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Combinatorial cell-specific regulation of GSK3 directs cell differentiation and polarity in *Dictyostelium*

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SUMMARY

In *Dictyostelium*, the interaction of secreted cAMP with specific cell surface receptors regulates the activation/de-activation of GSK3, which mediates developmental cell patterning. In addition, *Dictyostelium* cells polarize in response to extracellular cAMP, although a potential role for GSK3 in this pathway has not been investigated. Previously, we had shown that ZAK1 was an activating tyrosine kinase for GSK3 function in *Dictyostelium* and we now identify ZAK2 as the other tyrosine kinase in the cAMP-activation pathway for GSK3; no additional family members exist. We also now show that tyrosine phosphorylation/activation of GSK3 by ZAK2 and ZAK1 separately regulate GSK3 in distinct differentiated cell populations, and that ZAK2 acts in both autonomous and non-autonomous pathways to regulate these cell-type differentiations. Finally, we demonstrate that efficient polarization of *Dictyostelium* towards cAMP depends on ZAK1-mediated tyrosine phosphorylation of GSK3. Combinatorial regulation of GSK3 by ZAK kinases in *Dictyostelium* guides cell polarity, directional cell migration and cell differentiation, pathways that extend the complexity of GSK3 signaling throughout the development of *Dictyostelium*.

KEY WORDS: cAMP, Cell polarity, Receptors, Protein phosphorylation, Chemotaxis, *Dictyostelium*

INTRODUCTION

The regulation of cell specification by GSK3 may be universal (Angers and Moon, 2009; Harwood, 2008; Kimmel et al., 2004; McNeill and Woodgett, 2010). For metazoans, this generally involves the functional repression of GSK3 by Wnt-mediated canonical signaling. The Wnts are a family of secreted glycoproteins, the canonical signals of which are transduced intracellularly by co-receptors of seven-transmembrane Frizzled (Fz) and LDL receptor-related proteins (LRP) 5/6 (Angers and Moon, 2009; Bhanot et al., 1996; McNeill and Woodgett, 2010; Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000; Yang-Snyder et al., 1996). There are, however, at least two multicellular systems, *C. elegans* and *Dictyostelium*, that use both activating and inhibitory paths for GSK3 regulation of cell fate determination (Angers and Moon, 2009; Hardin and King, 2008; Harwood, 2008; Kimmel et al., 2004; McNeill and Woodgett, 2010; Moon et al., 2002). GSK3 activation during endoderm/mesoderm specification in *C. elegans* is Wnt/Fz dependent. *Dictyostelium* use a distinct signal, secreted cAMP, that targets a family of specific cell-surface receptors (CAR1, CAR2, CAR3, and CAR4) and regulates GSK3 activity (Harwood, 2008; Kimmel et al., 2004). Signaling by both cAMP and Wnt also regulates cell polarity (Hardin and King, 2008; Harwood, 2008; Kimmel and Firtel, 2004; Kimmel and Parent, 2003; Kimmel et al., 2004; Schlesinger et al., 1999; Veeman et al., 2003; Walston et al., 2004).

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Although in certain aspects the cAMP/CAR and Wnt/Fz pathways appear functionally related, they are mechanistically distinct. GSK3 activity per se is not altered upon Wnt stimulation; rather, Wnt/Fz functions to disrupt association of GSK3 with the specific substrate β-catenin. By contrast, in *Dictyostelium*, signaling through distinct cAMP receptors serves to activate or inhibit the enzymatic activity of GSK3. These actions, respectively, involve the tyrosine phosphorylation of GSK3 via ZAK1 or de-phosphorylation by a PTPase (Harwood et al., 1995; Kim et al., 2002; Kim et al., 1999; Schilde et al., 2004; Strmecki et al., 2007).

Dictyostelium development is characterized by a succession of distinct phases. Early events regulate cell polarization and directed cell migration toward centers of cAMP signaling where cells form multicellular aggregates that differentiate into progenitor prespore and prestalk cells (Kimmel et al., 2004; Williams, 2006). After aggregation, precursor populations sort asymmetrically along a body axis. The anterior 20% is primarily prestalk, whereas the posterior 80% is highly enriched in prespore cells. However, the prestalk population is not homogeneous; prestalk A (pstA) and prestalk B (pstB) cell populations are identified by the expression of specific genes. During the commitment to terminal differentiation, the prepore and prestalk precursors differentiate into mature spores and stalk cells (Gaudet et al., 2008; Kimmel and Firtel, 2004; Williams, 2006).

We had shown that the cAMP/CAR3/ZAK1/GSK3 cascade positively regulates prespore gene expression and spore differentiation, but suppresses prestalk differentiation (Kim et al., 2002; Kim and Kimmel, 2000; Kim et al., 1999; Kimmel and Firtel, 2004). *car3*, *gsk3* and *zak1* nulls have impaired prespore/spore differentiation, and resistance to cAMP-mediated repression of pstB cell and stalk formation (Harwood et al., 1995; Kim et al., 2002; Kim and Kimmel, 2000; Kim et al., 1999; Kimmel and Firtel, 2004; Plyte et al., 1999; Schilde et al., 2004). CAR3 stimulation will activate ZAK1, which, in turn, will tyrosine phosphorylate and activate GSK3. However, biochemical and

genetic data indicate that additional components immediately upstream of GSK3 must be involved; limited, but reproducible, tyrosine phosphorylation and activation of GSK3 are evident in *zak1* nulls, suggesting the presence of an additional tyrosine kinase (Kim et al., 2002). Furthermore, regulation of pstA cells by ZAK1 and GSK3 is not identical (Harwood et al., 1995; Kim et al., 1999). We have identified a new activating tyrosine kinase, ZAK2, in the GSK3 pathway. Although both ZAK1 and ZAK2 can phosphorylate and activate GSK3, they function distinctly in control of the different cell populations. ZAK2 and ZAK1 regulate separate prestalk populations through the common target GSK3. Both kinases are required to activate prespore/spore differentiation via GSK3, but ZAK2 also appears to have an additional non-autonomous function. Finally, we extended our studies to examine the regulation of cell polarity in *Dictyostelium* by cAMP- and GSK3-mediated signaling. Results indicate that activation of GSK3 by ZAK1 is required for cell polarization and migration.

MATERIALS AND METHODS

Dictyostelium culture, development and differentiation

Dictyostelium wild-type and mutant cells were grown, developed on nitrocellulose filters and differentiated in shaking culture or in monolayers as described previously (Kim et al., 2002; Kim et al., 1999). Developing organisms with cell-specific *lacZ* reporter plasmids were fixed and stained as described previously (Richardson et al., 1994). Relevant DictyBase gene numbers are DDB0185150 for *GSK3*, DDB0185184 for *ZAK1* and DDB0229958 for *ZAK2*.

Isolation of *ZAK2* cDNA and generation of *zak2* nulls

ZAK2 cDNA was isolated as described (Kim et al., 1999). The blasticidin-resistance cassette was subcloned into the single *Eco*RV site (GATATC) at nucleotide 1506 within the C-terminal tyrosine kinase domain coding region of the *ZAK2* cDNA. Disruptants were screened by PCR using a 5′ primer at nucleotide 1440 (GGTGGTTCAATACTTTATATGGCACC-AGAG) and 3′ primer at nucleotide 1883 (CCACTACCATAGGTTG-ATGAGT). Disruption was confirmed by genomic Southern blot and loss of expression by a developmental northern blot hybridized with a full-length probe to ZAK2. Disruption was confirmed by genomic Southern blot and loss of expression was confirmed by a developmental northern blot hybridized with a full-length probe to ZAK2.

