Dictyostelium AMPKα regulates aggregate size and cell-type patterning

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Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.3810550.

1. Background

AMPK (5′ adenosine monophosphate-activated protein kinase) is a heterotrimeric serine/threonine kinase, comprising a catalytic (α) and two regulatory (β and γ) subunits, and is conserved from yeast to humans. It is both a nutrient and an energy sensor that helps maintain energy homeostasis. It plays a role in the regulation of cellular as well as whole-body energy metabolism and also participates in the cell-cycle and membrane excitability regulation. AMPK coordinates control of cell growth and autophagy, acting as a metabolic checkpoint, and inhibits cellular growth via suppression of the mTORC1 (mammalian target of rapamycin complex 1) pathway. It acts as a central regulator of cell growth, cell proliferation and development [1–5]. When activated, AMPK promotes energy-producing catabolic pathways while inhibiting anabolic pathways, such as cell growth and proliferation. Besides its role as an energy sensor, it also plays a role in cell differentiation and development of organisms [6]. In Caenorhabditis elegans, AMPKα promotes cell survival and arrests germline development during nutrient stress [7].

In the case of Dictyostelium discoideum, the size of multi-cellular structures formed is well regulated. Dictyostelium amoeba divides mitotically when food is abundant, but undergoes multi-cellular development upon starvation. Vegetative cells secrete prestarvation factor (PSF) that helps monitor cell density relative to the amount of available nutrients [8]. High PSF induces the expression of genes required for aggregation. When the food supply is depleted, PSF production declines and another cell density sensing factor called conditioned medium factor (CMF) begins to accumulate. Once the starving cells reach high cell density, CMF accumulates and the cells initiate aggregation via cAMP signal relay [9] to aggregate into groups of approximately 10^7 cells. Hohl & Raper [10] had earlier investigated several small-sized aggregate mutants and found them to be defective in either aggregation or cell number or mass sensing. It was

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observed that mutants defective in aggregation could be rescued by crowding of the cells, so that aggregation becomes unnecessary. The cell number sensor senses the number of cells present in a group, and if they are exceedingly high it breaks them into smaller groups. Earlier, Brock & Gomer [11] observed the smlA mutants that formed small-sized aggregates, due to the oversecretion of Countin A protein. The aggregates then form the migratory slugs where the anterior quarter region is composed of prestalk cells and the remaining posterior region of prespore cells. The ratio of the cell types remains constant regardless of the size of the multi-cellular structures formed. Prestalk cells are further divided into subtypes: pstA cells occupy the anterior 10% of the slug, pstAB cells occupy the core to the tip, pstO cells are found behind the pstA cells and anterior-like cells (ALCs) lie dispersed within the prespore region [12]. A number of genes play a role in cell-type proportioning and spatial patterning [13–16], thus there is a large selective pressure on the starved cells to form fruiting bodies for proper spore dispersal. Neither too long nor too short fruiting bodies are advantageous for the organism. A fruiting body is composed of two terminally differentiated cell types, namely the stalk (dead vacuolated) cells and the spore (viable) cells [17].

AMPK plays an important role in starvation responses and nutrient deprivation is necessary for the initiation of development in this organism. Earlier, Bokko et al. [18] investigated the role of AMPK in mitochondrial diseases and found overexpression to result in fruiting bodies with short, thick stalks and comparatively large sori, while antisense inhibition developed fewer and smaller fruiting bodies. This study was undertaken to explore the functions of AMPKα during development of D. discoideum, which was found to be involved in regulating the aggregate size. The ampkα− cells formed small-sized aggregates, which developed asynchronously and the spores formed displayed reduced viability. The developmental defects shown by ampkα− cells were cell autonomous as chimaeras formed with only 5% ampkα− mixed with 95% Ax2 cells caused the aggregation streams to break up. The conditioned medium (CM) collected from ampkα− cells caused the Ax2 cells to form small-sized aggregates. The ampkα− cells showed low cytosolic glucose levels during starvation and the small-aggregate phenotype could be corrected to a certain extent when developed in the presence of exogenous glucose. In chimaeras with Ax2 cells, the ampkα− cells showed a propensity towards the prestalk region and had lower tendency to form spores. Importantly, our results showed AMPKα to play a regulatory role in the spatial cell-type patterning as mutation caused an increase and mis-localization of the prestalk cells and a decrease in the prespore cells, ultimately resulting in fruiting bodies with small sorus and long stalk.

