

In-phase oscillation of global regulons is orchestrated by a pole-specific organizer

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Cell fate determination in the asymmetric bacterium *Caulobacter crescentus* (*Caulobacter*) is triggered by the localization of the developmental regulator SpmX to the old (stalked) cell pole during the G1→S transition. Although SpmX is required to localize and activate the cell fate-determining kinase DivJ at the stalked pole in *Caulobacter*, in cousins such as *Asticcacaulis*, SpmX directs organelle (stalk) positioning and possibly other functions. We define the conserved σ^{54} -dependent transcriptional activator TacA as a global regulator in *Caulobacter* whose activation by phosphorylation is indirectly down-regulated by SpmX. Using a combination of forward genetics and cytological screening, we uncover a previously uncharacterized and polarized component (SpmY) of the TacA phosphorylation control system, and we show that SpmY function and localization are conserved. Thus, SpmX organizes a site-specific, ancestral, and multifunctional regulatory hub integrating the in-phase oscillation of two global transcriptional regulators, CtrA (the master cell cycle transcriptional regulator A) and TacA, that perform important cell cycle functions.

cell cycle | *Caulobacter crescentus* | σ^{54} | SpmX | α -proteobacteria

Akin to the stem cell division of eukaryotes, the bacterium *Caulobacter crescentus* divides asymmetrically during each cell division cycle into progenies with distinct developmental and replicative fates. The motile swarmer cell progeny is characterized by the presence of a polar flagellum and pili, whereas the sessile stalked cell progeny is characterized by the presence of a polar stalk, which is a tubular extension of the cell envelope. The former is replication incompetent (naïve), residing temporarily in a G1-like phase. To enter S-phase and initiate replication, this cell must undergo an obligate differentiation into the replicative stalked cell (1).

Underlying the cellular asymmetry is the unequal activation (phosphorylation) of the cell fate determinant, DivK, at the poles of the predivisional cell. Although the DivJ kinase phosphorylates DivK (DivK~P) at the old (stalked) cell pole, the phosphate is again removed by the PleC phosphatase at the new (swarmer) pole (2–4). Concomitant with the G1→S transition, the PleC-bearing swarmer pole is remodeled into a stalked pole, and polar PleC is substituted with DivJ. Thus, perturbations in the spatiotemporal dynamics of this system lead to alterations in the relative DivK~P levels and a commensurate cell fate dysfunction (5). Such perturbations occur when the localization factor SpmX is inactivated (5). SpmX localizes to the stalked pole during the G1→S transition and recruits DivJ to this site, enhancing its activity and therefore promoting DivK~P levels (Fig. 1A and B). In the absence of SpmX, DivJ is delocalized and poorly active, and DivK~P levels are reduced (5).

A known output of DivK activity is the master cell cycle transcriptional regulator A (CtrA) (1, 6–10). CtrA, an OmpR-like DNA-binding response regulator, oscillates during cell cycle: it is present in G1-phase, degraded at the G1→S transition, and is resynthesized later in the S-phase (11) (Fig. 1A). CtrA is essential for viability, and on phosphorylation by the essential cell cycle kinase, CckA, phosphorylated CtrA (CtrA~P) activates a plethora of developmental and cell division genes (12–14). It also acts negatively on the initiation of DNA replication by binding to the

origin of replication and restricting DNA replication to the correct daughter cell (15, 16). DivK~P down-regulates the accumulation of CtrA~P (1, 6–10) and thus CtrA-dependent functions and target genes.

Genome-wide transcription studies revealed that CtrA only regulates a subset of the cell cycle regulated genes at the level of transcription initiation (13, 14, 17, 18), indicating that additional transcription factors reinforce transcript oscillation during the cell cycle (19). TacA is a conserved DNA-binding response regulator that oscillates in-phase with CtrA. TacA is present in G1 cells, cleared during the G1→S transition, and resynthesized in (late) predivisional cells (5). This oscillation of TacA underlies a combination of cell cycle-regulated transcription and proteolysis (5, 20). Remarkably, the transcription of TacA is regulated directly by CtrA (5, 13), and both CtrA and TacA stability are controlled by the same protease (ClpXP; Fig. 1B), but at different levels in the regulatory hierarchy of proteolysis due to distinct cofactor dependencies (20).

TacA is also regulated at the level of phosphorylation by the histidine kinase/response regulator hybrid ShkA and the phosphotransfer protein ShpA (21). The active and phosphorylated form of TacA (TacA~P) controls stalk biogenesis, by acting on a subclass of σ^{54} -dependent genes (17, 22). Although the TacA regulon remains uncharted on a genome-wide scale, it is known

Significance

Although several studies have pointed towards the importance of the sigma factor, σ^{54} , in regulating virulence, biofilm formation, and cell cycle control in α -proteobacteria, knowledge on its activators and their regulation is incomplete. In this study, we demonstrate that the activity of a highly conserved σ^{54} -activator, TacA, is spatiotemporally coordinated with that of the master cell cycle transcriptional regulator A (CtrA) in *Caulobacter crescentus*. Remarkably, we find that the polar organizer/morphogen, SpmX, governs the in-phase oscillation of CtrA, via the cell fate-determining kinase DivJ, and TacA via a newly identified and conserved determinant, SpmY, which is recruited to the poles through SpmX. Most importantly, we show that the DUF2336 domain of SpmY is functionally conserved among the α -proteobacteria, revealing a possibly conserved mechanism to regulate TacA.