GSK3 kinase assay

The GSK3 peptide kinase assays and in vitro phosphorylation were as described previously (Kim et al., 2002; Kim et al., 1999). Whole-cell lysates were prepared, normalized by GSK3 western blot and the primed GS peptide was used as a specific substrate. Background kinase activities were corrected by LiCl treatment, a specific GSK3 inhibitor (Kim et al., 2002; Kim et al., 1999).

Prespore and prestalk A cell purifications

Wild-type cells expressing GFP with either the *cotB* or *ecmA* promoters were developed on the nitrocellulose filter for 15 hours. Slugs were harvested and mechanically dissociated in PBS and 40 mM EDTA (Chen et al., 2004). Cells were washed, resuspended at 10^6 cell/ml and sorted by fluorescence intensity using the FACS VantageSE (BD Biosciences). Cells with strongest signal (<5%) were positively selected as prespore specific (Chen et al., 2004). The positive and negative pools were confirmed by fluorescence microscopy and RT-PCR using *cotB* primers.

Sporulation in chimeras

Wild-type and *zak2*-null cells expressing *act15*/*lacZ* were grown and mixed at varying cells ratios with growing unmarked wild-type *zak2*-null cells. Cell mixes were plated for development and sori collected from terminal structures and disrupted. Cells were visualized under bright-field and fluorescence microscopy. Mature spores exhibited characteristic ovoid and phase bright properties.

Chemotaxis

For submerged experiments, log phase cells were analyzed at various cell densities. After 12 hours at 20°C, cell migration, streaming and aggregation were scored. For chemotaxis, log phase cells were differentiated with 50 nM pulses of cAMP for 7 hours and plated at 6×10^4 cells/cm^2. An Eppendorf Patchman micromanipulator with a glass capillary needle (Eppendorf Femtotip) filled with 100 nM cAMP solution was used to test cell responses, followed by time-lapse, digital recording and analysis by DIAS (Brzostowski et al., 2004; Soll, 1999).

RESULTS

Tyrosine kinase ZAK2 regulates GSK3 and cell pattern formation

We previously described the screening of bacterial expression libraries with anti-phosphotyrosine sera to isolate cDNAs for *Dictyostelium* tyrosine kinases (Kim et al., 1999). The procedure yielded multiple tyrosine kinases. We characterized the biochemical and cellular properties of tyrosine kinase ZAK1 in considerable detail (Kim et al., 2002; Kim et al., 1999) and also identified another tyrosine kinase, ZAK2, that has a very high amino acid sequence identity with ZAK1 (Fig. 1). ZAK2 was independently identified as DPYK4 (Adler et al., 1996), but its sequence was only partially characterized. Given its sequence and functional kinship with ZAK1, we suggest that ZAK2 is a more consistent nomenclature.

Both ZAK2 and ZAK1 contain an N-terminal serine/threonine-type (PSK) kinase domain, as well as a separate C-terminal tyrosine kinase (PTK) domain (Fig. 1A). Both domains possess all the conserved residues essential for their specific activities. This distinguishes the ZAK tyrosine kinase group from that of the Janus Kinases (JAKs). JAKs have active C-terminal PTKs, but inactive N-terminal PSKs (Hou et al., 2002). The PTK and PSK domains of ZAK1 and ZAK2 are highly related (Fig. 1A), although there is an AAT, tri-nucleotide expansion in ZAK1 that is absent in ZAK2. This repeat encodes an asparagine block [$N_{(40)}$] within the PSK activation loop of ZAK1 that causes low activity (Kim et al., 1999).

ZAK2 is expressed during the growth phase of *Dictyostelium* and throughout the major developmental stages (Fig. 1B). To assess the role of ZAK2 during the *Dictyostelium* life cycle, we disrupted the *ZAK2* gene by homologous recombination. *ZAK2* disruption was confirmed by genomic Southern (data not shown) and northern blot hybridizations (Fig. 1D) and RT-PCR (Fig. 1C) using gene-specific *ZAK2* and *ZAK1* probes.

We compared the development of wild-type and *zak2*-null cells. Gross morphology is largely similar between the two strains through ~12 hours of development. However, as differentiation and morphogenesis proceed, we observed dramatic defects in *zak2* nulls. The terminal fruiting body structure of wild-type cells is characterized by a sorus, or large spore mass, atop an elongated stalk of vacuolated cells. Terminally differentiated *zak2* nulls do not form as prominent sori and exhibit expanded stalk structures (Fig. 1D), indicating that ZAK2 plays a central and reciprocal role in control of spore and stalk cell differentiation. These morphological characteristics are similar to those of *zak1* and *gsk3* nulls, and suggest that ZAK2, like ZAK1 and GSK3, is required in a pathway for spore formation, and perhaps suppresses stalk cell differentiation (Kim et al., 1999). The sequence, structural and functional similarities between ZAK1 and ZAK2 also suggest that GSK3 is a regulatory target of ZAK2 in the control of development.

Dictyostelium and mammalian GSK3s have high sequence identity and are identically phosphorylated by ZAK1 in vitro (Kim et al., 2002; Kim et al., 1999). We, therefore, examined if rabbit

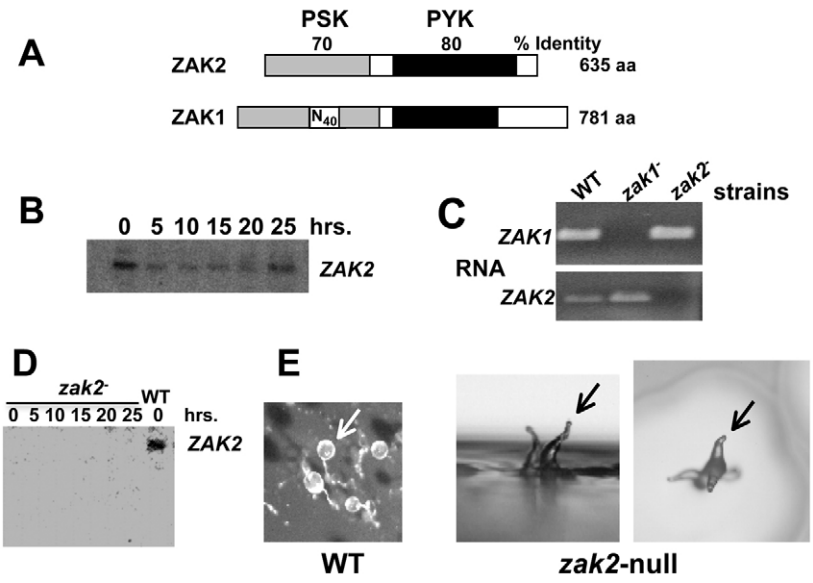

Fig. 1. Tyrosine kinase ZAK2 has a high sequence identity with ZAK1 and similarly regulates cell fate decisions. (**A**) A schematic diagram of the molecular architectures of ZAK2 and ZAK1. Both kinases possess an N-terminal serine/threonine kinase (PSK) domain, a short linker region and a C-terminal tyrosine kinase (PYK) domain. Asparagine repeat-block (N) in the activation loop of ZAK1 does not exist in ZAK2. (**B**) *ZAK2* is expressed throughout development. Equal amounts of total RNA for each developmental stage were analyzed by northern blot using a *ZAK2*-specific probe. (**C**) *ZAK2* mRNA is not expressed in *ZAK2* mutant cells. RNA was prepared from growing wild-type, *zak1*-null and *zak2*-null cells, and *ZAK1*- and *ZAK2*-specific primers were used for RT-PCR. (**D**) *ZAK2* mRNA is not expressed in *ZAK2* mutant cells. Equal amounts of total RNA for each developmental stage of *zak2*-null cells and from growing (0 hours). Wild-type cells were analyzed by northern blot using a full-length *ZAK2* probe. (**E**) Terminal differentiation (arrows) of wild-type and *zak2*-null strains after plating on bacterial lawns for 10 days.

