Precise timing of transcription by c-di-GMP coordinates cell cycle and morphogenesis in Caulobacter

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Bacteria adapt their growth rate to their metabolic status and environmental conditions by modulating the length of their G1 period. Here we demonstrate that a gradual increase in the concentration of the second messenger c-di-GMP determines precise gene expression during G1/S transition in Caulobacter crescentus. We show that c-di-GMP stimulates the kinase ShkA by binding to its central pseudo-receiver domain, activates the TacA transcription factor, and initiates a G1/S-specific transcription program leading to cell morphogenesis and S-phase entry. Activation of the ShkA-dependent genetic program causes c-di-GMP to reach peak levels, which triggers S-phase entry and promotes proteolysis of ShkA and TacA. Thus, a gradual increase of c-di-GMP results in precise control of ShkA-TacA activity, enabling G1/S-specific gene expression that coordinates cell cycle and morphogenesis.
he bacterial cell cycle is divided into three periods: cell division and before initiation of chromosome replication (B or G1); chromosome replication (C or S); and cell division (D or G2). Since chromosome replication and cell division (C and D periods) remain constant over a wide range of growth rates, the step committing cells to initiate chromosome replication largely determines bacterial proliferation rates. Bacteria like *Escherichia coli* or *Bacillus subtilis* can increase their growth rate by bypassing the B period and by initiating replication multiple times per division cycle. In contrast, *Caulobacter crescentus* strictly separates its cell cycle stages. An asymmetric division generates a sessile stalked (ST) cell, which directly reenters S-phase, and a motile swarmer (SW) cell that remains in G1 for a variable time depending on nutrient availability. Coincident with G1/S transition, motile SW cells undergo morphogenesis to gain sessility.

In *C. crescentus*, replication initiation is regulated by the cell cycle kinase CckA. CckA is a bifunctional enzyme that acts as a kinase during the replication initiation period in G1, but switches to being a phosphatase in S phase, resulting in the inactivation of CtrA and clearance of the replication block. In contrast, *Caulobacter crescentus* strictly separates its cell cycle stages. An asymmetric division generates a sessile stalked (ST) cell, which directly reenters S-phase, and a motile swarmer (SW) cell that remains in G1 for a variable time depending on nutrient availability.

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C-di-GMP, which accumulates in wild-type cells, reduces TacA and ShkA phosphorylation (Fig. 1e, f) and could still be partially stimulated by c-di-GMP in the absence of c-di-GMP (Fig. 2c) and could still be partially stimulated by c-di-GMP in the absence of c-di-GMP (Fig. 2d). The mutant strain lacking c-di-GMP formed stalks at low-phosphate concentrations, but failed to localize DivIV to the pole (Fig. 1c). Many cells displayed bipolar stalks, indicating that at low-c-di-GMP, the ShkA-TacA pathway controls stalk positioning through a path- way distinct from ShkA-TacA. Altogether, these results indicate that c-di-GMP acts upstream of and is required for the activity of the ShkA-TacA phosphorelay.

In vitro phosphorylation assays with purified ShkA or with all components of the phosphorelay demonstrated that c-di-GMP strongly and specifically stimulates ShkA autokinase activity (Fig. 1g, h). Moreover, purified ShkA bound c-di-GMP with a Kᵦ of 0.1 μM in a strain expressing a stabilized version of TacA and ShkA phosphorylation (Fig. 1f). Stalk formation is independent of the ShkA-TacA pathway when cells are grown in a low-phosphate medium. In agreement with this, a strain lacking c-di-GMP formed stalks at low-phosphate concentrations, but failed to localize DivIV to the pole (Fig. 1c). Many cells displayed bipolar stalks, indicating that at low-c-di-GMP, the ShkA-TacA pathway controls stalk positioning through a path- way distinct from ShkA-TacA. Altogether, these results indicate that c-di-GMP acts upstream of and is required for the activity of the ShkA-TacA phosphorelay.

C-di-GMP activates ShkA by binding to its pseudo-receiver domain. We next sought to dissect the mechanism of c-di-GMP-mediated ShkA activation. We devised a genetic selection (see "Methods") to isolate shkA mutations that restored spmX expression in a rcdG₀ background. Independent mutations were identified in two residues (D369, R371) within a short stretch of three highly conserved amino acids in the linker region between REC1 and REC2 (hereafter referred to as the "DDR" motif) (Fig. 2a; Supplementary Fig. 3). These results identified the REC1-REC2 linker region as a critical determinant of ShkA activation. We further characterized the D369N variant both in a wild-type background and in a strain expressing a stabilized version of TacA (TacA³⁴), and confirmed that TacA phosphorylation levels and c-di-GMP activators to a pseudo-receiver domain of ShkA autophosphorylation activity even in the absence of c-di-GMP (Fig. 2d). The mutant retained its ability to bind c-di-GMP in vitro (Supplementary Fig. 2c) and could still be partially stimulated by c-di-GMP.
**Fig. 1** C-di-GMP controls the ShkA-TacA phosphorelay in *C. crescentus*.  

**a** Schematic of the *C. crescentus* cell cycle with swarmer and stalked cells colored in blue and orange, respectively, and the G1- and S-phases of the cell cycle indicated in similar colors. Stage-specific kinase (Kin) and phosphatase (Pho) activities of CckA are indicated with the coloring referring to stage-related activities. DivK and PleD, that control the CckA switch are highlighted.  

**b** Schematic of the ShkA-ShpA-TacA phosphorelay.  

**c** Quantification of cells with stalks and SpmX-mCherry foci of strains expressing a chromosomal *spmX-mCherry* fusion and plasmid-driven *xyl::tacA* or *dgcZ*. EV, empty vector. The number of cells analyzed (*N*) is indicated above the graph. Also see Supplementary Fig. 1.  

**d** *spmX* promoter activity (plasmid pRKlac290-*spmX*) in indicated strains. IPTG indicates induction of *dgcZ* expression. Shown are mean values and standard deviations (*N* ≥ 2).  

**e** Phos-tag PAGE immunoblots of strains producing 3xFLAG-tagged ShkA or TacA from the native loci and encoding 3xFLAG-tagged ShkA or TacA at the native loci or the respective mutant alleles encoding degradation-resistant variants TacADD or ShkADD. Strains carried a plasmid for cumate-inducible expression of wild type or a catalytic mutant (AAL) of the phosphodiesterase (PDE) PA5295. Samples were harvested after addition cumate as indicated. PA5295AAL samples were harvested 4 h post induction. Relative phosphorylation (rel. phos.) of TacA and ShkA were determined by calculating the ratio of intensities of phosphorylated and unphosphorylated bands normalized to this value of the wild type with empty vector control.  

**f** Phos-tag PAGE immunoblots of strains encoding 3xFLAG-tagged ShkA or TacA at the native loci or the respective mutant alleles encoding degradation-resistant variants TacADD or ShkADD. Strains carried a plasmid for cumate-inducible expression of wild type or a catalytic mutant (AAL) of the phosphodiesterase (PDE) PA5295. Samples were harvested after addition cumate as indicated. PA5295AAL samples were harvested 4 h post induction. Relative phosphorylation (rel. phos.) was normalized to the PA5295AAL control. In vitro phosphorylation assays with purified components (4 μM) of the ShkA-ShpA-TacA phosphorelay with (+) or without (−) c-di-GMP (76 μM). Reactions were initiated as described for g. Source data are provided as a Source Data file.
both in vivo (Fig. 2b) and in vitro (Supplementary Fig. 2d). Thus, mutations in the DDR motif uncouple ShkA activity from c-di-GMP without interfering with c-di-GMP binding.