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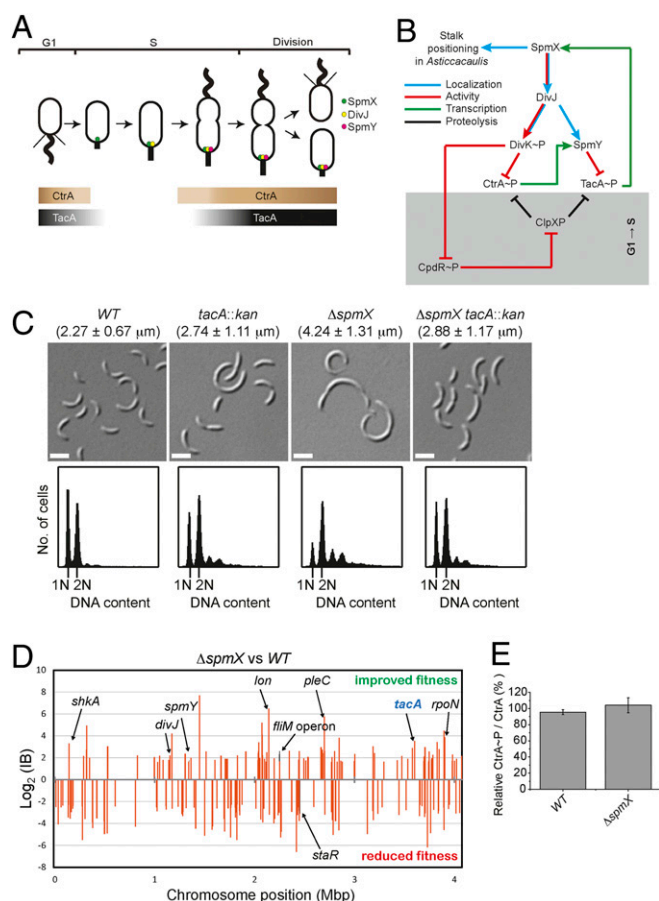


Fig. 1. Effects of TacA in the absence of SpmX. (A) Graphical representation of the subcellular localization of developmental regulatory factors SpmX, DivJ, and SpmY. At the initiation of the S-phase, SpmX (green) localizes to the stalked pole and recruits DivJ (yellow), which later recruits the newly discovered SpmY (magenta) to the same subcellular position. The graded bars indicate the time during which CtrA (brown) and TacA (black) are present during the cell cycle. (B) SpmX on localization to the stalked pole recruits and activates DivJ, which on activation initiates the phosphorylation of DivK (DivK~P). The DivK~P in turn indirectly inhibits the phosphorylation and activation of CtrA (CtrA~P). Additionally, DivK~P also inhibits the abundance of CtrA and TacA by activating the protease ClpXP through the dephosphorylation of CpdR, which happens coincident with the G1→S transition, resulting in the clearance of CtrA and TacA in the early S-phase cells. From this study, we show that the stalked-pole localized SpmX inhibits the activity of TacA through SpmY, and the localization of SpmY to the stalked-pole is dependent on the localization of DivJ by SpmX. In another related α -proteobacteria, *Asticcacaulis*, SpmX regulates the biogenesis and positioning of the stalk. (C) Differential interference contrast (DIC) micrographs and flow cytometry profiles of WT, the *tacA* mutant (*tacA::kan*), *spmX* mutant (Δ *spmX*), and *tacA spmX* double mutant (Δ *spmX tacA::kan*). Mean cell size \pm SD of at least 200 cells is given at the top of the image. (Scale bar, 2 μ m.) (D) Log₂ representation of the Tn-Seq profiles in Δ *spmX* vs. WT. See Dataset S1 for complete list and values. (E) Measurement of CtrA~P/CtrA levels in WT and Δ *spmX* cells. The values \pm SE are the average of three independent experiments.

that TacA targets two promoters directly, those driving expression of the stalk biogenesis regulator, StaR (21, 23), and SpmX (5).

Interestingly, in addition to its role in DivJ localization in *Caulobacter*, SpmX plays an instructive role in stalk placement at the subpolar or bilateral sites in the genus *Asticcacaulis* (24), showing that SpmX is multifunctional. Herein, we describe an additional and conserved regulatory role for SpmX. We identify an uncharacterized DUF2336 domain protein, SpmY, that depends on SpmX for localization to the *Caulobacter* stalked pole.

Moreover, we show that TacA is a global transcriptional regulator whose activity is curbed by SpmY and SpmX. Thus, SpmX emerges as a multifunctional polar organizer that controls two oscillating global regulators, CtrA and TacA, that reprogram transcription in the same cell cycle phase.

Results

TacA Activity Is Deregulated in Δ *spmX* Cells. Mutations in the DivJ/K phosphorylation pathway lead to an accumulation of G1 phase cells due to an increase in CtrA activity (7, 25). Surprisingly, no commensurate effect was obtained by the Δ *spmX* mutation that impairs DivJ/K phosphorylation. In fact, FACS analysis revealed a relative increase in G2 cells (2N chromosome) over G1 cells (1N chromosome) in the Δ *spmX* population compared with WT (Fig. 1C). Moreover in Δ *spmX* cells, LacZ-based promoter probe assays failed to reveal major changes on the activity of CtrA-regulated promoters, such as the *pilA* promoter (*P_{pilA}*) (Fig. S1A), a robust marker to report perturbations in CtrA~P via the DivJ/K pathway. To confirm this result, we determined the relative CtrA~P:CtrA levels in WT and Δ *spmX* cells by in vivo phosphorylation analysis (Fig. 1E) and found no measurable difference in the ratio. Because loss-of-function mutations in DivJ/K are known to elevate CtrA~P levels (21, 26), we speculated that the strong effects of SpmX on the cell cycle likely stems from a regulatory pathway other than CtrA.