2. Results

2.1. ampkα mRNA is expressed in prestalk/stalk cells

To determine the spatio-temporal mRNA expression patterns of ampkα, reverse transcriptase PCR (RT-PCR) and in situ hybridization analyses were performed. The ampkα transcript was present during growth and development, showing minimum levels in the vegetative cells and increased levels during multi-cellular development (figure 1ab). It remained more or less constant during later stages of development. The whole mount in situ hybridization analyses showed ampkα transcript to be localized in the tip of the mound and at the site of contact with the substratum corresponding to the prestalk cells (figure 1cA.A). As development proceeded towards culmination, the transcript was localized in the pstAB cells, stalk tube and the basal disc region (figure 1cB–E). The high expression levels in the basal disc suggest it to be present largely in the pstB cells as they are shed off during culmination. The sense probe did not show any evident staining (figure 1cA′–E′). In conclusion, ampkα transcript shows prestalk localization and is expressed throughout growth and development.

2.2. Successful creation of ampkα mutants

To investigate the functions of AMPKα, we successfully made both overexpressing and deletion mutants (electronic supplementary material, figures S1 and S2). The constitutive promoter, actin 15, was used to drive the expression of the fusion protein AMPKα–Eysp and was called AMPKα over-expressor (ampkαOE). Following transformation and blasticidin selection, 500 independent clones were isolated and screened.
ampk reached stationary phase at 12.8 × 10^6 cells ml⁻¹. (b) Dry weight of 5 × 10^7 cells harvested from Ax2, ampkOE, ampkOE, ampkOE and ampkOE. (c) Cell-size analysis of cells from various strains using FACS. (d) Time-specific developmental stages of Ax2, ampkOE, ampkOE and ampkOE analysed. Scale bar, 200 μm. (e) Enlarged image of a fruiting body produced by each strain. Scale bar, 50 μm. (f) A graph representing the viability of spores harvested from Ax2, ampkOE, ampkOE and ampkOE. The values represent mean ± s.e.; n = 4; ***p < 0.001, **p < 0.01 (Student's t-test).

Figure 2. Overexpression and/or disruption of ampk affect growth, development and spore viability. (a) Cell proliferation studies with ampkOE, ampkOE and ampkOE and their comparison with Ax2 cells (seeding density approx. 5 × 10^5 cells ml⁻¹). (b) Dry weight of 5 × 10^7 cells harvested from Ax2, ampkOE, ampkOE and ampkOE. (c) Cell- size analysis of cells from various strains using FACS. (d) Time-specific developmental stages of Ax2, ampkOE, ampkOE and ampkOE analysed. Scale bar, 200 μm. (f) Enlarged image of a fruiting body produced by each strain. Scale bar, 50 μm. (f) A graph representing the viability of spores harvested from Ax2, ampkOE, ampkOE and ampkOE. The values represent mean ± s.e.; n = 4; ***p < 0.001, **p < 0.01 (Student’s t-test).

for positional integration by PCR amplification of the genomic DNA. Four positive clones of ampk null were obtained having similar growth and development patterns. One of these clones, designated as ampkA, was used for further studies. Rescue (ampkA) was created by expressing the AMPK fusion protein in ampk cells. The growth and early developmental defects shown by ampkA were more or less rescued in ampkOE cells.

2.3. AMPKα suppresses cell proliferation and growth

To measure the rate of cell proliferation in liquid culture, Ax2, ampkOE, ampkA and ampkOE log phase cells were identically diluted into fresh media at a density of approximately 5 × 10^5 cells ml⁻¹ and monitored over several days. Ax2 cells reached stationary phase at 12.8 × 10^6 cells ml⁻¹ and cell proliferation of ampkOE cells was consistently lower when compared with all other strains and displayed a decline phase after 108 h. ampkgOE cells proliferated more slowly, reaching stationary phase tardily and at a much lower density of approximately 9.3 × 10^5 cells ml⁻¹. On the other hand, deletion of ampkA caused an increase in cell proliferation when compared with the Ax2 cells. The ampkA cells reached the stationary phase at a higher cell density of approximately 14.3 × 10^6 cells ml⁻¹ and displayed a decline phase comparatively at an earlier time-point (72 h). ampkgOE reached stationary phase at approximately 11.2 × 10^6 cells ml⁻¹. The increased proliferation defects shown by ampkgA cells could be corrected in ampkgOE up to a certain extent. Doubling times of Ax2, ampkgOE, ampkgA and ampkgOE were 12.1 ± 0.1, 25 ± 0.4, 9.75 ± 0.6 and 15 ± 1.5 h, respectively (figure 2a).

