A localized adaptor protein performs distinct functions at the Caulobacter cell poles

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Asymmetric cell division generates two daughter cells with distinct characteristics and fates. Positioning different regulatory and signaling proteins at the opposing ends of the predivisional cell produces molecularly distinct daughter cells. Here, we report a strategy deployed by the asymmetrically dividing bacterium Cau-
lobacter crescentus where a regulatory protein is programmed to perform distinct functions at the opposing cell poles. We find that the CtrA proteolysis adaptor protein PopA assumes distinct oligo-
meric states at the two cell poles through asymmetrically distrib-
uted c-di-GMP: dimeric at the stalked pole and monomeric at the swarmer pole. Different polar protein distributions at each cell pole recruit PopA where it interacts with and mediates the function of two molecular machines: the ClpXP degradation machinery at the stalked pole and the flagellar basal body at the swarmer pole. We discovered a binding partner of PopA at the swarmer cell pole that together with PopA regulates the length of the flagella filament. Our work demonstrates how a second messenger provides spatio-
temporal cues to change the physical behavior of an effector pro-
tein, thereby facilitating asymmetry.

Significance

Asymmetric cell division yields two distinct daughter cells by mechanisms that underlie stem cell behavior and cellular diversity in all organisms. The bacterium Caulobacter crescentus is able to orchestrate this complex process with less than 4,000 genes. This article describes a strategy deployed by Caulobacter where a regulatory protein, PopA, is programmed to perform distinct roles based on its subcellular address. We demonstrate that, depending on the availability of a second messenger molecule, PopA adopts either a monomer or dimer form. The two oligo-
meric forms interact with different partners at the two cell poles, playing a critical role in the degradation of a master transcription factor at one pole and flagellar assembly at the other pole.

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Cyclic-di-GMP Promotes PopA Dimerization In Vitro. PopA is a structural homolog of the diguanylate cyclase PdeA and binds c-di-GMP with an equilibrium dissociation constant $K_D$ of $\sim 2\mu M$ in vitro (21). A single amino acid mutation within the binding pocket of the GGDEF domain, R357G, prevents PopA from binding to c-di-GMP (21). As previously published, PopA$_{R357G}$-eYFP localizes to only the stalked pole, as opposed to the symmetric distribution to both poles in wild-type (WT) cells, and cannot assist ClpXP in CtrA degradation (21) (Fig. 1, B, Center). In a cell line completely lacking c-di-GMP (cdG$^0$), PopA-eYFP only forms a single focus at the stalked pole (30) (Fig. 1, B, Right). These observations suggest that PopA employs two distinct mechanisms to target the opposing cell poles: a c-di-GMP-dependent mechanism at the stalked pole and a c-di-GMP-independent mechanism at the stalked pole.

We considered the possibility that c-di-GMP controls the polar localization of PopA through dimerization. A weak self-association of PopA in Escherichia coli using a bacterial two-hybrid (BacTH) system has been reported (21). We reproduced PopA self-association using the BacTH system. Furthermore, we found that the mutant PopA$_{R357G}$, which does not bind c-di-GMP, failed to self-associate (Fig. 1C). It was previously reported that 0.31 $\mu M$ intracellular c-di-GMP is present in E. coli (31). The nonpolar intracellular c-di-GMP levels in Caulobacter were estimated to fluctuate between 0 (swarmer cells) to 0.5 $\mu M$ (stalked cells) during the cell cycle (29). This difference in self-association observed for PopA and PopA$_{R357G}$ in E. coli suggested that PopA–PopA interaction could be c-di-GMP dependent. To verify that PopA self-association is driven by c-di-GMP, we performed cross-linking studies with purified PopA using chemical cross-linker BS3 (Bis-sulfosuccinimidyl carbonate) (Fig. 1D). Cross-linked PopA dimers are seen upon the addition of c-di-GMP. The dynamic population of PopA increased as the PopA concentration was increased (increased (Fig. 1D)). Schalch-Moser (32), a student in the Urs Jenal laboratory (University of Basel, Basel, Switzerland), documented a nondenaturing gel electrophoresis experiment in her thesis showing that PopA dimerization increased as a function of c-di-GMP concentration in vitro.

**Results**

**Cyclic-di-GMP Promotes PopA Dimerization In Vitro.**

- **Fig. 1.** PopA subcellular distribution and proposed c-di-GMP-dependent oligomeric states. (A) Schematic of the *Caulobacter crescentus* cell cycle yielding a swarmer cell and a stalked cell upon division. The second messenger c-di-GMP (blue) is asymmetrically distributed, with low levels in motile swarmer cells and high levels in stalked cells (29). The swarmer cell cannot initiate DNA replication until it differentiates into a stalked cell by shedding its flagellum and pilus and building a stalk at the same site. De novo synthesis of c-di-GMP initiates during the swarmer-to-stalked cell transition, reaching peak concentration in stalked cells. The biogenesis of a new flagellum occurs at pole opposite the stalk. PopA (yellow circles) regulates the degradation of the master regulator CtrA at the stalked pole by ClpXP (21) yet is positioned at both cell poles. We propose that PopA adopts distinct oligomeric states at the opposing cell poles in response to local c-di-GMP concentration, dimeric at the stalked pole and monomeric at the opposite pole. (B) Subcellular localization of PopA-eYFP in a WT genetic background and in a mutant strain unable to synthesize c-di-GMP (cdG$^0$), PopA-eYFP only forms a single focus at the stalked pole (30) (Fig. 1, B, Right). These observations suggest that PopA employs two distinct mechanisms to target the opposing cell poles: a c-di-GMP-dependent mechanism at the stalked pole and a c-di-GMP-independent mechanism at the stalked pole.