We used the c-di-GMP independence of the D369N ShkA variant to identify residues involved in c-di-GMP binding. We reasoned that mutations in ShkA specifically interfering with c-di-GMP-mediated activation should be rescued when combined with D369N. In contrast, mutants causing more general kinase defects (ATP-binding, phosphotransfer, protein stability etc.) would not be recuperated when combined with D369N. An alignment of ShkA orthologs from \textit{C. crescentus} and related organisms revealed a total of 25 candidate residues for c-di-GMP binding distributed throughout the entire protein (Supplementary Fig. 3). Of all the mutants that severely affected ShkA activity (Supplementary Fig. 4a, b) and that were rescued in vivo (Fig. 2e) and in vitro (Fig. 2f) when combined with D369N, only one, Y338A, in REC1, interfered with c-di-GMP binding (Fig. 2g, h), while the other mutations likely affect c-di-GMP-mediated conformational changes required for activation. Thus, Y338 in REC1 is likely a part of the c-di-GMP binding site. Indeed, the purified REC1 domain alone was able to bind c-di-GMP, although with lower affinity than full-length ShkA (Fig. 2i). NMR spectroscopy with REC1 revealed a fold reminiscent of prototypical REC domains, except that helix α3 is not present irrespective of the presence or absence of c-di-GMP (Supplementary Fig. 4c–f). Upon the addition of c-di-GMP, REC1 shows chemical shift perturbations (CSPs), which cluster on the β5-α5 surface (Fig. 2j; Supplementary Fig. 4g). Importantly,
Fig. 2 C-di-GMP binds to the REC1 pseudo-receiver domain. a ShkA domain architecture drawn to scale (top) and alignment of the REC1-REC2 linker harboring the DDR motif (highlighted in green) of ShkA orthologs (bottom). Ccr, C. crescentus; Cse, Caulobacter segnis; Che, Caulobacter henrici; K31, Caulobacter sp. K31; Pzu, Phenyllobacterium zunicum; Abi, Asticcacaulis biprosthecium; Aex, Asticcacaulis excentricus; Bsu, Brevundimonas subovoides. Dimerization and histidine-phosphotransfer (DHP), catalytic and ATP-binding (CA) and receiver (REC1, REC2) domains are indicated. b Activity of the spmX promoter in indicated strains harboring plasmid pAK502-spmX. shkAD369N is expressed from the native chromosomal locus. Means and standard deviations are shown (N = 3). c Phos-tag PAGE immunoblots of indicated strains producing 3xFLAG-tagged TacA or TacAΔD369 from the native locus. d In vitro autophosphorylation assays of ShkA and ShkAΔD369N. Top: autoradiograph; bottom: Coomassie stain. β-Gal assays of ΔshkA mutant strains harboring plasmid pAK502-spmX and expressing different shkA alleles in trans from plasmid pQF with the indicated amino acid substitutions alone (WT, white bars) or in combination with D369N (blue bars). Shown are means and standard deviations (N = 3). e In vitro autophosphorylation assays of wild type or mutant ShkA with (10 μM) or without c-di-GMP. Reactions were run for 5 min. Top: autoradiograph; bottom: Coomassie stain. g Autoradiographs of purified ShkA and ShkA mutant variants (0.5 μM) UV-crosslinked with 10 μM [32P]c-di-GMP with or without addition of a 50-fold molar excess of non-labeled c-di-GMP. Top: autoradiograph; bottom: Coomassie stain. h Quantified autoradiographs of purified ShkA and ShkAΔD369A (0.5 μM) UV-crosslinked with increasing concentrations of [32P]c-di-GMP. Shown are mean values and standard deviations (N = 2). i Autoradiographs and Coomassie stain of the same gel of purified ShkA and the isolated REC1 domain (ShkAREC1) (0.5 μM) after UV crosslinking with 10 μM [32P]c-di-GMP (top). Quantified autoradiographs of purified ShkA and ShkAΔREC1 (0.5 μM) after UV crosslinking with increasing concentrations of [32P]c-di-GMP (bottom). Means and standard deviations are shown (N = 2). j Cartoon and surface representation of the ShkAREC1 homology model with NMR chemical shift perturbations (CSPs) upon c-di-GMP binding indicated by a blue-to-yellow gradient. k Conservation score of ShkA orthologs (see ‘Methods’). Source data are provided as a Source Data file.

these residues include Y338 and are well conserved (Fig. 2k; Supplementary Fig. 3). Some of the other residues implicated in c-di-GMP binding by NMR were also important for ShkA activity in vivo (Supplementary Fig. 4h). Altogether, these experiments revealed a REC1 pseudo-receiver domain of ShkA as the primary docking site for c-di-GMP, and identified residues directly involved in c-di-GMP binding and c-di-GMP-mediated activation of ShkA. In silico analysis revealed that pseudo-receiver domains, although often not annotated, are widespread among histidine kinases (Supplementary Fig. 5, Supplementary Data 1). We propose that pseudo-receiver domains have lost their original phosphorylation function but during evolution have adopted novel signal perception functions and may thus represent a large class of so-far unrecognized kinase input domains.

Similar to the two identified DDR mutants, the kinase activity of ShkA is also uncoupled from c-di-GMP when conserved residues of REC2 are mutated (Supplementary Fig. 2e) or when the C-terminal REC2 domain is removed from the catalytic core (DHp, CA, and REC1 domain) (Fig. 1h). Thus, the REC2 domain and the DDR linker motif inhibit ShkA autophosphorylation, maintaining it in an inactive state. We propose that c-di-GMP binding to REC1 overrides this auto-inhibition and activates the enzyme.

C-di-GMP defines a narrow window of ShkA activity during G1/S. The c-di-GMP-independent variants of ShkA allowed us to more carefully investigate the role of ShkA in the temporal control of events during G1/S. Introduction of the shkAΔD369N allele into the rcdG strain restored SpmX protein levels, stalk biogenesis, DivJ localization to the incipient stalked pole, and normal cell morphology (Fig. 3a, b; Supplementary Fig. 6a). Of note, shkAΔD369N failed to restore G2-specific processes in the rcdG strain like motility and type IV pili assembly (Supplementary Fig. 6b, c). Restoration of cell morphology and DivJ localization was entirely dependent on SpmX (Fig. 3b), arguing that c-di-GMP and ShkA contribute to cell cycle progression and morphogenesis via spmX expression control. Consistent with this notion, the shkAΔD369N allele rescued the synthetic-lethal phenotype of a strain lacking c-di-GMP and suffering from reduced DivK levels in a SpmX-dependent manner (Supplementary Fig. 6), indicating that ShkA influences the CckA kinase/phosphatase balance. A strain harboring the shkaΔD369N allele prematurely produced SpmX already in newborn G1 cells (Fig. 3c). Accordingly, the shkaΔD369N mutant exited G1 prematurely, as indicated by the strong reduction of G1 cells (Fig. 3d). Likewise, cells failed to properly arrest in G1 after entry into the stationary phase (Fig. 3d, e). Growth rate of the shkAΔD369N mutant was not increased in either PYE or M2G medium compared with wild type, suggesting that the overall length of the cell cycle (combined G1, S, and G2 phases) is not or only marginally affected (Supplementary Fig. 6). These results support a role of c-di-GMP, and the ShkA-TacA pathway, in the timely execution of the G1/S transition.

TacA was previously shown to be degraded by the ClpXP protease, a process that requires the adapter proteins RcdA and CpdR and depends on the C-terminal Ala–Gly degradation motif of TacA16. ShkA harbors the same C-terminal degradation signal and was also degraded upon S-phase entry about 10 min after the removal of TacA (Fig. 3f). When the C-terminal Ala–Gly motif was replaced by Asp residues (ShkAΔD37), ShkA and ShkA–P were stabilized and prevailed throughout the cell cycle (Fig. 3f). Sequential degradation of TacA and ShkA may be explained by their differential requirements for protease adapters. While TacA degradation by ClpXP depends on CpdR and RcdA16, ShkA degradation also requires PopA, the third member of the adapter hierarchy regulating ClpXP protease activity during the C. crescentus cell cycle (Fig. 3g, h). Because PopA needs to bind c-di-GMP to act as a protease adapter17, the ShkA-TacA pathway is confined to G1/S by the sequential c-di-GMP-dependent activation and c-di-GMP-mediated degradation of the ShkA kinase.

