Developmental Biology

Extracellular adenosine deamination primes tip organizer development in *Dictyostelium*

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During the development of the unicellular eukaryote Dictyostelium discoideum, cells aggregate into mounds, forming protrusions or tips, which then become the front of migrating slugs and the top of fruiting bodies. This **valuable** study identifies adenosine deaminase-related growth factor (ADGF) as a key regulator of tip formation and **convincingly** shows that ADGF catalyses the conversion of adenosine to ammonia, allowing ammonia to initiate tip formation, and then elucidates pathways upstream and downstream of ADGF. The authors discuss the intriguing possibility that mammalian ADGF may also similarly regulate development.

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Abstract

Ammonia is a morphogen in *Dictyostelium* and is known to arise from the catabolism of proteins and RNA. However, we show that extracellular adenosine deamination catalyzed by 'adenosine deaminase related growth factor' (ADGF), is a major source of ammonia, and demonstrate a direct role of ammonia in tip organizer development. The tip formed during early development in *Dictyostelium* functions analogously to the embryonic organizer of higher vertebrates. *Dictyostelium* cell lines carrying mutations in the gene *adgf* fail to establish an organizer, and this could be reversed by exposing the mutants to volatile ammonia. Interestingly, *Klebsiella pneumoniae* physically separated from the *Dictyostelium adgf* mutants in a partitioned dish, also rescues the mound arrest phenotype suggesting a cross-kingdom interaction that drives development. Both the substrate, adenosine and the product, ammonia regulate *adgf* expression, and ADGF acts downstream of the histidine kinase DhkD in regulating tip formation. Thus, the consecutive transformation of extracellular cAMP to adenosine, and adenosine to ammonia are integral steps during *Dictyostelium* development. Remarkably, in higher vertebrates, *adgf* expression is elevated during gastrulation and thus adenosine deamination may be a conserved process driving organizer development in different organisms.

Introduction

During early embryonic development, organizers play an important role in patterning and directing the differentiation of surrounding cells into specific tissues and organs. The embryonic organizer establishes the developmental polarity in vertebrates; and similarly, in *Dictyostelium*, the mound/slug tip acts as an organizer (Rubin and Robertson, 1975) playing a pivotal role in guiding collective cell migration and patterning. Despite numerous investigations on *Dictyostelium* tip formation, the processes by which the tip establishes and maintains the primary developmental axis remains elusive. Gaining insights into the mechanisms regulating tip organizer function will offer valuable insights on orchestrated cell movements and processes underlying development.

Dictyostelium transitions from a unicellular amoeba to a multicellular organism in response to starvation. The cells aggregate into a mound, which gives rise to a migrating slug and ultimately a fruiting body composed of spores and a dead stalk (Raper, 1940; Kessin, 2001). This process is regulated by diffusible signals including cAMP, adenosine, ammonia, and a chlorinated hexaphenone, 'differentiation-inducing factor' (DIF) (Bloom and Kay, 1988; Williams, 1988; Gross, 1994; Mahadeo and Parent, 2006). Importantly, the cell cycle phase soon after starvation is known to strongly influence the cell fate either as prestalk (pst) or prespore (psp) cells (Weeks and Weijer, 1994; Jang and Gomer, 2011).

Tip development in *Dictyostelium* depends on cAMP signaling in turn regulating protein kinase A (PKA) activity, adenosine signaling, and morphogenetic cell movements (Schaap and Wang, 1986; Mann and Firtel, 1993; Siegert and Weijer, 1995). Adenosine, a by-product of cAMP hydrolysis, acts as an inhibitory morphogen suppressing additional tip formation (Schaap and Wang, 1986).

Adenosine deaminases (ADA) catalyse the breakdown of adenosine to generate inosine and ammonia, and are conserved among bacteria, invertebrates, vertebrates including mammals (Cristalli et al., 2001). In humans, two isoforms of ADA are known including ADA1 and ADA2, and the *Dictyostelium* homolog of ADA2 is adenosine deaminase-related growth factor (ADGF). Unlike ADA that is intracellular, ADGF is extracellular and also has a growth factor activity (Li and Aksoy, 2000; Iijima et al., 2008). Loss-of-function mutations in *ada2* are linked to lymphopenia, severe combined immunodeficiency (SCID) (Gaspar, 2010), and vascular inflammation due to accumulation of toxic metabolites like dATP (Zhou et al., 2014; Notarangelo, 2016). In mice, *ada2* disruption leads to perinatal mortality (Wakamiya et al., 1995), and overexpression of *ada2* results in aberrant heart and kidney development (Riazi et al., 2005). In frogs, mutations in *ada2* manifest in reduced body size and altered polarity (Iijima et al., 2008). In *Drosophila*, certain isoforms of ADGF are known to play a pivotal role in cell proliferation by depleting extracellular adenosine (Zurovec et al., 2002), and loss of function of *adgfA* promotes melanotic tumour formation and larval death (Dolezal et al., 2005). Adenosine deaminases are known to interact with dipeptidyl peptidase IV (DPP), cluster of differentiation (CD26) expressed on T-cells and the adenosine receptor, A2AR (expressed on dendritic cells) to facilitate cell-cell signaling (Moreno et al., 2018). Thus, *adgf* plays a pivotal role in the regulation of cell proliferation and development in several organisms.

Four isoforms of ADA are annotated in the *Dictyostelium discoideum* genome (Eichinger et al., 2005) including adenosine deaminase (*ada*; DDB_G0287371), adenosine deaminase acting on tRNA-1 (DDB_G0278943), adenosine deaminase tRNA-specific (DDB_G0288099) and adenosine deaminase-related growth factor (*adgf*; DDB_G0275179), and their role in growth and development

is not known. In this study, we demonstrate that ammonia generated by ADGF plays a direct role in establishing the tip organizer, highlighting a novel link between nucleoside metabolism and developmental polarity in *Dictyostelium*.

Results

Differential regulation of *adgf* expression during growth and development

Dictyostelium cell lines carrying mutations in the gene *adgf* were obtained from the genome wide *Dictyostelium* insertion (GWDI) bank, and were subjected to further analysis to know the role of *adgf* during *Dictyostelium* development. To verify the insertion of blasticidin *(bsr)* resistance cassette in *adgf* mutant, a diagnostic PCR was carried out and the integration was validated (**Figures 1A** and **1B**), and qRT-PCR analysis confirmed the absence of *adgf* expression (**Figure 1C**). In vertebrates, adenosine deaminases are expressed in a tissue specific manner to control growth and development. To determine whether *adgf* expression is differentially regulated during *Dictyostelium* development, qRT-PCR was performed using RNA isolated at 0 h, 8 h, 12 h, 16 h, 20 h and 24 h post starvation. *adgf* expression peaks at 16 h (**Figure 1D**) implying an important role for *adgf* later in development. At this time point, the expression of the other three isoforms of ADA were not significantly different from wild-type (WT), suggesting that the loss of *adgf* (data not shown) function is not compensated by the other isoforms.

The predicted structures of *Dictyostelium* ADGF and human ADA2 share a strong structural similarity

The *D. discoideum adgf* gene is predicted to encode a protein of 543 amino acids, and belongs to the metallo-transferases superfamily (**Figure S1A**), having an ADA and an N-terminal domain similar to human ADA2 (**Figure S1B**). The ADGF sequence from the protein family database (*https://www.ebi.ac.uk/interpro/*) was analysed using the online tool SMART (Simple Modular Architecture Research Tool: *http://smart.embl-heidelberg.de*), to know the presence of structurally similar domains. The different domains of *Dictyostelium* ADGF (DdADGF) show a high degree of similarity with human ADA2. DdADGF shares 37.5% identity with human ADA2 and shares a sequence similarity of 59.5% with *D. fasciculatum*, 61.2% with *D. pupureum*, 53.6% with *D. lacteum* and 62.1% with *Polysphondylium pallidum* (*www.uniprot.org*). Multiple sequence alignment reveals the presence of an N-terminal signal sequence characteristic of extracellular proteins (*Figure S1C*).and conserved histidine and glutamine residues in the active site of both *D. discoideum* ADGF and human ADA2 (*Figure S1D*).

The phylogenetic relation of ADGF to the classic ADA subfamily has been reported previously (Maier et al., 2005). To determine the evolutionary relationship of DdADGF with that of other organisms, a phylogenetic analysis was carried out, and the amino acid alignment was created using MEGAX software (Kumar et al., 2018). The evolutionary history of ADGF was inferred using a Maximum Likelihood approach with Bootstrap analysis (100 iterations) as described by Felsenstein (1985). The resulting phylogenetic tree indicated that DdADGF is closely related to the ADGF proteins of other Dictyostelids, including *D. purpureum*, *Heterostelium pallidum,* and *Cavenderia fasciculata.* DdADGF forms a distinct clade, likely representing a distant relative of its vertebrate homolog (Figure S1E). Using the crystal structure of human ADA2, Cat eye syndrome critical region protein 1 (CECR1) as a template (PDB-3LGD), the structure of DdADGF was predicted by homology modeling (*https://alphafold.ebi.ac.uk/*) (Figures S1 F-H). Alignment of DdADGF with human ADA2 yielded a root mean square deviation (RMSD) value of 1 Å, indicating strong structural similarity (Eidhammer et al., 2000; Koehl, 2001).

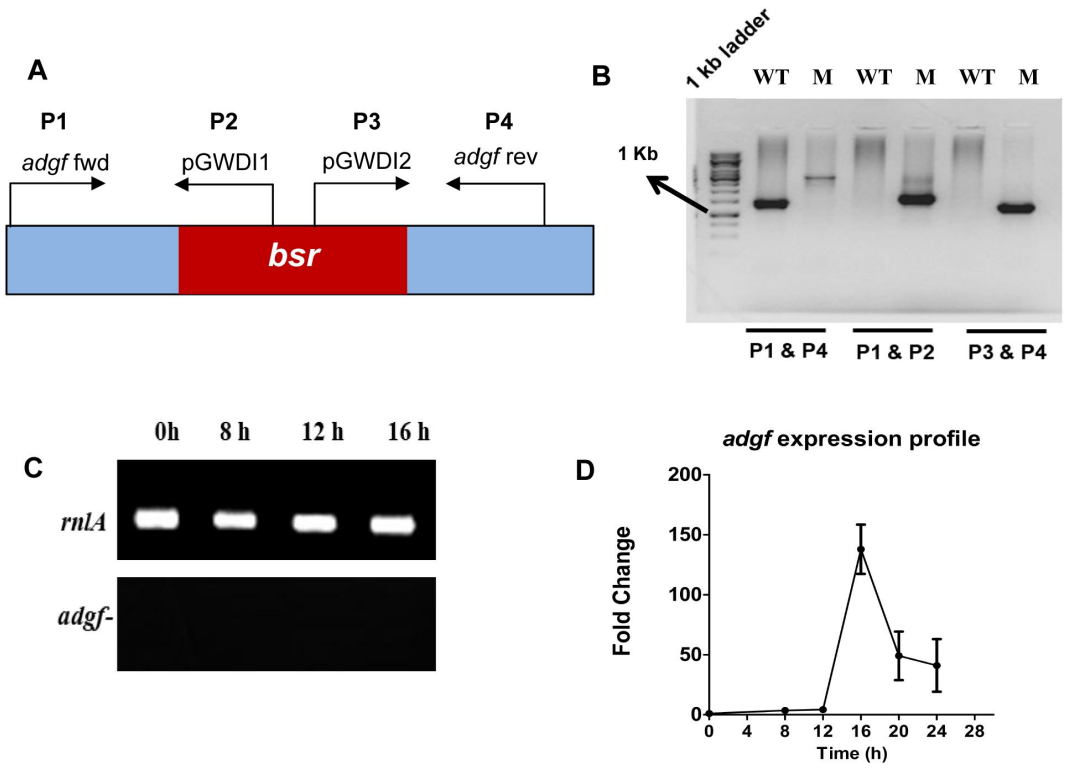

Figure 1.

adgf mutant validation

A) Schematic representation of *adgf* locus showing the relative positions of the primers (P1–P4) and the blasticidin resistance cassette (*bsr*). Primer P1 (*adgf* fwd) is at the start codon of *adgf*, and primer P4 (*adgf* rev) is 264 bp upstream of the stop codon, flanking the insertion site. Primers P2 (pGWD2) and P3 (pGWD1) are located within the *bsr* cassette. *bsr* insertion is in exon 2 of *adgf*. **B)** PCR analyses using P1 and P4 primers. A 1.4 kb shift in the *adgf* mutant. PCR using P1 and P2 primers showed an amplicon from the mutant (M) and not from the wild type (WT). PCR using P3 and P4 primers showed an amplicon with the *adgf* mutant while WT did not show any amplicon. **C)** Semi-quantitative RT-PCR of the internal control, *rnlA* and *adgf*$^{-}$. *adgf* expression during development in *Dictyostelium*. **D)** Total RNA was isolated from *Dictyostelium* during vegetative growth and development using TRIzol method. To quantify *adgf* expression, qRT-PCR was carried out with *rnlA* as a control and the fold change was calculated accordingly. Time points are shown in hours (bottom). Error bars represent the mean and SEM (n = 3).

adgf controls aggregate size in *Dictyostelium*

To understand the role of *adgf* during *D. discoideum* development, *adgf* mutants were plated at a density of 5×10^5 cells/cm^2 on non-nutrient phosphate buffered agar plates and monitored thereafter. In comparison to WT, the aggregates of *adgf*$^-$ were larger, and thus the number of aggregates were fewer (**Figure 2A**) than the WT. To determine the pathways impairing the tissue size in the mutant, RNA expression of countin (*ctn*) and small aggregates (*smlA*) were examined, and their levels were reduced significantly compared to controls (**Figure 2B**). Countin factor (CF) regulates group size by reducing the expression of cell-cell adhesion proteins cadherin (*cadA*) and contact site A (*csaA*) (Siu et al., 1985; Coates and Harwood, 2001). Compared to WT, both cell-to-cell adhesion and *cadA, csaA* expression were higher in the *adgf* mutant (**Figures 2C** and **2D**) suggesting that *adgf* regulates the overall size of the aggregates.

cAMP chemotaxis significantly influences cell-cell adhesion (Konijn et al., 1967), and to determine if chemotaxis is impaired in the *adgf*$^-$ lines, an under-agarose chemotaxis assay was carried out. The chemotactic activity was not significantly different between the two cell types (**Figure 2E**), suggesting that the increased mound size in the mutant is not due to altered chemotaxis.

adgf mutants form large, tipless mounds

Subsequent to plating on KK2 agar, WT cells formed mounds by 8-9 h, and culminate to form fruiting bodies by the end of 24 h. In contrast, the *adgf*$^-$ lines were blocked as rotating mounds with no tips till 30 h (**Figure 2F**; **Supplementary videos S1 and S2**). Such a mound arrest phenotype could be mimicked by adding the ADA specific inhibitor deoxycoformycin (DCF) to WT cells (**Figure 2G**). After 36 h, a fraction of *adgf*$^-$ mounds (34.77 ± 4%) formed fruiting bodies with bulkier spore sac (79.6 ± 6.3 mm^2) and stalk (**Figures 2H** and **2I**). This late recovery from the mound arrest may be due to the expression of other ADA isoforms after 36 h.

Reduced ADA activity leads to high adenosine levels in the *adgf* mutant

If *adgf* function is compromised, ADA enzyme activity is expected to be low. To verify this, total ADA activity in WT and *adgf*$^-$ mounds was measured using a commercial assay kit. This assay relies on the conversion of inosine (derived from ADA activity) to uric acid, and was measured spectrophotometrically. Although ADA activity dropped significantly in the mutant (**Figure 3A**), it was not completely abolished, and possibly, other isoforms may have a basal activity both at 12 h and 16 h.

ADGF quenches extracellular adenosine, and if blocked, as expected in *adgf*$^-$, the mutants will have increased extracellular adenosine. Using a commercial kit, the total adenosine levels were measured, and both at 12 h and 16 h, the levels were elevated (**Figure 3B**), and this difference was highly significant at 16 h. *adgf* expression at this time point was also high in WT cells. The elevated adenosine levels at 16 h may result from reduced ADA activity and increased expression of genes such as 5' nucleotidase (*5'nt*) and phosphodiesterases (*pdsA* and *regA*), all involved in adenosine formation (Headrick and Willis, 1989; Godecke, 2008). Hence, their expression levels were examined. Although the expression of both *5'nt* and *regA* were enhanced at 8 h, *pdsA* levels remained unaltered. Interestingly, the expression of all three genes trended lower at 12 h but were significantly upregulated at 16 h (**Figure 3C**), suggesting an important role of these genes at a specific time in development.