GSK3β were also tyrosine phosphorylated by the purified, bacterially expressed tyrosine kinase domain of ZAK2. GSK3β was incubated with or without ZAK2 in the presence of [γ-^{32}P]ATP and tyrosine-specific phosphorylation monitored (Fig. 2A). Although there is an inherent tyrosine auto-phosphorylation activity in GSK3β, this assay has proven effective for identifying tyrosine trans-phosphorylation of GSK3 by heterologous protein tyrosine kinases (Kim et al., 1999). Indeed, GSK3 tyrosine phosphorylation is significantly enhanced by the presence of ZAK2 similar to that observed with ZAK1 (Kim et al., 1999).

The ability of ZAK2 to phosphorylate GSK3 in vitro suggests that ZAK2 also regulates GSK3 in vivo. We, thus, examined the kinase activity of GSK3 throughout development of *zak2* nulls. Wild-type, *zak2*-null and *gsk3*-null cells were developed and whole-cell extracts prepared at representative time points. GSK3 kinase activity was measured (Fig. 2B) using a pre-phosphorylated peptide that behaves as a GSK3-specific substrate (Plyte et al., 1999); activity was normalized to GSK3 levels by western blotting using α-GSK3 antibody. As has been previously observed (Kim et al., 2002; Kim et al., 1999; Plyte et al., 1999), wild-type cells exhibit a twofold activation of normalized GSK3 activity during development. GSK3 activation during development was significantly compromised in *zak2* nulls compared with wild type. We had previously shown that *zak1* nulls also had comparably reduced levels of GSK3 activation during development (Kim et al., 1999). The data indicate that the remaining elevated GSK3 activity observed in *zak2* nulls is the result of ZAK1 expression and its ability to phosphorylate and activate GSK3; unfortunately, we were unable to establish a cell line that was deficient for both *ZAK1* and *ZAK2*. Nonetheless, these data suggest that ZAK1 and ZAK2 function collectively to regulate GSK3 during *Dictyostelium* development.

Cell specific patterns of gene expression

We next compared the relative expression of *ZAK2* and *ZAK1* mRNAs in prespore and prestalk cells, the major progenitor cell types of *Dictyostelium* development. Specificity and relative efficiency of *ZAK2* and *ZAK1* mRNA amplification by RT-PCR was established using RNA from growing wild-type, *zak2*-null and *zak1*-null strains as controls (see Fig. 1C).

Prespore cells were isolated by FACS from a population of differentiated *Dictyostelium* that expressed GFP from the prespore-specific *cotB* promoter (*cotB*/*GFP cells*), and relative levels of prespore *ZAK2* and *ZAK1* mRNA were assayed by semi-quantitative RT-PCR (Fig. 3A). We show that *ZAK1* mRNA

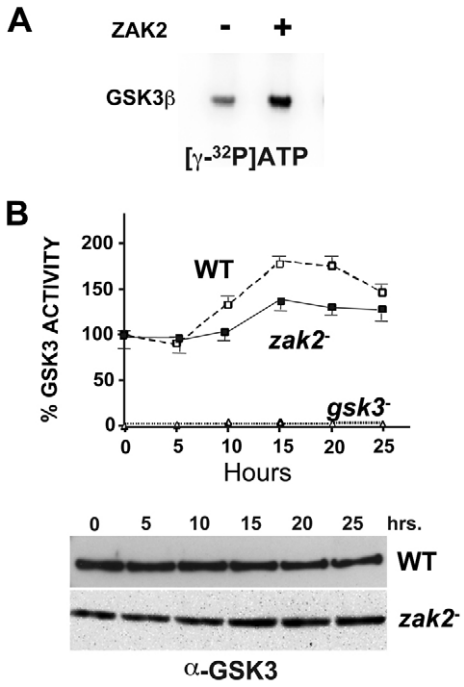

Fig. 2. GSK3 is phosphorylated and activated by ZAK2. (**A**) Purified mammalian GSK3β was incubated in the absence (–) or presence (+) of purified, recombinant ZAK2 tyrosine kinase domain and [γ-^{32}P]ATP, and then analyzed by gel electrophoreses. SDS gels were then incubated with 1 N KOH at 55°C for 90 minutes, which hydrolyzes phosphoserine and phosphothreonine, but does not affect phosphotyrosine (Kim et al., 1999). (**B**) Whole-cell lysates were prepared from wild-type, *zak2*-null and *gsk3*-null cells at each developmental stage, and GSK3 kinase activities were measured using a GSK3-specific peptide substrate. Values were normalized by western blot assay using α-GSK3 (lower panel). Results are mean±s.e.m.

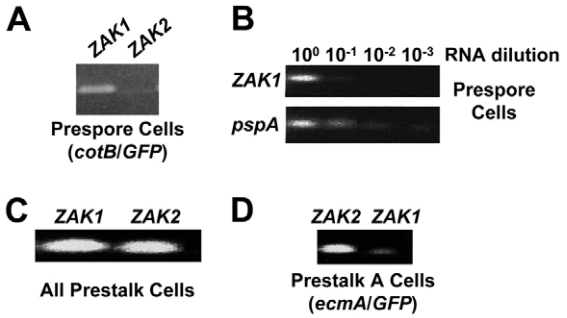

Fig. 3. Cell-specific patterns of *ZAK1* and *ZAK2* gene expression. (**A**) *ZAK2* is expressed at relatively low levels in prespore cells. RNA was prepared from purified wild-type prespore cells, and *ZAK1*- and *ZAK2*-specific primers were used for semi-quantitative RT-PCR to determine relative abundance of the respective mRNAs. (**B**) *ZAK1*- and *pspA*-specific primers were used for semi-quantitative RT-PCR to amplify a dilution series of RNA from prespore cells. (**C**) *ZAK1* and *ZAK2* mRNAs are expressed at similar levels in the total prestalk population. RNA was prepared from cells that did not express *cotB/GFP*, and *ZAK1*- and *ZAK2*-specific primers were used for semi-quantitative RT-PCR to determine relative abundance of the respective mRNAs. (**D**) Expression of *ZAK2* is relatively enriched in prestalk A cells. RNA was prepared from purified wild-type prestalk A cells and *ZAK1*- and *ZAK2*-specific primers were used for semi-quantitative RT-PCR to determine relative abundance of the respective mRNAs.

expression in prespore cells is enriched ~10-fold in comparison with *ZAK2* mRNA. The quality of the RT-PCR design was confirmed using sequentially diluted prespore RNA samples to amplify mRNAs from *ZAK1* and the prespore marker *pspA* (Fig. 3B). In agreement with these conclusions, recently published data of global gene expression in *Dictyostelium* using RNA-seq (Parikh et al., 2010) predict more than a 10-fold enrichment of *ZAK1* mRNA sequences in prespore cells in comparison with *ZAK2* (*pyk4*) mRNA in prespore cells at the threshold of RNA-seq detection.

