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Adenylate cyclase A acting on PKA mediates induction of stalk formation by c-di-GMP at the *Dictyostelium* organizer

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ABSTRACT
Coordination of cell movement with cell differentiation is a major feat of embryonic development. The *Dictyostelium* stalk always forms at the organizing tip by a mechanism that is not understood. We previously reported that c-di-GMP, synthesized by diguanylate cyclase A (DgcA), induces stalk formation. We here used transcriptional profiling of *dgca*-structures to identify target genes for c-di-GMP, and used these genes to investigate the c-di-GMP signal transduction pathway. We found that knock-down of PKA activity in prestalk cells reduced stalk gene induction by c-di-GMP, while PKA activation bypassed the c-di-GMP requirement for stalk gene expression. c-di-GMP caused a persistent increase in cAMP, which still occurred in mutants lacking the adenylate cyclases ACG or ACR, or the cAMP phosphodiesterase RegA. However, both inhibition of adenylate cyclase A (ACA) with SQ22536, and incubation of a temperature-sensitive ACA mutant at the restrictive temperature prevented c-di-GMP induced cAMP synthesis as well as c-di-GMP induced stalk gene transcription. ACA produces the cAMP signals that coordinate *Dictyostelium* morphogenetic cell movement and is highly expressed at the organizing tip. The stalk-less *dgca*- mutant regained its stalk by expression of a light-activated adenylate cyclase from the ACA promoter and exposure to light, indicating that cAMP is also the intermediate for c-di-GMP *in vivo*. Our data show that the more widely expressed DgcA activates tip-expressed ACA, which then acts on PKA to induce stalk genes. These results explain why stalk formation in Dictyostelia always initiates at the site of the morphogenetic organizer.

Significance statement
Organizers are small groups of cells in developing embryos that secrete signals to control behaviours like cell differentiation or cell movement of larger groups. In Dictyostelia, the apical tip is the site where differentiation of the fruiting body stalk initiates. The cause of tip-specific stalk formation was unclear, but Chen and co-workers show here that the more widely produced stalk-inducing signal c-di-GMP activates cAMP synthesis by adenylate cyclase A, which is specifically expressed at the apical tip. cAMP next activates cAMP-dependent protein kinase, which then triggers stalk differentiation.
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INTRODUCTION
Aggregative multicellularity resulting in fruiting body formation is the most common evolutionary transition from a unicellular to a multicellular life style. While in most aggregating organisms, the fruiting bodies consist entirely of either spores or cysts, or have stalks consisting of secreted matrix, the Dictyostelia additionally evolved somatic cells. Stalk cells are the ancestral somatic cells of Dictyostelia and their differentiation starts at the tip of emerging fruiting structures, with prespore cells moving up along the stalk to form the spore head. The model *Dictyostelium discoideum* additionally differentiates into three more somatic cell types, that form disc and cup-shaped structures to support the stalk and spore head (1).

Similar to animals, but unlike plants and fungi, *D. discoideum* development consists of an integrated programme of coordinated cell movement and cell differentiation. This programme is initiated by starvation, which causes cells to collect into aggregates, using secreted cAMP pulses, produced by ACA, as a chemoattractant (2). Secreted cAMP produced by the adenylate cyclases ACG and ACR additionally induces differentiation of prespore cells (3). The prespore cells in turn synthesize the polyketide DIF-1, which causes differentiation into precursors of some somatic cell types (4). In cell monolayers, DIF-1 induces the differentiation of vacuolated cells, which are phenotypically identical to stalk and basal disc cells (5). However, in normal development, DIF-1 is only required for lower cup and basal disc differentiation (6).

D.discoideum uses the diguanylate cyclase DgcA to synthesize cyclic diguanylate (c-di-GMP) (7), a well-known second messenger in prokaryotes with a major role in triggering biofilm formation (8). *Dictyostelium dgcA* is expressed in prestalk cells and *dgca* null mutants form normal migrating slugs, but do not initiate fruiting body formation. This defect is due to lack of stalk cell differentiation, and is restored by externally applied c-di-GMP. C-di-GMP

also induces stalk cell differentiation in cell monolayers, indicating that c-di-GMP is a secreted signal that triggers stalk cell differentiation (7). The mode of action of c-di-GMP is unknown, as are the c-di-GMP regulated genes that cause stalk cell differentiation.

We previously used a reporter gene fused to a region of the *ecmB* promoter, which directs expression in stalk cells, as a marker for c-di-GMP induced stalk gene expression (7). *EcmB* is a commonly used stalk marker, but is also expressed in the basal disc and upper and lower cup from other promoter regions (1). Because absolute expression levels from cells transformed with reporter constructs depend on plasmid copy number, such markers are less suited for comparing gene expression levels between mutants. They are also unsuitable for use in mutants generated by overexpression of genes under the same selectable marker. To identify stalk genes that are directly regulated by c-di-GMP, we performed high-throughput RNA sequencing of wild-type and *dgca-* multicellular structures. We validated candidate stalk genes by examining their expression pattern and up-regulation by c-di-GMP, and then used the genes as markers to investigate the signal transduction pathway of c-di-GMP. Our results point to crucial roles for ACA and PKA as intermediates for c-di-GMP induced stalk gene expression.