The ShkA-TacA pathway limits gene expression to G1/S. To carefully assess the contribution of ShkA activation and degradation for the temporal control of spmX, the spmX promoter was fused to the fluorescent protein Dendra2. The photoconvertible properties of Dendra2 allowed determining both “ON” and “OFF” kinetics of spmX promoter activity during the cell cycle (Fig. 4b; Supplementary Fig. 7a). These experiments revealed that spmX promoter activity peaks during G1/S roughly 15–30 min after passing through the predivisional stage (Fig. 4b). The spmX promoter was active in cells progressing through G1/S, but not in newborn ST progeny that reenter S-phase immediately at the end of the asymmetric cell cycle (Fig. 4a, c; Supplementary Fig. 7a–c). Stage-specific expression of spmX required a combination of c-di-GMP oscillations during the cell cycle and TacA degradation: expression of combinations of dgaZ, a gene encoding a highly active DGC from E. coli19, and of tacAΔD (encoding stable TacA16) and shkAΔD369N (encoding constitutive ShkA) alleles resulted in a gradual loss of G1/S-specific spmX expression.

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were probed with anti-FLAG, anti-TacA, anti-SpmX and anti-CtrA antibodies. 

deviations (3xFLAG exponential or stationary phase after rifampicin treatment. 3xFLAG strains expressing the consequences of ShkA-TacA dysregulation using stable pathway to G1/S for accurate cell cycle progression, we examined through the G1 phase of the cell cycle. 

strictly limited to G1/S and SW progeny that need to passage directly evidence that the activity of the ShkA-TacA pathway is (Fig. 4c; Supplementary Fig. 7c). These experiments provided direct evidence that the activity of the ShkA-TacA pathway is strictly limited to G1/S and SW progeny that need to passage through the G1 phase of the cell cycle.

To investigate the importance of limiting the ShkA-TacA pathway to G1/S for accurate cell cycle progression, we examined the consequences of ShkA-TacA dysregulation using stable (TacADD, ShkADD), or constitutively active (ShkAD369N) variants or combinations thereof. All alleles increased overall spmX expression and showed additive effects when combined (Supplementary Fig. 8a). Cell division and cell morphology were normal in all strains that either retained TacA degradation or c-di-GMP-mediated ShkA activity control. However, when mutations that stabilize ShkA or TacA were combined with a

Fig. 3 C-di-GMP imposes precise temporal control of TacA activity during G1/S. a Representative phase-contrast micrographs of indicated strains. Arrows point to stalks. The scale bar represents 4 μm. b Quantification of cell length and polar DivJ localization (DivJ-mCherry) of indicated strains. Median values with interquartile ranges are shown in the graph and mean values and standard deviations are indicated above the graph. The number of cells analyzed is shown in brackets. **** indicates a P value of < 0.0001; ns, not significant (ordinary one-way ANOVA and Tukey’s multiple comparison test). c Immunoblots of synchronized cultures of C. crescentus strains expressing chromosomally encoded alleles were probed with anti-FLAG antibody. d Analysis of chromosome content by flow cytometry of indicated strains in exponential or stationary phase after rifampicin treatment. e Quantification of chromosome number of indicated strains. Shown are means and standard deviations (N = 3). f Phos-tag PAGE immunoblots of synchronized cultures of strains expressing chromosomally encoded 3xFLAG-tacA, 3xFLAG-shkA, 3xFLAG-tacADD, or 3xFLAG-shkADD alleles were probed with anti-FLAG antibody. g Phos-tag PAGE immunoblots of mixed cultures of indicated mutant strains expressing 3xFLAG-tacA probed with anti-FLAG antibodies. h Phos-tag PAGE immunoblots of mixed cultures of indicated mutant strains expressing 3xFLAG-shkA probed with anti-FLAG antibodies. Source data are provided as a Source Data file.
mutation constitutively activating ShkA, strains showed strong cell division and morphology aberrations, effects that were strictly dependent on an intact copy of spmX (Fig. 4d; Supplementary Fig. 8b). Thus, dysregulation of the SpmX morphogen leads to aberrant cell morphogenesis. Together, these experiments provide evidence that dysregulation of ShkA-TacA activity leads to severe cell cycle and morphological defects.

The diguanylate cyclase PleD activates ShkA during G1/S. The above data support a model in which an upshift of c-di-GMP stimulates ShkA kinase activity thereby initiating the G1/S-specific genetic program (Fig. 5a). If so, the G1/S transition should be kick-started by one of the C. crescentus diguanylate cyclases. Screening a spmX-lacZ reporter strain for transposon insertions with reduced lacZ expression identified mutations in pleD.
Accordingly, a ΔpleD deletion strain showed strongly reduced spmX expression (Fig. 5b). While a second diguanylate cyclase enzyme, DgcB, had a more modest effect, spmX promoter activity was almost completely abolished in a ΔpleD ΔdgcB double mutant, akin to a strain lacking c-di-GMP (Fig. 5b). Thus, PleD is the major diguanylate cyclase driving the G1/S-specific transcriptional program.

PleD phosphorylation is regulated by the antagonistic ST cell-specific kinase DivJ and the SW cell-specific phosphatase PleC (Figs. 1a and 5a). However, PleC but not DivJ, was required for spmX expression, and spmX expression was restored to normal levels in a strain lacking both PleC and DivJ (Fig. 5b). This indicated that neither DivJ nor PleC is responsible for the initial activation of PleD and for ShkA stimulation, and that the role of PleC is likely indirect. PleC phosphatase was previously shown to reduce DivK phosphorylation leading to the activation of CtrA. Because tacA is a direct target of CtrA, it was proposed that in a ΔpleC mutant, spmX expression is impaired because TacA fails to accumulate. Expression of tacA and spmX indeed required the PleC phosphatase (Fig. 5c, d). However, when the pleC deletion was combined with the constitutive shkA<sub>DD69N</sub> allele, SpmX protein levels and polar localization of DivJ, but not tacA expression, were fully restored (Fig. 5c, e; Supplementary Fig. 9), arguing that TacA levels are not the limiting factor in cells lacking PleC. Rather, ShkA activity and its stimulation by c-di-GMP are switched off in the ΔpleC mutant. Indeed, a constitutively active variant of PleD, PleD<sup>d</sup>, restored SpmX protein levels in the ΔpleC mutant, an effect that was entirely dependent on ShkA (Fig. 5f). These results suggest that c-di-GMP levels are limiting in a ΔpleC mutant. In line with this, LC-MS measurements showed that the ΔpleC mutant has significantly reduced c-di-GMP levels as compared with the isogenic pleC<sup>+</sup> strain (9.5 ± 1.3 µM vs. 16.3 ± 0.5 µM; N = 3).

The finding that the ShkA-TacA pathway is OFF in the ΔpleC mutant because c-di-GMP concentrations are limiting, together with the observation that PleD serves as the main c-di-GMP donor for ShkA activation, argues for the existence of an as yet unidentified PleD kinase, the expression of which will likely depend on the Ple-C-CckA-CtrA cascade. We speculate that activation of this kinase and its downstream target PleD represents a key event in the decision of C. crescentus to exit G1 (Fig. 5a).