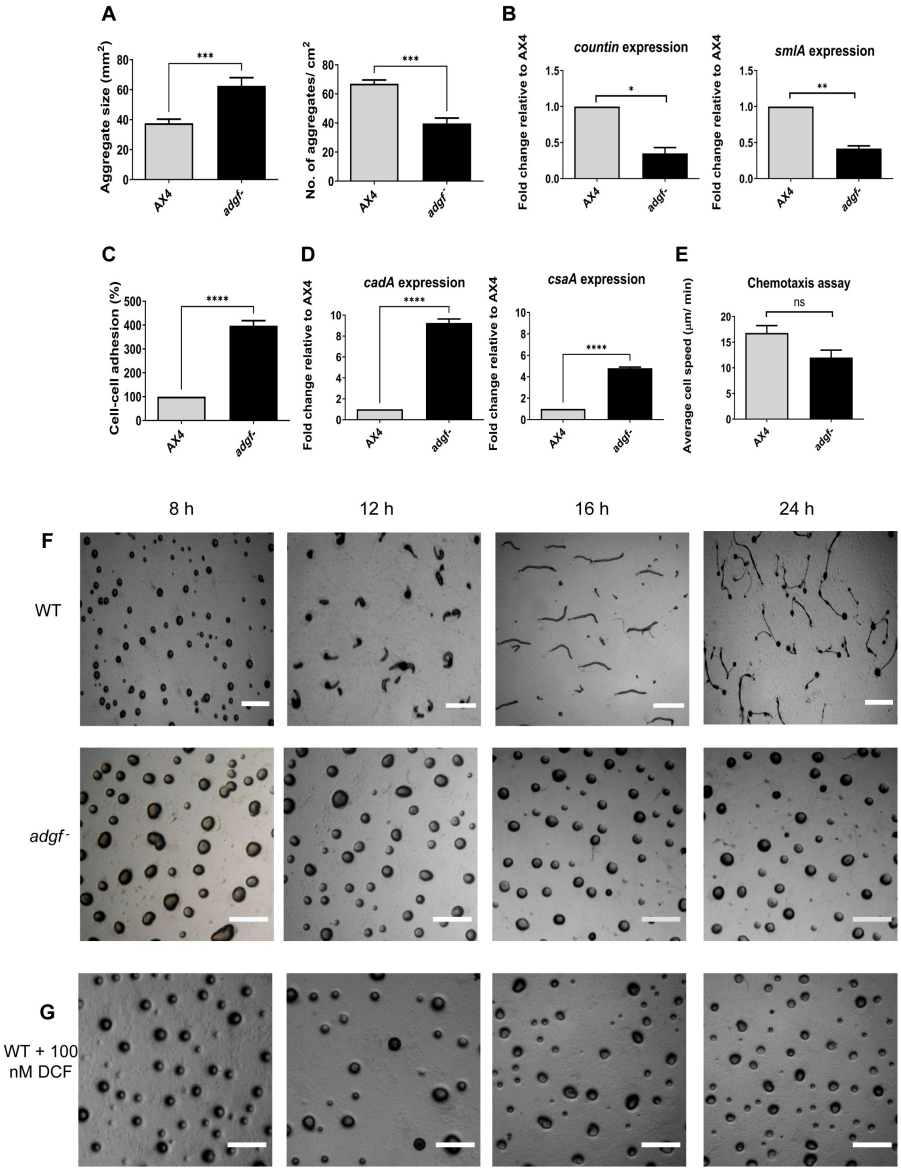

Figure 2.

Aggregates formed by *adgf* mutants were larger in size

A) The graph shows the mound size and the number of aggregates formed by WT and *adgf* ⁻. The values represent mean ± S.E; n=3. **B)** Expression levels of the genes, countin (*ctn*) and small aggregates (*smlA*) during aggregation in *adgf* ⁻ compared to WT. *rnlA* was used as the internal control. **C)** WT and *adgf* ⁻ cells were developed on KK2 agar, and after 16 h, the multicellular mounds/slugs were dissociated by vigorous vortexing in KK2 buffer. Individual cells were counted using a hemocytometer and resuspended in a phosphate buffer. Non-adherent single cells were counted 45 min after incubation. The percent cell-cell adhesion was plotted by normalizing the values to the non-adherent WT count to 100%. Error bars represent the mean ± SEM (n=3). **D)** qRT-PCR analysis of cadherin (*cadA*) and contact site (*csA*) during aggregation. The fold-change in RNA transcript levels is relative to WT at the indicated time points. (n=3). ns = not significant. Significance level is indicated as *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001. (Student's t-test). **E)** Under agarose chemotaxis assay. The average cell speed in response t o 10 µ M c AMP was r ecorded. The graph r eprese n t s t h e m e a n ± SE M (n=3). Developmental phenotype of *adgf* ⁻. **F)** WT and *adgf* ⁻ cells were washed, plated on 1% KK2 agar plates at a density of 5×10^5 cells/cm^2, incubated in a dark, moist chamber and images were taken at different time intervals. **G)** WT cells treated with 100 nM of DCF mimicked the mound arrest phenotype of the mutant. The time points are indicated in hours at the top of the figure. Scale bar: 2 mm; (n=3). **H)** WT and *adgf* ⁻ cells after 36 h of development. Scale bar: 0.5 mm; (n=3). **I)** Fruiting bodies of WT and *adgf* ⁻. Scale bar: 2 mm; (n=3).

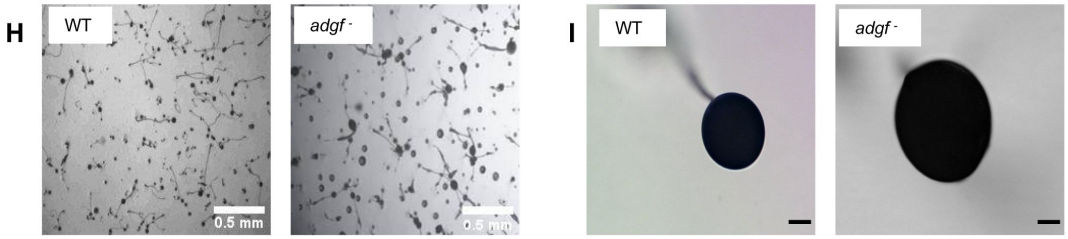

Figure 2. (continued)

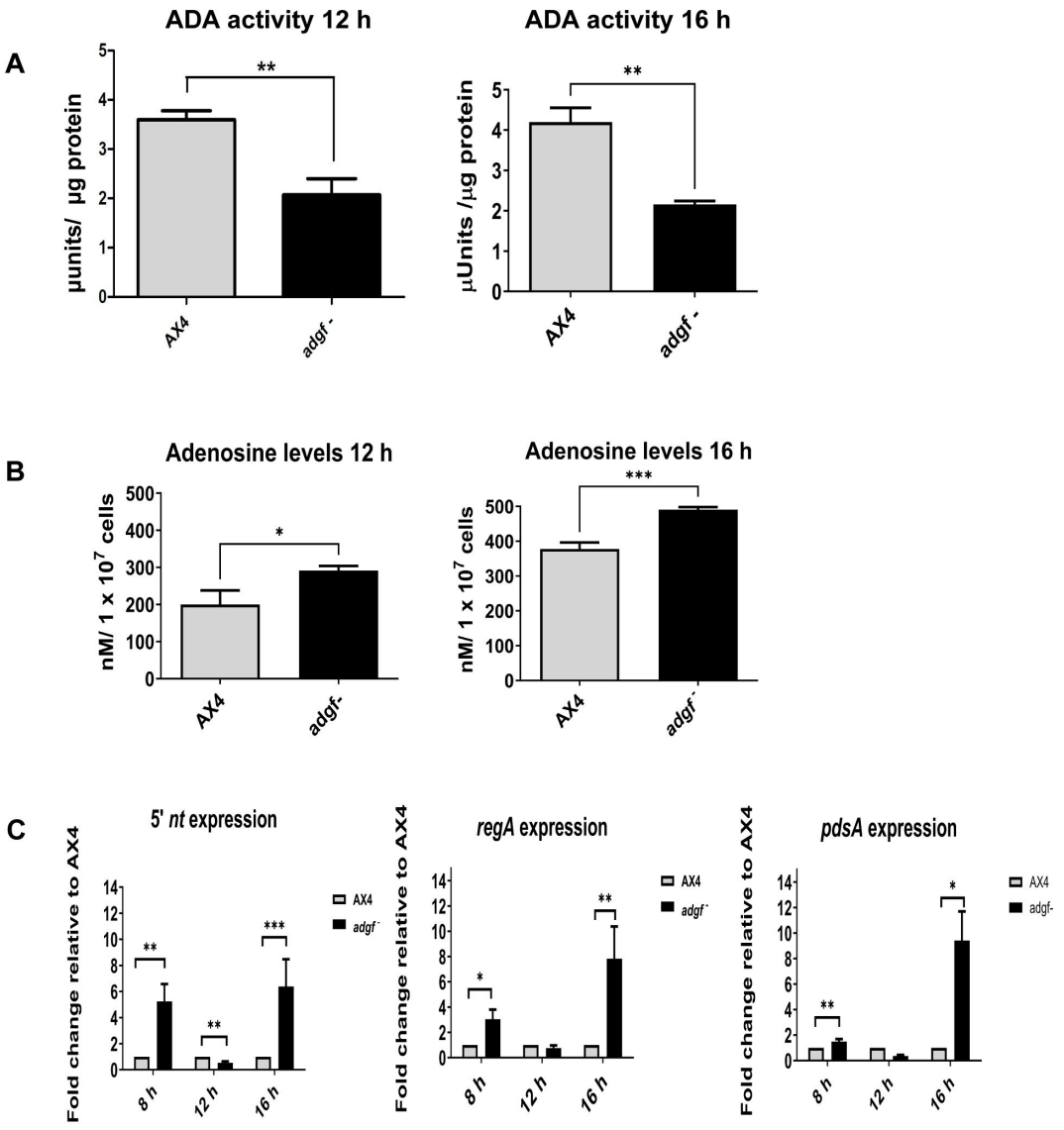

Figure 3.

adgf mounds have reduced ADA activity and high adenosine levels.

A) ADA activity in WT and *adgf* $^{-}$ harvested at 12 h and 16 h. The enzymatic assay for ADA was performed in *adgf* $^{-}$ with the corresponding WT control. Error bars represent the mean and SEM (n=3). Significance level is indicated as **p< 0.01. **B)** Quantification of adenosine levels in WT and *adgf* mutants at 12 h and 16 h. Level of significance is indicated as *p< 0.05, **p< 0.01, ***p< 0.001; (n=3). **C)** Expression profile of 5′ nucleotidase (*5′nt*) and phosphodiesterases (*regA*, *pdsA*) involved in cAMP-to-adenosine conversion. The fold-change in RNA transcript levels is relative to WT at the indicated time points. *rnlA* was used as an internal control. Error bars represent the mean and SEM (n=3).

Addition of ADA or overexpression of *adgf* cDNA restored tip development in *adgf*$^-$

adgf mutants carry significantly high levels of adenosine. By administering ADA enzyme on top of the *adgf*$^-$ mounds (**Figure 4A**), excess adenosine can be quenched resulting in ammonia formation, possibly rescuing the phenotype. Indeed, addition of 10 U ADA onto mutant mounds restored tip development. Besides, overexpression of WT *adgf* cDNA (driven by actin15 promoter) complemented the developmental defects of *adgf* mutants (**Figures 4B** and **4C**), confirming that the developmental block is the result of *adgf* gene disruption alone. However, *adgf* cDNA overexpression in WT cells does not result in any observable defects (**Figure 4D**). Taken together, these findings support an important function of *adgf* in tip development.

WT or its conditioned media (CM) restored tip formation in *adgf*$^-$ mounds

To determine whether *adgf* is necessary for tip formation in a cell-autonomous manner, WT and the mutant cells were mixed in different proportions and plated for development. In a mix of 50% WT: 50% mutant, the mound defects of *adgf*$^-$ were rescued, but with 20% WT and 80% *adgf*$^-$, the rescue was partial (31 ± 4 %) (**Figure 4E**) suggesting that the mound arrest phenotype of the mutant is due to the absence of some secreted factor(s).

Further, when developed in the presence of WT CM, *adgf*$^-$ cells formed tipped mounds and eventually fruiting bodies (**Figure 4F**). Conversely, in the presence of *adgf*$^-$ CM, WT cells developed as large mounds with no tips (**Figure 4G**) and the size was comparable to the mounds formed by *adgf*$^-$. These findings imply that the developmental phenotype of *adgf*$^-$ is not due to a cell autonomous defect but due to faulty secreted factor signaling. While this finding is consistent with the involvement of a secreted factor, it is also possible that a membrane-bound extracellular factor may have a role in complementing the mutant phenotype.

Volatile ammonia rescued the mound arrest phenotype of *adgf*$^-$

If ADGF enzyme activity is compromised, ammonia levels are expected to be low. Hence, the concentration of ammonia from WT and *adgf*$^-$ mounds was measured using a commercial kit. Ammonia levels were significantly reduced in *adgf*$^-$ lines (**Figure 5A**). By mixing sodium hydroxide and ammonium chloride (Thadani et al., 1977), ammonia could be generated, and in such conditions, tip formation was restored in *adgf*$^-$ mounds (**Figure 5B**). All the rescue experiments involving ammonia were carried for a 3.5 h time period.

Physically separated WT restored tip development in the mutant

WT mounds are expected to release different set of volatiles including ammonia, possibly rescuing the mound arrest phenotype of the mutants nearby. To verify if this is correct, *adgf*$^-$ cells were developed in one half of a compartmentalized Petri dish, and WT cells on the other side. Interestingly, the mound arrest of *adgf*$^-$ was fully rescued (**Figure 5C**) in the presence of WT, although they were physically separated from each other. This suggests that volatiles, likely to be ammonia released from WT is sufficient enough to rescue the mutant phenotype.

The *adgf*$^-$ CM is expected to have high adenosine levels, possibly generating low or no ammonia. Addition of ADA to *adgf*$^-$ CM in one compartment of the partitioned dish led to partial rescue (57 % ± 2) of *adgf*$^-$ kept on the other side of the dish (data not shown) implying that the ammonia generated in such conditions may not be enough for a full rescue.

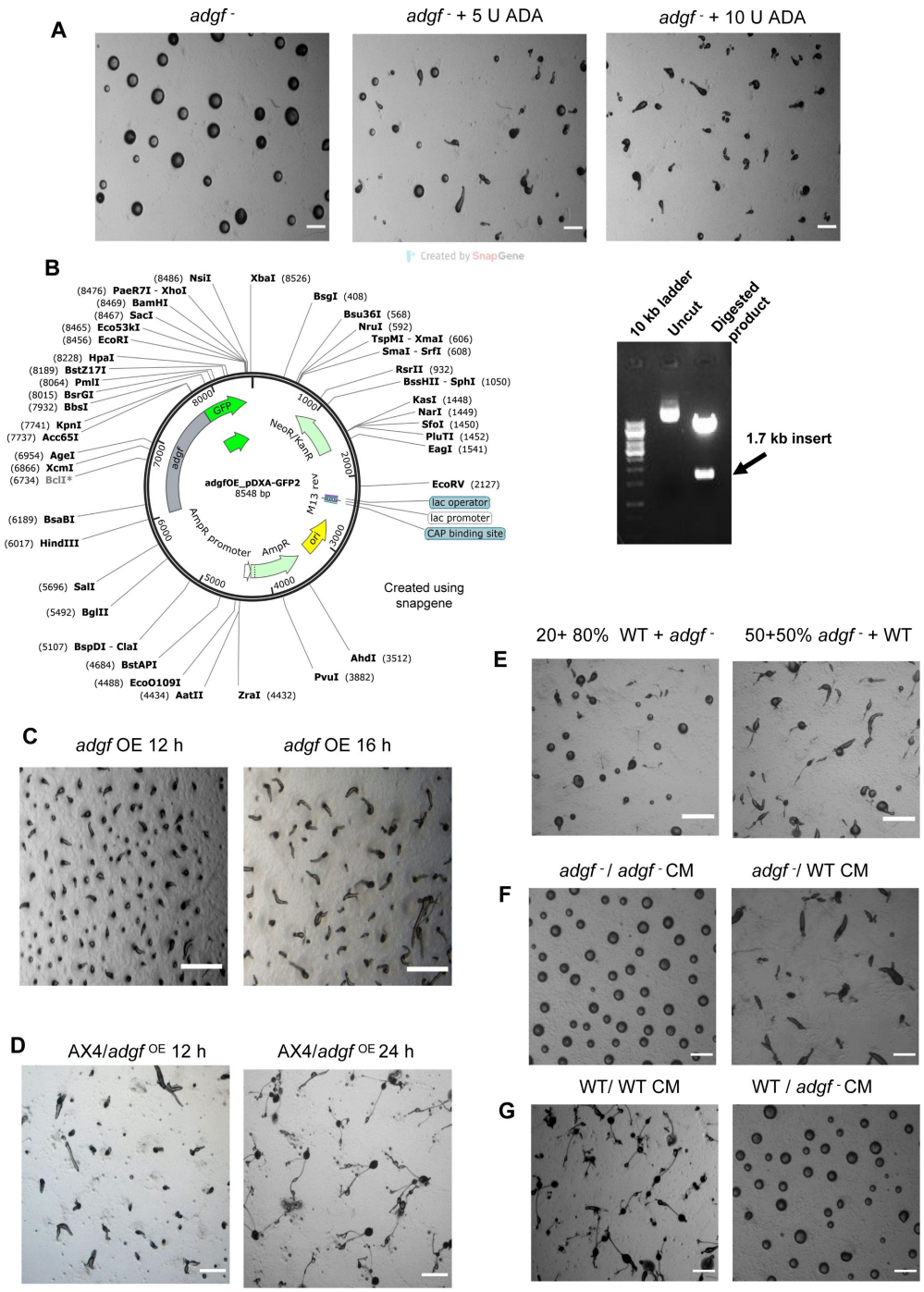

Figure 4.

Overexpression of *adgf* rescued the mound arrest phenotype

A) *adgf* ¯ mounds were treated with 5 U and 10 U ADA enzyme, and imaged at 16 h. Scale bar: 2 mm; (n=3). **B)** The full-length *adgf* gene was cloned in the vector pDXA-GFP2. The overexpression construct was verified by restriction digestion with HindIII and KpnI enzymes. **C)** *adgf* overexpression in the mutant rescued the mound arrest. **D)** Overexpression of *adgf* in WT background. Scale bar: 2 mm; (n=3). The time points in hours are shown at the top. WT cells mixed with *adgf* ¯ rescued the *adgf* mutant phenotype. **E)** Mixing of WT with *adgf* ¯ in a 1:4 ratio showed a partial rescue, and a full rescue of the *adgf* ¯ mound arrest phenotype in a 1:1 ratio with WT. **F)** Development of *adgf* mutants in the presence of *adgf* ¯ CM and WT CM on KK2 agar plates. WT CM rescued the mound arrest. **G)** Development of WT in the presence of WT CM and *adgf* ¯ CM on KK2 agar plates. *adgf* ¯ CM induced mound arrest in WT cells. Scale bar: 2 mm; (n=3).