RESULTS
Identification of target genes for c-di-GMP.
To identify genes that are regulated by c-di-GMP, we used high throughput RNA sequencing to compare all genes transcribed in *dgca-* mutants and control random integrants at the time point when the control cells just started to form fruiting bodies (Figure S1A). Due to their stalk defect, the *dgca-* mutants continue slug migration for days until they die (7). RNAs were isolated from two *dgca-* knock-out clones and two random integrants in two separate experiments, yielding four replicates each for *dgca-* and control. Cluster analysis of the gene expression profiles showed that the four knock-out (KO) and four random integrant (RI) samples formed two distinct clades (Fig. S1B), indicating reproducible differences between the expression profiles of *dgca-* and control cells. Statistical analysis showed that out of the 8939 detected mRNAs, 2565 were significantly up-regulated in the *dgca-* mutant, 2281 were down-regulated and 4093 genes remained unchanged (Fig. S1C). Gene ontology analysis showed that the genes down-regulated in *dgca-* were enriched in catalytic activity, oxidoreductase activity, RNA binding and carbohydrate binding (Fig. S1D). The latter is expected, since cellulose cell wall synthesis is a major aspect of the stalk (and spore) phenotype.

The 2281 genes that are down-regulated in the *dgca-* mutant are potential c-di-GMP target genes. However, only 390 genes are down-regulated more than 5x, suggesting that the greater majority of genes responded pleiotropically to the altered environment of the early fruiting body, rather than being direct targets for c-di-GMP. Additionally, because spore differentiation depends on the stalk being formed first, this set also contains spore genes that are indirectly up-regulated by c-di-GMP. Comparison with earlier expression profiling of prestalk and prespore cells in migrating slugs (9), shows that of the 2281 genes down-regulated in *dgca-*, 440 and 484 genes are >3-fold upregulated in prestalk and prespore cells, respectively (Dataset S1, sheet 4). To isolate experimentally useful stalk marker genes, we first selected genes that were at least 10-fold down-regulated in *dgca-* cells and had a read-count in control cells >50 (Dataset S1, sheet 6). From this set, we isolated genes that were at least 10x over-expressed in prestalk over prespore cells, and were strongly upregulated at the onset of fruiting body formation, as determined earlier (9). We also selected some highly expressed down-regulated genes that had stalk-enriched orthologues in another *Dictyostelium* species, *Polysphondylium pallidum*. This left us with 25 well-expressed potential target genes for c-di-GMP (Dataset S1, sheet 7, Fig. S1C). This set also contained *ecmB*, but not other DIF-1 regulated genes, such as *ecmA, staA(pDd26)* and *staB* (10, 11).

C-di-GMP regulation and expression patterns of putative c-di-GMP target genes.

The 5'intergenic regions of eight putative c-di-GMP target genes were fused to *LacZ* and transformed into wild-type cells. Cells were developed on nitrocellulose filters and at progressive stages of development, structures were stained with X-gal to visualize *LacZ* expression. Four genes, *DDB_G0271196*, *abcG18*, *DDB_G0277757* and *gluA* were exclusively or predominantly expressed in the stalk, with *DDB_G0271196*, further called *staC,* also being expressed late in the upper and lower cup of the mature spore head (Figure 1). We used quantitative real-time PCR to establish whether the four genes were up-regulated by c-di-GMP in wild-type cells. Recent studies showed that c-di-GMP induced stalk cell differentiation was strongly reduced in the *stlb-* and *dmta-* mutants that cannot synthesize DIF-1 (6, 12, 13) and we therefore also included these mutants in our analysis.

In wild-type cells, 1-3 µM c-di-GMP optimally induced stalk gene transcription, with transcription decreasing at 10 µM. However, *DDB_G0277757* and *gluA* were only up-regulated 5 and 2 fold, respectively. In the DIF-less mutants, upregulation of staC and abcG18 only occurred effectively at 10 µM c-di-GMP, suggesting that DIF-1 induces responsiveness of cells to c-di-GMP. The marginally up-regulated *staD* and *gluA* genes were even less upregulated by c-di-GMP in the absence of DIF. There was however no marked difference in fold upregulation of stalk transcripts by 10 µM c-di-GMP between wild-type and DIF-less mutants.

A second set of four genes was expressed very late in fruiting body formation, when spore formation was already completed (Figure 1). These genes were expressed in the upper- and lower cup of the spore head, but unlike the elliptical spores, the cells expressing these genes retained the irregular amoeboid shape (Fig. 1 arrows). The genes all encode proteins of unknown function and were named *cupA-D. CupC* (*DDB_G0282455*) is also expressed very late throughout the stalk, while *cupD* (*DDB_G0293854*) showed scattered expression earlier in development, and relatively high expression at the rear of migrating slugs. None of the genes were expressed in the basal disc. The *cup* genes were also inducible by c-di-GMP, but required higher concentrations than the two stalk genes, although this was less pronounced for *cupB* (*DDB_G0278537*). The *dmta-* and *stlb-* mutants required even higher c-di-GMP concentrations for *cup* gene induction, and also tended to show lower fold upregulation by c-di-GMP. Additional experiments using promoter-*lacZ* constructs showed that *cup* genes needed at least 100 µM c-di-GMP for optimal expression (Figure S2). Since *DgcA* is not expressed in the cup or spore population (7), the requirement for such high c-di-GMP concentrations for *cup* gene induction *in vitro,* could be artefactual, and not reflect regulation of *cup* genes in normal development.

Components of the c-di-GMP signal transduction pathway

To identify components of the c-di-GMP signal transduction pathway, we first investigated mutants with a similar phenotype as the *dgca-* mutant. A mutant that expresses a dominant-negative inhibitor of PKA (Pka-Rm) from the *ecmA* prestalk promoter shows, like *dgca-,* prolonged slug migration, but eventually forms gnarled erect structures without stalk cells (14). We first tested whether c-di-GMP could restore proper fruiting body formation of the *ecmA::Pka-Rm* slugs. However, unlike *dgca-* slugs, *ecmA::Pka-Rm* slugs did not form fruiting bodies when exposed to c-di-GMP (Fig. 2A), suggesting that PKA either acts downstream of c-di-GMP, or in parallel to the c-di-GMP pathway.