In the case of D. discoideum, cell proliferation (increase in the number of cells) and cell growth (increase in cell mass or size) are regulated independently. To determine the role of AMPKα in regulating growth, we measured the cell mass of Ax2, ampkgOE, ampkgA and ampkgOE cells. The average cell mass of Ax2 cells was 10.3 ± 0.2 mg and comparable to that observed previously [19]. The average cell mass of ampkgOE, ampkgA and ampkgOE were 8.5 ± 0.3, 12.3 ± 0.3 and 11.1 ± 0.2 mg, respectively. Change in the average cell mass of ampkgOE was insignificant compared with Ax2 cells (figure 2b).

To determine if AMPKα affects cell size, flow cytometry analyses were performed. Figure 2c shows ampkgA cells to be larger in size, whereas ampkgOE cells were smaller when compared with Ax2 cells. ampkgOE cells exhibited similar cell size as Ax2 cells. Taken together, the data suggest that AMPKα regulates both cell proliferation and growth of vegetative cells.

2.4. Disruption of ampkg gene results in small-sized aggregates, asynchronous development and reduced spore viability

To examine the role of AMPKα in multi-cellular development, we plated cells from the various strains at equal densities (5 × 10^5 cells ml⁻¹) on non-nutrient agar plates and allowed them to develop after synchronization. Ax2 cells completed their development by forming aggregation territories (mounds) by 12 h, slugs at 16 h and finally culminate into fruiting bodies at 24 h. ampkgA cells developed into smaller aggregates and, subsequently, not all aggregates pursued further development, with only few of them proceeding towards slug formation. Following that, not all slugs proceeded to early culminate at the same time, further added more asynchrony. Thus, the development of ampkgA cells appeared highly asynchronous, showing various developmental intermediates (aborted mound, slugs, early culminant and culminant) when compared with the Ax2 cells by 24 h of development (figure 2d). The fruiting bodies formed by ampkgA had smaller sori (spore heads) and longer stalks when compared with the Ax2 cells. In fact most of the fruiting bodies formed by ampkgA cells collapsed, which could be attributed to the longer stalks formed (figure 2d). By contrast, the fruiting
bodies formed by ampkαΔ cells showed large sori and short stalks when compared with the Ax2 cells (figure 2e). As only few fruiting bodies were formed by ampkαΔ cells, we checked the viability of the spores. The average viability of Ax2 spores was 84 ± 1.4% [20,21], of ampkαΔ spores was 71 ± 2.8% and of ampkαΔ+ spores was significantly reduced to 45 ± 1.8% (figure 2f). The reduced spore viability of ampkαΔ was rescued in the ampkαΔ+ strain, which displayed average spore viability of 78 ± 4.2% (figure 2f).

The early developmental defects (small-sized aggregates) shown by ampkαΔ were rescued by the exogenous expression of AMPKα, but the late developmental defects (asynchronous development) were still apparent. The ampkαΔ cells formed smaller (approx. 3.4-fold decrease) and comparatively more (approx. 1.8-fold increase) aggregation territories than Ax2 cells (figures 3a–d).

The above data suggest that an optimum level of AMPKα is necessary for proper development.

2.5. ampkαΔ cells exhibit changes in secreted factors

To investigate the role of secreted factors in the development of ampkαΔ phenotype (small-sized aggregates), we performed cell mixing experiments with the Ax2 cells in varying proportions (5–90%) and allowed them to develop. The Ax2 cells in the chimeras formed were not able to rescue the phenotypic defects caused by ampkαΔ cells, suggesting that the small-sized aggregate phenotype was not due to the lack of secreted factor(s). However, with only 5% addition of ampkαΔ cells to 95% Ax2 cells, there was a significant increase in aggregate numbers and decrease in aggregate size when compared with Ax2 cells alone (figure 4a; electronic supplementary material, figure S3a,b). The size of the aggregates in these cell mixtures was indistinguishable from size of those formed by ampkαΔ cells alone (figure 4a). The size and number of aggregates formed by Ax2 cells were affected by addition of only 5% mutant cells, thus it is possible that mutant cells secrete some factors that regulate group size.

2.6. Conditioned medium collected from ampkαΔ cells causes the Ax2 aggregates to break up

When cells are starved, they secrete factors that regulate aggregate size. To test the hypothesis that extracellular factors are involved in the phenotype observed by ampkαΔ cells, CM experiments were performed. When Ax2 cells were allowed to develop in the presence of CM collected from Ax2 cells, the aggregates formed were similar to the ones formed in the presence of buffer only (figure 4b, upper panel). On the other hand, when Ax2 cells were allowed to develop in the presence of the CM collected from ampkαΔ cells, the aggregates formed were small-sized and more in number (figure 4b, upper panel; electronic supplementary material, figure S3c,d). The small-aggregate phenotype was mimicked by the Ax2 cells when developed in the presence of ampkαΔ CM. The ampkαΔ aggregates were small in size, irrespective of their development in buffer, Ax2 CM or ampkαΔ CM (figure 4b, lower panel). Therefore, disruption of ampkαΔ caused oversecretion of some factors that could regulate the aggregate size.