To identify which regulatory pathway is perturbed in Δ *spmX* cells, we conducted comprehensive comparative transposon (*Tn*) deep-sequencing (Tn-Seq) analysis to uncover *Tn* insertions that confer improved competitive fitness to Δ *spmX* cells (Fig. 1D, Fig. S1B, and Dataset S1) and are thus overrepresented in Δ *spmX* vs. WT cells. This comparative analysis revealed that *Tn* insertions in the gene (*tacA*) encoding the poorly characterized σ^{54} -dependent activator TacA, or in genes such as *shkA*, *rpoN*, and *pleC* known to be required for TacA activity or TacA expression (5, 21), were overrepresented in Δ *spmX* vs. WT cells (Fig. 1D). This result suggested that high TacA activity leads to adverse effects in SpmX-deficient cells and that mutations in these genes mitigate these problems. These genes encode the kinase (ShkA) controlling TacA phosphorylation (27), the σ^{54} -component of RNA polymerase (RpoN) (17), or the PleC phosphatase that promotes TacA expression (28). We validated the Tn-Seq data in competition experiments with pairs of strains to determine whether a mutation either improves or reduces the fitness of Δ *spmX* cells relative to WT (Fig. S1B).

To confirm that inactivation of *tacA* (*tacA::kan*) ameliorates the Δ *spmX* defects, we imaged WT, *tacA::kan*, Δ *spmX*, and Δ *spmX tacA::kan* cells by differential interference contrast (DIC) microscopy and observed a reduction in the cell filamentation (Fig. 1C). Moreover, FACS analysis revealed a balanced G1:G2 cell (1N:2N chromosome) ratio in the Δ *spmX tacA::kan* double mutant vs. the Δ *spmX* single mutant (Fig. 1C). The slight filamentation of the Δ *spmX tacA::kan* double mutant and a *tacA* single mutant may be attributed to the effects due to the complete removal of TacA or to the effects on CtrA, through SpmX-dependent regulation of the DivJ-DivK pathway in *tacA* mutant cells. Ectopic expression of TacA from a vanillate inducible promoter on a plasmid rescued the developmental defects of the *tacA* single mutant, the balanced G1:G2 ratio was lost, and the cell filamentation increased when TacA was expressed in the Δ *spmX tacA::kan* double mutant (Fig. S1C and D). These results indicated that deregulated TacA abundance or activity contributes to the negative developmental effects in the Δ *spmX* cells via the TacA regulon.

SpmX Regulates TacA Activity. As the TacA regulon is largely unknown, apart from a few selected target promoters that were identified as TacA targets in vivo by quantitative chromatin immunoprecipitation (qChIP) (5, 21), we turned to ChIP-Seq (ChIP deep sequencing) using polyclonal antibodies to TacA to chart all TacA target sites on the *Caulobacter* genome (Fig. 24 and Dataset

S2). From the ChIP-Seq experiment, we found that TacA binds more than 125 putative target promoters (Dataset S2). This experiment also validated the previously established direct targets of TacA, including the promoters of the *staR* transcriptional regulator gene and of *spmX* (5, 18, 27), and MEME analyses predicted a consensus motif of (inverted) dyad symmetry [5'-tTCgCct-(N)₃-agGcGAa-3'] for TacA target promoters (Fig. 2B).

Having validated the *spmX* promoter (P_{spmX}) as a direct TacA target on a genome-wide scale and knowing that σ^{54} and TacA are required for activation of the P_{spmX} -*lacZ* promoter probe construct (5), we interrogated P_{spmX} -*lacZ* activity in Δ *spmX* cells and found it to be elevated compared with *WT* cells (150% of *WT* activity; Fig. 2C), and, as expected, this increase was dependent on TacA (Fig. S2A). Nevertheless, immunoblotting did not reveal any changes in TacA steady-state levels in *WT* vs. Δ *spmX* cells, indicating that the activity rather than the level of TacA is responsible for the increase in P_{spmX} activity (5) (Fig. 2D). By contrast, impairing TacA expression by the Δ *pleC* mutation (which curbs many CtrA-activated promoters, including the *tacA* promoter) resulted in reduction of P_{spmX} -*lacZ* activity to 10% of *WT* activity (Fig. 2C and D) as reported before (5, 26).

To test whether the levels of active TacA~P are altered in Δ *spmX* vs. *WT* cells, we conducted in vivo phosphorylation analyses of TacA using anti-TacA antibodies. We indeed observed that TacA~P levels are higher in the Δ *spmX* mutant than the *WT* cells (Fig. 2E). In further support of the conclusion that elevated TacA~P causes the increased P_{spmX} -*lacZ* activity in Δ *spmX* cells, we found that overexpression of the phosphomimetic (constitutively active) version of TacA [TacA(D54E)] (21) suffices to induce P_{spmX} -*lacZ* activity in the *WT* background (Fig. S2B). Together these results indicate that SpmX negatively regulates the activity of TacA and that TacA~P levels are elevated in Δ *spmX* cells.