To investigate whether PKA acts downstream of c-di-GMP, we compared c-di-GMP induction of target gene expression in AX2 cells, transformed with either *ecmA::PkaRm* (which cannot bind to cAMP) or with a control *ecmA::PkaRc* construct, which can additionally not bind to PkaC (14). Figure 2B shows that c-di-GMP induction of both the stalk and cup genes is 50-70 % reduced in PkaRm cells, with repression being stronger for cup genes than stalk genes. The incomplete inhibition by *ecmA::PkaRm* is likely due to the fact that not all prestalk cells express *ecmA*, since we found that even at 3-fold higher G418 selection than the 100 µg/ml used previously (14), 30% of terminal structures still showed a very thin stalk (Fig. S3).

To validate PKA involvement, we tested whether the membrane-permeant PKA activator 8Br-cAMP could bypass the c-di-GMP requirement for target gene induction. Figure 2C

shows that the stalk genes were induced equally effectively by 3 µM c-di-GMP and 10 mM 8Br-cAMP in *dgca-* cells. Combinatorial stimulation with c-di-GMP and 8Br-cAMP only slightly improved stalk gene induction (Fig. S4), indicating the 8Br-cAMP mimics rather than facilitates the effects of c-di-GMP.

For cup genes the induction by 8Br-cAMP was at least 10x more effective than induction by c-di-GMP. Combined with the inhibitory effects of PkaRm, the stimulatory effects of 8Br-cAMP indicate that PKA is likely to act downstream of c-di-GMP. However, the much greater effect of 8Br-cAMP than c-di-GMP on cup genes suggests that c-di-GMP is unlikely to be the primary signal for induction of cup genes.

Involvement of adenylate cyclases and RegA

To investigate how c-di-GMP increases PKA activity, we first tested the effect of c-di-GMP on cellular cAMP levels. Cells from dissociated slugs were incubated with the cAMP phosphodiesterase inhibitors dithiothreitol (DTT) and 3-isobutyl-1-methylxanthine (IBMX) (15), in the presence and absence of 2 µM c-di-GMP. In slugs, cAMP is produced by three adenylate cyclases, ACA, ACG and ACR (3, 16, 17). Addition of PDE inhibitors allows cAMP to accumulate to about 20 pmol/10^7 cells, while stimulation with c-di-GMP causes a further two-fold increase (Fig. 3A), which persisted for as long as c-di-GMP was present (Fig. S5).

Many signals that stimulate cell differentiation by activating PKA in *Dictyostelium* do so by indirectly inhibiting the intracellular cAMP phosphodiesterase RegA (18). Null mutants in either *regA*, *acgA* and *acrA* develop to the slug stage, but *acaA* null cells cannot aggregate and therefore do not acquire competence for c-di-GMP. To investigate whether the c-di-GMP induced cAMP increase was due to either inhibition of RegA or stimulation of ACG or ACR, we measured the effect of c-di-GMP on cAMP levels in *rega-*, *acga-* or *acra-* mutants. Figure 3B shows that all three mutants showed c-di-GMP induced cAMP accumulation similar to wild-type cells, indicating that neither RegA, ACG nor ACR mediates the effect of c-di-GMP. To test involvement of ACA, we used an adenylate cyclase inhibitor, SQ22536, which inhibits ACA *in vivo,* but not ACG or ACR (15). Figure 3A shows that SQ22536 almost completely inhibits the c-di-GMP induced cAMP accumulation, suggesting that c-di-GMP activates ACA. SQ22536 also inhibits c-di-GMP induced stalk gene expression, as measured in *abcG18::LacZ* and *staC::LacZ* transformed cells (Fig. 4A), supporting evidence that ACA and PKA mediate c-di-GMP induction of stalk cell differentiation.

Because pleiotropic effects of SQ22536 cannot be excluded, we used an *aca-* mutant transformed with a temperature-sensitive ACA variant (tsaca2) to validate involvement of ACA. This mutant develops normally at 22°C, but stops development when transferred to 28°C, a temperature where wild-type still forms normal fruiting bodies (19). After development of *aca-/tsaca2* cells into slugs at 22°C, the cells showed normal c-di-GMP-induced cAMP accumulation at 22°C. However, at the non-permissive temperature of 28°C, c-di-GMP induced cAMP accumulation was much reduced in the *aca-/tsaca2* mutant (Fig. 3C), but not in wild-type cells (Fig. 3D). c-di-GMP induction of stalk gene expression was also strongly inhibited at 28°C in *aca-/tsaca2* (Fig. 4B). This was not due to direct inhibition of gene expression by the higher temperature, since in wild-type, c-di-GMP-induced stalk gene expression was 1.5-2x higher at 28°C than at 22°C (Fig. 4C). Neither DIF-1, nor higher c-di-GMP concentrations could restore stalk gene expression in the *aca-/tsaca2* mutant at 28°C (Fig. S6). When combined, the results obtained with the ACA inhibitor and the *aca-/tsaca2* mutant show that ACA mediates c-di-GMP induced stalk gene expression.

Increasing cAMP or activating PKA in prestalk cells blocks slug- and fruiting body formation due to interference with chemotactic signalling (20-22). To prove nevertheless that c-di-GMP effects in intact slugs are solely mediated by cAMP, we fused the light-activated cyanobacterial adenylate cyclase mPAC, also when expressed in *Dictyostelium* (23) to the ACA promoter that directs tip-specific expression (24) (Fig. 5C). Figure 5A shows that in darkness the *dgca-/ACAt::mPAC* slugs continued to migrate, but that light triggered fruiting body formation and stalk cell differentiation (Fig. 5B) within 5 hours (Movie S1). *Dgca-* did not form fruiting bodies in darkness or in light (Fig. 5A) and an existing A15::mPAC construct

(23) was much less effective (Fig. S7). When combined, our experiments conclusively show that ACA mediates c-di-GMP induced stalk formation.

