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Activation and polar sequestration of PopA, a c-di-GMP effector protein involved in *Caulobacter crescentus* cell cycle control

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Summary

When *Caulobacter crescentus* enters S-phase the replication initiation inhibitor CtrA dynamically positions to the old cell pole to be degraded by the polar ClpXP protease. Polar delivery of CtrA requires PopA and the diguanylate cyclase PleD that positions to the same pole. Here we present evidence that PopA originated through gene duplication from its paralogue response regulator PleD and subsequent co-option as c-di-GMP effector protein. While the C-terminal catalytic domain (GGDEF) of PleD is activated by phosphorylation of the N-terminal receiver domain, functional adaptation has reversed signal transduction in PopA with the GGDEF domain adopting input function and the receiver domain serving as regulatory output. We show that the N-terminal receiver domain of PopA specifically interacts with RcdA, a component required for CtrA degradation. In contrast, the GGDEF domain serves to target PopA to the cell pole in response to c-di-GMP binding. In agreement with the divergent activation and targeting mechanisms, distinct markers sequester PleD and PopA to the old cell pole upon S-phase entry. Together these data indicate that PopA adopted a novel role as topology specificity factor to help recruit components of the CtrA degradation pathway to the protease specific old cell pole of *C. crescentus*.

Introduction

Dynamic localization of structural or regulatory proteins enables bacterial cells to sequester specific functions and processes to specific subcellular sites (Rudner and Losick, 2010). These include factors involved in cytokinesis and chromosome dynamics, as well as elements needed for the biosynthesis and operation of polar organelles like flagella and pili. In addition, many regulatory proteins like sensor histidine kinases, transcription factors and proteases as well as enzymes have specific addresses within the cell (Rudner and Losick, 2010). Although protein localization appears to represent a general regulatory feature of bacterial proteomes, the underlying sequestration mechanisms, structural determinants and polar receptors remain poorly understood.

The aquatic α-proteobacterium *Caulobacter crescentus* divides asymmetrically producing two genetically identical, but physiologically distinct daughter cells, a motile swarmer cell (SW) and a sessile stalked cell (ST). The two progeny inherit different replicative programmes, in that stalked cells can initiate DNA replication immediately after their birth, while chromosome replication is blocked in swarmer cells for an extended period termed G1. The establishment of swarmer and stalked cell specific programmes relies on the subcellular distribution of cell fate determinants and regulatory proteins that govern cell cycle progression and polar morphogenesis (Matroule *et al.*, 2004; Paul *et al.*, 2008; Tsokos *et al.*, 2011). For example, G1-S transition is facilitated by the co-ordinate recruitment of the replication initiation inhibitor CtrA and its cognate protease ClpXP to the stalked cell pole where CtrA is degraded (Jenal and Fuchs, 1998; McGrath *et al.*, 2006).

Caulobacter crescentus cell cycle progression and morphogenesis are co-ordinated by the bacterial second messenger cyclic di-GMP (Jenal and Malone, 2006). Several proteins involved in the production and sensing of the second messenger have been shown to dynamically position to specific cellular sites during the bipolar life cycle of *C. crescentus*. For example, the diguanylate cyclase PleD localizes to the stalked cell pole where it orchestrates flagellar ejection and biogenesis of stalk and holdfast. PleD is an unorthodox response regulator with two receiver domains (Rec1–Rec2) fused to a GGDEF diguanylate cyclase (DGC) output domain (Chan *et al.*, 2004). PleD activation and localization to the cell pole depends on the phosphorylation of its first receiver domain by the sensor histidine kinases PleC and DivJ (Aldridge *et al.*, 2003;