As the CM collected from ampkαΔ cells allowed the formation of smaller aggregates by the Ax2 cells, we also examined the levels of countin mRNA in the ampkαΔ cells (figure 5a). The expression of countin mRNA was significantly increased in ampkαΔ cells during development when compared with the Ax2 cells (figure 5a). Countin factor (CF) regulates group size by repressing cell-cell-adhesion proteins. The two major
cell-adhesion proteins expressed during early development of *Dictyostelium* are Gp24 and Gp80. High expression levels of gp24 were observed in *countin*2 cells, whereas gp80 expression levels were not significantly altered during early development [22]. The mRNA expression of *cadA* (gp24) was significantly decreased from streaming onwards in developing *ampkα*− cells resulting in decreased cell–cell adhesion, and subsequently resulting in the formation of small-sized aggregates (figure 5b). Overexpression of the Gp80 cell-adhesion protein, which causes aggregation streams to break up [23], was significantly increased at the mRNA level from mound onwards in the developing *ampkα*− cells, suggesting the possible reason for the break-up of streaming cells to result in small-sized aggregates (figure 5c).

2.7. Starving *ampkα*− cells show low glucose levels

CF appears to regulate cytosolic glucose levels and these in turn affect cell adhesion, suggesting that glucose levels are a part of the Countin signal transduction pathway [24]. In other words, glucose affects the group size in *Dictyostelium* and is the downstream target of CF. Adding glucose exogenously negates the effect of high extracellular CF on aggregate size and mimics the effect of depletion of CF. When Ax2 cells were allowed to
develop on non-nutrient agar plates containing 5 mM glucose [25], it resulted in the formation of large-sized aggregates (figure 6a, upper panel). In our studies, we too found 5 mM of glucose to be the best concentration (data not shown). Our earlier results showed high countin expression in the ampk2 cells; thus, it was possible that the CF could repress the internal glucose levels in these cells. When ampk2 cells were allowed to develop in the presence of 5 mM glucose, we observed large aggregates, but the size was still comparable to Ax2 (figure 6a, lower panel; electronic supplementary material, figure S4a,b). Therefore, we measured the cytosolic glucose levels in both the vegetative and starved cells. Cytosolic glucose levels in the vegetative cells of Ax2 (31.26 ± 1.8 nmol mg⁻¹ protein) and ampk2 (27.59 ± 2.8 nmol mg⁻¹ protein) were not significantly altered, but the starved ampk2 cells showed low glucose levels (6.98 ± 0.39 nmol mg⁻¹ protein) when compared with the Ax2 cells (11.2 ± 0.18 nmol mg⁻¹ protein) (figure 6b). Taken together, the data show increased countin expression and low glucose levels in the starved ampk2 cells to be responsible for regulating the aggregate size.

2.8. AMPKα regulates spatial cell-type patterning

As aberrant fruiting bodies were made by ampk2 cells, we were interested in elucidating the role of AMPKα in regulating cell-type differentiation (figure 7). We monitored mRNA levels of the cell-type-specific marker genes during development of both Ax2 and ampk2 cells. Expression levels of the prestalk-specific genes, ecma and cmb, were significantly increased in the ampk2 cells (electronic supplementary material, figure S5a,b) while the expression level of the prespore-specific gene, pspA (electronic supplementary material, figure S5c), was reduced when compared with the Ax2 cells. We attribute these differences to the formation of fruiting bodies with long stalks and small sori by the ampk2 cells. We substantiated our results by examining the spatial cell-type patterning using cell-type-specific promoters fused to the lacZ reporter in both the Ax2 and ampk2 cells. We found an increase in the staining region and/or mis-localization of prestalk-specific genes during the development of ampk2 cells (figure 7a–d), while the prespore-specific gene, pspA, staining region was reduced (figure 7e).

