Adenylate cyclase A acting on PKA mediates induction of stalk formation by cyclic diguanylate at the Dictyostelium organizer
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Figure 1. Gene expression profiling of *dgca*-mutants

A. *Experimental set-up.* Two *dgca*-knock-out (KO) clones and two random-integrants (RI) were developed for 22 h in two separate experiments, A and B. At this stage, the RI clones were in early to mid fruiting body formation. RNAs were isolated, enriched for mRNA and reverse transcribed to yield cDNA libraries, which were sequenced using the Illumina HiSeq2000 platform.

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**Legend:**

- **A:** Experimental set-up. Two *dgca*-knock-out (KO) clones and two random-integrants (RI) were developed for 22 h in two separate experiments, A and B. At this stage, the RI clones were in early to mid fruiting body formation. RNAs were isolated, enriched for mRNA and reverse transcribed to yield cDNA libraries, which were sequenced using the Illumina HiSeq2000 platform.

- **B:**

  - KO2 A
  - KO2 B
  - KO1 A
  - KO1 B
  - RI2 A
  - RI2 B
  - RI1 A
  - RI1 B

- **C:**

  - Up in KO: 2565
  - Down in KO: 2281

- **D:**

  - Small GTPase regulator activity
  - Diacylglycerol kinase activity
  - Acid phosphatase activity
  - Oxidoreductase activity, acting on paired donors
  - SNAP receptor activity
  - Hydrolase activity, acting on carbon-nitrogen
  - Metalloprotease activity
  - Oxidoreductase activity
  - NAD+ kinase activity
  - Monoxygenase activity
  - Cellulose binding
  - Carbohydrate binding
  - Metal ion transmembrane transporter activity
  - O-methyltransferase activity
  - Small GTPase regulator activity
  - Beta-glucosidase activity
  - Ceramidase activity
  - GTPase activator activity
  - Oxidoreductase activity, acting on paired donors
  - Transmembrane signaling receptor activity
  - Monoxygenase activity
  - Ethanolamine kinase activity
  - Diacylglycerol binding
  - Hydrolase activity, carbon-nitrogen (not peptide) bonds, linear amides
  - 4-Coumarate-CoA ligase activity
  - Polysaccharide binding
  - Hydrolase activity, carbon-nitrogen (not peptide) bonds, cyclic amides
  - mRNA binding
  - NAD binding
  - Carboxy-peptidase activity
  - Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds
  - Transaminase activity
  - Oxidoreductase activity, CH-OH donor group, NAD/NADP as acceptor
  - Adenyl nucleotide binding
  - Cellulose binding
  - Magnesium ion binding
  - Pyridoxal phosphate binding
  - Structural constituent of ribosome
  - Carbohydrate binding
  - RNA binding
  - Oxidoreductase activity
  - Catalytic activity

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**Legend:**

- **A:** A representative experiment showing RNA isolation.

- **B:** A summary of the two experiments performed (A and B).

- **C:** A scatter plot showing the log fold change of gene expression in KO and RI clones.

- **D:** A bar chart showing the number of differentially expressed genes.
B. **Sample clustering.** Gene expression across each of the samples was clustered via their per-sample pairwise Spearman correlation coefficients.

C. **Differential gene expression in KO vs RI.** Differentially expressed genes (FDR < 0.05) highlighted in red. Blue lines indicate a log$_2$ fold-change of 1, up or down; cyan lines delineate genes that are 5-fold up- or downregulated. Green diamonds mark 25 highly expressed prestalk genes that are potential targets for c-di-GMP. An orange diamond marks the position of the stalk marker ecmB.

D. **Gene Ontology Analysis**

A gene ontology analysis was performed using goseq (v 1.12.0) (34) with the *D. discoideum* Gene Ontology annotations [http://dictybase.org/Downloads/](http://dictybase.org/Downloads/) to test for molecular function enrichment among all genes that were differentially expressed in the *dgca*-mutant, and in the sets that were respectively up- or down-regulated. Molecular functions are shown, which were significantly enriched (p<0.05) after correction for false discovery rates using the Benjamini-Hochberg procedure. Gene functions that are enriched in more than one set have identically colour-coded descriptions.

![Figure S2. Extended c-di-GMP dose response curve](image)

**Figure S2. Extended c-di-GMP dose response curve**

Wild-type AX2 cells transformed with either the *abcG18::LacZ* or *cupA::lacZ* construct were developed to the slug stage. Slugs were dissociated into single cells and incubated in stalk salts at 10$^6$ cells/ml for 8 h with the indicated concentrations of c-di-GMP. Cells were lysed by three freeze-thaw cycles and incubated at 22°C with CPRG and Z-buffer until the samples with highest activity per transformed strain had reached an OD$_{574}$ of about 1.0. Data are expressed as percentage of activity obtained at 100 µM c-di-GMP, and represent means and SD of two experiments performed in triplicate.
Figure S3. Inhibition of stalk formation by ecmA::PkaRm
Phenotype of ecmA::PkaRm culminants. Wild-type AX2 and AX2, transformed with ecmA::PkaRm and selected at 300 µg/ml G418, were developed until erect fruiting structures had formed. Structures were transferred to a slide glass, squashed with a coverslip and photographed. About a third of PkaRm structures contained a thin stalk as shown in the image.