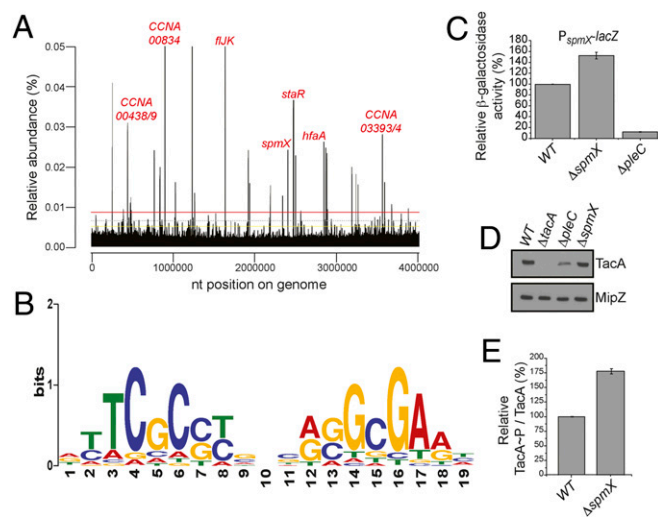


Fig. 2. TacA is a global transcriptional regulator controlled by SpmX. (A) Genome-wide occupancy of TacA on *Caulobacter* genome derived from ChIP-Seq using antibodies to TacA. The plot denotes the relative abundance of reads on each probe at various nucleotide (nt) positions on the genome. The horizontal red line denotes the cutoff applied to derive the TacA bound probes from the background. The complete list and the list of top targets are given in Dataset S2. (B) The deduced binding motif of TacA in *Caulobacter* as derived from the analysis of TacA bound probes from A using MEME. (C) The relative β -galactosidase activity (in percentage) of the P_{spmX} -*lacZ* reporter in *WT*, Δ *spmX*, and Δ *pleC* cells. (D) Immunoblot analyses showing the levels of TacA protein in *WT*, Δ *tacA*, Δ *pleC*, and Δ *spmX* cells. MipZ was used as the loading control. (E) Measurement of TacA~P/TacA levels in *WT* and Δ *spmX* cells. The values \pm SE in C and E are the average of at least three independent experiments.

A Genetic Screen Identifies a SpmX-Dependent Regulator. If SpmX curbs TacA~P levels through an unknown regulator, then deletion of such a regulator should yield a phenotype similar to that of the Δ *spmX* mutant. Therefore, we designed a genetic screen using a TacA-dependent reporter to identify additional developmental regulators that may be under the control of SpmX. The production of the stalk-specific protein StpX (23) seemed a suitable indirect proxy for this (Fig. 3A), as StpX is not known to interfere with cell cycle or developmental processes in *Caulobacter*. StpX is under the control of the transcriptional regulator StaR (18), which in turn is directly activated at the level of transcription by TacA (5, 27). To test the suitability of StpX expression as a proxy, we measured the activity of the *stpX* promoter (P_{stpX}), using a P_{stpX} -*lacZ* fusion in Δ *spmX* cells and found it to be elevated (Fig. S3A). Moreover, immunoblotting with antibodies to StpX revealed a higher abundance of StpX in the Δ *spmX* cells compared with *WT* (Fig. 3B). Having validated the effect of a Δ *spmX* mutation on StpX abundance, we used a *stpX-gfp* strain, producing StpX-GFP from the native chromosomal locus (*stpX::stpX-gfp*), as a reporter to probe for Tn5 mutations that cause an increase in StpX-GFP fluorescence. We uncovered a Tn5 mutation in CCNA_01280 (henceforth referred to as *spmY*; Fig. 3C) with the desired properties (elevated StpX-GFP levels and fluorescence). The *spmY* gene is predicted to encode a 361-residue protein with a domain of unknown function (DUF2336; Fig. 3C) that is highly conserved among the α -proteobacteria. Next, we engineered an in-frame deletion in *spmY* (Δ *spmY*) and found the resulting Δ *spmY* mutation to phenocopy the *spmY::*Tn5 transposon mutation (Fig. 3C and D). Expression of *spmY* from the xylose-inducible promoter (P_{xyl}) at the chromosomal *xylX* locus (*xylX::P_{xyl}-spmY*) rescued the phenotype of Δ *spmY* mutant (Fig. S3B). The Δ *spmY* mutant phenocopied the Δ *spmX* mutant with aberrant stalks, a decrease in G1 cells (1N chromosome), and the defect in swarming ability (Fig. 3D and E and Fig. S3D and F). Next we analyzed if the activity of TacA is increased in the Δ *spmY* mutant using the P_{spmX} -*lacZ* reporter assay as a readout. This analysis revealed an increase in P_{spmX} -*lacZ* activity in Δ *spmY* cells resembling that of Δ *spmX* cells (Fig. 3F), indicating that the TacA activity is indeed high in the Δ *spmY* mutant. Moreover, the P_{stpX} activity and StpX protein abundance were high in the Δ *spmY* single mutant and the Δ *spmX* Δ *spmY* double mutant (Fig. 3B and Fig. S3A) without affecting TacA protein levels (Fig. S3E). Finally, *tacA* inactivation in the Δ *spmY* background (Δ *spmY* *tacA::kan*) restored the 1N:2N chromosome ratio (Fig. S3C).