The spatial distributions of prestalk- and prespore-specific genes were compared in the Ax2 and ampk2 developmental structures (figure 7a–e). In the ampk2 mound, the spatial distribution of ecma/lacZ was aberrant, as it was dispersed all over the mound (figure 7b(E)) rather than only at the tips as in the case of the Ax2 mound (figure 7b(E)). The spatial localization of cmb/lacZ was also strikingly aberrant as it was precociously expressed in the ampk2 loose aggregates (figure 7d(M')) when compared with Ax2 (figure 7d(M)) and the staining region of pspA/lacZ was decreased in the ampk2 mound (figure 7e(Q)). The spatial localizations of these genes were also compared in the Ax2 and ampk2 slugs. In Ax2 slugs, the spatial localization of ecma/lacZ was found in the anterior region and also distributed throughout the slugs (the anterior-like cells (ALCs)) (figure 7a(B)), whereas the cmb/lacZ staining was expanded in the anterior region of ampk2 slugs and also more pronounced at the rear-guard region (figure 7a(F)). The cma/lacZ staining displayed in the anterior region was expanded and aberrant as it was also found in the prespore region of ampk2 slugs (figure 7b(P')), and the cmb/lacZ spatial localization was expanded in the tip region of ampk2 migratory slugs (figure 7c(Y)) that normally is occupied by pstA cells in Ax2 (figure 7c(J)) but was absent in the rear-guard region (figure 7c(Y)). The cmb/lacZ expression was found in the anterior region of slugs that was normally occupied by pstA cells and also the staining region was expanded in the rear-guard region (figure 7d(N')), and the pspA/lacZ staining was reduced in ampk2 slugs (figure 7e(R')).

In the ampk2 early culminant, cma/lacZ expression was mis-localized as it was also found in the prespore/spore region (figure 7b(G)), cmb/lacZ was absent in the prespore/spore region and the basal disc (figure 7c(K)), cmb/lacZ was absent in the stalk region (figure 7d(O)) and the staining region displayed by pspA/lacZ was reduced.
ampk cells contributed only to 1.22 ± 0.78%, 2.8 ± 0.32%, 5.8 ± 0.32%, 14 ± 4.15% and 44 ± 9.5%, respectively (figure 8b). The Ax2 and ampkα− cells when developed individually could form fruiting bodies on their own. Our data suggest that AMPKα is a critical component responsible for the maintenance of the prestalk/prespore ratio as well as the boundary between prestalk/prespore regions.

3. Discussion

During the past several years numerous studies have explored the importance of AMPK in various aspects, but still it needs to be investigated to uncover many unsolved issues. AMPK, a central regulator of metabolism, is a nutrient and energy sensor that is regulated reciprocally upon starvation by either energy or glucose [27–30]. Mammalian AMPK and its homologue present in plants and yeast are known to be activated in response to glucose depletion [31,32] and regulate expression of genes that permit growth on alternative carbon sources [33,34]. Previous findings have shown that AMPK is activated upon decrease in ATP/ADP or ATP/AMP levels [35]. The change in ATP/ADP level could be the consequence of various factors like glucose depletion, hypoxia, reactive oxygen species, etc. [29,36]. In Dictostelium, multi-cellular development programme is initiated upon nutrient starvation and cells undergo various metabolic changes to survive and further differentiate. The major energy sources for starving cells result from the cellular degradation of RNA and protein, but glycolysis and gluconeogenesis contribute minimally [37].

We have characterized AMPKα from D. discoideum and show its involvement in the regulation of aggregate size and cell-type patterning. Dictostelium AMPKα, like other AMPK homologues, is activated upon starvation (electronic supplementary material, figure S6), suggesting its role in nutrient sensing. The mRNA is expressed at all stages of development.
and is localized in the dying cell population, suggesting it to be involved in stalk cell differentiation and also to play a role in cell differentiation.

3.1. AMPKα regulates aggregate size

One of the most interesting aspects of development is how a large number of cells organize themselves into structures of specific sizes and shapes. In this study with *D. discoideum*, we have identified AMPKα in regulation of aggregate size. We show the mutant *ampkα*2 cells to have a defect in aggregate size determination, which causes the formation of a high density of aggregates containing small numbers of cells. We found (data not shown) that when plated at higher density for development, they formed smaller aggregates, showing there is a defect in the size-determination mechanism rather than an inability to form aggregates. In fact, the defect was not limited only to the aggregate size-determination mechanism, as the mutants also showed defects in cell proliferation, development and differentiation of prespore and prestalk cells. It is known that CF (a 450 kDa complex of five proteins), a component of CM, is secreted in moderate amounts by the Ax2 cells, and regulates the number of cells present within the group and ultimately controls the size of the aggregates. Disruption of the *countin* gene does not allow the aggregation stream to break up, thus resulting in the formation of large aggregates and subsequently larger fruiting bodies, while oversecretion of Countin protein causes the aggregation streams to break up resulting in smaller aggregates and ultimately smaller fruiting bodies [11,38]. The small aggregates are due to an increased amount of secreted factor by the *ampkα* cells, as *ampkα* phenotype could be imitated by developing the Ax2 cells in the presence of the CM collected from the mutant cells, but it also could not be rescued by starving *ampkα* cells in the presence of Ax2 CM. The ability of 5% *ampkα* cells in a population of 95% Ax2 cells to cause the entire population to form small aggregates suggests that the mutant phenotype is not due to the lack of secreted factor. As AMPK is a serine/threonine kinase and regulates the expression of a variety of proteins, it possibly could regulate the group size by regulating *countin* expression which involves levels of intracellular glucose and also represses the cell-adhesion proteins. The *ampkα* cells exhibit increased *countin* expression, which reduces the glucose levels and exhibits aberrant cell-adhesion expression to maintain the group size. From our studies, we conclude that in wild-type cells, AMPK represses Countin and subsequently the glucose levels and cell-adhesion proteins to regulate the aggregate size (figure 9). The *ampkα* cells developed into comparatively larger aggregates in the presence of exogenous glucose, but the size still remained comparable to wild-type. Therefore, we could say that decreased glucose levels were not the only cause of small-sized aggregates, but there may also exist some other factors that directly or indirectly regulate aggregate size.