Relative quantification of *ZAK2* and *ZAK1* expression in prestalk cells is technically more complex. In agreement with RNA-seq analyses (Parikh et al., 2010), *ZAK2* and *ZAK1* mRNA levels are very similar in the total prestalk cell population (Fig. 3C). However, differences are observed in the subpopulations of prestalk (pst) cells, which are characterized by distinct gene expression patterns. The pstA cells are defined by the presence of *ecmA* mRNA expression and represent the largest pst cell population. pstA cells were isolated by FACS from a population of differentiated *Dictyostelium* expressing GFP from the *ecmA* promoter (*ecmA*/*GFP* cells), and relative levels of *ZAK1* and *ZAK2* mRNA assayed by semi-quantitative RT-PCR (Fig. 3D). Here, we see that *ZAK*2 expression in the pstA cells is significantly enriched in comparison with that of *ZAK1*; the prestalk *ZAK1* mRNA expression is, thus, primarily restricted to cells that do not express *ecmA* (e.g. prestalk B cells). Although we were unable to obtain a sufficiently pure population of pstB for analyses, these conclusions are consistent with our previous data that indicate that ZAK1 has a significant function in the regulation of *ecmB* expression, but not of *ecmA* gene expression (Kim et al., 1999).

ZAK1 and ZAK2 regulate distinct prestalk subpopulations

Despite the restriction of ZAK2 and ZAK1 expression to different pst subpopulations, the similarity of the terminal phenotypes of *zak1*- and *zak2*-null cells, and their shared capacity to regulate GSK3 activity during late development suggest some commonality in control of development. ZAK1 activation of GSK3 suppresses prestalk differentiation, but promotes prespore differentiation (Kim et al., 1999). Data from Fig. 1D suggest that *zak2* nulls are also hyperactive for stalk differentiation. We, thus, investigated whether ZAK2, like ZAK1, was involved in a signaling pathway that represses stalk differentiation. *Dictyostelium* will form stalk cells in monolayer culture when treated with the DIF-1 inducing factor, but stalk differentiation is inhibited by cAMP (Berks and Kay, 1988). The majority of wild-type, *zak2*-null and *zak1*-null cells form stalk cells in monolayer culture in the presence of DIF-1 (Fig. 4A). However, only wild-type cells were sensitive to inhibition by cAMP (Fig. 3A). *zak2*, *zak1* (Kim et al., 1999) and *gsk3* nulls (Harwood et al., 1995; Schilde et al., 2004) were all resistant to cAMP inhibition of stalk differentiation (Fig. 4A), indicating a related signaling path.

Multicellular development of *Dictyostelium* is characterized by specific cell pattern formation. At the slug stage, the differentiated cells are organized along an anterior-posterior axis, with the anterior zone comprised primarily of prestalk cells; the prespore cells dominate the posterior region. We were interested to determine the roles of ZAK1 and ZAK2 in the spatial regulation of gene expression in the two primary prestalk sub-groups, prestalk A (pstA) and prestalk B (pstB) cells, which are distinguished, respectively, by the expression patterns of *ecmA* and *ecmB* (Gaudet et al., 2008; Williams, 2006).

In wild-type cells, *ecmA* (as visualized with a *lacZ* reporter) is expressed throughout the entire anterior zone (Fig. 4B), with minimal expression through the posterior of the slug. *ecmB* expression is restricted to a narrow cone within the center of the anterior prestalk region (Fig. 4C). *gsk3* nulls have aberrant patterning of both prestalk subtypes (Harwood et al., 1995; Schilde et al., 2004). In *gsk3* nulls, both *ecmA* and *ecmB* expression is expanded from the anterior zone throughout the posterior region (Fig. 4B,C). *zak2* nulls have a disrupted *ecmA* expression pattern that is similar to that of *gsk3* nulls, but an *ecmB* pattern like that of wild type (Fig. 4B,C). By contrast, *zak1* nulls have a completely reciprocal phenotype (Fig. 4B,C); *ecmB* expression in *zak1* nulls is expanded, as with *gsk3* nulls, but *ecmA* expression is similar to the wild-type pattern. Thus, although ZAK2 and ZAK1 both negatively regulate prestalk differentiation, they are selective for the different subtypes: the pstA gene *ZAK2*, which is preferentially expressed in pstA cells, regulates expression of *ecmA*, while the *ZAK1* regulates expression of *ecmB* and is preferentially expressed in pstB cells.

We next examined the role of ZAK2 on overall levels of prestalk gene expression (Fig. 5A). Wild-type and *zak2*-null cells were differentiated in shaking culture under conditions where exogenous cAMP induces the expression of prestalk genes *ecmA* and *ecmB*, and prespore genes *psA* and *cotB*. RNA was isolated prior to and post cAMP-induction and probed on northern blots. Consistent with the expansion of *ecmA*-expressing cells in *zak2* nulls, we see a dramatic increase in global levels of *ecmA* mRNA compared with wild type or internally normalized to *ecmB* expression (Fig. 5A), highlighting the preferential role of ZAK2 in prestalk A determination. We conclude that ZAK1 and ZAK2 regulate distinct prestalk cell pathways. In addition, *zak2* nulls correspondingly exhibited a dramatic relative reduction in prespore gene expression (Fig. 5A), in agreement with the reduction of spore differentiation during normal development (Fig. 1D).

Although *ZAK2* and *ZAK1* expression is enriched in different pst populations, we have postulated that they have similar mechanistic kinase functions. We were, thus, interested to determine whether

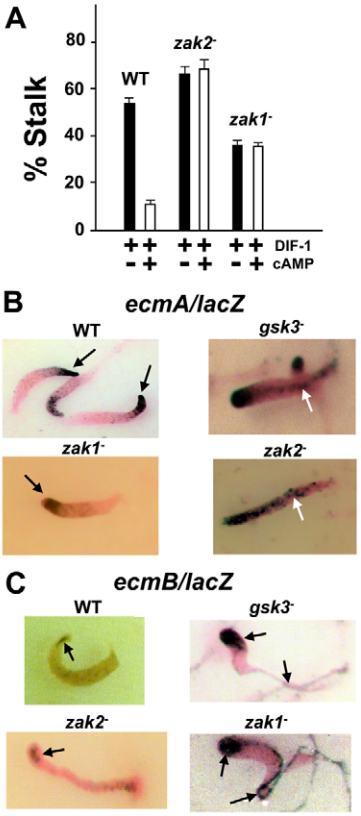

Fig. 4. Differential regulation of prestalk subclasses by ZAK2, ZAK1 and GSK3. (**A**) Loss of cAMP-dependent stalk inhibition in *zak2* nulls. Cells were incubated with 5 mM cAMP for 20 hours in monolayer cultures, and then treated with 100 nM DIF-1 alone or DIF-1 plus cAMP for additional 20 hours. The percentage of vacuolated stalk cells was determined for each cell line. Three separate assays were repeated, and values are presented as the mean±s.e.m. (**B**) *ecmA* patterning. Wild-type, *gsk3*-null, *zak1*-null and *zak2*-null cells were marked with *ecmA/lacZ*, developed to the slug stage on nitrocellulose filters, and stained for β-galactosidase activity. Wild-type and *zak1*-null slugs show primary *ecmA* expression at the anterior 20%, with little staining at the rear (black arrows). By contrast, *gsk3* and *zak2* nulls exhibit extended *ecmA* expression in the posterior prespore region (white arrows). (**C**) *ecmB* patterning. Wild-type, *gsk3*-null, *zak1*-null and *zak2*-null cells were marked with *ecmB/lacZ*, developed to the slug stage on nitrocellulose filters and stained for β-galactosidase activity. Wild-type and *zak2*-null slugs show primary *ecmB* expression in the central core of the anterior region (arrows). By contrast, *gsk3*- and *zak1*-nulls exhibit expansion of *ecmB* expression throughout the anterior region, as well as precocious stalk-like expression through the rear of the slugs (arrows).