Figure S4. Combinatorial effects of c-di-GMP and 8Br-cAMP on stalk gene induction
Wild-type AX2 cells, transformed with abcG18::LacZ, were developed to the slug stage. Slugs were dissociated into single cells and incubated in stalk salts at 10^6 cells/ml for 6 h with 0 or 3 µM c-di-GMP, 10 or 30 mM 8Br-cAMP or 3 µM c-di-GMP combined with 10 or 30 mM 8Br-cAMP. Cells were lysed and assayed for β-galactosidase activity. Data are expressed as percentage of β-galactosidase activity induced by 3 µM c-di-GMP. Means and s.e.m. of three experiments, performed in triplicate are presented.
Figure S5. Prolonged c-di-GMP effects and cycloheximide effects on cAMP levels

A. Prolonged c-di-GMP effect. Dissociated wild-type slugs were incubated for 5 h at 10^8 cells/ml with the PdsA/RegA inhibitors DTT and IBMX only, or with additionally 1 µM c-di-GMP, which was added either once at the start of the experiments or at 1 h intervals during the experiment. At the indicated time points, reactions were terminated and cAMP was assayed by isotope dilution assay. Means and s.e.m. of two experiments, performed in triplicate.

B. Cycloheximide effects. Dissociated wild-type slugs were preincubated for 30 min in the presence or absence of 500 µg/ml cycloheximide and each sample was subsequently incubated in the presence or absence of 1 µM c-di-GMP (with cycloheximide remaining present). At the indicated time points, reactions were terminated and cAMP was assayed by isotope dilution assay. Means and s.e.m. of three experiments, performed in triplicate. The pretreatment and presence of cycloheximide treatment reduced overall adenylate cyclase activity, but not its stimulation by c-di-GMP. This, and its rapid effect on cAMP levels, indicates that c-di-GMP activates ACA activity and not ACA protein synthesis.

Figure S6. Effects of DIF and 10 µM c-di-GMP on stalk gene expression in aca-/tsaca2 cells.

Aca-/tsaca2 slugs, developed at 22°C, were dissociated and incubated for 6 h at 22°C or 28°C with 0, 3 or 10 µM c-di-GMP or a combination of 3 µM c-di-GMP and 100 nM DIF, as
indicated. RNAs were isolated and abcG18 and staC transcript levels were measured by qRT-PCR. Data represent means and s.e.m. of two experiments performed in triplicate.

**Figure S7. Effect of transformation of A15::mPAC on dgca-development**

The dgca- mutant was transformed with a fusion construct of the constitutive Dictyostelium actin 15 promoter and the light activated adenylate cyclase mPAC from Microcoleus chthonoplastes. Two plates each of dgca- and dgca-/A15::mPAC cells were developed for 16 h in darkness until slugs had formed. Subsequently, 1 plate of each was incubated for up to 48 h in darkness and the other under ambient room lighting. Developing structures were photographed after a total incubation period of 40 or 64 h. A few fruiting bodies that were formed in light treated dgca-/A15::mPAC cells at 64 h are indicated by arrows. Bar: 1 mm.

**Figure S8. Correlation between dgcA dependent genes and early culmination genes**

For all genes that were significantly upregulated in control random integrants (RI) compared to dgca knockouts (KO), upregulation was expressed as the sum of normalized reads in the four RI replicates, divided by the sum of normalized reads in the four RI and four KO replicates (the result has to be >0.5 for upregulated genes, see Dataset S1, sheet 8). For the same genes, normalized reads at 16 h and 20 h were retrieved from a duplicate developmental time course of wild-type Dictyostelium cells, which were at those time points at the migrating slug and early-mid culmination stages, respectively (10). Upregulation in
culminants over slugs was similarly calculated, and plotted against upregulation in random integrants. Linear regression indicates a weakly supported positive correlation between temporal and \( dgca \) induced upregulation. However, many genes that are highly upregulated in RI over KO cells show poor upregulation or even down-regulation in culminants over slugs and \textit{vice versa}. This indicates that selection of putative c-di-GMP target genes on the basis of upregulation in culmination would yield many false positives. The stalk (red) and cup genes (green) that were used in this study and selected using a combination of criteria (see main text) are highly upregulated under both experimental conditions.

LEGEND TO SUPPORTING MOVIE

Movie S1. Fruiting body formation in \( dgca \)- and \( dgca-/ACAt::mPAC \) slugs. \( Dgca \)- and \( dgca-/ACAt::mPAC \) cells were plated on agar, on either side of a thin barrier of overhead foil, and incubated for 16 hours in darkness. The agar plate was then transferred to a dissection microscope and while being exposed to room lighting and trans-illumination with a cold light source from below, images were taken at 10 min intervals for 23 h and converted into a movie file. The barrier sagged a bit towards the \( dgca \)- slugs during imaging.