- **Fig. 2.** PopA Diffuses as a Smaller Complex in the Absence of c-di-GMP. Next, we assessed c-di-GMP–dependent dimerization in vivo using a diffusion-based estimate of sizes of the diffusing molecules (33–35). According to the Stokes–Einstein equation (Eq. 1), the diffusion coefficient of spherical particles undergoing Brownian diffusion, $D$, senses the hydrodynamic radius ($R_h$) and viscosity of the solution ($\eta$) (36, 37). Proteins in different oligomeric states, but in the same cellular environment, hence, should exhibit different diffusion coefficients. The PopA polar foci are made up by mostly immobilized PopA molecules; however, there is a diffuse cytoplasmic population of PopA that shuttles between the two polar regions in the cell body. By analyzing the behavior of the diffusive PopA, we were able to isolate the impact of c-di-GMP on PopA diffusion. Using live-cell single-particle tracking with 20 ms exposures, we measured the apparent early-time diffusion coefficients of PopA-eYFP (77 KDa), the PopA$_{R357G}$-eYFP mutant unable to bind c-di-GMP in a WT background, and PopA-eYFP in a

- **Fig. 3.** Cyclic-di-GMP Promotes PopA Dimerization In Vitro. We provide in vivo and in vitro evidence that PopA assumes a binding partner of PopA at the swarmer pole that regulates flagellar filament length. We propose that the asymmetric distribution of c-di-GMP drives PopA in two populations that mediate the function of two different molecular machines—the flagellar basal body at the swarmer pole and the ClpXP degradation machinery at the stalked pole.

**Results**

**Cyclic-di-GMP Promotes PopA Dimerization In Vitro.**

- **Fig. 1.** PopA subcellular distribution and proposed c-di-GMP-dependent oligomeric states. (A) Schematic of the *Caulobacter crescentus* cell cycle yielding a swarmer cell and a stalked cell upon division. The second messenger c-di-GMP (blue) is asymmetrically distributed, with low levels in motile swarmer cells and high levels in stalked cells (29). The swarmer cell cannot initiate DNA replication until it differentiates into a stalked cell by shedding its flagellum and pilus and building a stalk at the same site. De novo synthesis of c-di-GMP initiates during the swarmer-to-stalked cell transition, reaching peak concentration in stalked cells. The biogenesis of a new flagellum occurs at pole opposite the stalk. PopA (yellow circles) regulates the degradation of the master regulator CtrA at the stalked pole by ClpXP (21) yet is positioned at both cell poles. We propose that PopA adopts distinct oligomeric states at the opposing cell poles in response to local c-di-GMP concentration, dimeric at the stalked pole and monomeric at the opposite pole. (B) Subcellular localization of PopA-eYFP in a WT genetic background and in a mutant strain unable to synthesize c-di-GMP (cdG$^0$), PopA-eYFP only forms a single focus at the stalked pole (30) (Fig. 1, B, Right). These observations suggest that PopA employs two distinct mechanisms to target the opposing cell poles: a c-di-GMP-dependent mechanism at the stalked pole and a c-di-GMP-independent mechanism at the stalked pole.

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strain (cdG\textsuperscript{0}) completely depleted of c-di-GMP (30) to evaluate the effect of c-di-GMP on oligomeric states of PopA (Fig. 1B):

\[ D = \frac{k_B T}{6\pi\eta R_h} \quad \text{[1]} \]

In order to avoid the confounding effect of the variation in cell cycle, we first collected trajectories of PopA proteins in predivisional cells outside the polar region. The ensemble early-time diffusion coefficient for PopA was calculated by analyzing the mean-square displacement (MSD) from pooled trajectories (Fig. 2A). The average diffusion coefficient for PopA-eYFP in a WT background was 0.615 \pm 0.0176 \mu m/s\textsuperscript{2} (error is SD determined from 20 bootstrapped samples of individual tracks) (Fig. 2B). The diffusion coefficients of PopA\textsubscript{R357G}-eYFP in an otherwise WT background and PopA-eYFP in cdG\textsuperscript{0} background were 0.785 \pm 0.0109 and 1.01\pm 0.0144 \mu m/s\textsuperscript{2}, respectively (Fig. 2B). The slower diffusion of PopA-eYFP in a background with WT levels of c-di-GMP suggests that it is diffusing as a bigger complex.

The difference in diffusion coefficients of PopA\textsubscript{R357G}-eYFP and PopA-eYFP in cdG\textsuperscript{0} (both representing PopA without interaction with c-di-GMP) prompted us to investigate the effective cellular viscosity of the three cell lines in order to infer information about the hydrodynamic radii. The Stokes–Einstein equation (Eq. 1) suggests that if we track the same noninteracting test particle in various cell lines, the difference in diffusion coefficient would reflect the difference in cellular viscosity. We therefore performed a control based on single-particle tracking and MSD analysis of free HaloTag protein (33 kDa) labeled with JF549 in three of these isogenic cell lines (WT, WT bearing a PopA\textsubscript{R357G} variant, and a cdG\textsuperscript{0} strain) and extracted the relative viscosities to be 1.00 \pm 0.008, 0.972 \pm 0.010, and 0.812 \pm 0.018, respectively (SI Appendix, Fig. S1). The low cellular viscosity of cdG\textsuperscript{0} cells may be accounted for by the fact that these cells display strong morphological abnormalities (Fig. 1B) with the cells being elongated and erratically shaped (30).