ZAK1 could substitute for ZAK2 in pstA cells. *zak2* nulls were engineered to overexpress ZAK1 using the *ecmA* promoter (*zak2*$^{-}$[*ecmA*/*ZAK1*] cells). *ZAK1* expression would track the induction of endogenous *ecmA* gene expression. Cells were developed on solid substrata and *ecmA* mRNA expression was monitored by northern blot assay. Expression level differences between parental and engineered strains are not always easily interpretable. However, clear temporal differences between the *zak2*$^{-}$ and *zak2*$^{-}$[*ecmA*/*ZAK1*] strains are observed. The *zak2*-null strain shows continuous accumulation of *ecmA* mRNA through late development. However, once ZAK1 becomes induced in the *zak2*$^{-}$[*ecmA*/*ZAK1*] cells, *ecmA* expression is rapidly repressed

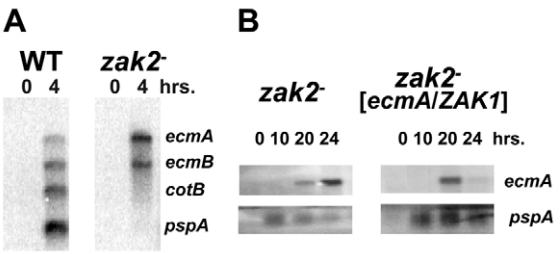

Fig. 5. ZAK2 regulation of gene expression in prestalk A and prespore cells. (**A**) mRNA expression patterns. Log phase wild-type and *zak2*-null cells were washed, resuspended, treated with 50 nM cAMP pulses for 5 hours, and then incubated with 300 μM cAMP for 4 hours. RNA was isolated before and after treatment with 300 μM cAMP. *ecmA* and *ecmB* are prestalk- and *cotB* and *psA* are prespore-specific markers used for northern analyses. (**B**) ZAK1 can substitute for ZAK2 in control of *ecmA* expression. *zak2* nulls and *zak2* nulls expressing *ecmA/ZAK1* (*zak2*$^{-}$[*ecmA/ZAK1*]) were developed on solid substrata and *ecmA* and *pspA* expression was determined by northern blot hybridization.

(Fig. 5B). Thus, ZAK1 is functionally equivalent to ZAK2 to negatively regulate *ecmA*. In parallel, we show that prespore *pspA* gene expression is not disrupted in *ZAK1*-expressing cells. Data may suggest that overexpression of ZAK1 in pstA cells promotes prespore gene expression (see below).

ZAK2 regulates prespore/spore differentiation

Although ZAK2 is expressed very poorly in prespore cells, data from Fig. 1D and Fig. 5A nonetheless indicate that ZAK2 regulates prespore/spore differentiation. To examine this directly, we differentiated wild-type and *zak1*-null cells into spores in monolayer culture at low cell density. Under these conditions, cell-cell interactions are minimized and spore formation requires stimulation by 8-Br-cAMP (Kay, 1987). Although wild-type cells form spores at a high efficiency when treated with 8-Br-cAMP, spore differentiation of *zak2*-nulls is largely refractory (Fig. 6A). Similar results were observed with *zak1* nulls (Fig. 5A) (Kim et al., 1999), as well as with *gsk3* nulls (Harwood et al., 1995; Kim et al., 1999; Schilde et al., 2004).

Developmental northern blot analyses were used to examine expression of the prespore-specific makers *pspA* and *cotB* throughout the developmental cycle of *Dictyostelium* on solid substrata. Compared with wild type, *zak2* nulls have severely reduced expression of both prespore genes during development on solid substrata (Fig. 6B), data that are qualitatively similar to that seen when cells are differentiated in shaking culture using exogenous cAMP (Fig. 5A). These data are also similar and consistent with that previously observed for *zak1*- and *gsk3*-null strains (Harwood et al., 1995; Kim et al., 1999). Thus, ZAK2, ZAK1 and GSK3 are required to direct prespore/spore fates during *Dictyostelium* development.

Non-autonomous regulation of prespore/spore differentiation by ZAK2

We have previously shown that kinase-inactive variants of GSK3 (GSK3-DN) will inhibit endogenous GSK3 signaling and function as dominant-negative mutants (Kim et al., 2002; Kim et al., 1999). We were interested to determine how disruption of GSK3-signaling in pstA or psp cells impacted developmentally regulated gene in all cells.

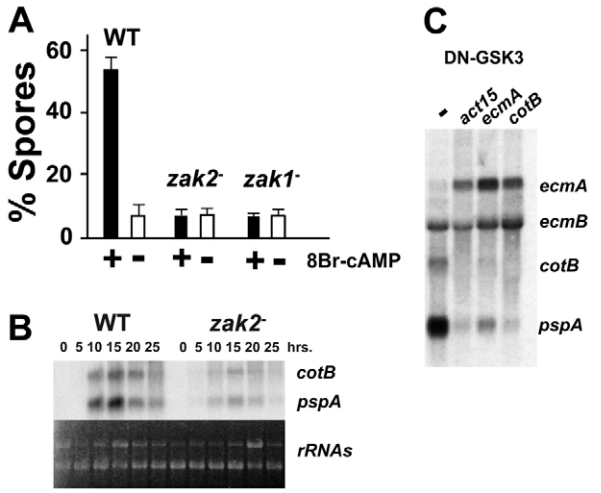

Fig. 6. ZAK2 is required for prespore/spore cell patterning. (**A**) Spore formation is reduced in *zak2* nulls. Wild-type, *zak1*-null and *zak2*-null cells were differentiated in monolayer culture for 48 hours in the presence or absence of 15 mM 8-Br-cAMP. The percentage of phase-bright elliptical spores was determined for each cell line. Three separate assays were repeated, and values are presented as the mean±s.e.m. (**B**) Prespore gene expression is reduced in *zak2* nulls. Prespore cell fate was analyzed by northern blot hybridization. Wild-type and *zak2*-null cells were developed on nitrocellulose filters, harvested at the indicated each times and RNAs prepared. *cotB* and *psA* are prespore-specific markers. (**C**) Non-autonomous regulation of prespore differentiation via GSK3. *car4* nulls and *car4* nulls expressing the kinase-inactive GSK3 (DN-GSK3) using the *act15*, *ecmA* and *cotB* promoters were differentiated in culture using cAMP. RNA was isolated 4 hours post-treatment and analyzed by northern blot hybridization. *ecmA* and *ecmB* are prestalk- and *cotB* and *psA* are prespore-specific markers.

car4-nulls exhibit enhanced prespore gene expression and reduction in expression of prestalk markers (Fig. 6C) (Ginsburg and Kimmel, 1997), but ubiquitous expression of GSK3-DN using the *act15* promoter reverses these developmental patterns; prestalk *ecmA* gene expression is activated, whereas expression of prespore markers *pspA* and *cotB* is repressed (Fig. 6C) (Kim et al., 2002; Kim et al., 1999). Not surprisingly, a similar phenotype is observed when GSK3 signaling is specifically inhibited in prespore cells using *cotB*/*GSK3-DN*. Prespore gene expression (i.e. *pspA* and *cotB*) is inhibited and there is a cell-autonomous enhancement of prestalk *ecmA* gene expression and parallel decrease in prespore gene expression (Fig. 6C) (Kim et al., 1999).