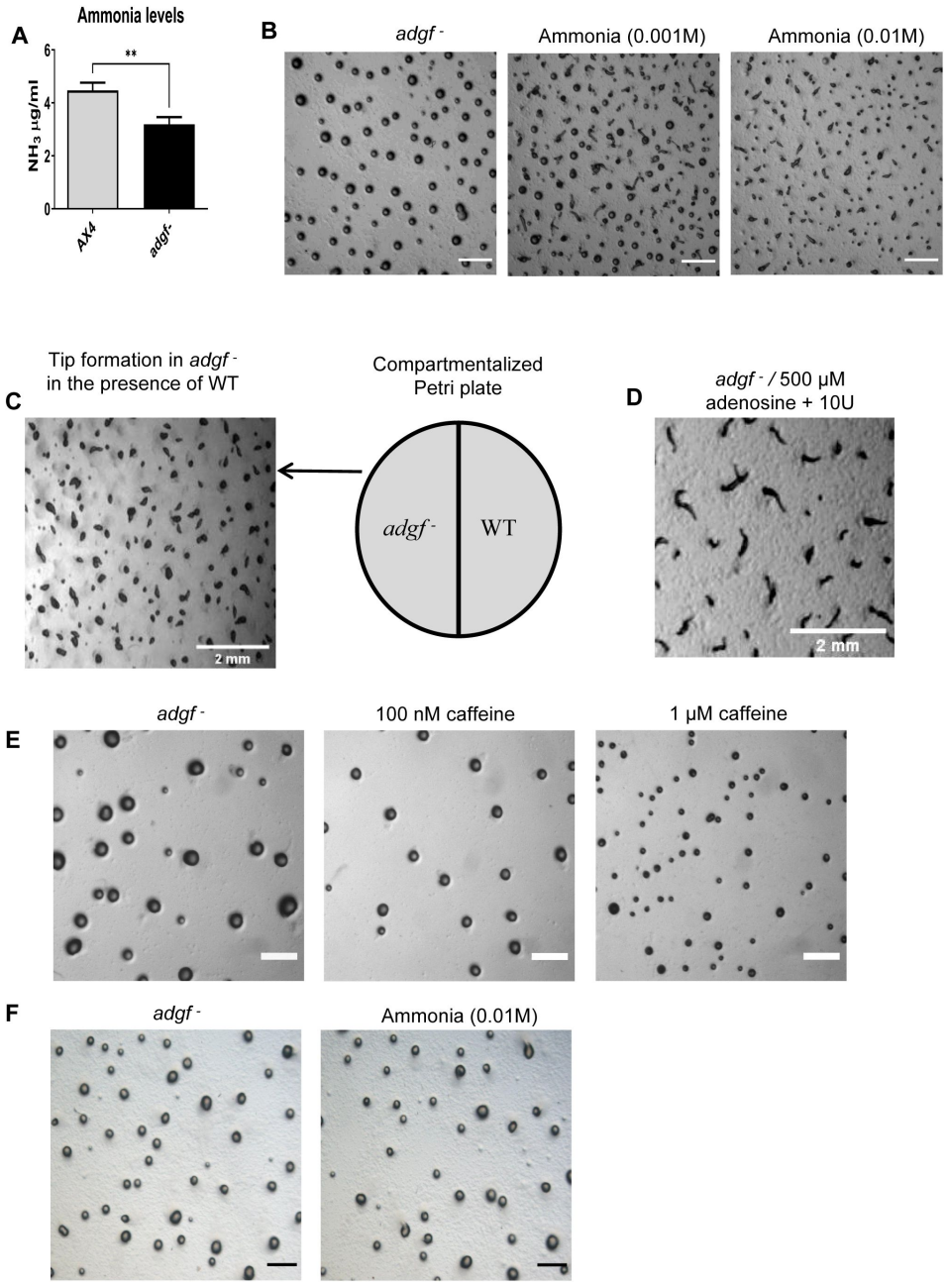

Figure 5.

Adenosine deamination reaction rescues the mound arrest of *adgf* $^{-}$

A) Quantification of ammonia using the ammonia assay kit. WT and *adgf* $^{-}$ mounds were harvested and lysed using a cell lysis buffer. Cell debris was removed by centrifugation, and the supernatant was used to quantify ammonia. **B)** Treatment of *adgf* $^{-}$ mounds with ammonia. Ammonia was generated by mixing 2 ml of NH_4Cl and 2 ml of 1N NaOH. The mixture was poured on top of the lid and the KK2 plates with the mounds were inverted and sealed thereafter. Images were taken 3.5 h post treatment. Dose dependent effect of ammonia on the rescue. Scale bar: 2 mm; (n=3). **C)** WT and *adgf* $^{-}$ cells on either side of a compartmentalized Petri dish led to tip fornation in *adgf* $^{-}$. **D)** *adgf* $^{-}$ cells on one side and KK 2 buffer containing adenosine and ADA on the other s ide of the compartmentalized dish, rescued the mound defect. Caffeine rescues the large mound size of *adgf* mutant. **E)** *adgf* $^{-}$ cells were treated with different concentrations of caffeine (100 nM, 1 µM) while plating, and images were taken during mound stage. Scale bar: 2 mm; n=3. **F)** Exposure to ammonia does not rescue the mound size of *adgf* mutant. *adgf* $^{-}$ mounds were exposed to 0.01 M ammonia and images were captured 3.5 h post chemical treatment. Scale bar: 2 mm; (n=3).

Adenosine deamination alone drives the rescue of *adgf*$^{-}$

To know if adenosine deamination is exclusively responsible for the rescue of the mutant, 10 ml cold KK2 buffer mixed with different concentrations of adenosine (10 µM, 0.1 mM, 1 mM) was added in one half of the compartmentalized dish, and the other side of the dish had the mutant on phosphate buffered agar arrested at the mound stage. Addition of ADA enzyme (10 U) to the buffered solution containing adenosine led to a full rescue after 3.5 h (**Figure 5D**), strongly indicating that volatile ammonia generated from adenosine deamination alone is rescuing the defect. Protein/RNA catabolism also generates ammonia in *Dictyostelium* (White and Sussman, 1961; Hames and Ashworth, 1974; Schindler and Sussman, 1977; Walsh and Wright, 1978), but their levels were not significantly different between WT and *adgf* mutants (Figure S2A-B) suggesting that ammonia released from adenosine deamination plays a direct role in tip formation.

Caffeine, not ammonia rescued the mound size of *adgf*$^{-}$

Given that adenosine levels were elevated in the mutants, we attempted to rescue the large mound size observed in the *adgf* mutants by treating them with the adenosine receptor antagonist, caffeine (Costenla et al., 2010; Ribeiro and Sebastiao, 2010). Caffeine rescued this early developmental defect in a dose-dependent manner (**Figure 5E**), suggesting that *adgf* may be one of the regulators of group size in *Dictyostelium*. Since ammonia levels were reduced in the mutant, we tested whether exposure to ammonia could rescue the large mound size of the *adgf* mutant. Exposing the mutants to 0.01 M ammonia soon after plating or six hours after plating, had no effect on the mound size of the mutants (**Figure 5F**), suggesting that while some early effects may be mediated through adenosine receptors, the later effects appear to be independent and are likely to be influenced by ammonia.

Faulty expression of cAMP relay genes in *adgf*$^{-}$

cAMP signaling is crucial for tip development and determining cell fate in *Dictyostelium* (Schaap and Wang, 1986; Saxe et al., 1993; Firtel, 1996; Singer et al., 2019). Hence, the expression of genes involved in cAMP relay were measured in WT and *adgf*$^{-}$ cells by qRT-PCR. Total cAMP levels and *acaA* gene expression were both low in the *adgf*$^{-}$ lines (**Figures 6A** and **6B**). Treating the cells with the pkA activator 8-Br-cAMP or the activator of adenyl cyclase, cyclic-di-GMP (Wang and Schaap, 1985; Chen and Schaap, 2012), reversed the *adgf*$^{-}$ mound arrest phenotype (**Figures 6C and D**), and increasing the cyclic-di-GMP dose from 0.5 mM to 1 mM resulted in multi tipped phenotype (**Figure 6E**). These observations reinforce that the mound arrest phenotype of the *adgf*$^{-}$ lines is due to faulty cAMP signaling. A transient increase in cAMP levels can be achieved by blocking the phosphodiesterase (*pde4*) activity. When treated with a known PDE4 inhibitor, 3-Isobutyl-1-methylxanthine (IBMX) (Bader et al., 2007; Siegert and Weijer, 1989), there was no effect on tip formation in *adgf*$^{-}$ mounds (**Figure 6F**), although caffeine restored tip formation (**Figure 6G**).

Circular instead of spiral cAMP waves in *adgf*$^{-}$ mounds

Low *acaA* expression, reduced cAMP levels and enhanced cell-cell contacts in *adgf*$^{-}$ is likely to impair cAMP wave propagation, and to ascertain if this is true, the cAMP signal propagation in WT and *adgf*$^{-}$ mounds were compared using dark field optics. The cAMP wave propagation was spiral in WT, and in contrast, the waves were circular in the *adgf* mutant, suggesting that the cAMP relay is impaired (**Figure 6H**; **Supplementary videos S3 and S4**). While this strongly suggests a defect in the mutant's ability to sustain spiral wave formation, the possibility of short-lived or transient spiral waves in the mutant cannot be excluded.

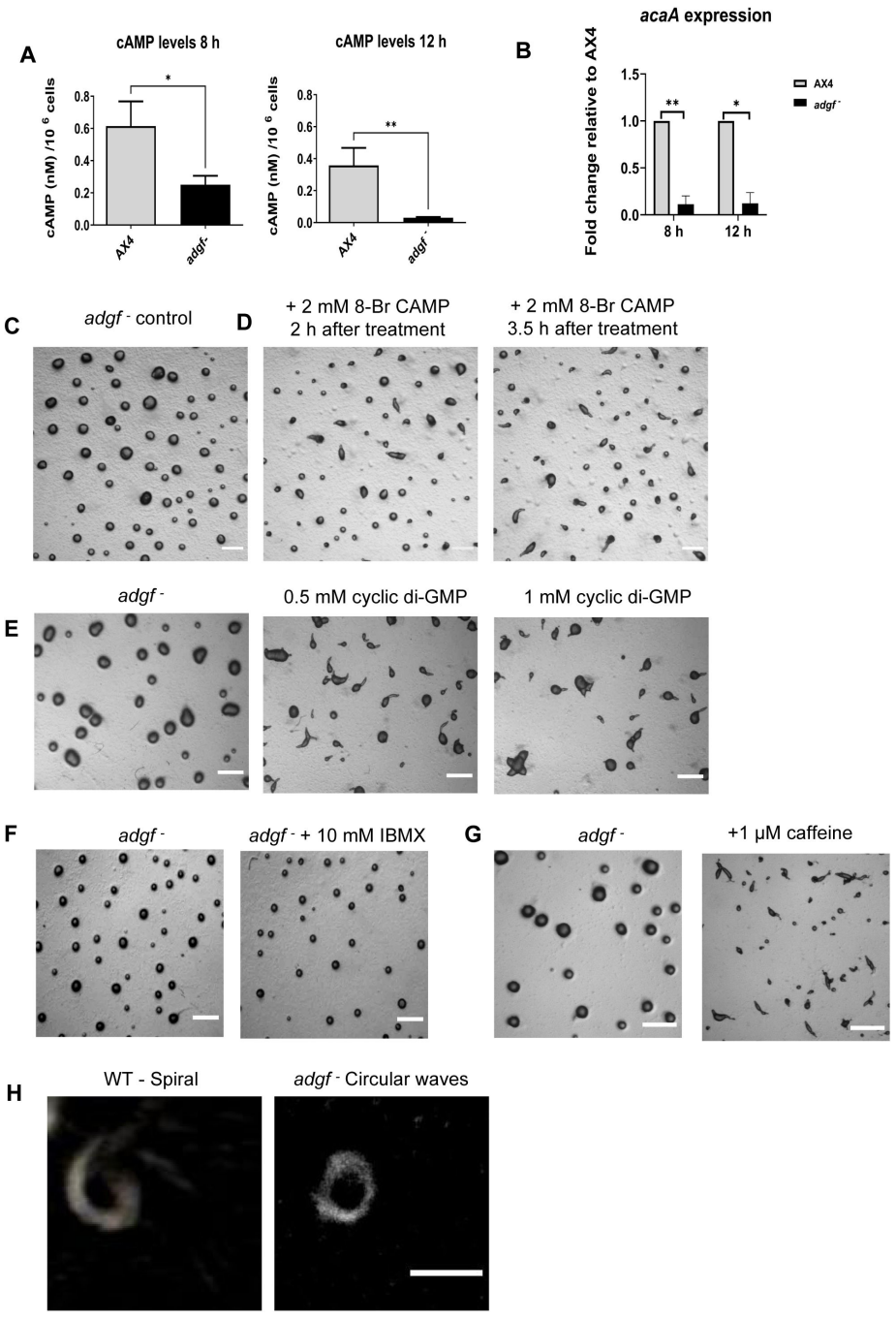

Figure 6.

Impaired cAMP signaling in *adgf* ¯

A) Total cAMP levels in WT and *adgf* ¯ mounds were quantified using cAMP-XP assay kit (Cell signaling, USA). Level of significance is indicated as *p< 0.05, **p< 0.01; (n=3). **B)** *acaA* expression was quantified using qRT-PCR. The error bars represent the mean ± SEM (n=3). **C)** *adgf* ¯ mounds. **D)** Time course of *adgf* ¯ mounds treated with 8-Br-cAMP and imaged at different intervals. Scale bar: 2 mm; (n=3). Treatment with cyclic di-GMP and caffeine rescues the mound arrest phenotype. **E)** Addition of cyclic-di-GMP restored tip formation in *adgf* ¯ 3.5 h after the treatment. Scale bar: 1 mm; (n=3). **F)** PDE inhibitor (IBMX) treatment failed to rescue the *adgf* ¯ mound arrest. Scale bar 1 mm; (n=3). **G)** *adgf* ¯ mounds treated with caffeine formed tips 3.5 h post treatment. Scale bar: 2 mm; (n=3). Altered cAMP wave pattern in *adgf* mutants. **H)** Optical density waves depicting cAMP wave generating centers in WT and *adgf* ¯. WT shows spiral and *adgf* ¯ exhibits circular wave propagation.

Ammonia restores tip formation by regulating *acaA/pde4* expression

To find if *adgf* expression is regulated by the substrate or the product, WT cells were treated with different concentrations of adenosine (100 nM, 1 µM, 0.5 mM), plated on KK2 agar plates and harvested at 16 h for RNA isolation. Independently, WT mounds on KK2 agar plates were exposed to different concentrations of volatile ammonia (0.1 mM, 1 mM, 10 mM) for 3 h and thereafter, the mRNA expression levels of *adgf*, *acaA* and *pde4* were examined. With 100 nM adenosine, the expression of both *adgf* and *acaA* decreased, but at higher adenosine concentrations (1 µM, 0.5 mM), the expression levels of these two genes were comparable to controls (**Figure 7A**). Interestingly, *pde4* expression decreased gradually with increasing adenosine concentrations (100 nM to 0.5 mM), but increased steadily with increasing ammonia levels.

Exposure of WT mounds to 0.1 mM ammonia, led to decreased *adgf* expression but exposure to 1 mM and 10 mM ammonia, respectively caused a 3 and 2.3-fold upregulation of *acaA* expression. These observations suggest that ammonia may be rescuing the mound arrest phenotype by enhancing *acaA* expression and thus cAMP levels. In similar conditions, a considerable increase in *pde4* expression was observed (**Figure 7B**), which may be necessary for controlling cAMP levels in response to ammonia treatment. In conclusion, the expression of *adgf* is influenced both by the substrate adenosine, as well as the product ammonia in a dose dependent manner. To ascertain if ammonia exposure indeed increases the cAMP levels in the mutant thereby restoring the phenotype, cAMP levels were measured in the *adgf* mutant mounds following the rescue with ammonia. Indeed, the cAMP levels were higher in the rescued mounds compared to untreated *adgf* controls (**Figure 7C**), supporting the idea that ammonia promotes tip development by restoring the cAMP signaling.

adgf^{-} cells sort to prestalk in a chimera with WT

To determine if *adgf* favours the differentiation of one cell type over the other, the expression of pst and psp markers were examined in the mutant by realtime PCR. While the expression of pst markers, *ecmA* and *ecmB* were significantly upregulated, the psp-specific *pspA* gene expression was reduced in the mutant (**Figure 7D**), suggesting that WT *adgf* favours psp expression.

To know the cell type preference of the *adgf* mutants in a chimera with WT, a fraction (20%) of any one cell type was labelled with a cell tracker stain 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DIL), and the development was tracked thereafter. In the slugs that formed after mixing labelled WT or *adgf*^{OE} cells with 80% unlabelled *adgf*$^{-}$ cells, the fluorescence was largely confined to the psp region (**Figures 8B-C**). Conversely, when *adgf*$^{-}$ cells stained with DIL were mixed with unlabelled WT or *adgf*^{OE}, the fluorescence was restricted to the pst part of the slug (**Figure 8A**). In the chimeric slugs, consistently, labelled WT or *adgf*^{OE} cells occupy the psp, whereas the *adgf*$^{-}$ cells end up in the pst region. Thus, the distribution of cell types in the mound/slug is significantly influenced by *adgf*, as the mutant cells sort out in a mixture with WT cells and differentiate to pst cells.