After taking into account the differences in cellular viscosity, the measured diffusion coefficients suggest that the relative hydrodynamic radii of PopA-eYFP, PopA\textsubscript{R357G}-eYFP, and PopA-eYFP in a cdG\textsuperscript{0} background are 1.00 \pm 0.0287, 0.806 \pm 0.0111, and 0.752 \pm 0.0106, respectively, values that can be cubed to obtain estimates of the relative molecular weights: 1.00 \pm 0.0086, 0.524 \pm 0.0218, and 0.426 \pm 0.017 (Fig. 2C, left and right axes, respectively). Since PopA\textsubscript{R357G} is unable to bind c-di-GMP, and cdG\textsuperscript{0} cells completely lack c-di-GMP, the relative molecular weights support the possibility that WT PopA dimerizes in the presence of c-di-GMP, leading to slower diffusion (Fig. 2D). The c-di-GMP concentration in Caulobacter was previously measured by a fluorescence resonance energy transfer-based sensor to be around 500 nM in predivisional cells (29). The fact that we were able to detect such a clear difference in the ensemble diffusion indicates that the K\textsubscript{d} of PopA binding c-di-GMP in vivo is likely smaller than what was measured in vitro using binding assays.

The c-di-GMP concentration fluctuates during the cell cycle. The swarmer cell has minimum cytosolic c-di-GMP. The synthesis of c-di-GMP begins during the swarmer-to-stalked transition leading to peak c-di-GMP concentrations in stalked cells (29) (Fig. 1A). We performed single-particle tracking in swarmer and stalked cells isolated from synchronized populations of cells to determine the cytoplasmic diffusional behavior of PopA-eYFP in low and high c-di-GMP environments using pooled MSD analysis as above. After correcting for different viscosities of two cell types (SI Appendix, Fig. S2), we estimated the relative hydrodynamic radii of PopA-eYFP, which can be cubed to estimate relative molecular weights of 0.448 \pm 0.0289 and 1.00 \pm 0.0582 for swarmer and stalked cells, respectively (Fig. 2E). Since the c-di-GMP concentration peaks in the stalked cells and reaches a minimum in the swarmer cells, our measurements support the hypothesis that PopA-eYFP dimerizes in the presence of c-di-GMP in Caulobacter (Fig. 2F).

**PopA Preferentially Dimerizes at the Stalked Pole.** To directly visualize the dimerization of PopA within the polar regions in vivo,
we implemented a BiFC assay (38). We divided eYFP into two nonfluorescent fragments, eYFP\(^{N}\) and eYFP\(^{C}\), as described by Kerppola (38), and fused them to the C-terminal ends of PopA or to PopA\(_{ΔR357G}\) that cannot bind to c-di-GMP (Fig. 3A). Fluorescent signal would indicate PopA dimerization. We then introduced either PopA-eYFP\(^{N}\)/PopA-eYFP\(^{C}\) or PopA\(_{ΔR357G}\)-eYFP\(^{N}\)/PopA\(_{ΔR357G}\)-eYFP\(^{C}\) into a ΔpopZ deletion strain. We were able to detect fluorescent signals from dimeric PopA-eYFP\(^{N}\)/PopA-eYFP\(^{C}\) predominantly at the stalked pole, demonstrating PopA dimerization in vivo (Fig. 3B). No fluorescence was detected in the cells containing the PopA\(_{ΔR357G}\)-eYFP\(^{N}\)/PopA\(_{ΔR357G}\)-eYFP\(^{C}\) constructs, suggesting that it was the PopA-PopA interaction but not the eYFP\(^{N}\)/eYFP\(^{C}\) interaction that drove the dimerization (SI Appendix, Fig. S1). In addition, we quantified the fluorescence complementation in predivisional cells, as PopA are bipolarly localized in these cells. We measured the fluorescence intensity ratio of BiFC at the stalked pole vs. the swarmer pole. Less than 19.5% of the cells (ST-to-SW BiFC signal is at or below 1) exhibit higher fluorescence signal at the swarmer pole (514 cells), while most cells show stronger BiFC signal at the stalked pole (Fig. 3C). Upon chromophore maturation, the eYFP fragments stay as a stable complex (39). Therefore, the fluorescence complementation observed at the stalked pole could be caused by a small fraction of previously formed PopA dimers. Our results showed that in vivo PopA dimersize in a c-di-GMP-dependent manner, and dimeric PopA preferentially localizes to the stalked pole.