TacA overexpression, or SpmX or SpmY inactivation, impairs motility of *WT* cells (Fig. 3E and Fig. S3G) (5). If SpmX and SpmY act in the same pathway, they should exhibit common epistatic relationships. To explore this, we resorted to motility suppressor genetics, reasoning that a Δ *spmX* motility suppressor should also restore motility of Δ *spmY* cells. We isolated a motility suppressor of Δ *spmX* cells (*Materials and Methods*) and indeed found that this allele also improved the swarming motility of Δ *spmY* cells (Fig. 3E). Interestingly, the suppressor allele, *ctrA*(T170A), was identified previously as a gain-of-function mutation that promotes the expression of motility functions and augments the activity of many CtrA-activated promoters, presumably by enhancing CtrA's ability to compete against the negative regulators acting on the same promoters (13). Consistent with this, we conducted LacZ-based promoter-probe assays of CtrA-activated reporters in Δ *spmX* *ctrA*(T170A) double mutant cells vs. Δ *spmX* or *WT* cells and found that CtrA activity indeed was elevated (Fig. S1A). Moreover, comparative ChIP-Seq analysis using antibodies to CtrA also revealed that CtrA(T170A) occupancy at target promoters is increased compared with *WT* CtrA in Δ *spmX* cells (Fig. 3G and Datasets S3-S5). This comparison not only revealed an increase in CtrA occupancy at the promoters of several class II

flagellar genes such as *fliL* and *fliO*, the flagellar regulatory genes *flbT* and *flaF*, and the chemotaxis genes *motA*, *motB*, and *cheW*, but also that of *spmY* (*P_{spmY}*) itself (Fig. 3 *G* and *H*).

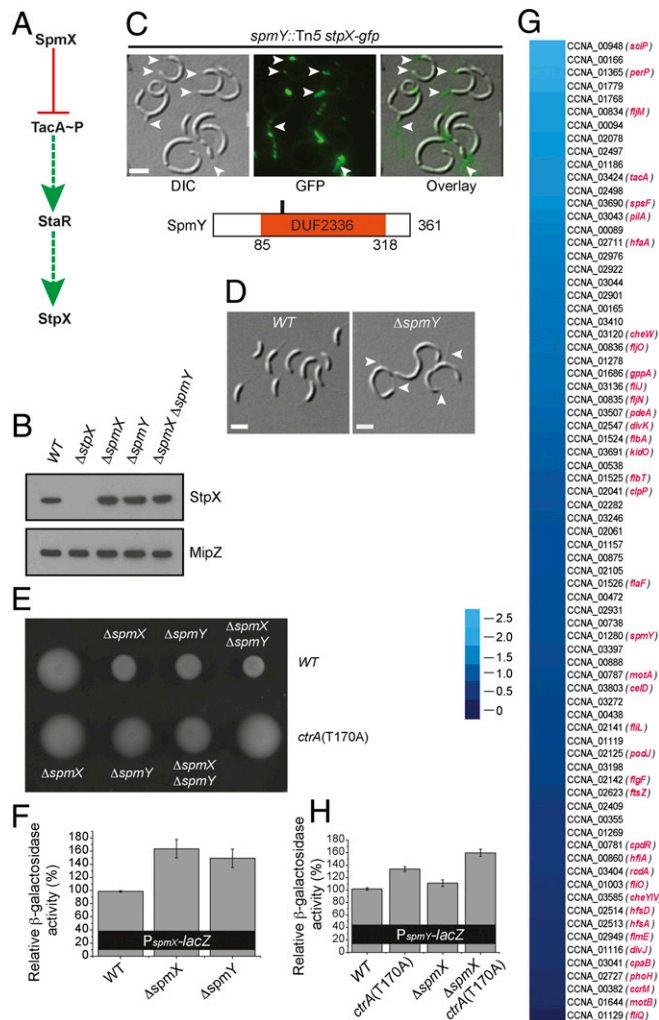


Fig. 3. SpmX regulates TacA activity through SpmY. (A) Schematic summarizing the regulation of the production of StpX. TacA activates the transcription of StaR that in turn activates the transcription of StpX. The activity of TacA is under the control of SpmX. Bold red line, negative posttranslational regulation; dashed green line, positive transcriptional regulation. (B) Immunoblot analyses showing the levels of the StpX protein in WT, Δ spmX, Δ spmY, and Δ spmX Δ spmY cells. MipZ was used as the loading control. (C) DIC and fluorescence micrographs showing the ectopic stalks (arrowheads) and the localization of StpX-GFP in these ectopic stalks in cells harboring a transposon mutation in *spmY* (*spmY::Tn5*) and producing *stpX-gfp* from the native *stpX* promoter. The block diagram shows the domain organization of the SpmY protein. The DUF2335 spans from amino acids 85–318. Black vertical line denotes the position of Tn5 insertion in the strain *spmY::Tn5*. (D) DIC microscopy images of WT and Δ spmY *Caulobacter* cells. Arrowheads indicate stalks. (E) Swarm plate showing the motility of cells in WT, Δ spmX, Δ spmY, Δ spmX Δ spmY, Δ spmX *ctrA*(T170A), Δ spmY *ctrA*(T170A), Δ spmX Δ spmY *ctrA*(T170A), and WT *ctrA*(T170A) genetic backgrounds. (F) Relative β -galactosidase activity (in percentage) of *P_{spmX}-lacZ* in WT, Δ spmX, and Δ spmY cells. (G) Comparative ChIP-Seq using antibodies to CtrA denoting the occupancy of CtrA on the chromatin of Δ spmX *ctrA*(T170A) vs. Δ spmX cells. The color key indicates the degree by which the occupancy of CtrA is altered in selected targets by the *ctrA*(T170A) mutation in Δ spmX, expressed as \log_2 ratio. See Dataset S5 for the complete list. (H) Relative β -galactosidase activity (in percentage) of *P_{spmY}-lacZ* in WT, WT *ctrA*(T170A), Δ spmX, and Δ spmX *ctrA*(T170A) cells. Values (\pm SE) in F and H are the average of at least three independent experiments. (Scale bars, 2 μ m.)