Further, when *adgf*$^{-}$ slugs were stained with the pst marker, neutral red (Yamamoto and Takeuchi, 1983), the staining was intense in the pst region and anterior-like cells (ALCs), and such an intense coloration was not apparent in WT especially in the ALCs (**Figure S3A**). To further investigate if reduced ADA activity affects cell type specific marker expression or their pattern in slugs, pst (*ecmA*-GFP, *ecmO*-GFP) or psp (*pspA*-RFP) lines were treated with the ADA inhibitor DCF. While there was no change in pst/psp patterns in the fraction of mounds and slugs that developed after a long delay, the *ecmA*-GFP fluorescence was intense when compared to WT (Figures S3B). Similar to NR staining pattern in *adgf* mutant slugs, a prominent expression of *ecmA*-GFP was

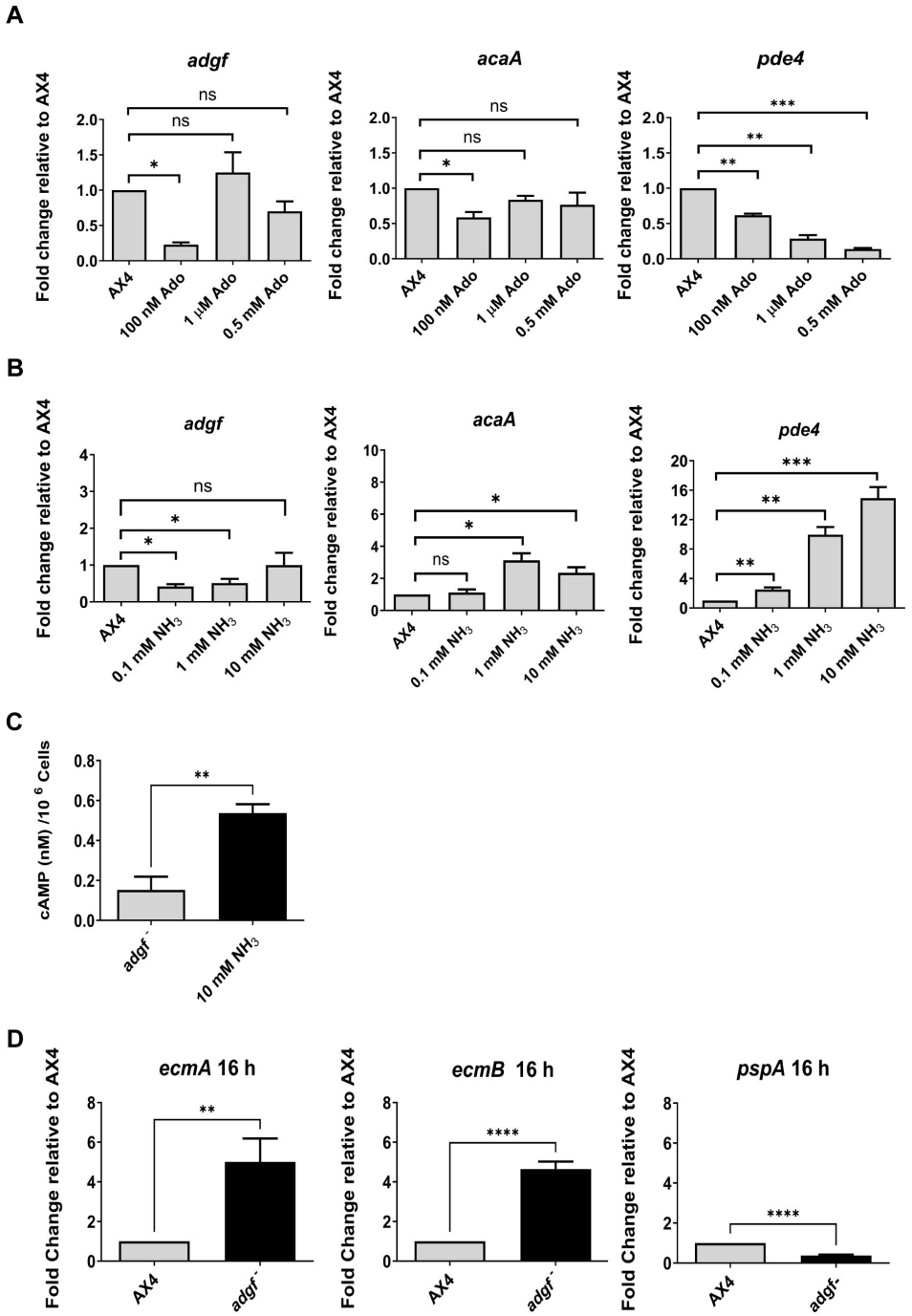

Figure 7.

Expression levels of *adgf*, *acaA* and *pde4* in response to adenosine and ammonia treatment

A) Expression levels of *adgf*, *acaA* and *pde4* in response to adenosine treatment (100 nM, 500 nM, 1 µM). **B)** And ammonia treatment (0.1 mM, 1 mM, 10 mM). Level of significance is indicated as *p<0.05, **p<0.01, and ***p<0.001; (n=3). **C)** cAMP levels in *adgf* mutants rescued with ammonia. Level of significance is indicated as **p< 0.01; (n=3). Expression levels of prestalk (pst), *ecmA, ecmB* and prespore (psp), *pspA* cell type markers in *adgf* $^{-}$. The expression profile of **D)** pst (*ecmA*, *ecmB*) and psp (*pspA*) specific markers in WT and *adgf* $^{-}$ were quantified using qRT-PCR. Level of significance is indicated as **p<0.01 and ****p<0.0001; (n=3). Three independent biological replicates were performed and the error bars represent the mean and SEM. The fold-change in RNA transcript levels is relative to WT at the indicated time points. *rnlA* was used as the internal control (n=3). ns = not significant.

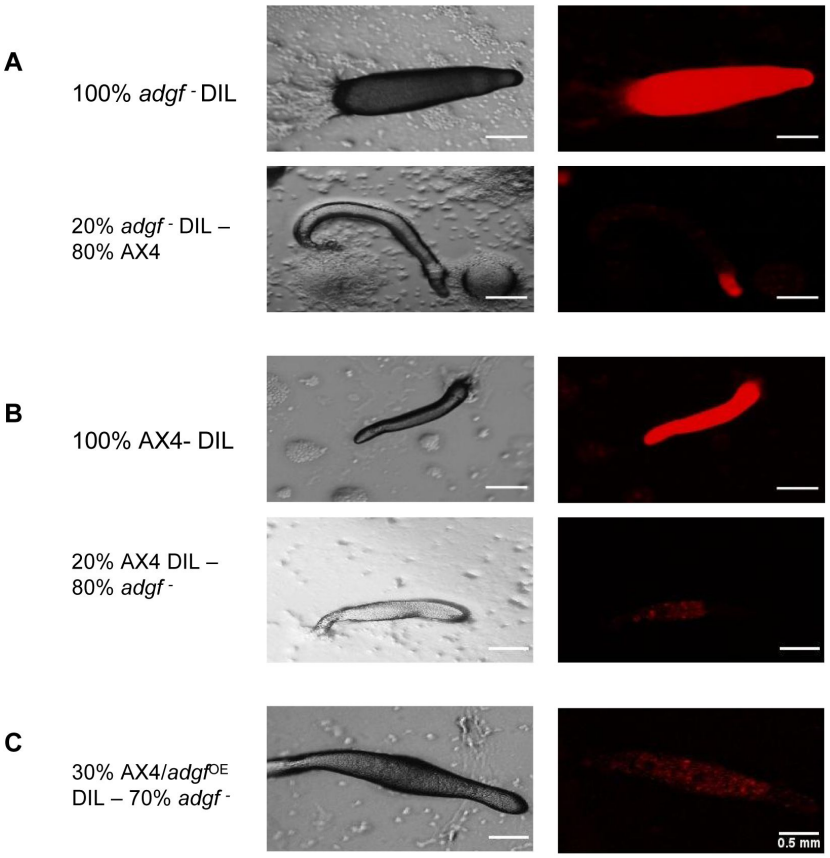

Figure 8.

Mixing of WT cells with *adgf* ⁻ following DIL staining

A-C) DIL labelled cells were mixed with unlabelled cells and plated on KK2 agar. Images were captured during the migrating slug stage. The left panel shows bright field, and the right panel shows the corresponding fluorescence images. Scale bar: 0.5 mm; (n=3).

observed in the ALC region also. Visually, there was no change in *ecmO* or *pspA* marker expression in DCF treated slugs (Figures S3C and S3D) suggesting that impaired ADGF activity affects tip specific expression.

To know if *adgf* expression is differentially regulated between the two major cell types, psp-GFP tagged WT slugs were disaggregated, and using the fluorescence activated cell sorter (FACS), GFP-negative prestalk (pst-GFP^{-}) and GFP-positive prespore (psp-GFP^{+}) populations were obtained (**Figures 9A-E**), and RNA was isolated from both cell populations. Real time PCR analysis reveals that *adgf* expression is 4.95-fold higher in the psp-GFP^{+} than pst-GFP^{-} cells (**Figure 9F**). The preferential sorting of WT cells to psp region and the *adgf* mutants (with reduced psp expression) to the pst part of the slug also reinforces the differential expression of *adgf*. Thus, cells with higher *adgf* expression preferentially sorted to the psp region and the absence of *adgf* in mutant cells may hinder their ability to adopt a prespore fate, leading to their preferential sorting in the pst region within the chimera.

adgf acts downstream of the histidine kinase, *dhkD*

In an effort to identify the pathway by which *adgf* acts during tip development, we selected a number of mutant lines with similar mound arrest phenotype such as cAMP receptor B (*carB* $^{-}$), LIM domain containing protein (*limB* $^{-}$), mound mutant (*mndA* $^{-}$) and histidine kinase (*dhkD* $^{-}$) (Saxe et al., 1993; Carrin et al., 1996; Chien et al. 2000; Singleton and Xiong, 2013), and treated the mounds with ADA enzyme. Treatment with 10 U ADA or exposure to ammonia restored tip formation in *dhkD* $^{-}$ mounds and not others tested (Figures S4A and S4C). An increase in ADA concentration (20 U), resulted in the development of multiple tips in *dhkD*$^{-}$ (**Figures 10A-C**). Further, in the *dhkD* mutant mounds that were rescued by ammonia, cAMP levels were significantly elevated compared to untreated *dhkD* $^{-}$ controls (**Figure S4D**). These findings imply that ammonia induced tip formation increased cAMP levels, thereby restoring the phenotype, and *adgf* acts downstream of *dhkD* in controlling tip development. Similarly, we tested few mutants with multi-tipped phenotype, such as tipped mutant (*tipA* $^{-}$), culinB (*culB* $^{-}$), autophagy mutants (*atg7* $^{-}$, *atg8*$^{-}$, and *atg9*$^{-}$) (Stege et al., 1999; Wang and Kuspa, 2002; Otto et al., 2003; Otto et al., 2004, Tung et al., 2010) by adding the ADA inhibitor, DCF (1 mM) to the cell suspension /agar plates and if rescued, those mutants are likely to be in the same pathway as that of *adgf* in controlling tip development. However, DCF treatment had no impact on the phenotype of the mutants with multiple tips (Figure S4B).

Impaired expression of other deaminases also results in aberrant tip formation

Several pathways control ammonia levels during development, and knockouts in other deaminases (2-aminomuconate deaminase: DDB_G0275081, adenosine monophosphate deaminase: DDB_G0292266, dCTP deaminase: DDB_G0293580, threonine deaminase: DDB_G0277245, N-acetyl glucosamine deaminase: DDB_G0286195, glucosamine-6-phosphate deaminase: DDB_G0278873) show a partial mound arrest phenotype (Figures S5A-F) although the expression of some of these candidates is stronger during development, suggesting a prominent and unique role of adenosine deamination in tip development.

High adenosine levels and other related purines do not block tip development

ADA catalyses the conversion of adenosine/deoxy adenosine respectively to inosine/ deoxy inosine. As *adgf* mutants have high adenosine levels, we investigated if the mound arrest could be mimicked in WT by treating with adenosine. However, addition of adenosine does not lead to mound arrest in WT (Figure S6A). It is possible that ADA/ADGF converted adenosine analogues

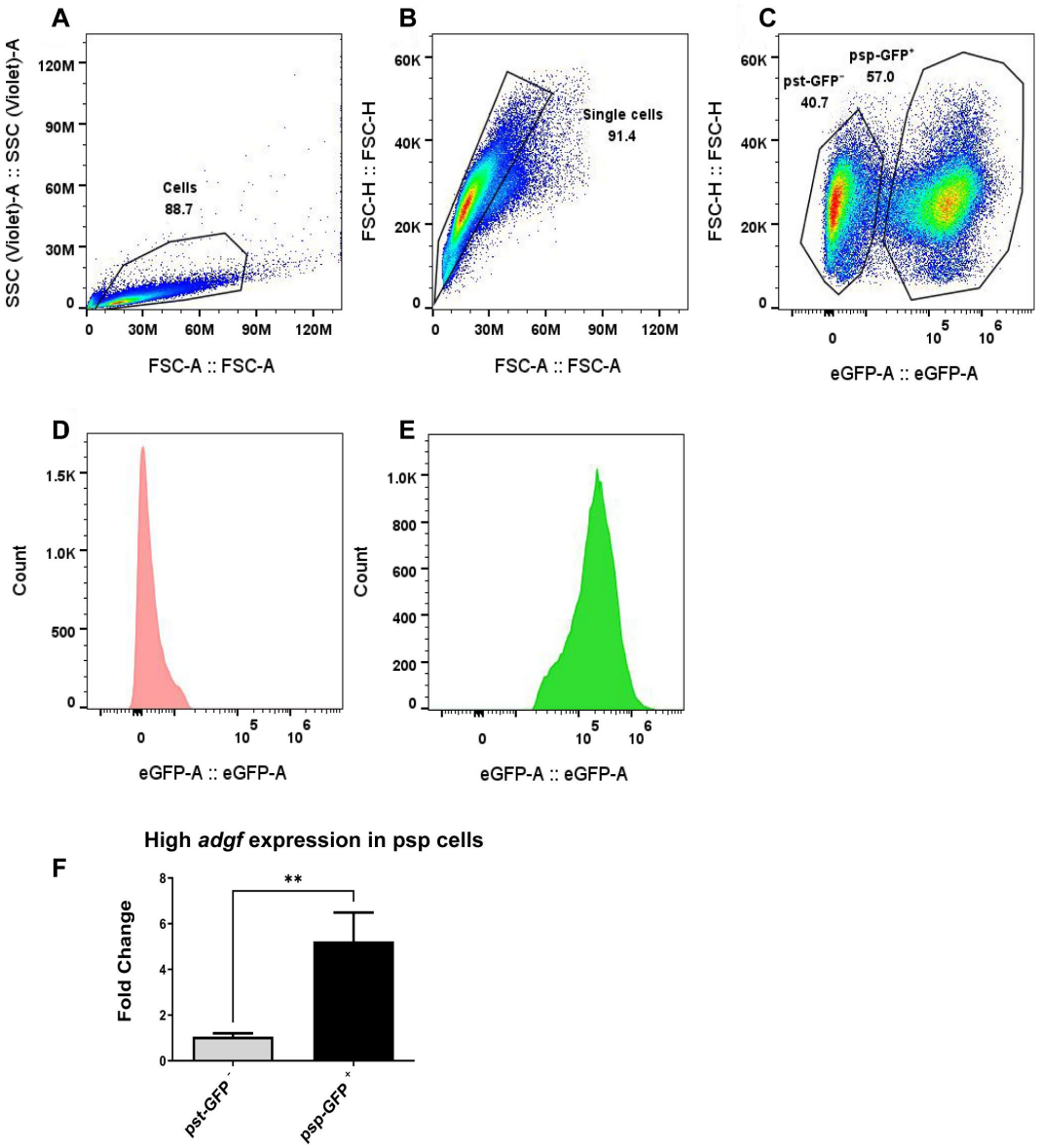

Figure 9.

Sorting of pst-GFP^{-} and psp-GFP^{+} *Dictyostelium* cells by fluorescence activated cell sorter

A) Forward scatter (FSC) vs. side scatter (SSC) plot used to gate total cells. **B)** Singlets were gated based on FSC-H vs. FSC-A to exclude doublets and aggregates. **C)** GFP fluorescence profile of gated single cells reveals two populations: pst-GFP^{-} and psp-GFP^{+}, corresponding to pst and psp cells, respectively. **D)** GFP^{-} (pst) and **E)** GFP^{+} (psp) fractions. **F)** *adgf* expression in FACS sorted samples was quantified by qRT-PCR. Level of significance is indicated as **p<0.01 (n=3).

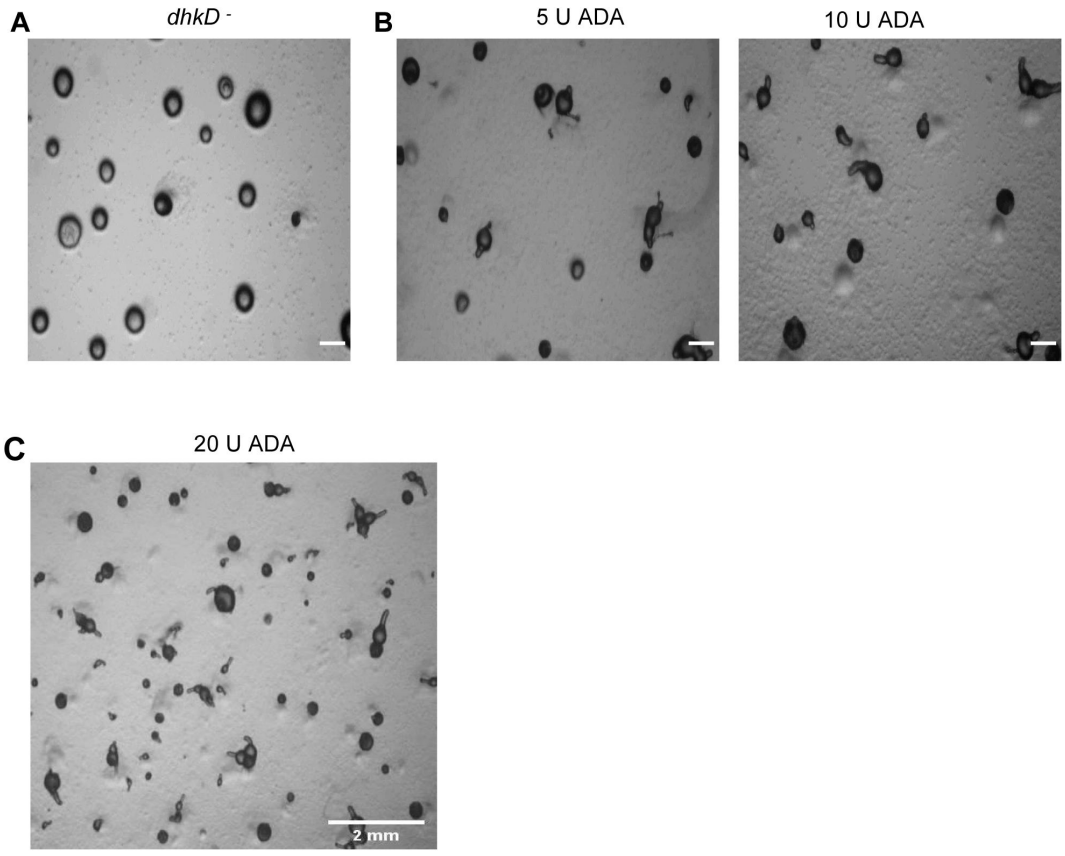

Figure 10.

adgf acts downstream of the histidine kinase *dhkD*

A) *dhkD* mutants on KK2 agar plates. **B)** 5 U, 10 U ADA rescued the mound arrest phenotype in a dose dependent manner. Scale bar: 1 mm; (n=3). Images were taken 3.5 h post treatment. **C)** Addition of 20 U ADA led to formation of multiple tips. Scale bar: 2 mm; (n=3).

to inosine analogues, which in some manner affected development. Hence, WT cells were treated with adenosine analogue (2'-deoxyadenosine) or guanosine (10 μM each), and they do not cause a mound arrest phenotype either. Similarly, treating *adgf*$^-$ mounds with inosine does not restore tip formation (Figures S6B and S6C) suggesting that the blocked tip development is due to faulty adenosine deamination alone.