**PopA Targets the Opposing Poles through Different Polar Organizing Proteins.** We now dissect the mechanisms that target PopA to the two distinct cell poles. PodJ functions as a polar organizing protein upon its localization to the flagella-bearing pole of the swarmer cell (40–42). Consistent with Duerrig et al., rather than localizing in a bipolar pattern, in 81% of predivisional cells PopA loses its swarmer pole foci in a ΔpopZ strain (21, 22) (Fig. 4A). We also observed that PopA-eYFP formed a single focus at the pole opposite to the stalk in ΔpopZ background (Fig. 4C). As popZ deletions lead to the mislocalization of many client proteins (50) and have pleotropic effects on cell growth, we further explored PopA-eYFP localization in a ΔpopZ overexpression strain (43). When PopZ is overproduced, the PopZ microdomain expands into a large volume (43), and we find PopA-eYFP fills the entire PopZ-rich region (Fig. 4D). Together, these results suggest that PopZ recruits PopA to the stalked pole. To determine whether PopZ interacts with PopA, we performed a BacT assay by expressing PopA/PopZ and PopA\(_{ΔR357G}/\)PopZ in *E. coli*. We found that PopA interacts with PopZ, whereas no interaction was observed between PopA\(_{ΔR357G}\) and PopZ (Fig. 4B, right column). The difference between the capability of PopA and PopA\(_{ΔR357G}\) to interact with PopZ suggests that the interaction between PopA and PopZ is c-di-GMP dependent, which is consistent with the observation that PopA\(_{ΔR357G}\) is unable to localize to the stalked pole.

**PopA Performs Distinct Functions at the Two Opposing Cell Poles.** As the swarmer cell differentiates into a stalked cell, the composition of the pole bearing the flagellum changes dramatically. Morphologically, the flagellar filament and hook are ejected, pili are retracted, and the biogenesis of the stalk is initiated at the site vacated by the flagellum (Fig. 1A). This transition is accompanied by the assembly of a ClpXP proteolytic complex at the pole that specifically degrades the chemotaxis apparatus, the TacA protein (51), and the CtrA global regulatory transcription factor (52). The PopA protein at the stalked pole functions as a ClpXP adapter protein for the targeted degradation of CtrA (24). We have shown here that PopA exists as a dimer in the presence of c-di-GMP at the stalked pole. However, when PopA localizes to the flagella-bearing stalked pole that lacks c-di-GMP, it does so as a monomer.

To explore the function of PopA monomers at the swarmer pole, we identified proteins that interact with PopA through BacT screening and coimmunoprecipitation of (3xFLAG-tag)-PopA (Materials and Methods). In addition to the previously known interaction partners at the stalked pole (CtrA, ClpX, RcdA), we identified four interaction partners of PopA. These include FlgG, FlfM, and FlfN [all three are flagellum switch complex proteins (53–56)] and CCNA_01529 (hereafter referred to as “SmrF” for “swarmer regulator of flagellum”), which is a small (122-residue)

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**Fig. 3.** PopA preferentially dimerizes at the stalked pole. (A) Schematic of a bimolecular fluorescence complementation assay (BiFC) to image protein–protein interactions. EYFP was split into N- and C-terminal fragments and fused to the C-terminal ends of proteins X and Y. Association between X and Y brings the two eYFP fragments into proximity, allowing the chromophore to mature and become fluorescent. (B) PopA BiFC signals. To assess oligomerization, both X and Y were PopA. PopA-eYFP\(^{N}\) and PopA-eYFP\(^{C}\) constructs were expressed from the xyI and van promoters, respectively, on the chromosome in a ΔpopA strain. Cells were induced with 0.03% xylose and 0.5 mM vanillate for 2 h before imaging. The white arrowheads indicate the stalked poles, and two field of views are shown. (Scale bars: 2 μm.) (C) Log-frequency plot of the ratio of BiFC intensity between stalked and swarmer poles calculated from 514 predivisional cells that contained at least one fluorescent focus. Only 19.5% of the population had a brighter swarmer pole BiFC signal.

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protein comprising a single FlaG-like domain (Pfam database) with unknown function. Notably, PopA interactions with these four proteins are c-di-GMP independent as the PopA<sub>R357G</sub> mutant bound effectively to these four proteins (Fig. 5A), consistent with the observation that the flagellated pole of the swarmer cell is devoid of c-di-GMP (29). In addition, the SmrF protein showed weak interaction with FliG and FliN and a lack of interaction with FliM (Fig. 5A).

**PopA Recruits SmrF to Regulate Flagellar Assembly.** While the function of the FlaG-like proteins remains elusive, the gene is always present in proximity to genes encoding flagellar assembly proteins (57–59) or flagellin (59, 60), suggesting a function related to the flagellum. In Caulobacter, smrF lies within the same operon, and immediately downstream of the flagellin encoding gene, fljK (61, 62), suggesting that its function might be related to flagella biogenesis. FljK is the major flagellin forming the majority of Caulobacter flagella filament, which is composed of six flagellins proteins in total (63, 64).

**Fig. 4.** PodJ and PopZ both regulate PopA localization. (A) PopA-eYFP was expressed from the native locus in a ΔpodJ strain. The white arrowheads indicate the swarmer poles with missing PopA-eYFP foci. From 543 predivisional cells, 80% cells had single foci at the stalked pole, while 18% exhibit bipolar foci. (Scale bars: 2 μm.) (B) BacTH assays indicate positive PopA–PodJ and PopA<sub>R357G</sub>–PodJ interactions in vivo; positive PopA–PopZ interactions and the absence of interaction between PopA<sub>R357G</sub> and PopZ in vivo. (C) PopA-eYFP was expressed from the native locus in a ΔpopZ strain and the stalked-pole marker protein StpX was fused to a mCherry fluorescent protein. The white arrowheads indicate the stalked poles with missing PopA-eYFP foci. From 248 predivisional cells, 87% cells had single foci at the stalked pole (swarmer pole) during the cell cycle. The white arrows indicate the swarmer poles. (Scale bars: 2 μm.) Among 47 mixed-population cells, 83% of them showed more than one expanded PopZ region (magenta), in which PopA and PopZ always colocalize.