**Discussion**

*C. crescentus* SW cells are born with low levels of c-di-GMP<sup>11,20</sup>. This is imposed by two cell type-specific regulators, the phosphodiesterases PdeA<sup>21</sup> and the phosphatase PleC, which maintains the diguanylate cyclase PleD in its inactive, unphosphorylated form<sup>8</sup> (Fig. 5a). During the G1/S transition, PdeA is proteolytically removed<sup>22</sup> and PleD is activated by phosphorylation. This results in a gradual increase of c-di-GMP<sup>11,20</sup>, which leads to a series of accurately timed events prompting exit from G1, cell morphogenesis and entry into S-phase. First, ShkA is allosterically activated resulting in TacA phosphorylation and the expression of a large group of G1/S-specific genes that orchestrate the morphological restructuring of the motile SW cells into sessile ST cells<sup>13,14</sup>. At this stage, c-di-GMP levels are high enough to activate ShkA-TacA but have not reached the peak levels needed to trigger the CckA cell cycle switch and S-phase entry<sup>8</sup>. This leads to the execution of the morphogenenic program before cells commit to chromosome replication and division. The next step is then catalyzed by the expression of one of the G1/S-specific proteins, the morphogen SpmX, which is responsible for the polar sequestration and activation of Div<sup>12</sup>. This, in turn, leads to the production of more c-di-GMP via reinforced activation of PleD and, together with DivJ-mediated phosphorylation of DivK, switches CckA into a phosphatase and ultimately licenses replication initiation<sup>7–9</sup> (Fig. 5a). ShkA binds c-di-GMP with five- to tenfold higher affinity than CckA<sup>9,17</sup> explaining how the ShkA-TacA pathway and the CckA switch can be sequentially activated. Thus, at least four kinases form a hierarchical cascade (HK → ShkA → Div → CckA) that is responsible for the accurate temporal control of events during G1/S. The activity and timing of this cascade is coordinated by the second messenger c-di-GMP, a stepwise increase of which enforces consecutive cell cycle steps by modulating the activity of ShkA and CckA, respectively (Fig. 5a).

By contributing to the CckA phosphatase switch via SpmX and DivJ, the ShkA-TacA pathway initiates its own termination. The CckA phosphatase activates a protease adapter cascade that includes CpdR and PopA<sup>16</sup> and that leads to the consecutive degradation of TacA and ShkA by the ClpXP protease (Fig. 5a). This negative feedback constitutes an intrinsic, self-sustained timer that shuts down ShkA-TacA activity as soon as c-di-GMP has reached peak levels required to activate the CckA phosphatase and the PopA protease adapter, thereby irreversibly committing cells to S-phase. Because TacA not only controls genes involved in cell cycle progression but also regulates morphological restructuring of the motile SW cells into sessile ST cells<sup>13,14</sup>, accurate temporal control of this pathway secures the tight coordination between replicative and behavioral processes.

Our results show that the diguanylate cyclase PleD is largely responsible for the c-di-GMP upshift during G1-S transition. We postulate that the initial event leading to PleD activation during G1/S must be executed by a kinase other than DivJ or PleC, and that DivJ is part of a positive feedback loop that reinforces PleD
activity upon S-phase entry (Fig. 5a). Although the nature of this kinase is currently unknown, we speculate that its expression or activity is CtrA-dependent and that it plays a key role in orchestrating exit from G1 as it may not only serve to activate PleD and provide the initial boost of c-di-GMP but may also contribute to the activation of DivK, a factor required for the CckA kinase/phosphatase switch. The essential nature of DivK but not of DivJ, its only known activating kinase, argues for the presence of a non-activated kinase.

We postulate that in its default state, the ShkA kinase is inhibited by the C-terminal REC2 domain and that c-di-GMP binding liberates the kinase by interfering with this off-state conformation. A ShkA variant lacking the REC2 domain is active without c-di-GMP13. Similarly, mutations of residues important for REC2 function lead to constitutive, c-di-GMP-independent autokinase activity. Thus, the conserved DDR motif in the REC1-REC2 linker likely serves to lock ShkA in the inactive state when no c-di-GMP is present. REC2 may be closely tethered to REC1 in the inactive state through an interaction of the DDR linker motif with REC1, a conformation that may prevent the productive interaction of the catalytic CA with the DHp domain. We hypothesize that binding of c-di-GMP to REC1 interferes with this tethering, thereby liberating REC2 and facilitating the productive interaction between CA and DHp for autophosphorylation and eventually for phosphotransfer between DHp and REC2. This model is strongly supported by an accompanying structural analysis of ShkA22.

These findings demonstrate that degenerate REC domains, also called pseudo-receiver domains, can function as docking sites for small regulatory molecules. Hybrid histidine kinases with pseudo-receiver domains, can function as docking sites for small regulatory molecules. Hybrid histidine kinases with pseudo-receiver domains, can function as docking sites for small regulatory molecules.
The product was digested with NdeI/EcoRI and cloned into pQF digested with Asel/EcoRI.

PQF-shkA(R74A): the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 9970/9971. The product was digested with NdeI/EcoRI and cloned into pQF digested with Asel/EcoRI. This shkA allele contains a second mutation, A451V. PQF-shkA(W113A): the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 9906/9907. The product was digested with NdeI/EcoRI and cloned into pQF digested with Asel/EcoRI. This shkA allele contains a second mutation, A451V.

PQF-shkA(D125N): the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 10009/10010. The product was digested with NdeI/EcoRI and cloned into pQF digested with Asel/EcoRI.

PQF-shkA(R128A): the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 10005/10006. The product was digested with NdeI/EcoRI and cloned into pQF digested with Asel/EcoRI.

PQF-shkA(R130A): the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 10007/10008. The product was digested with NdeI/EcoRI and cloned into pQF digested with Asel/EcoRI.

PQF-shkA(R177A): the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 9910/9911. The product was digested with NdeI/EcoRI and cloned into pQF digested with Asel/EcoRI. This shkA allele contains a second mutation, P233L.

PQF-shkA(F230A, F234A): the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 9732/9733. The product was digested with NdeI/EcoRI and cloned into pQF digested with Asel/EcoRI.

PQF-shkA(D129N?): the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 9974/9975. The product was digested with NdeI/EcoRI and cloned into pQF digested with Asel/EcoRI.

PQF-shkA(R324A): the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 9912/9913. The product was digested with NdeI/EcoRI and cloned into pQF digested with Asel/EcoRI.

PQF-shkA(D325N): the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 10013/10014. The product was digested with NdeI/EcoRI and cloned into pQF digested with Asel/EcoRI.

PQF-shkA(C469A): the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 9978/9979. The product was digested with NdeI/EcoRI and cloned into pQF digested with Asel/EcoRI.

Methods
Growth conditions. Caulobacter crescentus was grown in PYE (0.2% [w/v] bacto peptone, 0.1% [w/v] yeast extract, 0.8 mM MgSO4, 0.5 mM CaCl2) or defined M2G (12.2 mM Na2HPO4, 7.8 mM KH2PO4, 9.3 mM NH4Cl, 0.5 mM MgSO4, 0.5 mM CaCl2, 20 µM FeSO4, 0.2% [w/v] g-glucose) medium at 30 °C. Growth in low phosphate medium (PYEtis) was performed by replacing the phosphate buffer components in M2G with 20 mM Tris-HCl (pH 7.0). Standard plates contained 1.5% [w/v] agar. Motility was assayed on PYE plates containing 0.3% agar. Escherichia coli DH5α, DH10B, or TOP10 were used for cloning and were routinely cultivated in LB-Miller at 37 °C. When appropriate, media were supplemented with antibiotics at the following concentrations unless stated otherwise: liquid/solid media for C. crescentus contained 1.5% [w/v] agar. Motility was assayed on PYE plates containing 0.3% agar. Escherichia coli DH5α, DH10B, or TOP10 were used for cloning and were routinely cultivated in LB-Miller at 37 °C. When appropriate, media were supplemented with antibiotics at the following concentrations unless stated otherwise: liquid/solid media for E. coli; in µg ml−1: kanamycin (5/ 20; 30/50), oxytetracycline (2.5/5; 12.5/25), chloramphenicol (1/2; 20/30), gentamycin (1/5; 20/20), spectinomycin (25/50; −/−), streptomycin (5/5; −/−), ampicillin (1/2; 100/100), nalidixic acid (15/20; −/−). Isopropyl-β-D-thiogalactopyranoside (IPTG), 4-hydroxy-3-methoxybenzoic acid (vanillate), and xylose stocks were prepared in ddH2O at concentrations of 1 M, 200 mM, and 20 [w/v], respectively, and used at final concentrations of 300 µM, 250 µM, and 0.2% in C. crescentus, if not otherwise stated. 4-isopropylbenzoic acid (cumarate) stocks (100 mM) were prepared in 100% ethanol and used at a final concentration of 100 µM. Note that for experiments in which shkA alleles were provided on plasmid pQF in trans no cumate was present since already leaky expression of the wild-type allele could complement the ΔshkA null mutant phenotype.