Cross kingdom rescue of the *adgf*$^-$ mound arrest phenotype

Just like WT *Dictyostelium* rescuing the mound arrest phenotype of the *adgf* mutant, we examined if bacteria physically separated from the mutants would rescue the phenotype. To check this, *Klebsiella pneumoniae* and *adgf* mutants were incubated adjacent to each other within a compartmentalized KK2 agar plate. After 12 h incubation period, tip formation was restored in the mutants while in the same time frame, the mounds in controls failed to form tips. Possibly, *Klebsiella* on KK2 plates with no nutrients would die releasing ammonia thereby restoring tip development in *Dictyostelium* (Figure S7).

Discussion

Dictyostelium ADGF is likely to be a secreted growth factor

Multiple sequence alignments, experiments with conditioned media and cell mixing with WT suggests that ADGF is secreted. ADGF is also known to interact with 5'-adenosine monophosphate (AMP) and deoxyadenosine. AMP deaminase, previously characterized in the mollusk *Helix pomatia*, has been identified as a member of ADGF family (Tzertzinis et al., 2023). Studies on the characterization and expression of ADGF in Pacific abalone has shown that it is a secreted protein critical for embryonic and larval development (Hanif et al., 2022). The crystal structure of human ADA2 reveals the presence of a catalytic- and two unique ADA2-specific domains with novel folds, responsible for protein dimerization and interaction with cell surface receptors. Furthermore, the presence of a number of N-glycosylation sites, conserved disulfide bond, and a signal peptide indicate that ADA2 is specifically adapted to function in the extracellular environment (Zavialov et al., 2010).

Possible reasons for increased mound size in the *adgf* mutant

Enhanced cell adhesion influences cohesion and can impact the mound size (Roisin-Bouffay et al., 2000). The expression of *cadA* and *csaA* genes were upregulated in the *adgf* mutant, possibly leading to increased adhesion and larger mound formation. While over-secretion of *ctn* leads to stream breaking and small aggregate formation (Brock and Gomer, 1999), disruption of the *ctn* gene prevents this process, inducing large aggregate formation. *ctn* in turn regulates *smlA* (Brock et al., 2003), impacting the overall aggregate size. Thus, *adgf* mutants that have reduced *ctn* and *smlA* expression manifest in large mound formation. Indeed, cells treated with adenosine are known to form large aggregates (Schaap and Wang, 1986; Jaiswal et al., 2012), and the first genetic evidence supporting the previous work is from *adgf* mutants, which carry excess extracellular adenosine and form large aggregation streams.

Pathways generating ammonia in *Dictyostelium*

Ammonia can come from a variety of sources both within and outside the cells and this can be from dead cells also. Proteolysis during starvation is believed to be the main source of volatile ammonia in *Dictyostelium* (Hames and Ashworth, 1974; Schindler and Sussman, 1977; White and Sussman, 1961), while RNA degradation is also attributed to yield ammonia during starvation (Walsh and Wright, 1978). During development, amino acids, total protein and RNA levels are reported to reduce with a significant increase in ammonia levels, thus equivocating the

source of volatile ammonia (Hames and Ashworth, 1974). The highly acidic, autophagic vesicles in pst cells (Gross, 2009) are believed to catalyze the breakdown of proteins and RNA, also generating ammonia.

Furthermore, several deaminases or enzymatic reactions in *Dictyostelium* may also generate ammonia (Supplementary Table S1). The annotated *D. discoideum* genome contains five evolutionarily conserved family of ammonium transporter/methylammonium permease/rhesus protein (Amt/Mep/Rh) encoding genes (Eichinger et al., 2005), *amtA*, *amtB*, *amtC*, *rhgA*, and *rhgB*, which help in regulating ammonia levels during growth and development. *amtA* and *amtC* antagonistically control developmental processes and are involved in ammonium sensing or transport (Follstaedt et al., 2003; Kirsten et al., 2005; Singleton et al., 2006).

Role of ammonia during *Dictyostelium* development

Ammonia is known to inhibit aggregation (Schindler and Sussman, 1979; Williams et al., 1984), affect aggregate territory size (Thadani et al., 1977), aggregate density (Feit, 1988), cell fate (Gross et al., 1988), culmination (Davies et al., 1993) and fruiting body size in *Dictyostelium* (Lonski, 1976). Ammonia promotes psp over pst differentiation (Newell et al., 1969; Sternfeld and David, 1979; Gross et al. 1983; Oyama and Blumberg, 1986), and favors prolonged slug migration called 'slugging' over culmination by suppressing DIF biosynthesis (Neave et al., 1983). Ammonia plays a crucial role in orienting cell masses, accelerating the movement of aggregating cells (Bonner et al., 1986), and prevents the developmental transition of slugs to fruiting bodies (Bradbury and Gross, 1989; Wang and Schaap, 1989). Enzymatic removal of ammonia causes the quick transition from slug to fruiting thus controlling the morphogenetic pathways (Schindler and Sussman, 1977).

Beyond its broad effects on aggregation and culmination, ammonia also reinforces positional information by elevating intracellular cAMP levels, favoring prespore over prestalk differentiation (Bradbury and Gross, 1989; Riley and Barclay, 1990; Hopper et al., 1993). Ammonia is known to influence rapid patterning of *Dictyostelium* cells confined in a restricted environment (Sawai et al., 2002). In *adgf* mutants that have low ammonia levels, both neutral red staining and the prestalk marker *ecmA/ ecmB* expression are higher than the WT, and the mound arrest phenotype can be reversed by exposing the *adgf* mutant mounds to ammonia. As a gas, ammonia can diffuse generating a gradient. The slime sheath at the back of the slug is believed to prevent the diffusion of ammonia (Gross, 2009), and ammonia escaping through the slug front neutralizes the acidic vesicles in prestalk cells (Bonner, 1952). Thus, a rise in the pH of these acidic vesicles and the cytoplasm (Poole and Ohkuma, 1981; Gross et al, 1983; Davies et al 1993), is known to increase the speed of chemotaxing amoebae (Siegert and Weijer, 1989; Van Duijn and Inouye, 1991), favouring collective cell movement (Bonner et al., 1988, 1989), and tipped mound development. Thus ammonia actively promotes the transition from mound to tipped mound formation.

Our results (**Figure 6A**) also show that the amount of ammonia released from adenosine is in the same order of magnitude as that from other sources (Yoshino et al., 2007). It is interesting that a mutation in *adgf* manifests in arrested tip development, although *adgf* expression was found to be higher in psp than pst cells. If a threshold concentration of ammonia is not present, collective cell movement influencing tip formation may be blocked at the mound stage. Increased *adgf* expression in psp than pst cell types suggest that low extracellular adenosine (Weijer and Durston, 1985; Schaap and Wang, 1986) and high levels of ammonia influence psp cell fate. The decision between the formation of the tip (pst cells) and the ALC is controlled by the tip's production of ammonia, which prevents the migration of ALCs towards the tip (Sternfeld and David, 1982; Feit et al., 1990).

In slugger mutants, ammonia is known to inhibit tip formation (Gee et al., 1994), but not in WT NC4. Yoshino et al., (2007) have also reported ammonia inhibiting both aggregation and tip formation in WT cells. However, ammonium chloride was used as a source of ammonia, and the potential interference from chloride ions cannot be ruled out. A mutant impaired in mitochondrial function, *midA*$^{-}$, is reported to accumulate high levels of ammonia that inhibited development (Torija et al., 2006). Notably, these cells were developed with overhead light, a condition that may influence ammonia sensitivity and the developmental phenotype. However, ammonia exerts no effect on tip formation in AX4 (data not shown). After the tips are established, the slugs/ fruiting bodies move away from ammonia, reflecting a process by which fruiting bodies position themselves from other structures to increase the possibility of spore dispersal (Kosugi and Inouye, 1989). A gaseous signal can act over long distances in a short time and for instance ammonia promotes synchronous development in a colony of yeast cells (Palková et al., 1997; Palková and Forstova, 2000). The slug tip is known to release ammonia probably favouring synchronized development of the entire colony of *Dictyostelium*. However, after the tips are established ammonia exerts negative chemotaxis probably helping the slugs to move away from each other ensuring equal spacing of the fruiting bodies (Feit and Sollitto, 1987). Taken together, these findings suggest that ammonia acts as both a local and long-range regulatory signal, integrating environmental and cellular cues to coordinate multicellular development.

Cells carrying high ATP, its derivatives cAMP (Bagorda et al., 2009; Singer et al., 2019) and adenosine (Schaap and Wang, 1986), end up in the tip (Hiraoka et al., 2022). Further, 5'-nucleotidase promoter activity is high in pstAB cells (Ubeidat et al., 2002) that significantly covers the tip region suggesting high adenosine levels in the tip. However, extracellular 5'-AMP can be derived from multiple sources such as hydrolysis of cAMP, ATP and RNA (Carpousis et al., 1999).

In *adgf* mutants, ammonia levels may not be sufficient enough to neutralize the acidic vesicles and hence, NR staining is intense in the pst region and the ALCs (Bonner, 1952). Ammonia has been shown to differentially suppress cAMP chemotaxis in ALC and pst cells in *Dictyostelium* (Feit et al., 2001).

Ammonia's effect on cAMP signaling in *Dictyostelium*

Exposing the cells to ammonia is known to increase intracellular cAMP levels in *D. discoideum* (Riley and Barclay, 1990; Feit et al., 2001), but Schindler and Sussman, (1977) and Williams et al., (1984) reported that high ammonia levels inhibit the synthesis and release of cAMP. However, these experiments use ammonium carbonate (Schindler and Sussman, 1977), or ammonium chloride (Williams et al., 1984) as a source of ammonia, and the possibility of carbonate and chloride ions interfering with development cannot be ruled out. Ammonia is reported to show no effect on cAMP levels in *D. discoideum* slugs (Schaap et al., 1995), but in *D. mucoroides*, high ammonia levels are known to block the production of extracellular cAMP. The effect of ammonia on aggregation seems to be species specific notably in *P. violaceum, P. pallidum, and D. mucoroides* resulting in wider aggregation territories, while it was not observed in other species such as *D. discoideum* and *D. purpureum* (Bonner and Hoffman, 1963).

Adenylate cyclase (AC) activity is regulated by various factors (Steer, 1975). At physiological levels, ammonium ions increase AC activity in the rat brain by 40% (Yeung et al., 1989), but lowered its activity in the liver by about 30% (Wiechetek et al., 1979). Ammonia has been shown to increase the activity of AC-G in *Dictyostelium* sori (Cotter et al., 1999). High ammonia levels by altering the pH, can affect the activity of numerous enzymes whose activity is pH dependent and thus the activity of AC can be impacted by pH variations. *adgf* mutants with low ammonia levels have reduced cAMP levels, and increase in ammonia causes a significant increase in *aca*A expression. In rat brain, ammonia is known to interact with manganese (Rivera-Mancía et al., 2012; Lu et al., 2020), that is known to increase *aca*A expression in *Dictyostelium* (Loomis et al., 1979; Khachatrian et al., 1987). Possibly, ammonia interacts with metal ions like

manganese to form 'ammine complexes' (Lipkowski and Galus, 1973), particularly in aqueous environments or under specific conditions where the appropriate ligands are available, and enhances cAMP levels, thus rescuing the mound defects of the mutant.

Tip organizer development in *Dictyostelium* depends on the differentiation and appropriate sorting of pst and psp cells (Kay et al., 1978; Williams et al., 1989; Saxe et al., 1993; Williams, 2006), a process that relies on signaling of different morphogens including cAMP, adenosine, DIF and ammonia (Bloom and Kay, 1988; Williams, 1988; Riley and Barclay, 1990; Gross, 1994). These morphogens modify one another's effects, and determine the choice of the differentiation pathway as well as the spatial arrangement of cells (Bloom and Kay, 1988). Thus, the rescue of the *adgf* mutant upon exposure to ammonia is likely due to cAMP signaling and cell-cell contact.

Possible reasons for reduced cAMP levels in the *adgf* mutant

Higher *pde4* and reduced *acaA* expression may result in low cAMP levels in the mutant. ADA significantly regulates adenosine levels, thus reducing the activation of adenosine-mediated receptors (Van Haastert, 1983). Two classes of adenosine receptors including adenosine alpha- and beta-receptors are known to be expressed in *Dictyostelium* (Theibert and Devreotes, 1984). When adenosine concentrations are high, beta-receptors bound with adenosine inhibit the binding of cAMP to its receptors, thereby inhibiting cAMP signaling (Newell, 1982; Van Haastert, 1983; Theibert and Devreotes, 1984). High adenosine levels in the *adgf* mutant may also reduce cAMP levels via a similar mechanism.

The mound/slug tip is believed to carry high adenosine levels restricting additional tip formation (Wang and Schaap, 1985), and our results also suggest that *adgf*$^-$ cells with high adenosine end up as pst cells.

Distorted cAMP waves in mutant

Collective cell movement within mounds is essential for cell sorting and the progression from mounds to finger structures (Kellerman and McNally, 1999). In WT mounds, cAMP waves propagate as spirals, while in the mutants, the wave propagation was in concentric circles. Initially, the cAMP waves arise as concentric circles, that, upon symmetry breaking, form spiral waves (Siegert and Weijer, 1995). With successive wave propagation, the circular ring distorts, and one end curls towards the pulsatile center, creating a spiral wave around the organizing center. In *adgf* mutants, however, defective cAMP relay prevents this transition. A surge in phosphodiesterase inhibition is thought to regulate this shift from concentric to spiral wave propagation (Palsson and Cox, 1996), and indeed the phosphodiesterase expression was higher in the *adgf* mutant possibly distorting the wave propagation.

The mound/slug tip of *Dictyostelium* generates cAMP pulses (Traynor et al., 1992), and is known to suppress additional tip formation (Farnsworth, 1973). Adenosine, that inhibits additional tip formation represses *pde4* expression whereas ammonia, that promotes tip elongation, increases *pde4* expression. Thus, the restoration of cAMP signaling and spiral wave propagation possibly leads to the rescue of the *adgf*$^-$ mound arrest phenotype upon exposure to ammonia. Surprisingly adenosine exerts little effect on tip development in WT cells and studies of Inouye (1989) also reinforce our observations, showing that adenosine does not significantly influence the conversion of pst to psp cells in shaking culture conditions.

A robust cAMP relay is required for the transition from concentric to spiral wave propagation, with the corresponding changes in adenylate cyclase or phosphodiesterase activity (Siegert and Weijer, 1995; Palsson and Cox, 1996). Reduced *acaA* expression in the mutant could bias the wave propagation toward concentric rather than spiral geometry. However, further experiments will be required to ascertain this link.

Adenosine deamination drives tip organizer development

A crucial evidence supporting that adenosine deamination is singularly responsible for the rescue of *adgf*$^{-}$ mound arrest comes from experiments where partitioned dish with buffered solution containing adenosine on one side, and the mutants on the other half of the dish. Addition of ADA to the buffer containing adenosine resulted in *adgf* mutants forming tipped mound. Although ammonia's role in *Dictyostelium* development is well established, this report is the first to show a novel role of ammonia in tip development. If natural variants of *Dictyostelium* that fail to form tips exist in soil ecosystems, it is conceivable that these strains could still form fruiting bodies when in proximity to WT strains. This interaction would likely occur through volatile signals such as ammonia diffusing through the shared environment. These diffusible signals may help rescue or coordinate developmental processes among neighbouring cells. Notably, the development of an organism in its natural environment is strongly influenced by volatile compounds present in its surroundings (Schulz-Bohm et al., 2017).