**Fig. 5.** PopA recruits SmrF to the swarmer pole. (A) BacTH assays indicate positive interactions between PopA and four proteins FliG, FliM, FliN, and SmrF in vivo, all of which were independent of c-di-GMP. Weak association was observed for SmrF–FliG and SmrF–FliN. (B) SmrF is cell cycle regulated. Using immunoblots with anti-eYFP antibody, we determined the relative presence of SmrF-eYFP through the cell cycle starting from swarmer cells. SmrF-eYFP construct was the only copy in the cell and was expressed from the native promoter. The accumulation of SmrF starts in the late predivisional cell, coincidental with flagellin synthesis (61). (C) Time-lapse microscopy analyses every 30 min show SmrF-eYFP accumulated at the new cell pole (swarmer pole) during the cell cycle. The white arrows indicate the swarmer poles. (Scale bars: 2 μm.) (D) SmrF localization to the new pole requires PopA. Fluorescence images of SmrF-eYFP in WT, PopA<sub>R357G</sub>, and ΔPopA strains. In all strains, the chromosomal smrF locus was replaced with SmrF-eYFP. The white arrowheads indicate the swarmer poles. (Scale bars: 2 μm.) For over 250 synchronized swarmer cells, over 95% of the WT and PopA<sub>R357G</sub> have a single polar focus of SmrF-eYFP, which were completely lost in ΔPopA strains.
Construction of fusions of SmrF to eYFP allowed us to visualize the dynamic subcellular localization of SmrF during the cell cycle, where its abundance matches that of flagellar biosynthesis (Fig. 5B). Fluorescence imaging revealed that SmrF-eYFP only appeared at the flagellar pole in late predivisional cells, and the nascent daughter swimmer cells (Fig. 5C). In a strain bearing a PopA mutant (PopA<sup>ΔpopA</sup>) unable to bind to c-di-GMP, SmrF-eYFP retained the ability to localize to the flagellar cell pole (Fig. 5D, Middle). SmrF-eYFP was present as a diffuse signal in a ΔpopA strain, suggesting a direct or indirect interaction with PopA (Fig. 5D, Bottom). Using microscale thermophoresis (MST), we measured the <em>Kₐ</em> of PopA-SmrF and PopA<sup>ΔpopA</sup>-SmrF binding to be 5.6 ± 0.54 and 5.1 ± 0.72 μM, respectively (SI Appendix, Fig. S4). Our results suggest that PopA directly recruits SmrF to the swimmer pole.

The SmrF protein is degraded during the swimmer-to-stalked cell transition, coincident with cell cycle-controlled proteolysis of the CtrA master regulator (16) (SI Appendix, Fig. S5A). To determine the factors that affect SmrF proteolysis, we constitutively expressed SmrF-eYFP from PxylX promoter (65) in merodiploid strains containing the WT <em>smrF</em> gene. We measured the degradation of SmrF-eYFP in a ΔpopA strain, and in ClpX and ClpP depletion strains. We observed loss of proteolysis in all of these strains (SI Appendix, Fig. S5B), suggesting that SmrF degradation is performed by the ClpXP system using PopA as an adaptor, as is the case for CtrA proteolysis (16, 21). Thus, the cell cycle regulation of SmrF is maintained by the same proteolytic system that controls the critical regulatory proteins PdeA, TacA, and CtrA (24). During the swimmer-to-stalked transition, cells begin to synthesize c-di-GMP rapidly reaching peak levels in the stalked cells (29, 30). Increasing the amount of c-di-GMP allows PopA to dimerize and turn on its function as the proteolysis adaptor for ClpXP and the degradation of both CtrA and SmrF commence following the swimmer-to-stalked cell transition (21–24) (SI Appendix, Fig. S5A).

Although a <em>smrF</em> deletion strain or a SmrF overexpression strain did not exhibit viability defects in rich PYE or minimal M2G media (SI Appendix, Fig. S6A), and both strains exhibited normal sized swarm colonies on rich or minimal soft agar plates (SI Appendix, Fig. S6B), swimmer cells of a <em>smrF</em> deletion strain had longer flagella, while overproduction of SmrF protein produced shorter flagella compared to WT (Fig. 6A and B). While the length of the flagellum could be controlled by an increase in swimmer cell life span before it differentiates in to stalked cell, the deletion of <em>smrF</em> does not alter the swimmer cell life span as shown in SI Appendix, Fig. S7.

If the time of flagellum construction is constant, either an increase in flagellar expression or flagellin secretion, or both, can result in longer flagella. Accordingly, we asked whether SmrF serves as a regulatory protein for flagellin expression. However, SmrF and the FlaG superfamily proteins in general, are not known as DNA or RNA binding proteins. We entered the SmrF sequence into DRNApred to predict its DNA binding probability (67). We used fluorescently labeled fluorescence in situ hybridization (FISH) probes to investigate the spatial distribution of flagellin mRNAs and observed only diffuse fluorescent signal in the cytosol away from the polar regions (Fig. 6C), implying that flagellin mRNAs do not accumulate at the pole where SmrF forms loci. The absence of colocalization of SmrF and flagellin mRNA implies that they do not strongly interact. In addition, the PopZ polar microdomain excludes the ribosome translation machinery (50). Hence, it is unlikely that SmrF regulates flagellin on either the transcriptional or translational level.