Strains and plasmids. Plasmids are listed in Supplementary Data 2. Oligonucleotides used for plasmid construction were purchased from Sigma and are listed in Supplementary Data 3. Plasmids were constructed as follows:

pAK503: nptII without RBS and start codon was PCR-amplified from pAK405 using primers 8959/9495, the product was digested with BsgI/BssH1 and cloned into pAK501 cut with AsclI/XbaI.
pAK502-2ac: the spmX promoter and part of the codon sequence were PCR-amplified from C. crescentus gDNA using primers 10015/10016, the product was digested with XbaI/KpnI and cloned into pAK502 digested with the same enzymes.
pAK502-scmp: the spmX promoter and part of the codon sequence were PCR-amplified from C. crescentus gDNA using primers 8966/9064, the product was digested with XbaI/KpnI and cloned into pAK502 digested with the same enzymes.
pAK503-scmp: the spmX promoter and part of the codon sequence were PCR-amplified from C. crescentus gDNA using primers 8966/9064, the product was digested with XbaI/KpnI and cloned into pAK503 digested with the same enzymes. pNPTStet: tetA and tetR were PCR-amplified from pQF using primers 9552/9553, the product was digested with SpeI/Ndel and cloned into pNPTSts18 digested with AsclI/XbaI.
pNPTStet-ampG: part of ampG located downstream of shkA was PCR-amplified from C. crescentus gDNA with primers 9554/9555, the product was digested with SpeI/KpnI and cloned into pNPTStet digested with the same enzymes.
pET28a-shkA: shkA was PCR-amplified from C. crescentus gDNA with primers 9745/9746, the product was digested with NdeI/EcoRI and cloned into pET28a digested with the same enzymes.
pET28a-shkA(D369N): shkA(D369N) was amplified from C. crescentus strain U9618 by colony-PCR using primers 9745/9746, the product was digested with NdeI/EcoRI and cloned into pET28a digested with the same enzymes.
PQF-shkA: shkA was PCR-amplified from C. crescentus gDNA with primers 9745/9746, the product was digested with NdeI/EcoRI and cloned into pQF digested with Asel/EcoRI.
PQF-shkA(D369N): shkA(D369N) was amplified from C. crescentus strain U9618 by colony-PCR using primers 9745/9746, the product was digested with NdeI/EcoRI and cloned into pQF digested with Asel/EcoRI.
PQF-shkA(R26A): the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 9904/9905. The product was digested with NdeI/EcoRI and cloned into pQF digested with Asel/EcoRI.
PQF-shkA(H61A): the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 9968/9969.
The product was digested with NdeI/EcoRI and cloned into pQF digested with Ascl/EcoRI. This shkA allele contains a second mutation, A345V.

*pQF-shkA(D369N)*: the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 11177/11178. The product was digested with NdeI/EcoRI and cloned into pQF digested with Ascl/EcoRI.

*pQF-shkA(G337A):* a fragment harboring part of shkA was released from pQF-shkA(Y338A) by digestion with Ascl/PstI and cloned into PET28a-shkA digested with the same enzymes.

*pET28a-shkA(Y338A):* a fragment harboring part of shkA was released from pQF-shkA(Y338A) by digestion with Ascl/PstI and cloned into PET28a-shkA digested with the same enzymes.

*pET28a-shkA(D369N, Y338A):* a fragment harboring part of shkA was released from pQF-shkA(Y338A) by digestion with Ascl/PstI and cloned into PET28a-shkA digested with the same enzymes.

*pET28a-shkA(D369N, R324A):* a fragment harboring part of shkA was released from pQF-shkA(R324A) by digestion with Ascl/PstI and cloned into PET28a-shkA digested with the same enzymes.

*pET28a-shkA(R128A, R130A):* the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 11024/11025. The product was digested with NdeI/EcoRI and cloned into pQF digested with Ascl/EcoRI.

*pQF-shkA(L343A):* the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 11178/11180. The product was digested with NdeI/EcoRI and cloned into pQF digested with Ascl/EcoRI.

*pQF-shkA(I340A):* the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 11167/11168. The product was digested with NdeI/EcoRI and cloned into pQF digested with Ascl/EcoRI.

*pQF-shkA(L338A):* the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 11167/11176. The product was digested with NdeI/EcoRI and cloned into pQF digested with Ascl/EcoRI.

*pQF-shkA(G337A):* the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 1185/1186. The product was digested with NdeI/EcoRI and cloned into pQF digested with Ascl/EcoRI.

*pQF-shkA(Q351A):* the mutant allele was generated by SOE-PCR using pET28a-shkA as template and flanking primers 669/670 and mutagenic primers 11181/11182. The product was digested with NdeI/EcoRI and cloned into pQF digested with Ascl/EcoRI.

*pQF-shkA(H61A):* a fragment harboring part of shkA was released from pQF-shkA(H61A) by digestion with SpeI/PstI and cloned into pQF-shkA(D369N) digested with the same enzymes.

*pQF-shkA(D369N, R128A):* a fragment harboring part of shkA was released from pQF-shkA(R128A) by digestion with SpeI/PstI and cloned into pQF-shkA(D369N) digested with the same enzymes.

*pQF-shkA(D369N, R130A):* a fragment harboring part of shkA was released from pQF-shkA(R130A) by digestion with SpeI/PstI and cloned into pQF-shkA(D369N) digested with the same enzymes.

*pQF-shkA(D369N, R128A, R130A):* a fragment harboring part of shkA was released from pQF-shkA(R128A, R130A) by digestion with SpeI/PstI and cloned into pQF-shkA(D369N) digested with the same enzymes.

*pQF-shkA(D369N, R128A):* a fragment harboring part of shkA was released from pQF-shkA(D369N, R128A) by digestion with SpeI/PstI and cloned into pQF-shkA(D369N) digested with the same enzymes.

*pQF-shkA(D369N, R130A):* a fragment harboring part of shkA was released from pQF-shkA(D369N, R130A) by digestion with SpeI/PstI and cloned into pQF-shkA(D369N) digested with the same enzymes.

*pQF-shkA(D369N, Y338A):* a fragment harboring part of shkA was released from pQF-shkA(D369N, Y338A) by digestion with SpeI/PstI and cloned into pQF-shkA(D369N) digested with the same enzymes.

*pQF-shkA(D369N, R324A):* a fragment harboring part of shkA was released from pQF-shkA(D369N, R324A) by digestion with SpeI/PstI and cloned into pQF-shkA(D369N) digested with the same enzymes.