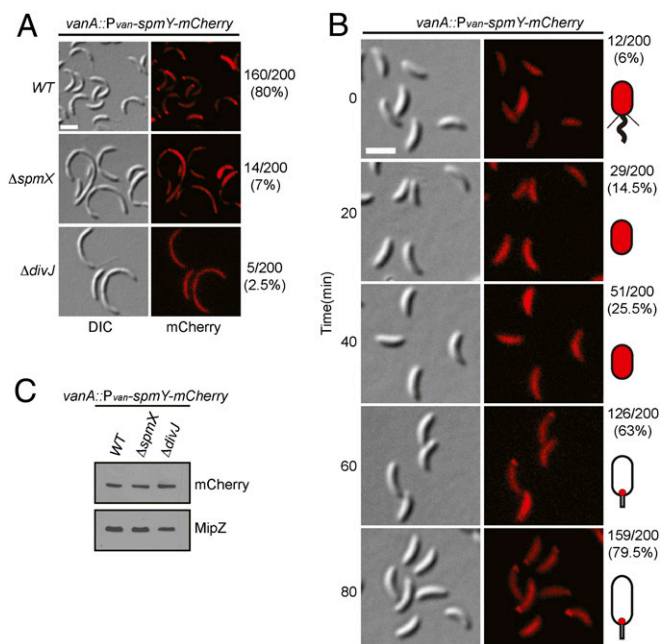


Fig. 4. Spatial regulation of SpmY. (A) DIC and fluorescence micrographs showing the localization pattern of SpmY-mCherry in WT, Δ spmX, and Δ divJ cells. SpmY-mCherry is localized to the stalked pole in the WT cells and is diffused in the Δ spmX and the Δ divJ cells. (B) Time course DIC and fluorescence micrographs of synchronized population of WT cells producing SpmY-mCherry showing the localization pattern of SpmY-mCherry during cell cycle. In A and B, number of cells (of 200) having a polar SpmY-mCherry foci is indicated, and their percentage is given in brackets. (C) Immunoblot showing the abundance of SpmY-mCherry protein in WT, Δ spmX, and Δ divJ cells. MipZ was used as the loading control. In all these experiments, *spmY-mCherry* was produced from the vanillate inducible promoter (*vanA*) on the chromosome (*vanA::P_{van}-spmY-mCherry*). (Scale bars, 2 μ m.)

As the *ctrA*(T170A) mutation did not reduce the filamentation of the Δ spmX and Δ spmY mutants (Fig. S3I) or TacA activity (assessed using the *P_{spmX}-lacZ* reporter) of Δ spmX, Δ spmY, and Δ spmX Δ spmY cells (Fig. S3H), we concluded that SpmX and SpmY act in the same pathway or on the same target(s) and that the imbalance caused by the loss of SpmX or SpmY can be partially offset by augmenting the CtrA regulon, without restoring normal TacA activity.

SpmX Recruits SpmY to the Stalked Pole Through DivJ. To test whether SpmY localizes to the same subcellular site (the stalked pole) as SpmX (5), we engineered a strain expressing SpmY translationally fused to the N terminus of the red fluorescent protein, mCherry, from the vanillate inducible *P_{van}* promoter at the chromosomal *vanA* locus (*vanA::P_{van}-spmY-mCherry*). Live-cell fluorescence microscopy revealed that SpmY-mCherry indeed localizes to the stalked pole (Fig. 4A). Imaging of synchronized populations revealed that the polar recruitment of SpmY-mCherry occurs later in S-phase (*T* = 60 min; Fig. 4B), well after that of SpmX during the G1→S transition (5). Based on the fact that SpmX and DivJ localization precedes that of SpmY, we asked if SpmY requires SpmX, and/or DivJ to be positioned at the stalked pole. Indeed, SpmY-mCherry was no longer localized to the stalked pole in Δ spmX (Fig. 4A) or in Δ divJ cells (Fig. 4A). The localization defect of SpmY-mCherry in the Δ spmX, and the Δ divJ mutants was not due to the decrease in the SpmY-mCherry protein levels (Fig. 4C). These experiments supported the conclusion that SpmX recruits SpmY to the stalked pole via DivJ to control the activity of TacA.