To mimic the specific inhibition of ZAK2 function in pstA cells, we used the *ecmA*/*DN-GSK3* construct (Fig. 6C). As expected, inhibition of GSK3 signaling in prestalk A cells autonomously activated *ecmA* expression, but remarkably, specific inhibition of GSK3 in prestalk A cells also caused the repression of prespore genes. It is well established that prespore/spore differentiation is regulated through an interplay of cell-autonomous and non-autonomous signaling (Aubry and Firtel, 1999; Kimmel, 2005; Kimmel and Firtel, 2004). The *ecmA*/*DN-GSK3* results suggest a potential function for ZAK2/GSK3 to regulate a non-autonomous pathway for prespore/spore differentiation that is mediated by the action of pstA cells. These conclusions are consistent with the observed expression pattern of *ZAK2*, which has relatively low levels of expression in prepsore cells.

We next studied these postulated contributions of autonomy/non-autonomy by lineage tracing of GFP-marked cells during terminal differentiation of chimeras with varying mixtures of wild type or *zak2* nulls. Approximately 80% of wild-type cells form spores during normal development, whereas sporulation efficiency of developed *zak2* nulls is only ~30% (Table 1). *zak2* nulls that express GFP (*zak2*- [*act15*/*GFP*]) sporulate at similarly poor efficiencies (~35%) during chimeric development with a large (~40-fold) excess of unmarked *zak2*-nulls (Table 1). However, when GFP-marked *zak2*-null cells are co-developed with unmarked wild-type cells, the *zak2*-null sporulation efficiency was enhanced approximately twofold (Table 1). When normalized (Table 1), these sporulation efficiencies approximate those of wild type and suggest strongly that ZAK2 in wild-type cells regulates a non-autonomous pathway that stimulates the spore differentiation of cells that lack *ZAK2*.

We were also interested to determine the potential impact on wild-type sporulation in the presence of a large population of cells that lack ZAK2. In controls, GFP-marked wild-type cells (WT[*act15*/*GFP*]) sporulate at very high efficiency (~80%), but when mixed with a 90-fold excess of *zak2* nulls, wild-type sporulation is reproducibly (P<0.01) suppressed by ~25-30% (Table 1). These data are consistent with a dependency of wild-type sporulation on a ZAK2-regulated, non-autonomous signaling pathway.

Table 1. Non-autonomous regulation of spore differentiation by ZAK2

% Unmarked	% GFP *zak2*⁻	% Spores*	% GFP cells as spores†	Relative *zak2*⁻ sporulation efficiencies‡
100 *zak2*⁻	0	30±2%	NA	NA
90 *zak2*⁻	10	35±2%	40±3% (A)	1.00 in *zak2*⁻
90 Wild type	10	75±4%	75±5% (B)	1.88 in wild type (P<0.01)
97.5 zak2⁻	2.5	35±3%	30±3% (A)	1.00 in *zak2*⁻
97.5 Wild type	2.5	75±4%	75±4% (B)	2.50 in wild type (P<0.01)
% Unmarked	% GFP wild type	% Spores†	% GFP cells as spores†	Relative wild-type sporulation efficiencies
100 Wild type	0	85±5%	NA	NA
90 Wild type	10	75±5%	78±5% (A)	1.00 in wild type
90 *zak2*⁻	10	31±3%	55±4% (B)	0.71 in *zak2*⁻ (P<0.01)
99 Wild type	1	83±5%	80±4% (A)	1.00 in wild type
99 *zak2*⁻	1	31±3%	61±3% (B)	0.76 in *zak2*⁻ (P<0.01)

*Numbers derive from a minimum of 500 cells and multiple experiments and are expressed as mean±s.e.m.
†Numbers derive from a minimum of 500 cells and multiple experiments and are expressed as mean±s.e.m. Values A and B are used to calculate relative sporulation efficiencies.
‡Relative sporulation efficiency for control, GFP cells within each homogeneous [(GFP-*zak2*⁻)+(*zak2*⁻)] or [(GFP-wild type)+(wild type)] mixture is set at 1.00.
Relative sporulation efficiency for GFP cells within each heterologous [(GFP-*zak2*⁻)+(wild type)] or [(GFP-wild type)+(*zak2*⁻)] mixture is calculated as B/A (see † above).
NA, not applicable.

Still, the wild-type sporulation values in the presence of *zak2* nulls are not fully suppressed to that of *zak2* nulls. Although we suggest that ZAK2 functions significantly in a non-autonomous pathway for prespore differentiation, ZAK2 in wild-type cells probably has an inherent, cell-autonomous potential for spore differentiation. *ZAK2* is expressed during growth and early development, and we do not exclude the possibility that ZAK2 can function in an autonomous pathway during the initial phases of prespore/prestalk fate choice.

Tyrosine phosphorylation of GSK3 regulates cell polarity

In addition to its role in regulating cell differentiation, cAMP is required to organize multi-cell formation during early *Dictyostelium* development. Although a dependent role for GSK3 has not been evaluated, the recent linkage of GSK3 function to cell polarization and migration (Ciani et al., 2004; Eickholt et al., 2002; Etienne-Manneville and Hall, 2003a; Etienne-Manneville and Hall, 2003b; Gartner et al., 2006; Jiang et al., 2005; Schlessinger et al., 2007; Shi et al., 2004; Yoshimura et al., 2005; Zhou et al., 2004) makes this an interesting line of investigation.

Directional cell migration constitutes an essential and early stage for multicellular development in *Dictyostelium*. During early development, cells polarize and migrate directionally toward signaling centers that produce cAMP. Cells 'stream' in coordinated groupings toward these cAMP centers and aggregate into multicellular structures, the precursors for later development. The ability of cells to stream and aggregate at low cell density is characteristic of their capacity to polarize directionally and chemotax towards the cAMP signal. During careful comparison of the early developmental phenotypes of wild-type, *gsk3*-null, *zak1*-null and *zak2*-null cells, we noticed differences in the ability of cells to form multicellular aggregates.

To examine aggregation more directly, cells were placed in submerged culture at varying cell densities. Wild-type and *zak2*-null cells establish territorial streams at densities as low as 2×10^4 cells/cm^2 (Fig. 7A), ~1000 times lower cell density than that normally used during standard development on solid surfaces. However, *gsk3* and *zak1* nulls were both dramatically defective in directional cell movement and in chemotactic streaming at these cell densities. Time-lapse imaging confirmed that *gsk3* and *zak1* nulls do not coalesce by forming migration streams, but rather aggregate through random cell collision and adhesion (Fig. 7A). We also examined the functional link between GSK3 and ZAK1 in chemotaxis and aggregation using *gsk3* nulls that express either the GSK3 wild-type form or the Y214F variant (Kim et al., 2002). GSK3^{Y214F} is not phosphorylated or activated by ZAK1, has an inherent low activity compared with wild type, and acts as a partial dominant-negative during *Dictyostelium* development (Kim et al., 2002). At a density of 10^5 cells/cm^2 (Fig. 7B), *gsk3* nulls are unable to establish territorial streams. Re-expression of wild-type GSK3 in *gsk3*-nulls results in a near complete rescue of chemotaxis in submerged cultures (Fig. 7B), whereas cells that express GSK3^{Y214F} remain defective (Fig. 7B).