As PopA interacts with the flagellar motor switch proteins FlIG, FlIM, FlIN, and SmrF is targeted to the pole through direct binding to PopA, PopA likely recruits SmrF into the vicinity of the flagellum basal body in order to perform its regulatory role. In the discussion below, we consider the possibility that at the swimmer pole the PopA/SmrF complexes are involved in flagellin secretion by docking onto the flagellar basal body.

**Discussion**

We have demonstrated that PopA employs c-di-GMP concentration differences as spatial cues to perform distinct roles at the opposing cell poles by adopting different oligomeric states. PopA complexes at the two cell poles appear to be intrinsically different: Stalked pole localization of dimeric PopA is c-di-GMP dependent, while swimmer pole localization of monomeric PopA is c-di-GMP independent. PopA localized at the stalked pole in the presence of c-di-GMP functions as a ClpXP protease adapter enabling CtrA degradation, while PopA at the swimmer pole appears to regulate the final step in flagella biogenesis, filament assembly. In each case, PopA recognizes different proteins at each pole, and is an accessory factor that contributes to the function of a complex biomolecular machinery (Fig. 7).

*Caulobacter* employs multiple proteins to synthesize and hydrolyze c-di-GMP (30). By positioning pairs of antagonistic enzymes to the opposing poles, cells acquire asymmetrically distributed c-di-GMP concentrations in the polar regions: The diguanylate cyclase PleD is localized and activated at the stalked pole (27) while the phosphodiesterase PdeA is sequestered to the swimmer pole (28). We have provided evidence that the high local concentration of c-di-GMP at the stalked pole drives PopA to adopt a dimeric form and that the low local concentration of c-di-GMP at the swimmer pole allows PopA to remain monomeric. c-di-GMP can be detected in the cytosol outside the polar regions due to the facile diffusion of this small molecule (29), which appears to be sufficient to keep PopA in dimeric form (Fig. 1A).

Binding assays using BacTH system revealed that PopA self-association is c-di-GMP dependent. PopA<sup>ΔGSPG</sup>, which is unable to bind c-di-GMP, was unable to form dimers in an *E. coli* heterologous system (Fig. 1C). Diffusion analysis further revealed that the presence of c-di-GMP changes the diffusive properties of PopA (Fig. 2). In the absence of c-di-GMP, PopA diffuses in a complex half the size compared to when c-di-GMP is present, supporting the hypothesis that PopA exists in a dimeric form at one pole and in a monomeric form at the other. BiFC assays directly demonstrated PopA dimerization and its preferential dimerization at the stalked pole, which is the site of c-di-GMP synthesis (Fig. 3).

The intrinsically disordered protein PopZ is a polar organizing protein that accumulates at the two cells poles and form space filling microdomains of 100 to 200 nm (43–47). The microdomains selectively recruit and concentrate signaling proteins including PopA (Figs. 1 and 4). The primary enzymes that synthesize and hydrolyze c-di-GMP are differentially deployed also at the cell poles (27, 28) and have been shown to interact with PopZ (45). The two catalytic reaction centers create an environment for PopA such that the local concentration of c-di-GMP is higher at the stalked than at the swimmer pole. The limited availability of c-di-GMP at the swimmer pole prevents PopA from dimerizing.

Upon dimerization, PopA exposes a different surface to the cytosol allowing the protein to interact with a new set of binding partners. The distinct behavior of PopA polar sequestration at the two cell poles implies that PopA might be recruited by different proteins. In previous deletion studies, PopA stalked pole localization is PopZ dependent while swimmer pole localization is PodJ dependent (22). We showed that PopA, indeed, interacts with both PopD and PopZ. Moreover, the interaction between PopA and PodJ at the swimmer pole is c-di-GMP independent and that PopA and PopZ interaction at the stalked pole requires c-di-GMP (Fig. 4).

Changes in c-di-GMP concentration as a function of the cell cycle not only triggers the switch of PopA between monomeric and dimeric form but also activates signaling pathways important for the transition from swimmer-to-stalked cells (30). The
concentration of c-di-GMP peaks in the stalked cells and is reduced by hydrolysis in swarmer cells (29). Previously, only the stalked pole function of PopA was known. Here, we identified a protein, SmrF, that is sequestered to the swarmer pole by PopA. The deletion and overexpression of the \textit{smrF} gene did not show any viability or motility defects, but cells lacking \textit{smrF} construct exceptionally long flagella filaments. The \textit{smrF} gene belongs to the \textit{flaG} super family, which is prevalent among the alphaproteobacteria (\textit{Rhizobium}, \textit{Nitrobacter}) and is found in many other proteobacteria species (\textit{Rhodocyclales}, \textit{Pseudomonas}, \textit{Desulfovibrio}, \textit{Campylobacterales}). Nonmotile, flagellum-less bacteria such as \textit{Klebsiella pneumoniae}, \textit{Yersinia pestis}, \textit{Streptococcus}, and \textit{Shigella} do not have genes that belong to the \textit{flaG} superfamily. While the function of the FlaG proteins remains elusive and is unlikely to be a flagellin subunit, the gene is always present in proximity to flagellin genes, suggesting a related function to filament assembly. In \textit{Campylobacter jejuni}, a deletion of \textit{flaG} yields extremely long flagella (58), as is the case in \textit{Pseudomonas fluorescens}, bearing a mutation in \textit{flag} (59), and in \textit{Vibrio anguillarum}, bearing an insertion in \textit{flaG} open reading frame (60).