![Figure 8](http://rsob.royalsocietypublishing.org/)

**Figure 8.** Distribution of Ax2 and *ampkα*− cells in chimaeras. (a) RFP-tagged Ax2 cells and GFP-tagged *ampkα*− cells were mixed in varying proportions and allowed to co-develop. Both DIC and fluorescence images during development (migratory slugs, early culminants and culminants) were captured. GFP-labelled *ampkα*− cells populated the prestalk regions (rear-guard and ALCs) of the slugs, whereas RFP-labelled Ax2 cells occupied the prespore regions of the slugs. GFP-labelled *ampkα*− populate lower cup and basal disc of early culminant and culminant. (b) Percentage (%) spore count of *ampkα*− in chimaeric fruiting bodies were scored and represented. Scale bar, 100 μm; n = 3.
Figure 9. Model illustrates the role of AMPK in growth and development. AMPK inhibits cell growth, proliferation and size. It inhibits CF that represses the conversion of glucose from glucose-6-phosphate to control cellular events such as cell–cell adhesion, aggregation territory size, prestalk/prespore ratio and spatial cell-type distribution. AMPK also helps in maintaining the prestalk/prespore ratio and spatial distribution. In the above model, solid lines indicate known information, while dotted lines display proposed hypothesis from this study.

An enigmatic aspect of AMPKα is that disruption caused increase in cell growth and proliferation; thus, one would think that the absence of it is advantageous to the cell. A vital aspect of Dictyostelium development is formation of the fruiting body for maintaining its generation. The spores thus produced are a measure of one’s fitness and germinates upon favourable conditions. The ampka- and ampkαOE cells both formed aberrant fruiting bodies and the spores displayed reduced viability when compared with Ax2. Therefore, abnormally low or high levels of AMPKα reduce the ability of spores to germinate and an optimum level is necessary for efficient spore formation. The function of the fruiting body is to lift the spore mass as high off the ground as possible, for optimal spore dispersal. If the fruiting body is too large, it would collapse and the spores on the ground will not get dispersed. Therefore, there is a limit on the number of cells in the spore mass as well as in the stalk of a fruiting body. As the mutants do complete their development and form fruiting bodies, we believe that all other components required for development are below the acceptable range.

3.2. AMPKα helps maintain prestalk/prespore ratio and spatial cell-type patterning

In the case of Dictyostelium, cell differentiation is initiated when myxamoebae stop growing and dividing. During development, cells terminally differentiate into two main cell types, the stalk and the spore cells, whose precursors are prestalk and prespore, respectively, and are observed at earlier developmental stages and distinguished by marker genes. The ratio of prestalk and prespore cells remains constant regardless of the size of the slug, and any alteration in this would lead to aberrant morphogenesis and differentiation [15,39,40]. Previous reports with Dictyostelium showed cells grown in the presence of glucose (G+) cells had increased spore-to-stalk as well as prespore-to-prestalk ratio when compared with the cells grown in glucose-depleted conditions [41,42]. The study was also supported by other groups who showed that cells when grown in the absence of glucose (G-) developed fruiting bodies with long stalks and small sori when compared with the cells grown in glucose-rich conditions, attributed to the differences in spore-to-stalk ratio [42,43]. Cell-type-specific marker expression studies in ampkα- cells display an increase in prestalk-specific gene expression and concomitant reduction in prespore-specific gene expression, and also the spatial localization of these genes was altered. In our study, we found that starved ampkα- cells had low glucose levels and developed fruiting bodies with long stalks and small sori. Therefore, reduced glucose levels could be one of the causes for this inequitable fruiting body formation. Mixing of cell types in the ampkα- mutant suggest its role in maintaining the boundary between them. Thus, AMPKα assists in proportioning and patterning of prestalk/prespore cells.