DISCUSSION
Transcription profiling identified novel stalk genes and cup genes.
Comparison of the transcription profiles of wild-type and *dgca-* mutants, yielded 2281 genes that were down-regulated in *dgca-*, but only 390 genes were down-regulated more than 5x. Their stalk defect prevents *dgca-* cells from entering culmination and the question arises whether the *dgca-*/wild-type comparison merely identifies culmination-specific genes. However, there is only a weakly supported positive correlation between genes upregulated in wild-type over *dgca-* on one hand, and genes upregulated in culmination over slug stage, on the other (Fig. S8), indicating that DgcA affects a specific gene set. Comparison of cells stimulated *in vitro* with and without c-di-GMP is another approach to identify c-di-GMP target genes. However, other signals, such as DIF-1, induce genes like *ecmA* and *pdsA,* and even stalk cell differentiation *in vitro* (5, 25, 26), without being required for these processes *in vivo* (6, 26). Our approach to identify DgcA dependent genes *in vivo* and then test their expression pattern and c-di-GMP induction in *vitro* is more likely to yield developmentally relevant DgcA targets. DIF-1 is required for formation of the basal disc (6, 13) and because basal disc and stalk cells are phenotypically identical, the cells induced by DIF-1 *in vitro* are most likely basal disc cells.

Recent studies found strongly reduced c-di-GMP induction of the vacuolated stalk/basal disc cell type in *stlb-* and *dmta-* mutants *in vitro* (12). We show that stalk gene transcription in *stlb-* and *dmta-* mutants *in vitro* required 10-fold higher c-di-GMP concentrations than in wild-type cells (Fig. 1), suggesting that DIF-1 promotes responsiveness to c-di-GMP. In normal development, stalks are somewhat weaker in *stlb-* and *dmta-* mutants (6), suggesting that sufficiently high c-di-GMP concentrations are produced to counteract most of the reduced responsiveness.

In addition to stalk genes, the set of prestalk-enriched genes that was down-regulated in *dgca-* also contained genes that were expressed very late in the upper- and lower cup of the spore head. The cells expressing these genes remained amoeboid and likely serve to anchor the spore mass to the stalk. The upper and lower cup cells, identified here, differ from those identified by the expression patterns of the prestalk genes *ecmA* and *ecmB*, which appear much earlier when the stalk is just being formed, and for *ecmA* already in slugs (10). The promoter region that directs *ecmB* lower cup expression, also directs expression in the basal disc, while the upper cup population largely becomes part of the stalk (27). The cup genes identified in our study are not expressed in the basal disc, and the amoebas expressing these genes move to the upper cup position too late to be incorporated in the stalk.

ACA acting on PKA mediates c-di-GMP induced stalk gene expression.
C-di-GMP induction of stalk gene expression is reduced in mutants in which PKA activity is down-regulated, while PKA activation with 8Br-cAMP is equally effective as c-di-GMP in inducing stalk gene expression (Figs. 2, S4). This suggests that the effects of c-di-GMP are mediated by PKA, which was further confirmed by experiments showing that c-di-GMP induces a prolonged elevation of cAMP levels in slug cells (Fig. 3). The c-di-GMP induced cAMP increase occurred normally in *acra-*, *acga-* and *rega-* mutants, but was prevented by the ACA inhibitor SQ22536 and by down-regulation of ACA in a temperature sensitive ACA mutant (Fig. 3). SQ22536 and ACA down-regulation also prevented c-di-GMP induced stalk gene expression (Fig. 4). These data show that c-di-GMP induces stalk gene expression in cell suspension by cAMP synthesis by ACA, which in turn activates PKA.

Stalk formation of the stalk-less *dgca-* mutant was restored by heterologous expression of a light-activated adenylate cyclase from a tip-specific promoter in its slugs, followed exposure to light. This indicates that also *in vivo* cAMP synthesis, normally induced by c-di-GMP, triggers stalk formation. *DgcA* has a fairly broad expression domain throughout the anterior 30% of the slug (7), while *ACA* is highly expressed at just the tip 5%, where stalk formation initiates (17, 24). This suggest a simple model (Fig. 5D) for induction of stalk

formation, in which c-di-GMP, though present at the entire prestalk region, can only activate PKA and thereby stalk cell differentiation at the slug tip, where ACA is expressed. The interaction of c-di-GMP with ACA therefore explains why stalk formation always initiates at the tip.

METHODS
Cell culture
D.discoideum cells were grown in HL5 medium (Formedium, UK), supplemented with 10 µg/ml blasticidin for knock-out mutants, 20 µg/ml G418 for *aca-/tsaca2* mutants (19) and cells transformed with *ACAt::mPAC-YFP* or *LacZ* constructs, and 300 µg/ml G418 for cells transformed with *ecmA::PkaRm* or *ecmA::PkaRc* (14).

High throughput RNA sequencing
RNA isolation, cDNA library construction and high-throughput sequencing. Two *dgca* knock-out clones and two clones harbouring random integrations of the *DgcA* knock-out construct were plated at 10^6 cells/cm^2 on non-nutrient agar and incubated at 22°C until the random integrant clones had reached the early culminant stage. Total RNA was isolated from two separate experiments and enriched for mRNA, using poly-T linked magnetic beads. Bar-coded cDNA libraries were constructed using the Illumina TruSeqRNA v2 protocol and checked for quality using the Agilent TapeStation DNA D1K kit. The 8 bar-coded libraries were normalised to 10 nM and combined into one pool, which was sequenced as paired end 100 bp reads, using the Illumina HiSeq2000 platform. Across the pool, 38-63 million reads were generated per library (Dataset S1, Sheet 1). The raw data are available from the European Nucleotide Archive (study ID: E-MTAB-3829).
Differential Gene Expression Analysis. Sequencing data quality was checked with FastQC and reads were aligned to the *D. discoideum* genome (28) using Tophat v2.0.9, with parameters set at an average *Dictyostelium* intron length of 146 bp (29). Reads had an alignment rate of 90%, and read counts were generated with HTSeq-count v0.5.4p5 (30). Poorly expressed genes (expression <1 count/millions in >4 samples) were filtered out and differentially expressed genes were determined with edgeR v3.2.0 (31), using the exact test algorithm following 'TMM' normalisation. All genes with false discovery rate (FDR)< 0.05 are listed as differentially expressed genes (DEG) in Dataset S1, sheet 3.