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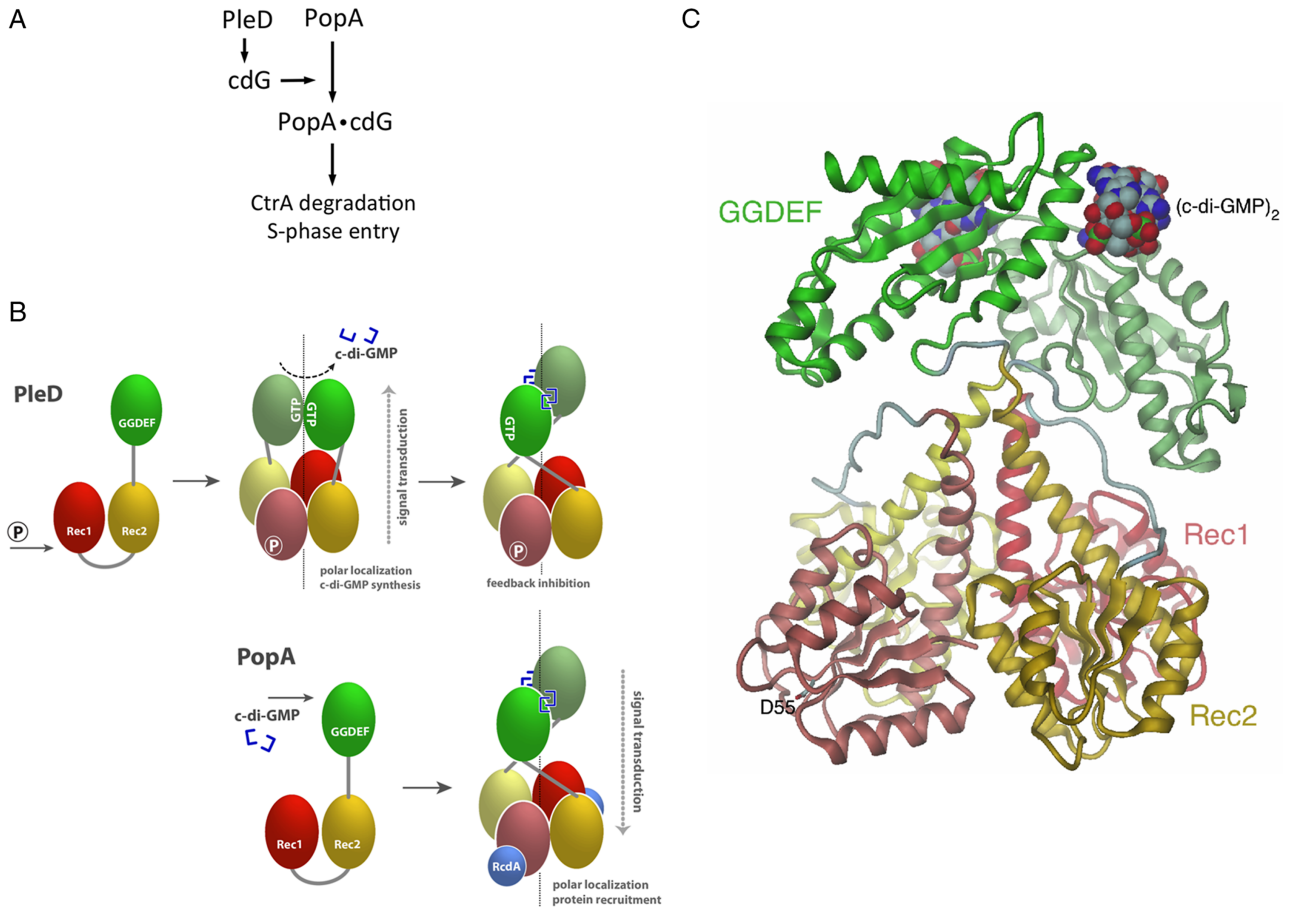