*pQF-shkA(D369N, R324A):* a fragment harboring part of shkA was released from pQF-shkA(D369N, R324A) by digestion with SpeI/PstI and cloned into pQF-shkA(D369N) digested with the same enzymes.
with primer pairs 3492 and 3493. The PCR product was cloned into pGEM-T Easy, sequenced and cut out with BamHI and KpnI. Both cut PCR products were ligated in a triparental mating using pNPTS138 digested with HindIII and BamHI. pET28a-His-shkA: shkA was PCR-amplified from C. crescentus gDNA with primers 5988/5486, the product was digested with NdeI/HindIII and cloned into pET28a digested with the same enzymes.

pET28a-His-shpA: shpA was PCR-amplified from C. crescentus gDNA with primers 6015/6016, the product was digested with HindIII/EcoRI and cloned into pET28a digested with the same enzymes.

pET28a-His-tacA-RD: the sequence of tacA encoding the receiver domain was PCR-amplified from C. crescentus gDNA with primers 7925/6013, the product was digested with HindIII/EcoRI and cloned into pET28a digested with the same enzymes.

pET28a-His-tacA: the sequence of tacA encoding the receiver domain was PCR-amplified from C. crescentus gDNA with primers 5988/5489. The product was digested with EcoRI/PsiI and ligated into pET28a digested with the same enzymes.

pET28a-His-shkA: the sequence of shkA encoding the REC1 domain was PCR-amplified from C. crescentus gDNA with primers 6101/6011, the product was digested with HindIII/EcoRI and cloned into pET28a digested with the same enzymes.

pET28a-His-shpA: the sequence of shpA encoding the REC2 domain was PCR-amplified from C. crescentus gDNA with primers 6109/6009, the product was digested with HindIII/EcoRI and cloned into pET28a digested with the same enzymes.

pMT687-tacA: tacA was PCR-amplified from C. crescentus gDNA with primers 5487/5484, the product was digested with NdeI/KpnI and cloned into pMT687 digested with the same enzymes.

pMT687-tacA(D54E): the mutant allele was generated by SOE-PCR using C. crescentus gDNA as template and flanking primers 5487/5490 and mutagenic primers 5488/5489. The product was digested with NdeI/KpnI and cloned into pMT687 digested with the same enzymes.

pPRLac290: pRLac290 digested with the same enzymes.

pPRLac290-RI: the promoter region of tacA was PCR-amplified from pRV-PAS295 with primers 9794/9875, the product was digested with HindIII/KpnI and cloned in pMT687 digested with the same enzymes.

pPRLac290-S29A: the promoter region of tacA was PCR-amplified from pPRLac290 digested with the same enzymes.

pPRLac290: pRLac290 digested with the same enzymes.

pPRLac290-staR: the promoter region of staR was PCR-amplified from C. crescentus gDNA with primers 6621/6622. The resulting product was digested with EcoRI/PsiI and ligated into pPRLac290 digested with the same enzymes.

pH100: pUC19 was digested with HindIII and PstI to integrate the following annealed oligos 5786/5903/5904/5905 downstream of the MCS.

pH107: pH107 digested with XbaI and its insert was replaced with the NdeI-PacI cassette of pH100 digested with 7284 and 7287 digested with NdeI/SpeI.

pH109: dendra2 was PCR-amplified from pDLH815 using primers 7503 and 7507, which also introduced a stop codon and the RBS of pQE70 upstream of the tacA promoter region.

pH111: the promoter region of spmX was PCR-amplified from C. crescentus gDNA with primers 6864 and 7512, digested with EcoRI/HindIII and ligated into pH109 digested with the same enzymes.

pH113: dendra2-ssrA was generated by PCR from pDLH815 using primers 7503 and 6478. The resulting product was digested with EcoRI/HindIII and ligated into pH109 digested with the same enzymes.

Plasmid DNA was delivered into C. crescentus by electroporation or triparental mating using L980 as helper strain. qCR30 was used for generalized transduction.

Synchronized cultures. Synchronized cultures were used for the experiments shown in Figs 4b, c, Supplementary Fig. 7, and for the experiments shown in Fig. 3c. Synchronized cultures are described above. Pellets were resuspended in 1X SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 10% [v/v] glycerol, 2% [w/v] SDS, 5% [v/v] β-mercaptoethanol, 0.025% [w/v] bromophenol blue) and boiled for 5–10 min before being loaded on 10–12.5% SDSPAGE or precast Mini-Protein TGE (Biorad) gels. Proteins were transferred from SDS-PAGE gels to PVDF-membranes (BioRad) using the Multiphor II transfer buffer (14.4 g glycine, 20% [v/v] ethanol, ddH2O) using a Biorad semi-dry system (20 V, 30 min. 30 min) or a Biorad wet blot system (80 V, 120 min, 4 °C) (Fig. 3c). For immunoblotting with Phos-tag gels, gels were kept at 4 °C and washed successively for 10 min with transfer buffer containing 10 mM EDTA and transfer buffer containing 0.1% (w/v) SDS, and transfer to PVDF-membranes (Immobilon-P, Millipore, 0.45 µm) was done using a Biorad wet blot system (80 V, 120 min, 4 °C). After transfer, membranes were blocked by incubation with blocking buffer for 1 h at room temperature. Blocking buffer was 1X PBS (137 mM NaCl, 27 mM KCl, 81 mM Na2HPO4, 18 mM KH2PO4) containing 0.1% (w/v) Tween20 and 5% (w/v) skimmed milk powder (for experiments shown in Fig. 3c) or TRST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% [w/v] Tween20) containing 5% skimmed milk powder (all other experiments). After blocking, membranes were incubated with the primary antibody in blocking buffer for 1 h at room temperature or overnight at 4 °C, followed by three washes in blocking buffer (5 min, room temperature) and incubation with the secondary antibody in blocking buffer for 1 h at room temperature. Membranes were washed three to four times with blocking buffer and three times in 1X PBS (for experiments shown in Fig. 3c) before addition of ECL detection reagent (KPL LumiGLO or LumigloGLO, SeraCare Life Sciences). Chemiluminescence was detected using a Fujifilm LAS-4000 Imaging System (Bedssoft) with automatic exposure time determination or by exposure of membranes to X-ray films (Fujifilm). Primary antibodies were used at the following dilutions: a-MreB (1:2000) (a gift from Régis Hallez), α-TacA (1:15000)12, α-SpmX (1:10000)12, α-PleC (1:5000)20, α-CtrA (1:5000)20, M2 α-FLAG (1:10,000) (Sigma). Secondary antibodies HRP-conjugated rabbit anti-mouse and swine anti-rabbit (Dako Cytomation, DK) were used at a 1:10,000 dilution.

Microscopy and image analysis. Cultures were grown in PYE and imaged in exponential phase (OD600 of 0.3–0.4) on 1% agarose PYE pads. Microscopy images were acquired using softWoRx 6.0 (GE Healthcare) on a DeltaVision system (GE Healthcare), equipped with a pco.edge sCMOS camera, and an UPlan FL N 100×/1.30 oil immersion objective (Olympus), Dwis-M-Cherry localization was analyzed using Fiji software package30 with the Microbel plugin31. Oufix2 was used to quantify cell length and data were analyzed in Prism 7 (GraphPad) with statistical testing using ordinary one-way ANOVA and Tukey’s multiple comparison test. SpmX-M-Cherry localization and stalk formation (Supplementary Fig. 1) was analyzed manually.

Time-lapse microscopy and image analysis. For Fig. 4 and Supplementary Fig. 7 spmX promoter activity during the cell cycle of single cells of C. crescentus wild type and selected mutants was analyzed using PspmX-Dendra2 and time-lapse microscopy. Strains were grown overnight in 5 ml PYE supplemented with appropriate antibiotics in a roller drum at 30 °C. On the day of the experiment, the cultures were diluted 50-fold in 5 ml PYE supplemented with 2.5 μg ml−1 oxytetracycline and if appropriate with 0.1 mM IPTG and allowed to grow in a roller drum at 30 °C until they reached an OD600 of 0.2. Cells were plated on 1% PYE medium agar plates and after 24 h incubation the plates were sealed with a cover of parafilm and placed on a base of frames (Thermo Fisher Scientific, 1.7 × 2.8 cm) and subject to time-lapse microscopy using softWoRx 6.0 (GE Healthcare) on a DeltaVision system (GE Healthcare), equipped with a pco.edge sCMOS camera, and an UPlan FL N 100×/1.30 oil objective (Olympus) using 0.3 s exposure time for phase-contrast, FITC and TRITC channels and a frame rate of 1 frame/15 min for different amounts of time.