SpmY Function and Localization Is Conserved. Bioinformatic analyses revealed that the DUF2336 domain of SpmY is highly conserved in the α -proteobacteria (Fig. 5A). To verify whether the function is also conserved, we tested if the putative SpmY ortholog from *Brevundimonas subvibrioides* (BRESU_RS07300, henceforth SpmY^{Bs}), having 68% similarity to *Caulobacter* SpmY, can functionally substitute for *Caulobacter* SpmY. Indeed, expression of SpmY^{Bs} from *P_{van}* on a plasmid (*pP_{van}-spmY^{Bs}*) in Δ *spmY* cells corrected the filamentation defect and the motility defect of the *Caulobacter* Δ *spmY* mutant (Fig. 5B and C). Moreover, SpmY^{Bs}-seGFP localized to the stalked pole in WT *Caulobacter*, similar to native SpmY-seGFP expressed in a similar fashion in *Caulobacter* (Fig. 5D and Fig. S4). Last, we also determined whether SpmY^{Bs} can down-regulate TacA activity in the Δ *spmY* mutant. Using the *P_{spmX}-lacZ* promoter probe plasmid, we indeed observed that expression of SpmY^{Bs} decreased the *P_{spmX}-lacZ* activity in the Δ *spmY* mutant, to the same extent as that conferred by the expression of the native *Caulobacter* SpmY (Fig. 5E). These experiments suggest that the function of SpmY proteins is conserved among the α -proteobacteria.

Discussion

Previous studies revealed that SpmX has been co-opted from its kinase regulatory role in *Caulobacter* to stalk biogenesis in other α -proteobacteria such as *Asticcacaulis biprosthecum* and

Asticcacaulis excentricus (24). Here we attribute an additional and previously unknown regulatory role to the ancestral function of SpmX in stalk biogenesis and DivJ regulation (5, 24), underscoring the role of SpmX as an important polar organizer in α -proteobacterial branches. SpmX regulates the uncharacterized SpmY protein of *Caulobacter* to curb TacA activity, and this function of SpmY is conserved in other α -proteobacteria. At the conceptual level, the co-option of SpmX for various regulatory roles is reminiscent of the repurposing of *Hox* genes during appendage development in arthropods and insects for the spatiotemporal regulation of morphogenesis (29, 30). For example, the *Hox* genes of paralog groups 9–13 are used in patterning of posterior axial morphology and in the regional specification of the limb field (30).

The pleiotropic defects caused by inactivation of the SpmX-dependent signaling cascades can be attributed to the disturbed regulation of the transcriptional regulator TacA and the DivJ/DivK cell cycle signaling cascade (4, 5, 8). In this study, we unearthed TacA as a global regulator, targeting transcription of several developmental and cell cycle components in *Caulobacter*. TacA abundance is itself cell cycle regulated at the level of transcription and proteolysis (5, 14, 20). As TacA activity is positively controlled by a His-Asp phosphorelay (21), our discovery that TacA activity is up-regulated in the absence of SpmX and SpmY unveils these two proteins as components of the cell cycle circuitry that are required to curb TacA activity, possibly through the ShkA-ShpA phosphorelay (21), at the right time in the cell cycle. The fact that the σ^{54} -dependent activator TacA is highly conserved (31) and that the regulatory function of SpmY is conserved among the α -proteobacteria supports our view that similar ancestral regulatory mechanisms control TacA activity among these relatives. Although the underlying mechanisms remains to be elucidated, the similarities in the regulation of the co-oscillating global regulators TacA and CtrA are striking. Both are regulated at the level of activity by a two-component phosphorelay system, and at the level of protein stability by regulated ClpXP-mediated proteolysis, during the same cell cycle phase (5, 11, 20). This regulation is in part reinforced by the localization of ClpXP to the stalked pole, the site where SpmX/DivJ/SpmY also colocalize in a hierarchical order (9, 32). With the DivJ kinase as a component of this system, SpmX not only serves as a localization/regulatory hub to potentially fine-tune the output of two global transcriptional regulators (CtrA and TacA), but also that of the second messenger cyclic-di-GMP that oscillates during the cell division cycle in various bacteria (33–35). Strikingly, in *Caulobacter*, DivJ regulates this oscillation of cyclic-di-GMP through the phosphorylation of the cyclic-di-GMP synthase/response regulator PleD (36), thus underscoring the role of SpmX as a multifunctional regulatory hub.

Materials and Methods

Strains and Growth Conditions. *C. crescentus* NA1000 (37) and derivatives were grown in PYE, M2G, or M5G (38) at 29 °C unless specifically mentioned. *Escherichia coli* S17-1 (39) and EC100D (Epicentre Technologies) were cultivated at 37 °C in LB. Motility was assayed on PYE (peptone yeast extract) plates containing 0.3% agar. Swarmer cell isolation, electroporation, biparental mating, and bacteriophage Φ Cr30-mediated generalized transduction were performed as previously described (38–41). Complete list of strains and plasmids used in this study are given in Tables S1 and S2.