Chemotactic aggregation is dependent upon both signal production and signal response. We therefore examined whether cells responded to an exogenous cAMP gradient. Consistent with the streaming/aggregation data (Fig. 7A,B), wild-type and *zak2*-null cells became highly polarized and migrated robustly toward a cAMP point source. By contrast, the *gsk3* and *zak1* nulls had extreme defects in polarity organization and in chemotactic movement (Fig. 7C), indicating a defect in chemotactic response.

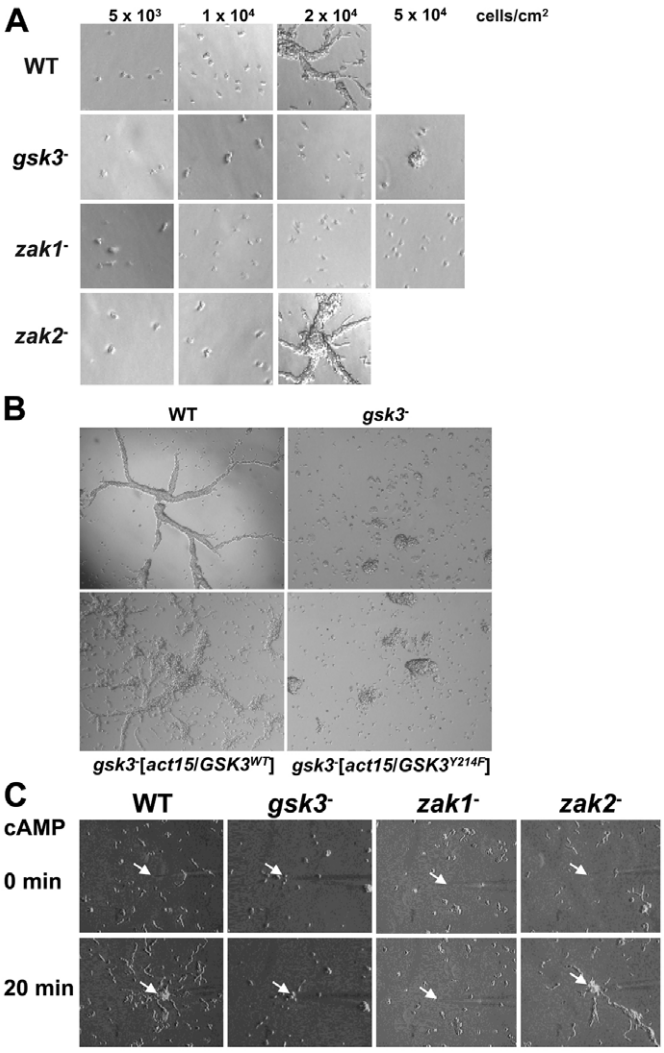

Fig. 7. The role of the ZAK and GSK3 kinases in cell polarity and chemotaxis. (**A**) Aggregation defects in *gsk3* and *zak1* nulls. Cells were plated at the indicated densities in submerged culture and photographed after 12 hours. Wild-type and *zak2*-null cells were able to form migration streams and aggregate. *gsk3* and *zak1* nulls were unable to migrate directionally in streams to form large aggregation territories at low cell densities. (**B**) The ZAK1 phosphorylation site Y214 of GSK3 is required for normal aggregation. Cells were plated at 5×10^4 cells/cm^2 in submerged culture and photographed after 12 hours. Wild types formed migration streams and aggregates. *gsk3* nulls and *gsk3* nulls expressing GSK3^{Y214F} (*gsk3*$^-$[*act15/GSK3*^{Y214F}]) were unable to migrate directionally in streams to form large aggregation territories. Small aggregates were sometimes observed due to 'random' collision and adhesion. *gsk3* nulls expressing wild-type GSK3 (*gsk3*$^-$[*act15/GSK3*^{WT}]) exhibit a near wild-type phenotype. (**C**) GSK3 and ZAK1, but not ZAK2, are required for efficient chemotaxis towards cAMP. Cells were cultured with 50 nM pulses of cAMP for 7 hours to ensure developmental competency. Cells were allowed to polarize and chemotax toward a pipette containing a 100 nM solution of cAMP (arrows). Images of chemotaxing cells were captured at 0 seconds and after a 20-minute exposure to the cAMP gradient.

To characterize the chemotactic defects more precisely, we dynamically imaged cellular movement toward a cAMP point source using DIAS software (Fig. 8). Chemotaxing wild-type cells are highly polarized and migrate in a directed manner (Fig. 8).

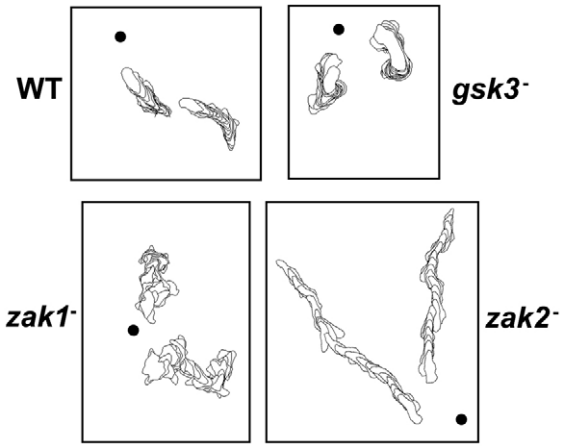

Fig. 8. ZAK1 and GSK3 kinases regulate cell polarity and chemotaxis. Wild-type, gsk3-null, zak1-null and zak2-null cells were differentiated in suspension culture, plated on plastic dishes, and allowed to chemotax toward a pipette containing a 1 μM solution of cAMP. Cell images were captured every 30 seconds for ~10 minutes. Individual cells were traced and analyzed using the DIAS software package. Cell tracings are arranged to demonstrate relative directional movement, polarity and distance traveled towards the cAMP point source (black dot), and are representative of at least five independent experiments. The dot indicates the orientation of the pipette relative to the cells, not the position of the tip.

Wild-type cells have a dominant anterior pseudopod that is regulated by F-actin polymerization and that extends persistently in the direction of the gradient; myosin II (myo II) assembly at the posterior and sides of the cells suppresses spurious lateral pseudopod formation and promotes contraction towards the gradient source (see Kimmel and Parent, 2003; Kimmel et al., 2004). By contrast, *zak1* nulls are very poorly polarized and their movement is characterized by the continuous formation of lateral pseudopods that often directs cells away from the gradient source (Fig. 8). As would be expected if GSK3 were in a regulatory path for chemotaxis downstream of ZAK1, *gsk3* nulls exhibit the most severe defects in migration (Fig. 8). *gsk3* nulls are minimally polarized and have highly restricted directional movement. They exhibit significant lateral pseudopod extension and difficulty in retraction of the cell posterior, a phenotype that further supports a role for ZAK1/GSK3 in control of polarity. By contrast, *zak2* nulls polarize and migrate well towards a cAMP source.

DISCUSSION

The terminal structure of asexual development of *Dictyostelium* is comprised of a sorus (a spore mass) atop a stalk of vacuolated cells. Stalk-like cells also form the base of the organism and the ‘cups’ that surround the sorus. These terminally differentiated cells derive from non-committed prespore and prestalk precursors. The pioneering work of Williams, Kay, Firtel and others have shown that progenitors to both prestalk and prespore populations are heterogeneous (Gaudet et al., 2008; Williams, 2006; Yamada et al., 2010). Spatially restricted expression of gene patterns has been used to define very specific classes of prestalk cells (e.g. pstA, pstB, pstO and pstU cells). Here, we have focused on three main progenitor classes, prestalk A, prestalk B and prespore cells, but further subdivisions within each can be made (Gaudet et al., 2008; Kimmel, 2005; Kimmel and Firtel, 2004; Williams, 2006). We show that regulation of tyrosine phosphorylation of GSK3 in *Dictyostelium* underscores a paradigm for cellular differentiation and chemotactic response. Thus, aspects of development from the formation of multicellular structures through terminal differentiation are dependent upon the precise regulation of GSK3.