For Fig. 4b, c, Supplementary Fig. 7, green Dendra2 was irreversibly photobleached to red in the latest detectable predivisional stage 15 min before the
first visible cell separation and birth of the swarmer cell using 2 s UV light (DAPI channel) leading to a conversion efficiency from green to red between 62–75%. Image processing and analysis was done using the Fiji software package30. From each predivisional and its offpring (swarmer and stalked cell) the total cell fluorescence and cell area was manually measured for every time point over the course of one cell cycle and if appropriate for up to three consecutive cell cycles and the integrated density (mean intensity normalised to cell area) determined. In case of growth of the swarmer cell after phototransformation the predivisional was normalised to the area of the swarmer or stalked cell. Integrated density values were then normalised for background fluorescence to obtain normalised fluorescence values over the cell cycle, plotted as arbitrary units (A.U.) in Supplementary Fig. 1c.

In order to determine the fraction of cells with induced spmX promoter activity in Fig. 5c, for individual cells the mean of all normalised fluorescence values over one cell cycle was calculated. If the mean normalised fluorescence was above 150 A.U., cells were scored as showing induced PspmX activity; if below 150 A.U. then cells were scored as non-induced. In Fig. 5g, normalised fluorescence values over the cell cycle of each cell type were set relative to the value of the late predivisional cell after phototransformation (t0 = 1). Green Dendra2 was irreversibly photoconverted to red only once in the latest detectable predivisional stage 15 min before the first visible cell separation and birth of the swarmer cell. Cells were normalized and evaluated as above. The time of the first visible cell separation was determined in the same way (green line) and the stalked cell (gray line) cells with slope ≤0.075 was plotted as fraction of cells with induced spmX promoter activity over time. For inferred OFF kinetics (blue dashed line), cells at different cell cycle stages were photoconverted once using 2 s UV light (DAPI channel), normalized as above and the increase of fluorescence within 15 min intervals was determined using the first time point after visible cell separation as reference. Fraction of cells scored as having induced spmX promoter activity over the cell cycle was plotted over time. Results were plotted using Prism 7 (GraphPad).

β-Galactosidase assays. For β-galactosidase assays strains harboring pAK502-based lacZ reporter plasmids were grown in 2 ml PYE supplemented with chloramphenicol and additional antibiotics as appropriate overnight at 30 °C in a drum roller and diluted the next day 20-fold in 2 ml of the same medium, followed by further incubation for an additional 4.5 h under the same conditions before sampling. β-Galactosidase assays were performed according to Miller33. For β-galactosidase assays strains harboring pRKlac290-based plasmids were grown overnight supplemented with the appropriate antibiotic and diluted 20-fold in 3 ml of PYE supplemented with 20 μg ml−1 carbenicillin and 20 μg ml−1 chloramphenicol at 30 °C. On the day of the experiment, the cultures were diluted 20-fold in 5 ml PYE supplemented with appropriate antibiotics and allowed to grow in a roller drum at 30 °C until they reached an OD600 of 0.3–0.5. 1.8 ml culture was spun and the pellet resuspended in 1.8 ml Z-buffer (0.06 M Na2HPO4, 0.04 M NaH2PO4, 0.5 M NaCl, 0.0025% [v/v] ThoninsX-100) and 0.8 ml were used for OD600 measurements and 1 ml transferred to a chloroform stable Eppendorf tube. Subsequently, 100 μl 0.1% SDS and 20 μl chloroform were added. Samples were vortexed for 10 s and incubated at room temperature for 30–60 min. From the top aqueous layer of the mixture three 200 μl (three technical replicates) were transferred to a 96-well plate, 25 μl ONPG (4 mg ml−1 in Z-buffer) were added to each well and consumption of ONPG was followed at 405 nm using a BioTek Instruments EL800 plate reader (20 reads at the fastest interval), β-galactosidase activity was calculated as the initial slope of the increase of OD600 over one time interval and for the OD600 and volume. Activities were normalized to the activity of a wild-type strain that was included in all assays.

Flow cytometry. Cultures were inoculated from a fresh colony and strains were grown in 2 ml PYE at 30 °C in a drum roller 20 h (stationary phase) or diluted back 20-fold in 2 ml of the same medium after overnight incubation, followed by incubation for an additional 4.5 h (exponential phase). Rifampicin was added to cultures at a final concentration of 25 μg ml−1 and cultures were incubated for 2 h under the same conditions before cells were being fixed in cold 70% ethanol. Cells were collected by centrifugation, resuspended in 0.5 ml FACS buffer containing 1.5 μl YPO-PRO-1 iodide (Thermo Fisher Scientific) and incubated for 2 h at room temperature in the dark. Data were acquired using a FACS Canto II (BD Biosciences) with >50,000 events recorded and analyzed with FlowJo software (FlowJo LLC). The gating strategy is outlined in Supplementary Fig. 10.

Mass spectrometry-based proteome analysis. 103 C. crescentus cells grown in PYE to exponential phase were collected, washed twice with phosphate buffer, resuspended in 50 μl lysis buffer (25 mM Tris-HCl pH 7.5, 1.1 mM EDTA, 50 mM sodium citrate, 0.1% [v/v] TritonX-100) containing 2.5 μl RNaseA solution (Sigma) and incubated at room temperature for 30 min, after which 50 μl were transferred to 1 ml FACS buffer containing 1.5 μl YPO-PRO-1 iodide (Thermo Fisher Scientific) and incubated for 2 h at room temperature in the dark. Data were acquired using a FACS Canto II (BD Biosciences) with >50,000 events recorded and analyzed with FlowJo software (FlowJo LLC). The gating strategy is outlined in Supplementary Fig. 10.

β-Galactosidase assays. For β-galactosidase assays strains harboring pAK502-based lacZ reporter plasmids were grown in 2 ml PYE supplemented with chloramphenicol and additional antibiotics as appropriate overnight at 30 °C in a drum roller and diluted the next day 20-fold in 2 ml of the same medium, followed by further incubation for an additional 4.5 h under the same conditions before sampling. β-Galactosidase assays were performed according to Miller33. For β-galactosidase assays strains harboring pRKlac290-based plasmids were grown overnight supplemented with the appropriate antibiotic and diluted 20-fold in 3 ml of PYE supplemented with 20 μg ml−1 carbenicillin and 20 μg ml−1 chloramphenicol at 30 °C. On the day of the experiment, the cultures were diluted 20-fold in 5 ml PYE supplemented with appropriate antibiotics and allowed to grow in a roller drum at 30 °C until they reached an OD600 of 0.3–0.5. 1.8 ml culture was spun and the pellet resuspended in 1.8 ml Z-buffer (0.06 M Na2HPO4, 0.04 M NaH2PO4, 0.5 M NaCl, 0.0025% [v/v] ThoninsX-100) and 0.8 ml were used for OD600 measurements and 1 ml transferred to a chloroform stable Eppendorf tube. Subsequently, 100 μl 0.1% SDS and 20 μl chloroform were added. Samples were vortexed for 10 s and incubated at room temperature for 30–60 min. From the top aqueous layer of the mixture three 200 μl (three technical replicates) were transferred to a 96-well plate, 25 μl ONPG (4 mg ml−1 in Z-buffer) were added to each well and consumption of ONPG was followed at 405 nm using a BioTek Instruments EL800 plate reader (20 reads at the fastest interval), β-galactosidase activity was calculated as the initial slope of the increase of OD600 over one time interval and for the OD600 and volume. Activities were normalized to the activity of a wild-type strain that was included in all assays.