ADA in organizer development and gastrulation in vertebrates

ada/adgf expression is found to be high during gastrulation stages in several vertebrates (Pijuan-Sala et al., 2019; Tyser et al., 2021), and collective cell migration during gastrulation and collective cell movement within the *Dictyostelium* mound are remarkably similar (Weijer, 2009), suggesting an overlooked role of ammonia in organizer development. The human homologue of *adgf*, CECR1, is a potential gene for the genetic disorder, Cat-eye syndrome and thus, *Dictyostelium* may also serve as a model to study this condition. Ammonia is crucial in regulating differentiation, metabolism, and gene expression (Stein et al., 2013; Liu et al., 2022). Ammonium has been shown to induce aberrant blastocyst differentiation, disrupt normal metabolic processes, alter pH regulation, and subsequently affect fetal development in mice (Lane and Gardner, 2003). Newt *Triturus* exposed to ammonia shows dorsalization of the ventral marginal zone highlighting the capacity of ammonia to alter embryonic axis formation (Yamada, 1950). Furthermore, research on avian embryos has revealed significant ammonia content in developing eggs, suggesting a potential role for ammonia in the energy metabolism during ontogenesis (Needham, 1926). While this raises the possibility of conserved mechanisms, further studies will be needed to determine whether adenosine deamination plays a role in organizer development across different systems.

dhkD functions upstream of *adgf*

The histidine kinase *dhkD*, a member of the two-component family of histidine kinases, appears to function upstream of *adgf*, adding to our understanding of the signaling cascade in *Dictyostelium*. Histidine kinases play essential roles in regulating developmental transitions, with *dhkC*, for example, initiating the late developmental program that ultimately leads to fruiting body formation (Singleton et al., 1998). This function of *dhkC* is regulated in part by ammonia levels, as low ammonia concentrations have been shown to inhibit the *dhkC* phospho-relay, thereby influencing the developmental outcomes (Kirsten et al., 2005). In *Dictyostelium*, several histidine kinases, including *dhkA*, *dhkB*, *dhkC*, and *dokA*, coordinate cAMP signaling to modulate the activity of PKA, a cAMP-dependent protein kinase that is essential for proper development (Anjard and Loomis, 2003). These findings suggest the interdependencies within the signaling network in regulating multicellular development in *Dictyostelium*.

Model of ADGF action

Ammonia generated from multiple sources is likely to be quenched rapidly by the intracellular acidic vesicles of pst cells, increasing its pH and favoring collective cell movement driving mound/slug tip development. However, the slime sheath at the back of the slug may prevent the diffusion of ammonia, and thus the major route of ammonia emission will be from the slug front (Farnsworth and Loomis, 1974). Although ammonia is likely to be formed from several sources

in *Dictyostelium*, a critical threshold concentration of ammonia may be necessary for tip development. The failure of even one pathway may result in a drop-in ammonia levels, exerting an effect on development (**Figure 11**). Thus, ammonia is likely to be important for the maturation of tip cells.

Our study shows that *adgf* acts downstream of *dhkD* and *in silico* studies have shown that *dhkD* interacts with *adgf*. Therefore, it is likely that in the presence of ammonia, *dhkD* activates the phosphorelay, in turn increasing intracellular cAMP levels, leading to tip formation. Histidine kinase *dhkC* is known to phosphorylate *regA* in *Dictyostelium* (Thomason et al.,1999). However, based on docking we found no direct interaction of *regA* with ADGF (data not shown). Thus, the integration of histidine kinase signaling ensures coordinated multicellular organization in *Dictyostelium* (Aoki et al., 2020). Previous work in other systems have found ADA to be interacting with CD 26 (dpp IV) (Tanaka et al., 1993), and docking DdADGF with AprA (dpp8) also supports this observation. However, adding ADA inhibitor to *aprA* mutant (dpp8), showed no effect on the phenotype (data not shown).

Material and methods

Bioinformatic analyses of ADGF

The genomic sequence and the protein sequence of ADGF were obtained either from dictybase (*http://dictybase.org*) or NCBI database (*https://www.ncbi.nlm.nih.gov/*). The ADA domain within the DdADGF was identified by SMART and BLAST (*http://smart.embl-heidelberg.de/*) (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*) (Altschul et al., 1990; Letunic and Bork, 2018) analyses. A phylogenetic tree was generated by aligning multiple amino acid sequences of ADGF from several taxa. Neighbour Joining approach and the MUSCLE alignment tool of the MEGAX programme were used for constructing the tree (Saitou and Nei, 1987). The tertiary structure of ADGF was obtained using the online programme alphafold (*https://alphafold.ebi.ac.uk/*) (Jumper et al., 2021), and PyMol was used for viewing the images. The expression profiles of *ada* and *ada2* during mouse and human gastrula development were retrieved from the *marionilab.cruk.cam.ac.uk/MouseGastrulation2018/* and *human-gastrula.net/* databases (Pijuan-Sala et al., 2019; Tyser et al., 2021), respectively. Protein-protein docking was carried out using High Ambiguity Driven Protein-Protein Docking (HADDOCK) version 2.4.

Culture and development of *Dictyostelium discoideum*

D. discoideum (WT-AX4) cells or the mutant *adgf* derived from AX4, (DBS0237637) were cultured in modified maltose-HL5 medium (Formedium, UK) with 100,000 U/L penicillin and 0.1 g/L streptomycin. Three independent mutants (GWDI_17_D_7, GWDI_47_C_1, GWDI_132_H_3; insertion in exon 2 in all three mutants) of the *adgf* gene (DDB_G0275179) were obtained from the GWDI bank (*https://remi-seq.org*), in *Dictyostelium* stock centre, North Western University, USA (Gruenheit et al., 2021). The mound defects were identical in all three and strain GWDI_47_C_1 alone was characterised further. The culture was raised as a monolayer in Petri plates or grown in an Erlenmeyer flask in shaking conditions at 150 rpm and 22 °C, to a cell density of 2-4×10^6 cells/ml. The cells were also grown on SM/5 agar plates supplemented with *Klebsiella pneumoniae* at 22 °C (2 g/L glucose, 2 g/L protease peptone, 0.4 g/L yeast extract, 1 g/L $MgSO_4.H_2O$, 0.66 g/L K_2HPO_4, 2.225 g/L KH_2PO_4, 1% Bactoagar, pH 6.4). For developmental assays, freshly starved cells were washed twice with ice cold KK2 buffer (2.25 g KH_2PO_4 and 0.67 g K_2HPO_4 per litre, pH 6.4), and plated on 1% non-nutrient KK2 agar plates at a density of 5×10^5 cells/cm^2 (Nassir et al., 2019). Thereafter, the plates were incubated in dark conditions at 22 °C for development.

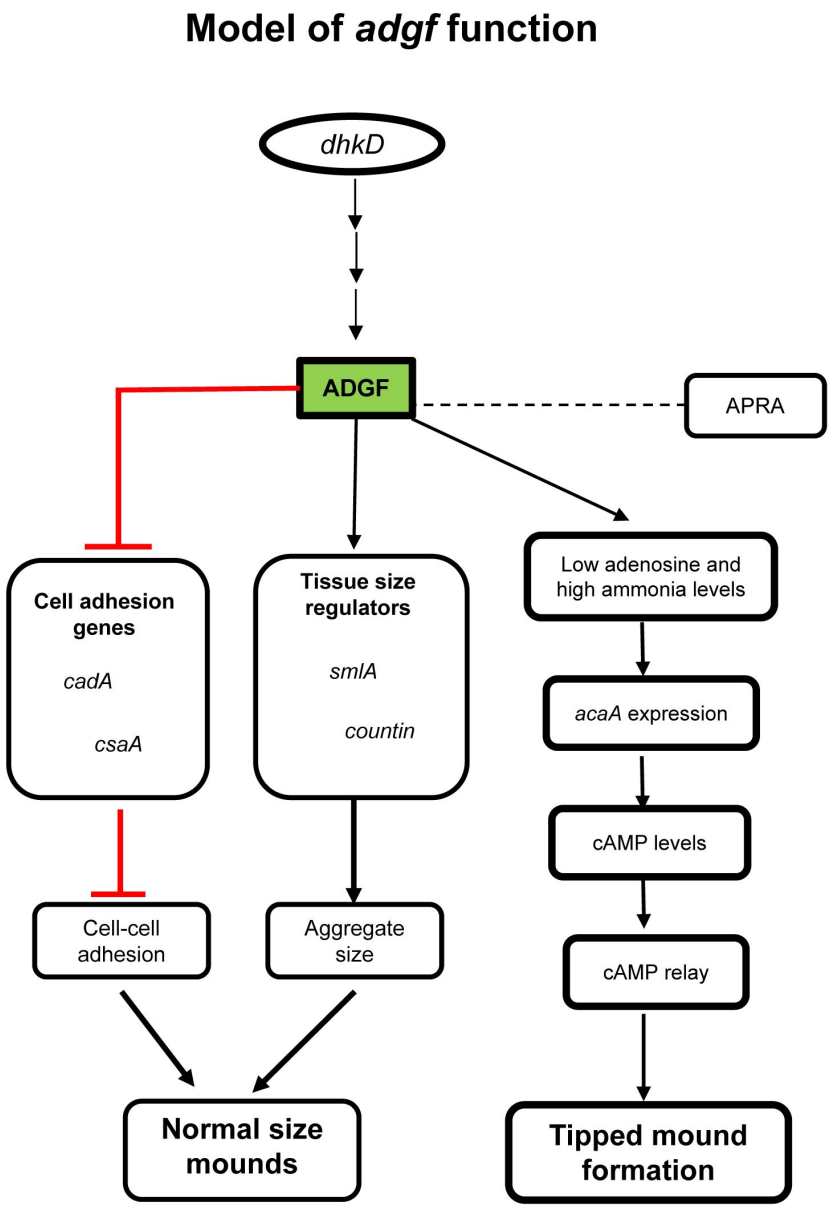

Figure 11.

Model illustrating the role of *adgf* in development

adgf suppresses the expression of genes involved in cell adhesion, *cadA* and *csaA,* and regulates the mound size and tip development by directly acting on adenosine, ammonia levels and cAMP signaling. Line ending in an arrow indicates that the previous gene/factor either directly or indirectly raises the activity or levels of the second; line ending in a cross-bar indicates inhibition. Dotted lines indicate ADGF interacting with APRA.

Dictyostelium genomic DNA isolation

WT and *adgf*$^-$ cells grown axenically were harvested, and the pellet was resuspended in 1 ml of lysis buffer (50 mM Tris-Cl, pH 8; 10 mM EDTA; 0.8% SDS). To this mixture, 200 µl of Nonidet P-40 (NP40), a non-ionic detergent, was added, vortexed and centrifuged at 12,000 g for 15 min at room temperature (RT). The resultant pellet was gently vortexed, resuspended in 500 µl of lysis solution containing 200 µg/ml Proteinase K, and incubated at 65 °C for 30 min. 300 µl of phenol: chloroform was added to this suspension, centrifuged at 18,000 g for 10 min at RT, and the resultant aqueous phase was extracted carefully. An equal amount of chloroform was added and centrifuged at 18,000 g for 10 min at RT. Following this, 750 µl of pure ethanol was added to the suspension, which was then centrifuged at 12,000 g for 15 min at 4 °C. Genomic DNA was precipitated by adding twice the volume of absolute ethanol and 1/10th the volume of 3 M sodium acetate. After a 10 min centrifugation at 15,000 g, the pellet was cleaned using 70% ethanol and stored at 4 °C. Electrophoresis was conducted in TAE buffer at 50 V using a Medox power pack system (India), and the integrity of DNA was confirmed on a 1% agarose gel.

Validation of the *adgf* mutant

To validate the blasticidin (*bsr*) resistance cassette insertion in the *adgf* mutant, WT and *adgf*$^-$ genomic DNA were isolated, and a diagnostic PCR was performed using the gene and *bsr* insert specific primers in accordance with the guidelines provided in the GWDI website. A qRT-PCR was carried out to confirm the absence of *adgf* expression in the mutant cells. The primers used for mutant validation are listed in Supplementary Table S2.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from WT and *adgf*$^-$ cells using Trizol reagent (Favorgen, USA) at specified intervals (every four hours from 0 to 24 h). cDNA was synthesized from the total RNA using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Japan). Random primers from the manufacturer were used to generate the cDNA from a template of 1 µg total RNA. qRT-PCR was performed using SYBR Green Master Mix (Thermo Scientific, USA) and 1 µl of cDNA.

The expression levels of *adgf*, *acaA*, cAMP receptor A (*carA*), phosphodiesterases (*pdsA*, *regA*), 5' nucleotidase (*5'nt*), extracellular matrix A (*ecmA*), extracellular matrix B (*ecmB*), prespore A (*pspA*), countin (*ctn*), and small aggregate (*smlA*) were quantified with a QuantStudio Flex 7 (Applied Biosystems, USA). The mitochondrial large RNA subunit (*rnlA*) served as an internal control. qRT-PCR data analysis was conducted according to the method described by Schmittgen and Livak (2008). The primer sequences used for qRT-PCR are provided in Supplementary Table S4.

Generation of *adgf* over expression construct

The full-length 1.7 kb *adgf* sequence was PCR-amplified using ExTaq polymerase (Takara, Japan) with WT genomic DNA as the template. The amplified product was then ligated into the pDXA-GFP2 vector at the HindIII and KpnI restriction sites. Both *adgf*$^-$ and WT cells were electroporated with this vector, and G418-resistant clones (10 µg/ml) were isolated for further analysis. The expression of *adgf* was confirmed by semi-quantitative PCR. The corresponding primer sequences are listed in Supplementary Table S3.

Transformation of Dictyostelium discoideum

WT and *adgf*$^-$ cells grown axenically were harvested, washed twice with ice-cold EP buffer (10 mM KH_2PO_4, 10 mM K_2HPO_4, 50 mM sucrose, pH 6.2), and resuspended in 100 µl of EP++ solution (10 K_2HPO_4 mM, 10 mM KH_2PO_4, 50 mM sucrose, 1 mM $MgSO_4$, 1 mM $NaHCO_3$, 1 mM ATP, 1 µM $CaCl_2$) containing 10 µg of plasmid vector (Nassir et al., 2019) in pre-cooled cuvettes (BioRad,

USA). The cells were electroporated using a BTX ECM830 electroporator (Harvard Apparatus, USA) at 300 V with 2 ms pulses and five square wave pulses at 5-second intervals. The cells were then transferred to a Petri dish with 10 ml of HL5 medium and incubated at 22 °C. After 24 hours, G418 (10 µg/ml) was added to the medium, and resistant colonies were selected for further analysis.

Preparation of conditioned media (CM)

WT and *adgf*$^-$ cells grown in HL5 medium were collected at the mid-log (ML) phase, resuspended in KK2 buffer at a density of 1×10^7 cells/ml, and incubated at 22 °C with shaking for 20 hours. The clarified supernatant obtained after centrifugation was used for the experiments.

Assay for ADA activity

Total ADA activity from *Dictyostelium* was determined as per the protocol of the manufacturer (Abcam, USA; Cat No: ab204695). For sample preparation, 5×10^5 cells/cm^2 were seeded onto KK2 agar plates and at the mound stage, ice cold ADA assay buffer was flooded, then vigorously pipetted to disrupt the mound integrity. The cell homogenate was agitated on a rotary shaker at 4 °C for 15 min and then centrifuged at 12,000 rpm for 10 min in a cold microfuge tube. The supernatant was subjected to the ADA assay. This ADA test relies on adenosine to inosine formation. The intermediate formed combines with the probe to produce uric acid, which is quantified at 293 nm. BCA (Bicinchonic acid) assay kit (Thermoscientific, USA) was used for determining the protein concentration. One unit of ADA activity is defined as the amount of enzyme that hydrolyses adenosine to yield 1 µmol of inosine per min under the assay conditions.

Adenosine quantification

Total adenosine levels from *Dictyostelium* mounds were measured according to the manufacturer's protocol, using the adenosine assay kit (Abcam, USA; Cat No: ab211094). Cells grown in HL5 were collected, washed and plated on KK2 agar plates. The cell lysis buffer was added to the plates with mounds and mixed thoroughly. 50 µl of the lysate was mixed with 2 U of ADA and was subjected to incubation for 15 min at RT. Adenosine quantification involves the use of ADA and after a series of enzymatic reactions, an intermediate is formed which reacts with the adenosine probe generating a fluorescent product. Using a spectrofluorometer (Perkin Elmer, USA; λEx = 544 nm/λEm = 590), the fluorescence intensity was measured, which is proportional to the concentration of adenosine. The adenosine levels were quantified using the adenosine standard curve.

Quantification of ammonia

Ammonia assay kit (Sigma-Aldrich, USA; Cat No: AA0100) was used for estimating the total ammonia levels. WT and *adgf*$^-$ cells developed on KK2 agar plates were sealed with parafilm and incubated at 22 °C. The mounds were collected using lysis solution, and the debris was removed by centrifugation at 10,000 g for 10 min. The supernatant was used for further analysis. For the ammonia assay, 1 ml of assay reagent was mixed thoroughly with either 100 µl of samples or standards, incubated for 5 min at RT, and the absorbance was measured at 340 nm. Then, 10 µl of L-glutamate dehydrogenase (GDH) solution was added to each cuvette, and after a 5-min incubation at 25 °C, the absorbance was measured again at 340 nm using a spectrophotometer (Eppendorf, Germany). In the presence of GDH, ammonia combines with α-ketoglutaric acid and reduced NADPH to produce L-glutamate and oxidised $NADP^+$. The decrease in absorbance at 340 nm is proportional to the ammonia concentration. The ammonia standard curve was used to calculate the ammonia levels.

Volatile ammonia generation

To generate ammonia, 1 ml of 1 N NaOH and 1 ml of NH_4Cl (concentrations used 0.1 mM, 1 mM, 10 mM) were mixed thoroughly (Thadani et al., 1977; Feit et al., 1990) and from this mix, 2 ml was aliquoted in the upper half of the Petri dish. The other half of the plate with the *adgf*$^-$ mounds on KK2 agar were inverted, sealed and incubated at 22 °C. To determine whether WT mounds, physically separated from the mutants could rescue the mound arrest, WT (1×10^6 cells/cm^2) and *adgf*$^-$ (5×10^5 cells/cm^2) cells were developed on KK2 agar plates on either side of compartmentalised and sealed Petri plates. *adgf*$^-$ cells developed on either side of the dish served as controls.

Quantification of cAMP

Using the cAMP-XP test kit and following the manufacturer's instructions, total cAMP levels were determined from both the WT and the mutant (Cell signaling, USA). WT and *adgf*$^-$ mounds were disrupted and collected in 1 ml ice cold KK2 buffer. After centrifuging the pellet, 100 µl of 1X lysis solution was added, and the mixture was incubated in ice for 10 min. Subsequently, 50 µl of lysate and 50 µl of HRP-conjugated cAMP solution were added to the test plates, which were then shaken horizontally at RT. After 3 h incubation, the wells were emptied and washed three times with 200 µl of 1X wash buffer. 100 µl of stop solution was added to halt the reaction, and the absorbance was measured at 450 nm using a spectrophotometer (Biorad, USA). The cAMP standard curve was used to determine the cAMP levels.

