M.R. Emmett, R.M. Caprioli, Micro-electrospray mass spectrometry: ultra-high-sensitivity analysis of peptides and proteins, J. Am. Soc. Mass Spectrom. 5 (7) (1994) 605–613.
[56] G.E. Reid, R.K. Rasmussen, D.S. Dorow, R.J. Simpson, Capillary column chromatography improves sample preparation for mass spectrometric analysis: complete characterization of human alpha-enolase from two-dimensional gels following in situ proteolytic digestion, Electrophoresis 19 (6) (1998) 946–955.
[57] S. Voruganti, J.C. Lacroix, C.N. Rogers, J. Rogers, R.L. Matts, S.D. Hartson, The anticancer drug AUY922 generates a proteomics fingerprint that is highly conserved among structurally diverse Hsp90 inhibitors, J. Proteome Res. 12 (8) (2013) 3697–3706.
[58] Q. Hu, R.J. Noll, H. Li, A. Makarov, M. Hardman, R. Graham Cooks, The Orbitrap: a new mass spectrometer, J. Mass Spectrom. 40 (4) (2005) 430–443.
[59] P.L. Courchesne, M.D. Jones, J.H. Robinson, C.S. Spahr, S. McCracken, D.L. Bentley, R. Luethy, S.D. Patterson, Optimization of capillary chromatography ion trap-mass spectrometry for identification of gel-separated proteins, Electrophoresis 19 (6) (1998) 956–967.
[60] N. Wang, L. Li, Exploring the precursor ion exclusion feature of liquid chromatography-electrospray ionization quadrupole time-of-flight mass spectrometry for improving protein identification in shotgun proteome analysis, Anal. Chem. 80 (12) (2008) 4696–4710.
[61] M.J. Schroeder, J. Shabanowitz, J.C. Schwartz, D.F. Hunt, J.J. Coon, A neutral loss activation method for improved phosphopeptide sequence analysis by quadrupole ion trap mass spectrometry, Anal. Chem. 76 (13) (2004) 3590–3598.
[62] D.N. Perkins, D.J. Pappin, D.M. Creasy, J.S. Cottrell, Probability-based protein identification by searching sequence databases using mass spectrometry data, Electrophoresis 20 (18) (1999) 3551–3567.
[63] R.D. Bjornson, N.J. Carriero, C. Colangelo, M. Shifman, K.H. Cheung, P.L. Miller, K. Williams, X!!Tandem, an improved method for running X!Tandem in parallel on collections of commodity computers, J. Proteome Res. 7 (1) (2008) 293–299.
[64] A. Keller, A.I. Nesvizhskii, E. Kolker, R. Aebersold, Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search, Anal. Chem. 74 (20) (2002) 5383–5392.
[65] D. Mika, J. Leroy, G. Vandecasteele, R. Fischmeister, PDEs create local domains of cAMP signaling, J. Mol. Cell. Cardiol. 52 (2) (2012) 323–329.
[66] A.J. Harwood, N.A. Hopper, M.N. Simon, D.M. Driscoll, M. Veron, J.G. Williams, Culmination in *Dictyostelium* is regulated by the cAMP-dependent protein kinase, Cell 69 (4) (1992) 615–624.
[67] J.G. Williams, A.J. Harwood, N.A. Hopper, M.N. Simon, S. Bouzid, M. Veron, Regulation of *Dictyostelium* morphogenesis by cAMP-dependent protein kinase, Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 340 (1293) (1993) 305–313.
[68] A. Errico, K. Deshmukh, Y. Tanaka, A. Pozniakovsky, T. Hunt, Identification of substrates for cyclin dependent kinases, Adv. Enzym. Regul. 50 (1) (2010) 375–399.
[69] R.J. Huber, The cyclin-dependent kinase family in the social amoebozoan *Dictyostelium discoideum*, Cell. Mol. Life Sci. 71 (4) (2014) 629–639.
[70] M.D. Houslay, G.S. Baillie, The role of ERK2 docking and phosphorylation of PDE4 cAMP phosphodiesterase isoforms in mediating cross-talk between the cAMP and ERK signalling pathways, Biochem. Soc. Trans. 31 (Pt 6) (2003) 1186–1190.
[71] M.E. Oates, P. Romero, T. Ishida, M. Ghalwash, M.J. Mizianty, B. Xue, Z. Dosztanyi, V.N. Uversky, Z. Obradovic, L. Kurgan, A.K. Dunker, J. Gough, D(2)P(2): database of disordered protein predictions, Nucleic Acids Res. 41 (Database issue) (2013) D508–D516.