DNA constructs
To study expression patterns of putative c-di-GMP target genes, complete 5'intergenic regions were amplified by PCR from *D. discoideum* gDNA using the primers listed in Table S1, which include XbaI and BglII sites in the forward- and reverse primers, respectively. Amplicons were digested with XbaI and BglII and cloned into the XbaI/BglII digested plasmid pDdGal17 (32). A fragment of 1,258 bp corresponding to *acaA* promoter 3 that directs tip-specific expression (33) was amplified using primer pair ACAtF_L and ACAtR_L with XbaI and BglII restriction sites for cloning into pDdGal17 and using primer pair ACAtF_mP and ACAtR_mP with SalI and HindIII sites for replacing the actin15 promoter in act15::mPAC-YFP (23). The amplicons were ligated into the plasmids after digesting with the appropriate enzymes. Constructs were verified by DNA sequencing and transformed into AX2 wild-type or *dgca-* cells.

B-galactosidase assays
Histochemical assay. Wild-type cells transformed with promoter-*LacZ* constructs were plated at 4x10^6 cells/cm^2 on nitrocellulose filters, supported by non-nutrient agar, and incubated at 22ºC until the desired developmental stages had been reached. Structures were fixed with 0.25% glutaraldehyde and stained with X-gal (34). Different developmental stages from cells transformed with the same constructs were stained for equally long periods, but for different constructs this could vary from 15 min to 24 hours.
Spectrophotometric assay. Cells transformed with promoter-*LacZ* constructs were developed into slugs, harvested and resuspended to 2x10^6 cells/ml and incubated as 90 µl aliquots in

microtiter plates. Plates were shaken at 170 rpm at 22°C for 6-8 hours. Cells were lysed by freeze-thawing, and incubated at 22°C with 30 µl of 2.5 x Z-buffer and 10 µl of 40 mM CPRG (chlorophenol red-β-D-galactopyranoside). The OD_{574} was measured at regular time intervals using a microtiter plate reader.

Quantitative transcript analysis by qRT-PCR

RNA was isolated from 10^7 cells using the RNeasy minikit (Qiagen) and DNA contamination was removed using the Turbo DNA-free™ kit (Ambion). RNA was transcribed into cDNA using the SensiFAST™ cDNA synthesis Kit (Bioline) and quantitative real-time PCR was performed using the iQ SYBR Green Supermix (Bio-Rad) on a Realplex2 Thermo-cycler (Eppendorf), and the oligonucleotide primers listed in Table S2. Gene expression levels were normalized to the expression level of the constitutively expressed genes *DDB_G0282429* and/or *DDB_G0280765* (9) in the same sample.

cAMP measurements

Slugs were dissociated by repeated pipetting and resuspended to 10^8 cells/ml. 25 µl aliquots of cells were mixed with 5 µl of variables (25 mM dithiothreitol (DTT), 5 mM 3-isobutyl-1-methylxanthine (IBMX), 12 µM c-di-GMP, 12 mM SQ22536, added either separately or in various combinations). The mixtures were shaken for 0 to 60 min at 22°C and 170 rpm. Reactions were terminated with 30 µl 3.5% perchloric acid. Samples were neutralized with 15 µl 50% saturated $KHCO_3$ and 30 µl cAMP assay buffer (4 mM EDTA in 150 mM K-phosphate, pH 7.5). Lysates were centrifuged for 15 min at 1000 x g, and cAMP was measured in 50 µl supernatant by isotope dilution assay (15).