Prestalk A cells express *ecmA* and differentiation is negatively regulated autonomously by a ZAK2/GSK3 pathway. Loss of *ZAK2* or *GSK3* or expression of the kinase-inactive GSK3 (DN-GSK3) in prestalk A cells increases *ecmA* expression (Fig. 4B, Fig. 5B, Fig. 6C). Although overexpression of ZAK1 in prestalk A cells of *zak2* nulls represses *ecmA* expression (Fig. 5B), ZAK1 normally has a limited function in prestalk A differentiation (Fig. 3D, Fig. 4B). Conversely, prestalk B differentiation is negatively regulated autonomously by ZAK1/GSK3. Loss of *ZAK1* or *GSK3* expands *ecmB* expression (Fig. 4C) and relieves inhibition by cAMP (Kim et al., 1999). Although re-expression of ZAK1 in prestalk B cells of *zak1* nulls represses *ecmB* expression, *ecmB* expression remains elevated in *gsk3* nulls that overexpress ZAK1 in prestalk B cells (Kim et al., 1999).

cAMP inhibition of stalk differentiation in monolayer culture has been previously shown to require functional GSK3 signaling. We suggest that *zak2*-null cells preferentially differentiate into a prestalk A population in these monolayers. As these *zak2*-null cells would be unable to activate GSK3-signaling, they would be insensitive to inhibition by cAMP. Likewise, pstB cells may be the more dominant population during differentiation of the *zak1*-null cells; they would also lack normal GSK3 signaling and would also be insensitive to inhibition by cAMP.

Prespore differentiation is positively regulated autonomously by ZAK1/GSK3. Loss of *ZAK1* or *GSK3* diminishes *pspA* and *cotB* expression (Kim et al., 1999). Expression of DN-GSK3 in wild-type prespore cells represses *cotB* expression (Fig. 6C). Re-expression of ZAK1 in prespore cells of *zak1* nulls induces *cotB*; however, *cotB* expression remains low in *gsk3* nulls that overexpress ZAK1 in prespore cells (Kim et al., 1999), indicating that GSK3 is required for ZAK1 regulation of prespore gene expression. Prespore differentiation is also positively regulated, but non-autonomously via the ZAK2/GSK3 pathway in prestalk A cells. *ZAK2* is expressed at relatively low levels in prespore cells, but loss of *ZAK2* or expression of DN-GSK3 in prestalk A cells represses *cotB* expression (Figs 5, 6). The non-autonomous regulation of prespore differentiation by ZAK2/GSK3 signaling was substantiated by comparing relative efficiencies of sporulation during chimeric development of wild-type and *zak2*-null cells. However, as ZAK2 is expressed in cells prior to the prespore differentiation, ZAK2 may function autonomously during the initial phases cell differentiation.

The loss of GSK3 signaling not only impairs cell fate determination, but also chemotactic movement. However, data indicate that these are functionally separate events. *zak1* nulls, which have severe defects in cAMP-mediated chemotaxis, establish proper anterior prestalk A patterning, whereas prestalk A patterning is aberrant in chemotactically competent *zak2* nulls. These data argue strongly that defects in cell pattern formation, as a consequence of restricted GSK3 signaling, do not result from simple abnormalities in chemotaxis towards cAMP.

Proteomic and microarray data indicate that ZAK1/GSK3 signaling regulates certain gene expression patterns during early *Dictyostelium* development (Strmecki et al., 2007). However, transcriptional misregulation in *zak1*- and *gsk3*-null cells is unlikely

to account for the entirety of their chemotaxis defects. Indeed a cotemporaneous study suggests that multiple chemoattractant signaling pathways are disrupted in *Dictyostelium* that lack GSK3 (Teo et al., 2010).

A common GSK3-dependent pathway that regulates both cell fate determination and cell polarity as in *Dictyostelium* may not be shared broadly among the metazoa. Canonical Wnt signaling is mediated by the effective inhibition of GSK3, although regulation is not by alterations in enzymatic activity as in *Dictyostelium*, but rather by disassembly of GSK3/substrate complexes (Angers and Moon, 2009; McNeill and Woodgett, 2010). Although Wnts can also direct planar cell polarity, the mechanisms involved are distinct from canonical signaling and do not generally involve coordinated GSK3 regulation.

Still, changes in GSK3 activity per se can influence cell polarization and specification in some developmental contexts. Mis-expression of GSK3 in *Drosophila* directs polarity defects (Tomlinson et al., 1997). In addition, localized inhibition of GSK3β can establish and maintain neuronal specification by altering microtubule assembly that can polarize the formation of axons and dendrites (Ciani et al., 2004; Eickholt et al., 2002; Gartner et al., 2006; Jiang et al., 2005; Schlessinger et al., 2007; Shi et al., 2004; Yoshimura et al., 2005; Zhou et al., 2004). Recent data indicate that a novel GSK3-mediated pathway involving Wnt signaling can polarize the microtubule cytoskeleton (Schlessinger et al., 2007). Microtubule polarization in *C. elegans* is similarly specified by Wnt regulation of GSK3 (Hardin and King, 2008; Schlesinger et al., 1999; Walston et al., 2004).

Although neuronal and astrocyte polarization and chemotactic cell movement in *Dictyostelium* appear unrelated, their shared dependence upon GSK3 may suggest some mechanistic commonality. Persistent directed movement of *Dictyostelium* toward a chemoattractant source involves antagonistic activities that localize specifically to the leading edge or rear of the chemotaxing cell (Kay et al., 2008; Kolsch et al., 2008; McMains et al., 2008). Continuous cell polarization is required to maintain directionality and to suppress spurious lateral movement away from the directional signal.

Non-autonomous regulation of cell patterning has been widely observed throughout *Dictyostelium* developmental cycle. The most mechanistically well described pathway involves prestalk control of a PKA-activity cascade within prespore cells that drives sporulation (Anjard and Loomis, 2005; Cabral et al., 2006; Wang et al., 1999). Prespore cells produce and secrete the SDF-2 (spore differentiation factor) precursor AcbA, which is inactive. Full-length AcbA is 84 amino acids, but is processed into the small active SDF-2 peptide by prestalk-specific transmembrane proteases. SDF-2 binding to DhkA surface receptors on prespore cells initiates the pathway for PKA activation and sporulation. *Dictyostelium* that lack the prestalk proteases cannot sporulate (Anjard and Loomis, 2005; Cabral et al., 2006; Wang et al., 1999). The non-autonomous role of ZAK2 in control of sporulation is more subtle. Sporulation persists in cells lacking ZAK2, but to a significantly lesser degree compared with wild-type controls. The defect, however, is rescued by co-development with wild-type cells. As ZAK2 is primarily a prestalk A marker, the data suggest the importance for narrow regulation of prestalk function. Expansion of prestalk cells may interfere with the regulatory balance that helps define normal prespore/prestalk ratios in *Dictyostelium* that are optimal for terminal differentiation.

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Competing interests statement
The authors declare no competing financial interests.

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