Genome-Wide Tn-Seq. *C. crescentus* WT and Δ *spmX* mutant strains were mutagenized with the *himar1* Tn delivered from an *E. coli* mini*himar1* donor strain by intergeneric conjugation as previously described (42). Collections of 100,000 kanamycin- and nalidixic acid-resistant clones were harvested, and chromosomal DNA was extracted. Genomic DNA was used to generate bar-coded ChIP-Seq libraries and submitted to Illumina HiSeq 2000 sequencing (Fasteris SA). Tn insertion-specific reads (50 bp long) were sequenced using the *himar1*-based Tn-Seq primer generating several million reads that were mapped to WT *C. crescentus* and processed through both Bowtie V0.12.9 and Samtools V0.1.18 algorithms to yield a BED file encompassing the Tn insertion coordinates. We imported these files into SeqMonk V0.23.0 (www.bioinformatics.babraham.ac.uk/projects/) to assess the total number of Tn

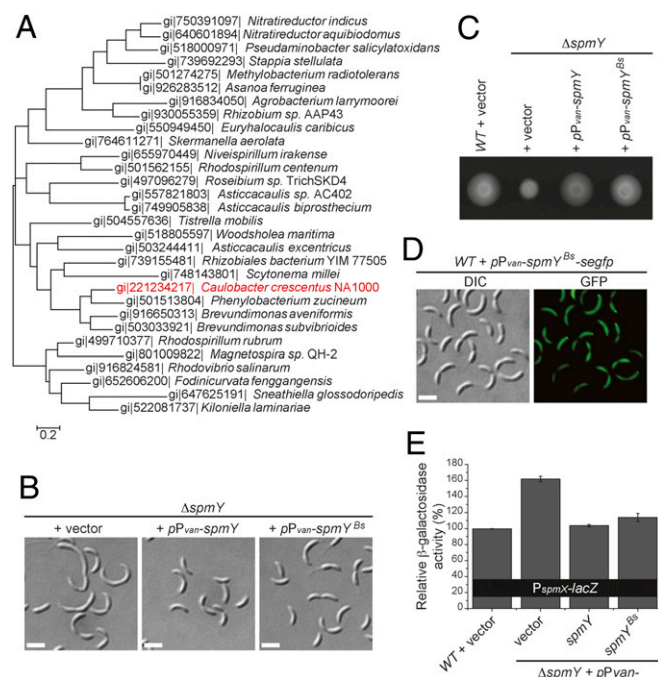


Fig. 5. The conserved activity of SpmY. (A) Phylogenetic tree depicting the conservation DUF2336 domain of SpmY among the α -proteobacteria. Scale bar indicates number of amino acid substitutions per site. (B) DIC microscopy images of Δ *spmY* harboring the vector (pMT335) or producing *Caulobacter* SpmY (*pP_{van}-spmY*) or *Brevundimonas subvibrioides* SpmY (*pP_{van}-spmY^{Bs}*) from the vanillate inducible promoter (*P_{van}*) on pMT335. (C) Motility assay of WT cells with the plasmid pMT335 or Δ *spmY* cells harboring pMT335 or *pP_{van}-spmY* or *pP_{van}-spmY^{Bs}*. The cells were spotted on PYE plates containing 0.3% agar and supplemented with 0.5 mM vanillate to induce the production of SpmY or SpmY^{Bs}. (D) DIC and fluorescence micrographs showing the localization of SpmY^{Bs}-seGFP in WT *Caulobacter*. *spmY^{Bs}-segfp* was produced from the *P_{van}* promoter on pMT335 (*pP_{van}-spmY^{Bs}-segfp*). (E) Relative β -galactosidase activity of *P_{spmX}-lacZ* in WT cells harboring pMT335 or Δ *spmY* cells of *Caulobacter* harboring pMT335 or *pP_{van}-spmY* or *pP_{van}-spmY^{Bs}*. Values (\pm SE) are the average of three independent experiments. The cells in B, D, and E were treated with 0.5 mM vanillate for 6 h to induce the production of SpmY or SpmY^{Bs} or SpmY^{Bs}-seGFP. (Scale bars in B and D, 2 μ m.)

insertion for each coding sequence (CDS). Datasets were exported into Microsoft Excel files for further analyses. First, datasets were normalized to CDS-*Tn* insertions per million overall reads count. To circumvent ratio issue for a CDS-*Tn* insertion value of 0, we increased every value by 1. In a second phase, we assigned for each CDS an insertion bias (IB) representing the ratio between normalized *Tn* insertions in Δ *spmX* vs. *WT* strains. Ultimately, we applied a log transformation to elicit a mean and a SD and to filter and select most significant IBs that diverge from the mean by 2 SDs. The implemented file was used to assemble a histogram of most significant IBs in function of the corresponding CDS along the *C. crescentus* NA1000 chromosome.

Genetic Screen for *Sp*mX-Dependent Regulators. For isolation of mutants that resemble a Δ *spmX* strain, *E. coli* S17-1 harboring the *Tn5* transposon delivery plasmid pIT2 (43) was conjugated with NR3330 strain (23) and grown on PYE agar supplemented with tetracycline (1 μ g/mL) and nalidixic acid (20 μ g/mL). The colonies generated were then inoculated onto M2G minimal media in 96-well plates. After a 16-h incubation at 29 °C, the plates were screened in a 96-well plate fluorescence reader for strains with elevated or comparable GFP fluorescence levels with respect to the Δ *spmX* strain harboring *stpX-gfp*. The selected

mutants were then subcultured into PYE and visualized using epifluorescence microscopy for the phenotype similar to Δ *spmX* cells and *StpX*-GFP localization. The selected mutants were further analyzed by immunoblotting for elevated *StpX*-GFP protein levels using the monoclonal GFP antibody (Living Colors JL-8; Clontech Laboratories).

Details of microscopy, mapping of transposon mutants, competition assay, in vivo 32 P labeling, ChIP-Seq, flow cytometry analysis, immunoblot, β -galactosidase assay, motility suppressor screen, and phylogenetic tree construction can be found in *SI Materials and Methods*.

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