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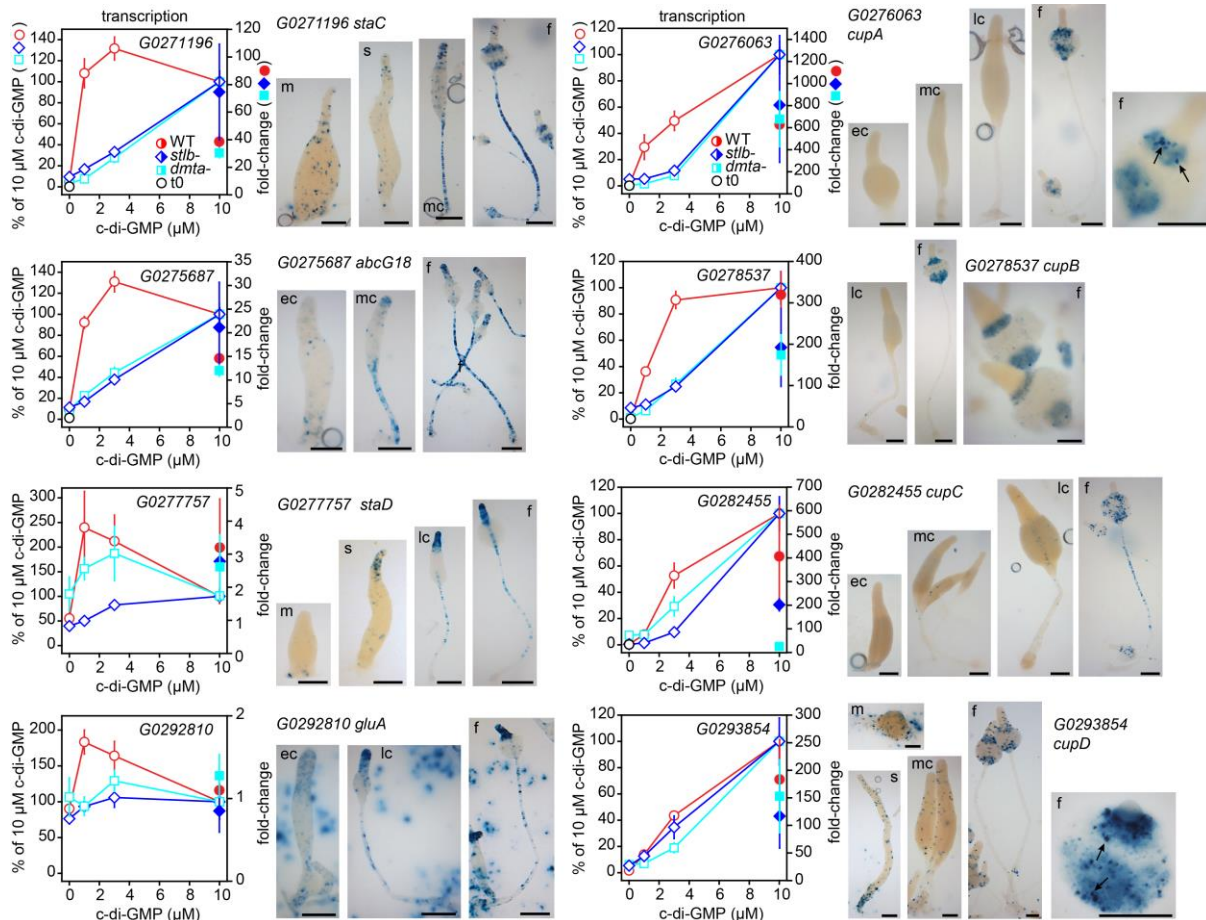
Figure 1. Expression patterns and c-di-GMP regulation of candidate c-di-GMP target genes

Expression patterns. Wild-type cells, transformed with *LacZ* fused to the promoters of 8 candidate c-di-GMP target genes, were incubated at 22ºC and stained with X-gal at different developmental stages. M: mound; s: slug; ec, mc, lc: early, mid and late culmination; f: fruiting body. Bars 100 µm.

C-di-GMP regulation. Wild-type, *dmta-* and *stlb-* slugs were dissociated, resuspended in stalk salts and incubated with 0, 1, 3, 10 µM c-di-GMP for 6 hours. Cells were harvested and total RNA was isolated and resuspended to 1 µg/µl. qRT-PCR reactions were performed using primers sets that hybridized to the coding regions of the candidate genes. Normalized transcript levels are expressed as percentage of fold-change upregulation by 10 over 0 µM c-di-GMP (open symbols), with fold-change at 10 µM shown as closed symbols. Means and s.e.m. of three experiments performed in triplicate. Gene identifiers are shown without DDB_ prefixes.
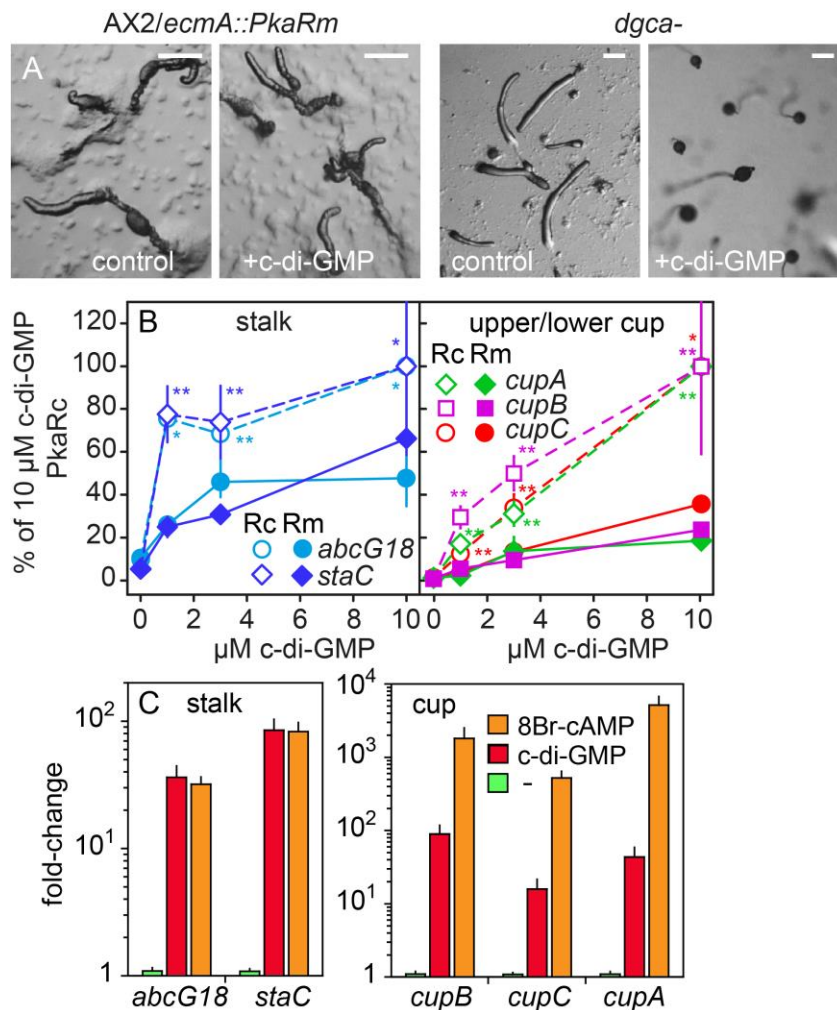
Figure 2. Effects of PKA inhibition and stimulation on c-di-GMP induced gene expression