Glucose has also been shown to play a role in the cell sorting behaviour of Dictyostelium cells. The differential preferences of cells in development and differentiation have been observed in various organisms using chimera study. Chimaeras are mixture of cells from two or more genetically different backgrounds. Several chimaeras in Dictyostelium show unequal apportionment of the genetically different cells into spore and stalk cells [21,44]; the one that gets a higher proportion of spores is called cheater and the other is called loser [45]. Khare & Shaulsky [46] have led this concept of multi-cellular bodies using competition to place their best cells into justified functions, so the question arises, do the mutant cells contribute equally in spore formation of chimaeras? ampka- cells form few fruiting bodies from a given number of cells when compared with the wild-type cells in pure populations; this result is sufficient to explain the competitive failure of mutant cells in contributing to spore formation in chimaeras. There are various reports that explain the role of glucose in the sorting behaviour of Dictyostelium cells during differentiation. In chimaeras, G+ cells preferentially become prespore cells of the slug and G- cells sort out to become prestalk cells [47–49]. Our study provides insights about the differential preferences of ampkα- cells that exhibit low glucose levels in the cell sorting behaviour. The ampka- cells participate mainly in prestalk-derived structures and have less proclivity to occupy the prespore/spore region within chimaeras in the presence of Ax2 cells. Previous studies showed deletion of ampk resulted in embryonic lethality and, therefore, its direct impact on cellular differentiation and lineage choice is still unknown [50]. Young et al. [51] showed direct impact of AMPKα in cell fate determination during differentiation. Further studies are still required to understand the direct impact of AMPKα on cell lineage tracing and differentiation in Dictyostelium.

4. Conclusion

Our results demonstrate that AMPKα regulates aggregate size in Dictyostelium. The fruiting bodies formed by ampkα- cells result from the differences in prestalk/prespore ratio. The results also suggest a role in cell-type differentiation and spatial patterning.

5. Material and methods

5.1. Growth and development of Dictyostelium discoideum

The Dictyostelium cells were grown and developed as described [52]. The logarithmic phase cultures (2.5–5 × 10^6 cells ml^{-1}) were identically diluted into fresh media at a density of...
approximately $5 \times 10^5$ cells ml$^{-1}$ and monitored over 6 days for measuring cell proliferation.

For development, exponentially growing cells were harvested, washed in $1 \times$ KK$_2$ buffer and spotted at a density of $5 \times 10^6$ cells ml$^{-1}$ on non-nutrient agar plates. The plated cells were incubated at 4°C for 4–6 h for synchronization of the development, followed by incubation at 22°C. Analyses of mound size were performed using NIS ELEMENTS AR v. 4.0.

5.2. RNA detection by RT-PCR and in situ hybridization analyses

RNA was isolated as described [52] from growing amoebae and at various developmental stages. RT-PCR reactions were performed using gene-specific primer pairs (electronic supplementary material, table S1). rilA (ig7) was used as an internal control.

The in situ hybridization studies were performed as described [52]. The probe was obtained by cloning a 744 bp PCR amplified region using the gene-specific primer pairs into XhoI/XbaI site of commercially available pBluescriptII phagemid (pBSIK§+) vector (electronic supplementary material, figure S7). It was followed by in vitro transcription into RNA, sense probe with T7 polymerase (template obtained after construct digestion with Xbal) and antisense probe with T3 RNA polymerase (template obtained after construct digestion with XhoI) were obtained. The probe was labelled with a DIG RNA labelling kit (Roche Diagnostics) and antisense probe with T3 RNA polymerase (template obtained after construct digestion with XbaI) were used as a control.

The list of primer pairs for other genes used in this study is given in the electronic supplementary material, table S1.

5.3. Preparation of construct and strains

5.3.1. ampkα overexpressing strain

A full-length $\alpha$mpkα gene was PCR amplified (electronic supplementary material, figure S1) from genomic DNA using the gene-specific primer pairs (electronic supplementary material, table S1) and cloned into act15/Acg-Eyfp vector (electronic supplementary material, figure S1) that contains the actin 15 promoter, enhanced yellow fluorescent protein (Eyfp) at the C-terminus, a G418 resistance marker (electronic supplementary material, figure S1) that contains the vector (electronic supplementary material, figure S1) that contains the vector (electronic supplementary material, figure S1) that contains the vector (electronic supplementary material, figure S1) that contains the vector (electronic supplementary material, figure S1) that contains the vector (electronic supplementary material, figure S1) that contains the vector (electronic supplementary material, figure S1) that contains the vector (electronic supplementary material, figure S1) that contains the vector (electronic supplementary material, figure S1) that contains the vector (electronic supplementary material, figure S1) that contains the vector (electronic supplementary material, figure S1) that contains the vector (electronic supplementary material, figure S1) that contains the vector (electronic supplementary material, figure S1) that contains the vector (electronic supplementary material, figure S1) that contains the vector. A sense probe was used as a control.