Visualisation of cAMP waves using dark field optics

5×10^5 cells/cm^2 were developed on KK2 agar plates under moist, dark conditions to monitor the propagation of cAMP waves. Using a Nikon DS-5MC camera mounted on a Nikon SMZ-1000 stereo zoom and Nikon Eclipse TE2000 inverted microscope, a time-lapse video of the aggregation was captured in real-time (Nikon, Japan). cAMP optical density waves were displayed by subtracting the image pairs (Siegert and Weijer, 1995) after processing the images with the NIS-Elements D program or ImageJ (NIH, USA).

Under agarose cAMP chemotaxis assay

The under-agarose cAMP chemotaxis assay (Woznica and Knecht, 2006; Singh and Insall, 2022) was carried out with cells obtained from WT and *adgf*$^-$ mounds. Briefly, the cells were starved in KK2 buffer at 1×10^7 cells/ml density. Three parallel troughs, each measuring 2 mm in width, were set up on a Petri dish containing 1% agarose. In the outer two troughs, 100 µl of WT and *adgf*$^-$ cell suspension was aliquoted respectively and 10 µM cAMP was added to the central trough. Cell migration toward cAMP was recorded every 30 s over a total duration of 20 min using the NIS-Elements D software and an inverted Nikon Eclipse TE2000 microscope (Nikon, Japan). 35 cells were tracked each time and subsequently analysed using ImageJ (NIH, USA).

Mixing WT with mutant cells

WT and *adgf*$^-$ cells cultured in HL5 medium, were harvested at the mid log (ML) phase by centrifugation, rinsed twice with ice-cold KK2 buffer. The cells mixed in different ratios (1:9, 2:8 and 1:1) were adjusted to a final density of 5×10^5 cells/cm^2, plated on 1% KK2 agar plates, and incubated at 22 °C for development.

Tracking cell fate after mixing

Dictyostelium cells harvested from fresh HL5 media were resuspended at a density of 1×10^6 cells/ml in KK2 buffer, followed by incubation at 22 °C for 1.5 h in shaking conditions with 0.2 µM DIL (Invitrogen, USA). DIL has been used extensively as a cell tracker in *C. elegans*, *Xenopus and* mice (Schultz and Gumienny, 2012; Xu et al., 2020; Erdogan et al., 2016). WT or *adgf*$^-$ cells

labelled with DIL were mixed with the unlabelled cells in a ratio of 1:9, 2:8 and 1:1 and plated for development. In the ratios mentioned, the smaller fraction represents the stained cells. DIL is a lipophilic dye that selectively stains the plasma membranes of living cells, allowing visualization and analysis of cell morphology.

Neutral red staining of mounds and slugs

WT and *adgf*$^-$ cells harvested from HL5 cultures were resuspended at a density of 1×10^7 cells per ml in KK2 buffer and treated with 0.005% neutral red (NR) solution for 15 min at RT in shaking conditions (Bonner, 1952). The stained cells were washed twice with KK2 buffer, plated on buffered agar plates and thereafter, NR stained slugs were observed using an upright microscope.

Cell-type specific expression analysis of *adgf*

To determine the differential expression of *adgf*, if any, between the two major cell types, psp-GFP cells in AX4 background were developed on KK2 agar plates at a density of 5×10^5 cells/cm² until they reached the slug stage, as described by Ratner and Borth (1983). Fluorescence was initially monitored using microscopy to confirm GFP expression. Slugs were then dissociated in 20 mM phosphate buffer containing 40 mM EDTA, pH 7.0 (Nadin et al., 2000), filtered through a 10-µm nylon mesh, chilled on ice, and using FACS (FACS Discover S8 Image Sorter (BD Biosciences, USA)) the two cell types were sorted. Sorting was performed at a rate of 10,000 cells/s with a 488 nm laser. GFP-positive prespore (psp) cells and GFP-negative prestalk (pst) cells were collected separately on ice over several hours. Total RNA was extracted from both cell populations, and the expression of *adgf* was analyzed using RT-PCR.

Cell-cell adhesion assay

After 14 h of development on phosphate buffered agar plates, WT and *adgf*$^-$ mounds were disaggregated by repeated pipetting and vortexing using ice-cold KK2 buffer. Dissociated cells were resuspended in KK2 buffer and incubated at 150 rpm for 45 min. Single and non-adherent cells were counted using a Neubauer chamber (Lam et al., 1981).

Treatment of *Dictyostelium* cells with different compounds

A highly specific ADA inhibitor, DCF that inhibits both extra and intra-cellular ADAs (Cha et al., 1975) was mixed with the WT cell suspension and plated on 1% non-nutrient KK2 agar plates at a density of 5×10^5 cells/cm^2. Of the different DCF concentrations tested (10 nM, 50 nM, 100 nM and 1 µM), 100 nM showed complete tip inhibition.

For the enzymatic rescue assay, 10 U of bovine ADA dissolved in KK2 buffer (Sigma-Aldrich, USA) was added onto mutant mounds. The concentrations of 8-Br-cAMP (2 mM), cAMP (0.1 mM, 0.5 mM), c-di-GMP (0.1 mM, 0.5 mM), adenosine (1 µM, 10 µM, 100 µM, 1 mM), caffeine (10 nM, 100 nM, 1 µM) and IBMX (0.5 mM) were based on previous publications (Chen et al., 2017; Chen and Schaap, 2012; Nassir et al., 2019; Siegert and Weijer, 1989) and these compounds were added independently either on top of the mounds or supplemented while plating. All the fine chemicals were from Sigma-Aldrich, USA except when mentioned.

Microscopy

Microscopy was performed using a Nikon SMZ-1000 stereo zoom microscope with epifluorescence optics, Nikon 80i Eclipse upright microscope, or a Nikon Eclipse TE2000 inverted microscope, connected with a digital sight DS-5MC camera (Nikon). Image processing was carried out using NIS-Elements D (Nikon) or Image J.

Statistical tools

Data analyses were carried out using Microsoft Excel (2016). Statistical significance was determined by paired or unpaired, two-tailed Student's t-test and ANOVA analysis (GraphPad Prism, version 7).

Data availability

NA

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Supplementary Results

Genes involved in autophagy and tipped mound formation do not show altered expression in the *adgf* mutant

Autophagy helps in maintaining cellular homeostasis by promoting the breakdown of damaged proteins and organelles (Mizushima et al., 2008), and ammonia is recognised to be a diffusible regulator of autophagy in human cells (Eng et al., 2010). To investigate if autophagy is impaired in the mutant, the expression of autophagy markers *atg8* and *atg18* were examined and there was no discernible change in the expression levels when compared to WT. The tipless to tipped mound transition as well as late developmental gene expression is regulated by *gbf*A, a G-box binding factor (GBF). However, in the *adgf* mutant the *gbf*A expression levels were comparable with no significant difference between the WT at 16 h (data not shown). Without the assay for autophagy as well as assay for GBF activity, these expression studies alone are not conclusive to rule out the role of these factors in tip formation.

ADA does not deaminate cytokinin

ADA is known to interact with 5'AMP (Hanif et al., 2022) and in *Dictyostelium*, 5'-AMP serves as a direct precursor of cytokinin, playing a significant role in cellular signaling and development (Taya et al., 1978). Although docking studies suggest low to moderate binding of ADGF with cytokinins (zeatin, dihydrozeatin, isopentenyl adenine), we carried a functional assay by mixing cytokinin and ADA with KK2 buffer in one side of the Petridish and found no rescue of the mutant in such conditions (data not shown), suggesting no functional activity between ADA and the versions of cytokinin tested.

Supplementary discussion

Plausible routes of caffeine action rescuing the mound arrest phenotype

The *adgf* mound arrest could be rescued by the addition of the adenosine antagonist, caffeine but treatment with the *pde4* inhibitor, IBMX failed to rescue the mound arrest. Caffeine is known to reduce adenosine levels in blood plasma (Conlay et al., 1988) and also increase ammonium levels in urine samples of rabbits (Bernheim and Bernheim, 1945). Thus, the mound rescue upon caffeine treatment may be a result of reduced adenosine and increased ammonia levels. With respect to caffeine action on cAMP levels, the reports are contradictory. Caffeine has been reported to increase adenylate cyclase expression thereby increasing cAMP levels (Hagmann, 1986) whereas Alvarez-Curto et al., (2007) found that caffeine reduced intracellular cAMP levels in *Dictyostelium*. Although, caffeine is moderately potent in inhibiting PDE enzyme activity, the *in vivo* concentrations are likely to be low to be associated with effective PDE inhibition (Burg and Werner, 1975; Daly, 1993).

Additional information

Author contributions

P.H. and R.B. conceptualized the work. P.H. performed all experiments. P.H. and R.B. analysed the data and wrote the manuscript.

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Additional files

Supplementary figures and tables

Supplemenatary videos

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Reviewer #1 (Public review):

Summary:

This work shows that a specific adenosine deaminase protein in Dictyostelium generates the ammonia that is required for tip formation during Dictyostelium development. Cells with an insertion in the adgf gene aggregate but do not form tips. A remarkable result, shown by several different ways, is that the adgf mutant can be rescued by exposing the mutant to ammonia gas. The authors also describe other phenotypes of the adgf mutant such as increased mound size, altered cAMP signaling, and abnormal cell type differentiation. It appears that the adgf mutant has defects the expression of a large number of genes, resulting in not only the tip defect but also the mound size, cAMP signaling, and differentiation phenotypes.

Strengths:

The data and statistics are excellent.

Comments on previous version:

Looks better, but I think you answered my questions (listed as weaknesses in the public review) in the reply to the reviewer but not in the paper. I'd suggest carefully thinking about my questions and addressing them in the Discussion (The authors have now done this).

https://doi.org/10.7554/eLife.104855.3.sa2

Reviewer #2 (Public review):

Summary:

The paper describes new insights into the role of adenosine deaminase-related growth factor (adgf), an enzyme that catalyses the breakdown of adenosine into ammonia and inosine, in tip formation during Dictyostelium development. The adgf null mutant has a pre-tip mound arrest phenotype, which can be rescued by external addition of ammonia. Analysis suggests that the phenotype involves changes in cAMP signaling possibly involving a histidine kinase dhkD, but details remain to be resolved.

Strengths:

The generation of an adgf mutant showed a strong mound arrest phenotype and successful rescue by external ammonia. Characterisation of significant changes in cAMP signaling components, suggesting low cAMP signaling in the mutant and identification of the histidine kinase dhkD as a possible component of the transduction pathway. Identification of a change in cell-type differentiation towards prestalk fate

Comments on previous version:

The revised version of the paper has improved significantly in terms of structure and clarity. The additional data on rescue of total cAMP production by ammonia (Fig. 7C) in the adgf-mutant and the 5-fold increased prespore expression of adgf RNA compared to prestalk cells (Fig 9) are useful data additions.

The link between changes in cAMP signaling (lower aca expression) and wave geometry (concentric waves rather than spiral waves) remains speculative.

I noted that Fig 6 contains different images than the previous version (Fig 7).

The statement "Interestingly, Klebsiella pneumoniae physically separated from the Dictyostelium adgf mutants in a partitioned dish, also rescues the mound arrest phenotype suggesting a cross-kingdom interaction that drives development" in the summary is rather overdone. All experiments were performed with axenic strains (no bacteria).

as is the sentence "Remarkably, in higher vertebrates, adgf expression is elevated during gastrulation and thus adenosine deamination may be a conserved process driving organizer development in different organisms"

The data supporting this in the supplementary information is hardly legible and poorly presented. What is shown is ADA expression in different tissues, not at different stages. I would suggest taking these figures out and concentrating the summary on the key mechanistic findings of the paper. (The authors have now done this.)

https://doi.org/10.7554/eLife.104855.3.sa1

Author response:

The following is the authors' response to the previous reviews.

> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *This work shows that a specific adenosine deaminase protein in Dictyostelium generates the ammonia that is required for tip formation during Dictyostelium development. Cells with an insertion in the ADGF gene aggregate but do not form tips. A remarkable result, shown in several different ways, is that the ADGF mutant can be rescued by exposing the mutant to ammonia gas. The authors also describe other phenotypes of the ADGF mutant such as increased mound size, altered cAMP signalling, and abnormal cell type differentiation. It appears that the ADGF mutant has defects in the expression of a large number of genes, resulting in not only the tip defect but also the mound size, cAMP signalling, and differentiation phenotypes.*
>
> *Strengths:*
>
> *The data and statistics are excellent.*
>
> *(1) Weaknesses: The key weakness is understanding why the cells bother to use a diffusible gas like ammonia as a signal to form a tip and continue development.*

Ammonia can come from a variety of sources both within and outside the cells and this can be from dead cells also. Ammonia by increasing cAMP levels, trigger collective cell movement thereby establishing a tip in Dictyostelium. A gaseous signal can act over long distances in a short time and for instance ammonia promotes synchronous development in a colony of yeast cells (Palkova et al., 1997; Palkova and Forstova, 2000). The slug tip is known to release ammonia probably favouring synchronized development of the entire colony of Dictyostelium. However, after the tips are established ammonia exerts negative chemotaxis probably helping the slugs to move away from each other ensuring equal spacing of the fruiting bodies (Feit and Sollitto, 1987).

It is well known that ammonia serves as a signalling molecule influencing both multicellular organization and differentiation in Dictyostelium (Francis, 1964; Bonner et al., 1989; Bradbury and Gross, 1989). Ammonia by raising the pH of the intracellular acidic vesicles of prestalk cells (Poole and Ohkuma, 1981; Gross et al, 1983), and the cytoplasm, is known to

increase the speed of chemotaxing amoebae (Siegert and Weijer, 1989; Van Duijn and Inouye, 1991), inducing collective cell movement (Bonner et al., 1988, 1989), favoring tipped mound development.

Ammonia produced in millimolar concentrations during tip formation (Schindler and Sussman, 1977) could ward off other predators in soil. For instance, ammonia released by Streptomyces symbionts of leaf-cutting ants is known to inhibit fungal pathogens (Dhodary and Spiteller, 2021). Additionally, ammonia may be recycled back into amino acids, as observed during breast cancer proliferation (Spinelli et al., 2017). Such a process may also occur in starving Dictyostelium cells, supporting survival and differentiation. These findings suggest that ammonia acts as both a local and long-range regulatory signal, integrating environmental and cellular cues to coordinate multicellular development.

> *(2) The rescue of the mutant by adding ammonia gas to the entire culture indicates that ammonia conveys no positional information within the mound.*

Ammonia reinforces or maintains the positional information by elevating cAMP levels, favoring prespore differentiation (Bradbury and Gross, 1989; Riley and Barclay, 1990; Hopper et al., 1993). Ammonia is known to influence rapid patterning of Dictyostelium cells confined in a restricted environment (Sawai et al., 2002). In adgf mutants that have low ammonia levels, both neutral red staining (a marker for prestalk and ALCs) (Figure. S3) and the prestalk marker ecmA/ ecmB expression (Figure. 7D) are higher than the WT and the mound arrest phenotype can be reversed by exposing the adgf mutant mounds to ammonia.

Prestalk cells are enriched in acidic vesicles, and ammonia, by raising the pH of these vesicles and the cytoplasm (Davies et al 1993; Van Duijn and Inouye 1991), plays an active role in collective cell movement during tip formation (Bonner et al., 1989).

> *(3) By the time the cells have formed a mound, the cells have been starving for several hours, and desperately need to form a fruiting body to disperse some of themselves as spores, and thus need to form a tip no matter what.*

Exposure of adgf mounds to ammonia, led to tip development within 4 h (Figure. 5). In contrast, adgf controls remained at the mound stage for at least 30 h. This demonstrates that starvation alone is not the trigger for tip development and ammonia promotes the transition from mound to tipped mound formation.

Many mound arrest mutants are blocked in development and do not proceed to form fruiting bodies (Carrin et al., 1994). Further, not all the mound arrest mutants tested in this study were rescued by ADA enzyme (Figure. S4A), and they continue to stay as mounds.

> *(4) One can envision that the local ammonia concentration is possibly informing the mound that some minimal number of cells are present (assuming that the ammonia concentration is proportional to the number of cells), but probably even a minuscule fruiting body would be preferable to the cells compared to a mound. This latter idea could be easily explored by examining the fate of the ADGF cells in the mound - do they all form spores? Do some form spores?*
>
> *Or perhaps the ADGF is secreted by only one cell type, and the resulting ammonia tells the mound that for some reason that cell type is not present in the mound, allowing some of the cells to transdifferentiate into the needed cell type. Thus, elucidating if all or some cells produce ADGF would greatly strengthen this puzzling story.*

A fraction of adgf mounds form bulkier spore heads by the end of 36 h as shown in Figure. 2H. This late recovery may be due to the expression of other ADA isoforms. Mixing WT and

adgf mutant cell lines results in a chimeric slug with mutants occupying the prestalk region (Figure. 8) and suggests that WT ADGF favours prespore differentiation. However, it is not clear if ADGF is secreted by a particular cell type, as adenosine can be produced by both cell types, and the activity of three other intracellular ADAs may vary between the cell types. To address whether adgf expression is cell type-specific, prestalk and prespore cells will be separated by fluorescence activated cell sorter (FACS), and thereafter, adgf expression will be examined in each population.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *The paper describes new insights into the role of adenosine deaminase-related growth factor (ADGF), an enzyme that catalyses the breakdown of adenosine into ammonia and inosine, in tip formation during Dictyostelium development. The ADGF null mutant has a pre-tip mound arrest phenotype, which can be rescued by the external addition of ammonia. Analysis suggests that the phenotype involves changes in cAMP signalling possibly involving a histidine kinase dhkD, but details remain to be resolved.*
>
> *Strengths:*
>
> *The generation of an ADGF mutant showed a strong mound arrest phenotype and successful rescue by external ammonia. Characterization of significant changes in cAMP signalling components, suggesting low cAMP signalling in the mutant and identification of the histidine kinase dhkD as a possible component of the transduction pathway. Identification of a change in cell type differentiation towards prestalk fate*
>
> *(1) Weaknesses: Lack of details on the developmental time course of ADGF activity and cell type type-specific differences in ADGF expression.*

adgf expression was examined at 0, 8, 12, and 16 h (Figure. 1), and the total ADA activity was assayed at 12 and 16 h (Figure. 3). Previously, the 12 h data was not included, and it's been added now (Figure. 3A). The adgf expression was found to be highest at 16 h and hence, the ADA assay was carried out at that time point. Since the ADA assay will also report the activity of other three isoforms, it will not exclusively reflect ADGF activity.