A. *EcmA::PkaRm rescue*. Wild-type AX2 cells, transformed with the prestalk-specific PKA inhibitor *ecmA::PkaRm,* and *dgca- cells* were developed into migrating slugs, overlayed with either 10 µl of water (control) or 1 mM c-di-GMP, and photographed after 10 h. Bar: 100 µm.
B. *PkaRm effects on gene induction.* AX2 cells transformed with *ecmA::PkaRm* or an inactive construct, *ecmA::PkaRc,* were developed into migrating slugs, which were dissociated and incubated with the indicated c-di-GMP concentrations. After 8 h, RNA was isolated and the levels of two stalk- and three cup-specific transcripts were determined by qRT-PCR. Data are expressed as percentage of transcript levels in the PkaRc controls, treated with 10 µM c-di-GMP. Mean and s.e.m. of two experiments, performed in triplicate. Significant differences between gene induction in Rm and Rc cells, as determined by a rank sum test, are indicated by * for P<0.05 and ** for P<0.005.
C. *8Br-cAMP effects on gene induction.* Dissociated *dgca-* slugs were incubated without additives, with 3 µM c-di-GMP or with 10 mM 8Br-cAMP. After 6 h, RNA was isolated and levels of stalk- and cup-specific transcripts were determined by qRT-PCR. Data are expressed as fold-change relative to untreated controls and represent means and s.e.m. of two experiments in performed triplicate.
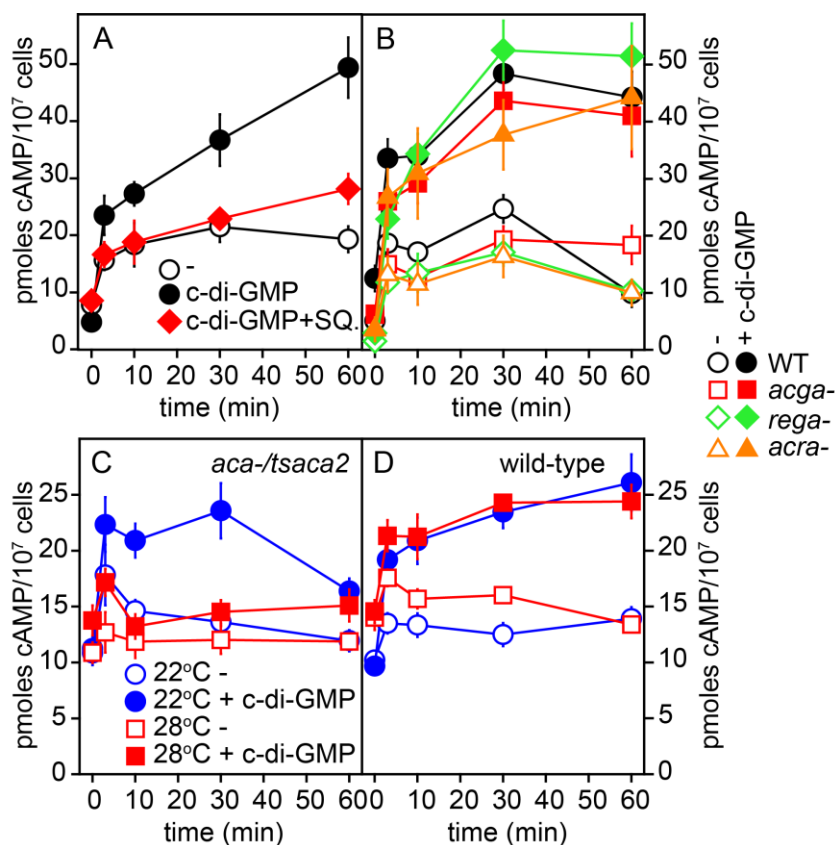
Figure 3. Effect of c-di-GMP on cAMP levels

A. *c-di-GMP effects on cAMP.* Dissociated wild-type slugs were incubated at 10^8 cells/ml with the PdsA/RegA inhibitors DTT and IBMX, 2 µM c-di-GMP and 2 mM of the ACA inhibitor SQ22536 as indicated. At 0, 3, 10, 30 and 60 min, reactions were terminated and cAMP was assayed by isotope dilution assay.

B. *Rega-, acra- and acga- mutants.* Null mutants in the intracellular PDE RegA and the adenylate cyclases ACR and ACG were developed to migrating slugs, dissociated and incubated with DTT/IBMX in the presence and absence of 2 µM c-di-GMP, followed by cAMP assay.