The rescue strain was made by expressing an AMPKα–Eyfp fusion construct into ampkα$^{-}$ cells.

5.4. Cell dry weight

Nearly $5 \times 10^7$ log phase cells were washed and vacuum dried at 55°C. After 1 h, the weight of dry pellets was scored.

5.5. Flow cytometry

To determine cell size, a BD FACS Calibur flow cytometer with CELL QUEST software was used. About $1 \times 10^7$ log phase cells were harvested, washed in $1 \times$ KK$_2$ buffer and re-suspended in 1.5 ml buffer (0.9% NaCl, 2% sucrose, 5 mM EDTA in KK$_2$ buffer). Cells were fixed by adding 75% chilled ethanol drop-wise and incubated for 30 min at 22°C and stored at 4°C. Immediately before analysis, 1 $\times 10^7$ cells were washed, re-suspended in KK$_2$ buffer, and incubated at 37°C for 30 min in 10 µg ml$^{-1}$ RNase A (Sigma-Aldrich, USA) followed by incubation at room temperature in 50 µg ml$^{-1}$ propidium iodide (Sigma-Aldrich).

5.6. Conditioned medium experiments

CM was prepared as described previously [54]. Briefly, log phase cells of Ax2 and ampkα$^{-}$ were starved and re-suspended at a density of $1 \times 10^7$ cells ml$^{-1}$ and kept under shaken conditions for 20 h, at 22°C. Cells were pelleted down and the supernatant was further clarified by centrifugation. The clarified supernatant thus prepared or CM was used immediately. To check the effect of CM on aggregate size, Ax2 and ampkα$^{-}$ cells were starved on dialysis membranes, which were placed on Whatman filters (two to three layers) soaked in Ax2 CM or ampkα$^{-}$ CM. Cells starved on Whatman filters (two to three layers) soaked in $1 \times$ KK$_2$ buffer were used as a control.

5.7. Spore viability assays

Spore viability assay was followed as described [21]. Spores from mature fruiting bodies were harvested in spore buffer (40 mM KH$_2$PO$_4$, 20 mM KCl, 2.5 mM MgCl$_2$) washed twice by centrifugation at room temperature and counted in a haemocytometer. Aliquots of 100 spores were mixed with a suspension of bacteria (Klebsiella aerogenes) and grown for 5 days. The per cent viability of spores was measured by counting the number of clear plaques formed on the bacterial lawns divided by total spores plated followed by multiplication with 100. Three independent experiments in triplicate were performed.

5.8. Glucose assay

Glucose assay was performed as described [25] with minor modifications. Log phase cells were harvested and re-suspended at a density of $8 \times 10^6$ cells ml$^{-1}$ and kept under shaken conditions for 6 h at 22°C. Cells were harvested and lysed by the freeze–thaw method. Glucose assay was performed as per instructions given by the manufacturer (GAHK20; Sigma-Aldrich). Protein levels were measured using Bradford reagent (BIO-RAD).
5.9. Development of chimaeric mixtures
Ax2 and ampkα− cells were transfected with pTX-RFP and pTX-GFP, respectively, and selected at 40 μg ml−1 of G418. GFP- and RFP-marked cells were mixed in varying percentages (5–75%) and developed on non-nutrient agar plates. Both DIC and fluorescent images were captured on a Nikon SMZ-1500 microscope. Individual spore heads were picked on a glass-slide and photographed, both under brightfield and under fluorescence using a Nikon eclipse 80i fluorescence microscope. Red and green fluorescent spores were counted from the photographs. A minimum of 10–15 fruiting bodies developed from each mixture per individual experiment was counted.

5.10. β-Galactosidase staining
β-Galactosidase staining was performed as described [52]. Images were captured using a Nikon AZ100 microscope.

5.11. Statistical analysis
The statistical analyses were performed (mean standard deviation and standard error) and values were plotted in graph using Microsoft EXCEL-2013 and GraphPad PRISM. p-Values of less than 0.05 were considered as significant.

References
3. Inoki K et al. 2006 TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. Cell 126, 955–968. (doi:10.1016/j.cell.2006.06.055)