Flow cytometry. Cultures were inoculated from a fresh colony and strains were grown in 2 ml PYE at 30 °C in a drum roller 20 h (stationary phase) or diluted back 20-fold in 2 ml of the same medium after overnight incubation, followed by incubation for an additional 4.5 h (exponential phase). Rifampicin was added to cultures at a final concentration of 25 μg ml−1 and cultures were incubated for 2 h under the same conditions before cells were being fixed in cold 70% ethanol. Cells were collected by centrifugation, resuspended in 0.5 ml FACS buffer containing 1.5 μl YPO-PRO-1 iodide (Thermo Fisher Scientific) and incubated for 2 h at room temperature in the dark. Data were acquired using a FACS Canto II (BD Biosciences) with >50,000 events recorded and analyzed with FlowJo software (FlowJo LLC). The gating strategy is outlined in Supplementary Fig. 10.

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primer 9058. In one strain (AKS50), the transposon mapped within ORIGIN at position 2 695 825 of the NA1000 reference genome37. **Protein expression and purification.** For isothermal titration calorimetry (ITC) and in vitro phosphorylation experiments shown in Fig. 2, proteins were expressed and purified as follows. E. coli Rosetta 2(DE3) cells were used to express proteins from pET28a and pET32b expression plasmids. Cells were grown in LB-Miller supplemented with the appropriate antibiotics to an OD<sub>600</sub> of 0.4 for 4 h at 30°C. Proteins were purified on an AKTA-purifier 10 system (GE Healthcare) using 1 ml HisTrap HP columns (GE Healthcare) followed by size exclusion chromatography (HiLoad 16/60 Superdex 200) using the following buffers: lysate buffer (wash buffer supplemented with protease inhibitor, Dnase 1 [NEB]), wash buffer (20 mM HEPES-KOH pH 8.0, 0.5 M NaCl, 10% glycerol, 20 mM imidazole, 1 mM DTT), elution buffer (20 mM HEPES-KOH pH 8.0, 500 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DTT), storage buffer (20 mM HEPES-KOH pH 8.0, 50 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM DTT), MgCl₂ was added to reaction mixtures immediately prior to experiments to a final concentration of 5 mM. For all other experiments using purified ShkA, mutant variants thereof and ShkAREC₁, except NMR experiments (see below), proteins were expressed in E. coli BL21(DE3) grown in LB-Miller at 37°C in 500-ml cultures with IPTG induction (1 mM) at an OD<sub>600</sub> of 0.5-0.8 followed by incubation for 4 h. Cells were harvested by centrifugation (5000 x g, 20 min, 4°C), washed once with 20 ml of 1X PBS, flash-frozen in liquid N<sub>2</sub> and stored at −80°C until purification. For purification, the pellet was resuspended in 8 ml of buffer A (2X PBS containing 500 mM NaCl, 20 mM β-mercaptoethanol, 2 mM imidazole) supplemented with DnaseI (AppliChem) and Complete Protease inhibitor (Roche). After one passage through a French press cell, the supernatant was ultra-centrifuged (100,000 x g, 30 min, 4°C) and the supernatant was mixed with 800 µl of Ni-NTA slurry, flash-frozen with buffer A, and incubated for 1-2 h on a rotary wheel at 4°C. Ni-NTA agarose was loaded on a polystyrene column and washed with buffer A, followed by wash buffer B, and then buffer A, after which protein was eluted with 2.5 ml of buffer A containing 500 mM imidazole. The eluate was immediately loaded on a PD-10 column pre-equilibrated with kinase buffer (10 mM HEPES-KOH pH 8.0, 50 mM KCl, 10% glycerol, 0.1 mM EDTA, 5 mM MgCl₂, 5 mM β-mercaptoethanol). The protein was then eluted with 3.5 ml of kinase buffer further used, unless otherwise mentioned. Additional nucleotides were added as indicated in the figures or figure legends. Kinase reactions contained 5 µM ShkA and were run for 3.5 min at room temperature if not otherwise mentioned and stopped by addition of 5X SDS sample buffer and stored on ice, then run on 12.5% SDS-PAGE or precast Mini-Preoan TGX (Biorad) gels. Gel colors were developed using a phosphor screen for 0.5-3 h and then scanned using a Typhoon FLA 7000 imaging system (GE Healthcare), after which gels were stained with Coomassie Brilliant Blue.

**Nuclear magnetic resonance (NMR) spectroscopy.** All NMR spectra were recorded at 25°C on a Bruker Avance-700 spectrometer equipped with a cryogenically cooled triple-resonance probe. ShkA<sub>REC₁</sub> protein samples were prepared in 25 mM Tris pH 7.2 with 50 mM KCl and 2 mM MgSO₄ in 95%/5% H₂O/D₂O. For the sequence-specific backbone resonance assignment of 950 µM [13C,15N]-ShkA<sub>REC₁</sub>, the following NMR experiments were recorded: 2D [15N, 13C]-TROSY, 2D [15N, 31P]-TROSY, 3D HNCA and 3D HNCACB. For the c-di-GMP binding experiments a series of 2D [13C,15N]-TROSY spectra of 200 µM [U-13C, 15N]-ShkA<sub>REC₁</sub> were recorded with c-di-GMP concentrations of 0 µM, 20 µM, 50 µM, 100 µM, 150 µM, 200 µM and 400 µM. CSPs (ΔΔHN) of amide moieties were calculated as ΔΔHN = (δ - δ<sub>ref</sub>) - 0.2 · Δδ<sub>15N</sub> - 0.2 · Δδ<sub>13C</sub> where Δδ<sub>15N</sub> (Δδ<sub>13C</sub>) are the amide proton and amide nitrogen chemical shift differences to the reference spectrum, respectively. Combined secondary chemical shifts of Ca and Cβ were calculated relative to the random-coil values of Kjaergaard and Poulson39.

**C-di-GMP extraction and quantifications.** Strains AKS371 and AKS412 were grown in 2 ml PYE overnight, diluted 40-fold the next day in 20 ml PYE and grown for 6 h until reaching an OD<sub>600</sub> of ~0.35. 14 ml were spun down (11,000 × g, 5 min, 4°C), the pellet was washed once in 1 ml dH₂O (16,000 × g, 2 min, 4°C) and snap-frozen in liquid nitrogen. Metabolites were extracted by resuspending the sample stock and lyophilizing the ice-cold extraction solvent. The lyophilized residue was extracted with 200 µl of a mixture of acetone (40% [v/v]) acetonitrile, 40% [v/v] methanol, 20% [v/v] dH₂O and incubation at 95°C for 10 min. Extrakts were passed on the a column with 200 µl extraction solvent and the pooled extracts (700 µl) were stored overnight at −80°C. Remaining debris was removed by centrifugation (16,000 × g, 10 min, 4°C), the supernatant was transferred to a fresh microcentrifuge tube and solvent was removed using a SpeedVac. LC-MS-based c-di-GMP quantification was performed by the Zentrale Forschungseinrichtung (ZFA) Metabolomics, Medizinische Hochschule Hannover, Germany, and cellular concentrations were calculated as described before42. Experiments were performed in biological triplicates and values given are means and standard deviations.

**Sequence alignments, structure prediction, and modeling.** The C. crescentus ShkA sequence was blasted against the nonredundant protein sequences (nr) database using default settings and the first 500 hits were aligned in Geneious using the Geneious Alignment algorithm with default settings (Supplementary Data 1). The alignment was manually checked for the presence of the DDR motif in the REC1-REC2 linker and the sequences that harbored this motif (see Fig. S3A) were submitted to the Biological Magnetic Resonance Data Bank under the following accession code: 27768 [http://www.bmrb.wisc.edu/data_library/summary/index.php?aid=27768]. All proteomics raw data and results associated with the manuscript were deposited with the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD012739 [https://www.ebi.ac.uk/pride/archive/projects/PXD012739]. The source data underlying Figs. 1d, 2c, 3c, e and b-5 and Supplementary Figs. 2a, 2c, e, 4b and 8a are provided as Source Data files. The source data underlying Fig. 4c and Supplementary Fig. 7c are deposited on Zenodo [https://doi.org/10.5281/zenodo.3574247].
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Additional information

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