C/D. *Tsaca2 activation by c-di-GMP. Aca-/tsaca2* (C) or wild-type (D) cells were developed to slugs at the permissive temperature (22ºC), followed by 40 min of incubation at either 22ºC or 28ºC. Structures were then dissociated and incubated at 22ºC or 28ºC with DTT/IBMX in the presence and absence of 2 µM c-di-GMP, and assayed for cAMP at the indicated time intervals.
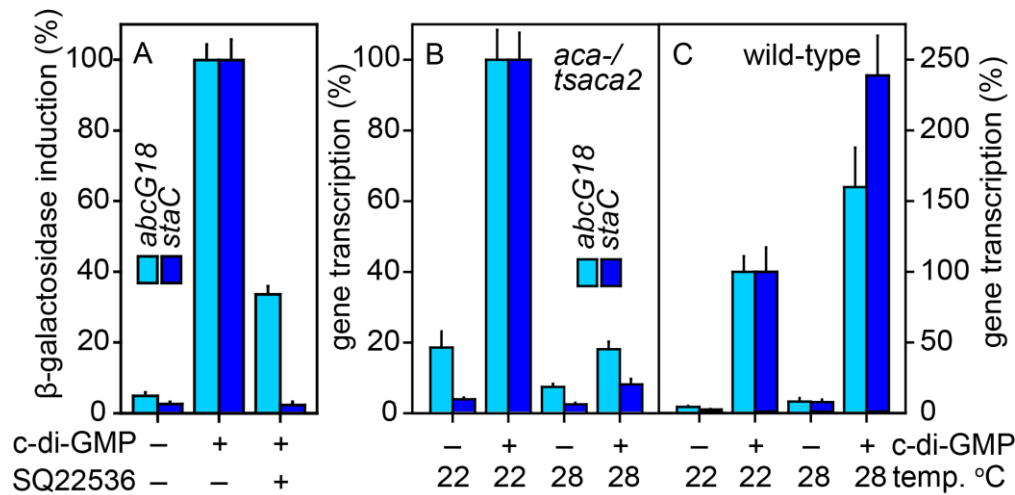
Figure 4. Effect of ACA inhibition on stalk gene induction

A. *SQ22536 effects on stalk genes. Dgca-* cells transformed with either *staC::LacZ* or *abcG18::LacZ* were developed to slugs, dissociated and incubated with 3 µM c-di-GMP in the presence and absence of 2 mM SQ22536. β-galactosidase activity was measured after 6 h. Data are expressed as percentage of activity obtained with only 3 µM c-di-GMP.

B/C. *Gene expression in aca-/tsaca2 and wild-type. Aca-/tsaca2* (B) or wild-type AX2 (C) slugs, developed at 22ºC, were dissociated and incubated in the presence and absence of 3 µM c-di-GMP at either 22ºC or 28ºC for 6 h. RNAs were isolated and *abcG18* and *staC* transcript levels were measured by qRT-PCR. Panels represent means and s.e.m. of three (A,B) or two (C) experiments performed in triplicate.
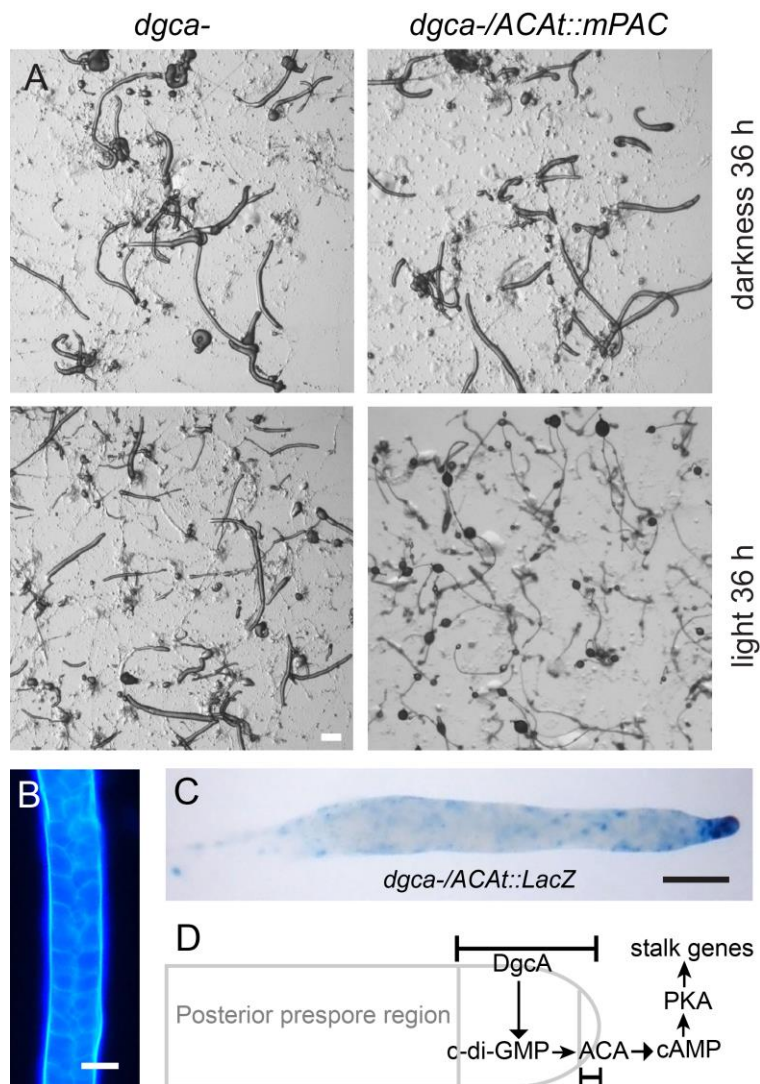
Figure 5. Rescue of *dgca-* culmination by a light activated adenylate cyclase

A. Two plates each of *dgca-* and *dgca-/ACAt::mPAC* cells were developed for 16 h in darkness until slugs had formed. Subsequently, 1 plate of each was incubated for 20 h in darkness and the other under ambient room lighting. The experiment was repeated twice with the same result. Bar: 200 μm.

B. Segment of the stalk of a light-exposed *dgca-/ACAt::mPAC structure* stained with the cellulose dye Calcofluor. Bar: 10 μm.

C. *Dgca-* cells, transformed with an *ACAt::LacZ* fusion construct, were developed to slugs and stained with X-gal to confirm the tip-specificity of expression from the *ACAt* promoter.

D. Model for induction of stalk formation. DgcA is expressed throughout the anterior quart of the slug (7), but its target ACA is only expressed at the tip. c-di-GMP therefore only induces stalk formation at the tip by activating ACA and thereby PKA.