The spatial localization of FlaG proteins in other species is unknown. In \textit{Caulobacter}, the cytosolic FlaG ortholog, SmrF, is recruited to the swarmer pole by PopA (Fig. 5D). We provide evidence that SmrF does not change the swarmer cell life span or motility and is unlikely to regulate the transcription of flagellin genes. As is well known, \textit{Caulobacter} tethers the origin of its single circular chromosome to the pole, and each gene locus has a defined subcellular address (68). The locations of flagellin genes are not near either pole (68); hence SmrF cannot colocalize with flagellin gene loci. In addition, a FISH experiment (Fig. 6C) demonstrated that flagellin mRNAs distribute evenly throughout swarmer cells. Finally, as PopA also interacts with all three components of the flagellum switch complex (Fig. 5A) that surrounds the secretion pore, it is possible that PopA recruits SmrF to physically block the secretion of flagellin monomers. A definition of the underlying mechanism is a topic of ongoing research.

Here, we demonstrated that the asymmetric distribution of c-di-GMP in the predivisional cell translates into the formation of two distinct PopA oligomeric states, which in turn interact with two different cellular machineries, the ClpXP degradation machinery at the stalked pole and flagellum basal body at the swarmer pole (Fig. 7). During the swarmer-to-stalked transition, c-di-GMP synthesis triggers the dimerization of PopA and switches PopA function from flagellar regulation to proteolysis adaptor. The degradation of SmrF by the ClpXP ensues. Interestingly, SmrF overexpression does not lead to delayed CtrA degradation.
(SI Appendix, Fig. S7), suggesting that SmrF does not compete with CtrA as a substrate of the ClpXP.

Studies have demonstrated that throughout evolution the availability of second messenger molecules can modulate protein activities (10, 69–71). Our analysis of PopA expands the repertoire of signaling schemes of second messenger sensing proteins by demonstrating how ligand availability can toggle distinct oligomeric states to achieve multiple functional states. Collectively, these studies of second messenger binding proteins suggest that second messenger molecules can provide spatial cues for proteins to adopt different forms and functions that enable asymmetric cell division.

Materials and Methods

Cell Preparation. All Caulobacter strains used for imaging were grown in minimal media M2G at 28 °C and collected at midlog phase (72) onto 1.5% (wt/vol) agarose pads made with M2G. For single-molecule tracking experiments, cells were washed three times with fresh M2G. Synchronized swarmer cells were collected by density centrifugation using Percoll (73). Synchronized swarmer cells were recovered in M2G at 28 °C for 5 min before imaging or let grow for 30 min to reach stalked cell stage before imaging (73). For single-molecule tracking experiments, 100-nm fiducial markers (Molecular Probes; 540/560 carboxylate-modified FluoSpheres) at around 1 nM were added to the cells before imaging.

Protein Purification. His6-PopA, His6-PopA<sub>R357G</sub>, were expressed from the pET28b vectors (21), and His6-SmrF was expressed from the pACYCDuet-1 vectors indicated in SI Appendix, Table S1. All proteins were purified using the following protocol adapted from Smith et al. (23). Proteins were expressed in E. coli BL21 cells (C2566H; NEB) grown to OD<sub>600</sub> of 0.4 at 37 °C in LB broth. The 0.4 mM isopropyl-β-D-thiogalactoside was used to induce protein expression overnight at 16 °C. Cells were harvested and the cell pellets were resuspended in lysis buffer (50 mM Tris·HCl, pH 8.2, 100 mM KCl, 1 mM MgCl<sub>2</sub>, 10% glycerol, 2 mM 2-mercaptoethanol) supplemented with protease inhibitors (Santa Cruz Biotechnology) and benzonase nuclease (Sigma). Cells were lysed using five passes through an EmulsiFlex (Avestin). Lysates were treated by centrifugation at 4 °C and incubated with Ni-NTA agarose resin for 1 h at 4 °C after adding 20 mM imidazole. We used 5 mL of NTA slurry/1 L of initial cell culture when purifying PopA, and 1 mL of NTA slurry/1 L of initial cell culture when purifying SmrF. The protein bound Ni-NTA resin was washed five times at 4 °C. The proteins were eluted with 10 mL of lysis buffer/1 L of initial culture supplemented with 300 mM imidazole using Poly-Prep chromatography columns (Bio-Rad). Ten millimolar EDTA was added to all eluted proteins before they were buffer exchanged into storage buffer, 25 mM Hepes-KOH, pH 8.0, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 15 mM NaCl, 5% (vol/vol) glycerol, and 1 mM DTT, before freezing in liquid nitrogen and storage at −80 °C.