Mixing WT and adgf mutant cell lines results in a chimeric slug with mutants occupying the prestalk region (Figure. 8) suggesting that WT adgf favours prespore differentiation. To address whether adgf expression is cell type-specific, prestalk and prespore cells will be separated by fluorescence activated cell sorter (FACS), and thereafter, adgf expression will be examined in each population.

> *(2) The absence of measurements to show that ammonia addition to the null mutant can rescue the proposed defects in cAMP signalling.*

The adgf mutant in comparison to WT has diminished acaA expression (Fig. 6B) and reduced cAMP levels (Fig. 6A) both at 12 and 16 h of development. The cAMP levels were measured at 8 h and 12 h in the mutant.

We would like to add that ammonia is known to increase cAMP levels (Riley and Barclay, 1990; Feit et al., 2001) in Dictyostelium. Exposure to ammonia increases acaA expression in WT (Figure. 7B) and is likely to increase acaA expression/ cAMP levels in the mutant also (Riley and Barclay, 1990; Feit et al., 2001) thereby rescuing the defects in cAMP signalling. Based on the comments, cAMP levels will also be measured in the mutant after the rescue with ammonia.

> *(3) No direct measurements in the dhkD mutant to show that it acts upstream of adgf in the control of changes in cAMP signalling and tip formation.*

cAMP levels will be quantified in the dhkD mutant after treatment with ammonia. The histidine kinases dhkD and dhkC are reported to modulate phosphodiesterase RegA activity, thereby maintaining cAMP levels (Singleton et al., 1998; Singleton and Xiong, 2013). By activating RegA, dhkD ensures proper cAMP distribution within the mound, which is essential for the patterning of prestalk and prespore cells, as well as for tip formation (Singleton and Xiong, 2013). Therefore, ammonia exposure to dhkD mutants is likely to regulate cAMP signalling and thereby tip formation.

> ***Reviewer #1 (Recommendations for the authors):***
>
> *(1) Lines: 47,48 - "The gradient of these morphogens along the slug axis determines the cell fate, either as prestalk (pst) or as prespore (psp) cells." - many workers have shown that this is not true - intrinsic factors such as cell cycle phase drive cell fate.*

Thank you for pointing this out. We have removed the line and rephrased as “Based on cell cycle phases, there exists a dichotomy of cell types, that biases cell fate as prestalk or prespore (Weeks and Weijer, 1994; Jang and Gomer, 2011).

> *(2) Line 48 - PKA - please explain acronyms at first use.*

Corrected

> *(3) Line 56 - The relationship between adenosine deaminase and ADGF is a bit unclear, please clarify this more.*

Adenosine deaminase (ADA) is intracellular, whereas adenosine deaminase related growth factor (ADGF) is an extracellular ADA and has a growth factor activity (Li and Aksoy, 2000; Iijima et al., 2008).

> *(4) Figure 1 - where are these primers, and the bsr cassette, located with respect to the coding region start and stop sites?*

The primer sequences are mentioned in the supplementary table S2. The figure legend is updated to provide a detailed description.

> *(5) Line 104 - 37.47% may be too many significant figures.*

Corrected

> *(6) Line 123 - 1.003 Å may be too many significant figures.*

Corrected

> *(7) Line 128 - Since the data are in the figure, you don't need to give the numbers, also too many significant figures.*

Corrected

> *(8) Figure 3G - did the DCF also increase mound size? It sort of looks like it did.*

Yes, the addition of DCF increases the mound size (now Figure. 2G).

> *(9) Figure 3I - the spore mass shown here for ADGF - looks like there are 3 stalks protruding from it; this can happen if a plate is handled roughly and the spore masses bang into each other and then merge*

Thank you for pointing this out. The figure 3I (now Figure. 2I) is replaced.

> *(10) Lines 160-162 - since the data are in the figure, you don't need to give the numbers, also too many significant figures.*

Corrected.

> *(11) Line 165 - ' ... that are involved in adenosine formation' needs a reference.*

Reference is included.

> *(12) Line 205 - 'Addition of ADA to the CM of the mutant in one compartment.' - might clarify that the mutant is the ADGF mutant*

Yes, revised to 'Addition of ADA to the CM of the adgf mutant in one compartment.'

> *(13) Lines 222-223 need a reference for caffeine acting as an adenosine antagonist.*

Reference is included.

> *(14) Figure 8B - left - use a 0-4 or so scale so the bars are more visible.*

Thank you for the suggestion. The scale of the y-axis is adjusted to 0-4 in Figure. 7B to enhance the visibility of the bars.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *The paper describes new insights into the role of ADGF, an enzyme that catalyses the breakdown of adenosine in ammonia and inosine, in tip formation in Dictyostelium development.*
>
> *A knockout of the gene results in a tipless mound stage arrest and the mounds formed are somewhat larger in size. Synergy experiments show that the effect of the mutation is non-cell autonomous and further experiments show that the mound arrest phenotype can be rescued by the provision of ammonia vapour. These observations are well documented. Furthermore, the paper contains a wide variety of experiments attempting to place the observed effects in known signalling pathways. It is suggested that ADGF may function downstream of DhkD, a histidine kinase previously implicated in ammonia signalling. Ammonia has long been described to affect different aspects, including differentiation of slug and culmination stages of Dictyostelium development, possibly through modulating cAMP signalling, but the exact mechanisms of action have not yet been resolved. The experiments reported here to resolve the mechanistic basis of the mutant phenotype need focusing and further work.*
>
> *(1) The paper needs streamlining and editing to concentrate on the main findings and implications.*

The manuscript will be revised extensively.

Below is a list of some more specific comments and suggestions.

> *(2) Introduction: Focus on what is relevant to understanding tip formation and the role of nucleotide metabolism and ammonia (see https://doi.org/10.1016/j.gde.2016.05.014) .leading). This could lead to the rationale for investigating ADGF.*

The manuscript will be revised extensively

> *(3) Lines 36-38 are not relevant. Lines 55-63 need shortening and to focus on ADGF, cellular localization, and substrate specificity.*

The manuscript will be revised accordingly. Lines 36-38 will be removed, and the lines 55-63 will be shortened.

In humans, two isoforms of ADA are known including ADA1 and ADA2, and the Dictyostelium homolog of ADA2 is adenosine deaminase-related growth factor (ADGF). Unlike ADA that is intracellular, ADGF is extracellular and also has a growth factor activity (Li and Aksoy, 2000; Iijima et al., 2008). Loss-of-function mutations in ada2 are linked to lymphopenia, severe combined immunodeficiency (SCID) (Gaspar, 2010), and vascular inflammation due to accumulation of toxic metabolites like dATP (Notarangelo, 2016; Zhou et al., 2014).

> *(4) Results: This section would benefit from better streamlining by a separation of results that provide more mechanistic insight from more peripheral observations.*

The manuscript will be revised and the peripheral observations (Figure. 2) will be shifted to the supplementary information.

> *(5) Line 84 needs to start with a description of the goal, to produce a knockout.*

Details on the knockout will be elaborated in the revised manuscript. Line number 84 (now 75). Dictyostelium cell lines carrying mutations in the gene adgf were obtained from the genome wide Dictyostelium insertion (GWDI) bank and were subjected to further analysis to know the role of adgf during Dictyostelium development.

> *(6) Knockout data (Figure 1) can be simplified and combined with a description of the expression profile and phenotype Figure 3 F, G, and Figure 5. Higher magnification and better resolution photographs of the mutants would be desirable.*

Thank you, as suggested the data will be simplified (section E will be removed) and combined with a description of the expression profile and, the phenotype images of Figure 3 F, G, and Figure 5 (now Figure. 2 F, G, and Figure. 4) will be replaced with better images/ resolution.

> *(7) It would also be relevant to know which cells actually express ADGF during development, using in-situ hybridisation or promoter-reporter constructs.*

To address whether adgf expression is cell type-specific, prestalk and prespore cells will be separated by fluorescence activated cell sorter (FACS), and thereafter, adgf expression will be examined in each population.

> *(8) Figure 2 - Information is less directly relevant to the topic of the paper and can be omitted (or possibly in Supplementary Materials).*

Figure. 2 will be moved to supplementary materials.

> *(9) Figures 4A, B - It is shown that as could be expected ada activity is somewhat reduced and adenosine levels are slightly elevated. However, the fact that ada levels are low at 16hrs could just imply that differentiation of the ADGF- cells is blocked/delayed at an earlier time point. To interpret these data, it would be necessary to see an ada activity and adenosine time course comparison of wt and mutant, or to see that expression is regulated in a celltype specific manner that could explain this (see above). It would be good to combine this with the observation that ammonia levels are lower in the ADGF- mutant than wildtype and that the mutant phenotype, mound arrest can be rescued by an external supply of ammonia (Figure 6).*

In Dictyostelium four isoforms of ADA including ADGF are present, and thus the time course of total ADA activity will also report the function of other isoforms. Further, a number of pathways, generate adenosine (Dunwiddie et al., 1997; Boison and Yegutkin, 2019). ADGF expression was examined at 0, 8, 12 and 16 h (Fig 1) and the ADA activity was assayed at 12 h, the time point where the expression gradually increases and reaches a peak at 16 h. Earlier, we have not shown the 12 h activity data which will be included in the revised version. ADGF expression was found to be highly elevated at 16 h and adenosine/ammonia levels were measured at the two points indicated in the mutant.

> *(10) Panel 4C could be combined with other measurements trying to arrive at more insight in the mechanisms by which ammonia controls tip formation.*

Panel 4C (now 3C) illustrates the genes involved in the conversion of cAMP to adenosine. Since Figure. 3 focuses on adenosine levels and ADA activity in both WT and adgf mutants, we have retained Panel 3C in Figure. 3, for its relevance to the experiment.

> *(11) There is a large variety of experiments attempting to link the mutant phenotype and its rescue by ammonia to cAMP signalling, however, the data do not yet provide a clear answer.*

It is well known that ammonia increases cAMP levels (Riley and Barclay, 1990; Feit et al., 2001) and adenylate cyclase activity (Cotter et al., 1999) in D. discoideum, and exposure to ammonia increases acaA expression (Fig 7B) suggesting that ammonia regulates cAMP signaling. To address the concerns, cAMP levels will be quantified in the mutant after ammonia treatment.

> *(12) The mutant is shown to have lower cAMP levels at the mound stage which ties in with low levels of acaA expression (Figures 7A and B), also various phosphodiesterases, the extracellular phosphodiesterase pdsa and the intracellular phosphodiesterase regA show increased expression. Suggesting a functional role for cAMP signalling is that the addition of di cGMP, a known activator of acaA, can also rescue the mound phenotype (Figure 7E). There appears to be a partial rescue of the mound arrest phenotype level by the addition of 8Br-cAMP (fig 7C), suggesting that intracellular cAMP levels rather than extracellular cAMP signalling can rescue some of the defects in the ADGF- mutant. Better images and a time course would be helpful.*

The relevant images will be replaced and a developmental time course after 8-Br-cAMP treatment will be included in the revised manuscript (Figure. 6D).

> *(13) There is also the somewhat surprising observation that low levels of caffeine, an inhibitor of acaA activation also rescues the phenotype (Figure 7F).*

With respect to caffeine action on cAMP levels, the reports are contradictory. Caffeine has been reported to increase adenylate cyclase expression thereby increasing cAMP levels (Hagmann, 1986) whereas Alvarez-Curto et al., (2007) found that caffeine reduced intracellular cAMP levels in Dictyostelium. Caffeine, although is a known inhibitor of ACA, is also known to inhibit PDEs (Nehlig et al., 1992; Rosenfeld et al., 2014). Therefore, if caffeine differentially affects ADA and PDE activity, it may potentially counterbalance the effects and rescue the phenotype.

> *(14) The data attempting to asses cAMP wave propagation in mounds (Fig 7H) are of low quality and inconclusive in the absence of further analysis. It remains unresolved how this links to the rescue of the ADGF- phenotype by ammonia. There are no experiments that measure any of the effects in the mutant stimulated with ammonia or di-cGMP.*

The relevant images will be replaced (now Figure. 6H). Ammonia by increasing acaA expression (Figure. 7B), and cAMP levels (Figure. 7C) may restore spiral wave propagation, thereby rescuing the mutant.

> *(15) A possible way forward could also come from the observation that ammonia can rescue the wobbling mound arrest phenotype from the histidine kinase mutant dhkD null mutant, which has regA as its direct target, linking ammonia and cAMP signalling. This is in line with other work that had suggested that another histidine kinase, dhkC transduces an ammonia signal sensor to regA activation. A dhkC null mutant was reported to have a rapid development phenotype and skip slug migration (Dev. Biol. (1998) 203, 345). There is no direct evidence to show that dhkD acts upstream of ADGF and changes in cAMP signalling, for instance, measurements of changes in ADA activity in the mutant.*

cAMP levels will be quantified in the dhkD mutant after ammonia treatment and accordingly, the results will be revised.

> *(16) The paper makes several further observations on the mutant. After 16 hrs of development the adgf- mutant shows increased expression of the prestalk cell markers ecmA and ecmB and reduced expression of the prespore marker pspA. In synergy experiments with a majority of wildtype, these cells will sort to the tip of the forming slug, showing that the differentiation defect is cell autonomous (Fig 9). This is interesting but needs further work to obtain more mechanistic insight into why a mutant with a strong tip/stalk differentiation tendency fails to make a tip. Here again, knowing which cells express ADGF would be helpful.*

The adgf mutant shows increased prestalk marker expression in the mound but do not form a tip. It is well known that several mound arrest mutants form differentiated cells but are blocked in development with no tips (Carrin et al., 1994). This is addressed in the discussions (539). To address whether adgf expression is cell type-specific, prestalk and prespore cells will be separated by fluorescence activated cell sorter (FACS), and thereafter, adgf expression will be examined in each population.

> *(17) The observed large mound phenotype could as suggested possibly be explained by the low ctn, smlA, and high cadA and csA expression observed in the mutant (Figure 3). The expression of some of these genes (csA) is known to require extracellular cAMP signalling. The reported low level of acaA expression and high level of pdsA expression could suggest low levels of cAMP signalling, but there are no actual measurements of the dynamics of cAMP signalling in this mutant to confirm this.*

The acaA expression was examined at 8 and 12 h (Figure. 6B) and cAMP levels were measured at 12 and 16 h in the adgf mutants (Figure. 6A). Both acaA expression and cAMP levels were reduced, suggesting that cells expressing adgf regulate acaA expression and cAMP levels. This regulation, in turn, is likely to influence cAMP signaling, collective cell movement within mounds, ultimately driving tip development. Exposure to ammonia led to increased acaA expression (Figure. 7B) in in WT. Based on the comments above, cAMP levels will be measured in the mutant before and after rescue with ammonia.

> *(18) Furthermore, it would be useful to quantify whether ammonia addition to the mutant reverses mound size and restores any of the gene expression defects observed.*

Ammonia treatment soon after plating or six hours after plating, had no effect on the mound size (Figure. 5G).

> *(19) There are many experimental data in the supplementary data that appear less relevant and could be omitted Figure S1, S3, S4, S7, S8, S9, S10.*

Figure S8, S9, S10 are omitted. We would like to retain the other figures

Figure S1 (now Figure. S2): It is widely believed that ammonia comes from protein (White and Sussman, 1961; Hames and Ashworth, 1974; Schindler and Sussman, 1977) and RNA (Walsh and Wright, 1978) catabolism. Figure. S2 shows no significant difference in protein and RNA levels between WT and adgf mutant strains, suggesting that adenosine deaminaserelated growth factor (ADGF) activity serves as a major source of ammonia and plays a crucial role in tip organizer development in Dictyostelium. Thus, it is important to retain this figure.

Figure S3 (now Figure. S4): The figure shows the treatment of various mound arrest mutants and multiple tip mutants with ADA enzyme and DCF, respectively, to investigate the pathway through which adgf functions. Additionally, it includes the rescue of the histidine kinase mutant dhkD with ammonia, indicating that dhkD acts upstream of adgf via ammonia signalling. Therefore, it is important to retain this figure.

Figure S4 (now Figure. S5): This figure represents the developmental phenotype of other deaminase mutants. Unlike adgf mutants, mutations in other deaminases do not result in complete mound arrest, despite some of these genes exhibiting strong expression during development. This underscores the critical role of adenosine deamination in tip formation. Therefore, let this figure be retained.

Figure S7 (now Figure. S8): Figure S8 presents the transcriptomic profile of ADGF during gastrulation and pre-gastrulation stages across different organisms, indicating that ADA/ADGF is consistently expressed during gastrulation in several vertebrates (Pijuan-Sala et al., 2019; Tyser et al., 2021). Notably, the process of gastrulation in higher organisms shares remarkable similarities with collective cell movement within the Dictyostelium mound (Weijer, 2009), suggesting a previously overlooked role of ammonia in organizer development. This implies that ADA may play a fundamental role in regulating morphogenesis across species, including Dictyostelium and vertebrates. Therefore, we would like to retain this figure.

> *(20). Given the current state of knowledge, speculation about the possible role of ADGF in organiser function in amniotes seems far-fetched. It is worth noting that the streak is not equivalent to the organiser. The discussion would benefit from limiting itself to the key results and implications.*

The discussion is revised accordingly by removing the speculative role of ADGF in organizer function in amniotes. The lines “It is likely that ADA plays a conserved, fundamental role in regulating morphogenesis in Dictyostelium and other organisms including vertebrates” have been removed.

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