Cross-Linking Assay. We cross-linked PopA by adapting a protocol that was used to study a paralogous protein PleD (74). The purified PopA (at 0.1, 0.25, 0.5, and 1 mg/mL concentration) was incubated with or without 1 mM c-di-GMP at 4 °C overnight. A water-soluble cross-linker BS3 (Abcam) was added at 2.5 mM final concentration for 30 min at room temperature to cross-link the protein. The reaction was quenched by adding Tris-HCl, pH 8.0, 50 mM final concentration. After separation on 4–15% precast polyacrylamide gel (Bio-Rad) and transfer to a PVDF membrane (GE Healthcare), PopA monomeric and dimeric forms were detected by staining with a polyclonal anti-PopA antibody (Josman). Secondary goat-anti-rabbit immunoglobulin G with HPR (Abcam; ab205718) conjugation was used for visualization.

MST Binding Assays. PopA and PopA<sub>R357G</sub> were fluorescently labeled on lysine residues with N-hydroxysuccinimide functionalized Atto-488 (Sigma-Aldrich). The dye conjugate was dissolved in dry DMSO to make stock solution. The conjugation reaction was performed in the dark using 1.5 mg/mL protein and a tenfold molar ratio of dye to protein at room temperature with gentle shaking for 15 min. Each protein was labeled with one to two dye molecules on average. Proteins were buffer exchanged into the same storage buffer through overnight dialysis to remove unlabelled dye molecules. The labeled proteins were diluted to 2 μM and flash frozen in liquid nitrogen and storage at −80 °C. Direct binding between fluorescently labeled PopA or PopA<sub>R357G</sub> with c-di-GMP (Axora), unlabeled PopA or PopA<sub>R357G</sub> as well as SmrF was probed in twofold serial dilutions using MST (75) (NanoTemper Technologies) (75, 76).

All proteins used for MST were dialyzed into MST buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT with 0.025% Tween 20) overnight. For each binding assay, a twofold serial dilution was made for each protein target and c-di-GMP in MST buffer. Fluorescently labeled PopA (or PopA<sub>R357G</sub>) was added at 25 nM, mixed, and incubated at room temperature for 15 min in the dark. The protein mixtures were then loaded into Standard 15% precast polyacrylamide gel. In this equation, B<sub>L</sub> represents the concentration of protein complexes, [B<sub>0</sub>] represents total binding sites of the fluorescent ligand, [L<sub>0</sub>] represents the amount of added ligand, and K<sub>d</sub> represents the dissociation constant.

BacTH Assay. Protein pairs were fused in frame to the C or N terminus of the T18 and T25 subunits of the adenylate cyclase. Paired constructs were expressed in the E. coli BTH101 cell line, which is depleted of any activity of adenylate cyclases. Cells were then plated on MacConkey agar. Magenta colonies indicate protein–protein interaction. We used the self-associating leucine zipper, T25-zip and T18-zip, as a positive control and T18 and T25 alone as a negative control (77).

Transmission Electron Microscopy. For flagellum visualization, cells were grown in M2G media to OD<sub>600</sub> of 0.5 with gentle shaking at 30 °C. Two
micromolar of cell culture were applied to glow-discharged, carbon-coated copper grids (EMS, FC300-Cu-25) and negatively stained with 0.3% (wt/vol) uranyl acetate. The grids were then washed three times with ddH2O. Centrifugation damages the flagellum integrity and must be avoided during preparation. Data were collected using a JEM1400, a JEOL 120-keV transmission electron microscope at various magnification settings. The length of flagellum was quantified by manually tracing the flagellum in ImageJ.

**Co-immunoprecipitation.** One liter of Caulobacter cells expressing PopA-3XFLAG and GapR-3XFLAG (nonpolar protein control) were cultured in M2G minimal media at 28 °C, and cells were harvested at midlog phase. All following steps were conducted at 4 °C. The pelleted cells were then washed three times with IP buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, protease inhibitor, and benzamidine nuclease) to remove remaining growth media. The cells were resuspended in 20 μL of IP buffer, and DSP (Lomant's reagent, Thermo Fisher, dissolved in DMSO) was added to a final concentration of 2 mM. The mixture was incubated for 2 h with nutation. The cross-linking reaction was terminated by adding Tris-Cl, pH 7.5, at a final concentration of 20 mM and incubated for 15 min. The cells were then lysed using an EmulsiFlex (Avestin) until lysates cleared. Cellular debris was removed from the lysates by centrifugation and the lysates were incubated with anti-FLAG magnetic beads (Sigma) overnight with nutation. The next morning, the magnetic beads were washed in 50 mM Tris, 100 mM NaCl, 0.5% Tween-20, and 0.1% SDS, and proteins were eluted by 200 μL of 0.1 mg/mL 3XFLAG peptide (Sigma). The protein mixture was denatured and run on SDS polyacrylamide gel before being sent to the Stanford University Mass Spectrometry facility for protein identification.

**FISH.** We adapted a smFISH protocol to visualize flagellin mRNA in Caulobacter cells (78). FISH probes targeting flagellin sequences were designed using SAGEmap (79). After order and ordering 20 from the Stanford RNA i-lab, FJk and FjI, were targeted by 13 and 11 probes, respectively. Probes were 20 nucleotides long with GC content ranging from 40 to 55% with 3’ amino modifier C7. Twenty-five nanomoles of each probe were synthesized and nucleotides long with GC content ranging from 40 to 55% with 3’ amino

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