Fig. 1. The c-di-GMP effector protein PopA is a structural homologue of the PleD diguanylate cyclase.
A. A schematic of the role of PleD and PopA in *C. crescentus* S-phase entry control.
B. Model for the signal transduction mechanisms proposed for PleD and PopA. In PleD the signal travels from the N-terminal Rec1 domain (phosphorylation) to the C-terminal catalytic GGDEF domain. Phosphorylation of the first receiver (Rec1) leads to dimerization of PleD via the receiver stem and the subsequent activation of the C-terminal DGC domain. Continuous production of c-di-GMP results in feedback inhibition through product-mediated cross-linking of two catalytic GGDEF domains in a non-productive arrangement. The direction of signal transduction is reversed in PopA, where signal input occurs through c-di-GMP binding to the GGDEF domain and the Rec1 domain interacts with the downstream component RcdA. The model proposes that PopA is active as a dimer, which results from c-di-GMP mediated cross-linking of its GGDEF domains.
C. Homology model of dimeric PopA based on the structure of activated PleD (Wassmann *et al.*, 2007). The Rec1, Rec2, and GGDEF domains are coloured red, yellow and green respectively. The position of the $(c\text{-di-GMP})_2$ dimers (shown in ball representation) have been inferred from the template structure. The position of the presumable phosphoryl-acceptor D55 is marked.

Paul, 2004; Paul *et al.*, 2008). Localization of activated PleD to the cell pole helps to recruit PopA, a c-di-GMP effector protein that, upon binding c-di-GMP, localizes to the same subcellular site prior to S-phase entry (Aldridge *et al.*, 2003; Paul, 2004; Duerig *et al.*, 2009) (Fig. 1A). PopA then recruits CtrA to the same pole via the mediator protein RcdA that facilitates CtrA localization and eventually degradation by the polar ClpXP protease (Jenal and Fuchs, 1998; McGrath *et al.*, 2006; Chien *et al.*, 2007; Duerig *et al.*, 2009).

PleD and PopA share the same domain architecture arguing that they originated through gene duplication. However, the two proteins have diverged in terms of both their function and their regulation. Catalytic activation of PleD and localization to the cell pole requires Rec1–Rec2 mediated dimerization (Chan *et al.*, 2004; Paul *et al.*, 2007) (Fig. 1B). In addition, the enzymatic activity of PleD is subject to allosteric product inhibition whereby two $(c\text{-di-GMP})_2$ dimers cross-link the two catalytic GGDEF domains of the dimer to form a catalytically incompetent arrangement (Fig. 1B). This is accomplished by two complementary c-di-GMP binding sites on adjacent PleD molecules, the primary and secondary I-sites (Christen *et al.*, 2006; Wassmann *et al.*, 2007). The GGDEF domain of PopA lost its catalytic activity and adopted a role as c-di-GMP effector by utilizing the allosteric I-site as high-affinity binding site

for the second messenger (Duerig *et al*., 2009). In accordance with this novel signal input, PopA appears to have lost input control by phosphorylation as its Rec1 domain lacks several key residues of the conserved phospho-switch (Gao and Stock, 2009). PopA dynamically localizes to both swarmer and stalked cell poles of the *C. crescentus* predivisional cell. While its role at the flagellated pole seems to promote motility, localization to the incipient stalked cell pole serves to regulate S-phase entry (Duerig *et al*., 2009). PopA localization to the stalked pole depends on c-di-GMP binding to the conserved RxxD I-site motif (Duerig *et al*., 2009). During the swarmer-to-stalked cell transition the c-di-GMP concentration increases resulting from the activation of PleD and several other DGCs (Paul *et al*., 2008; Abel *et al*., 2011; 2013). This upshift controls the *C. crescentus* motile-sessile switch and promotes PopA localization to the stalked pole.

Little is known about how c-di-GMP activates PopA and which domain(s) encode the positional information for localization and pole discrimination. Likewise, it is unclear if PopA and its paralogous DGC PleD that sequentially sequester to the same cell pole share a common localization mechanism. Here we provide evidence that PopA not only exploits the allosteric I-site for activation but, akin to feedback control of PleD, uses secondary binding sites to couple c-di-GMP binding to cellular localization. We demonstrate that while the molecular address for polar localization of PleD is contained entirely within the Rec1–Rec2 dimerization stem, it is the C-terminal GGDEF domain that directs PopA to the stalked pole. In agreement with this, distinct receptor proteins guide PleD and PopA to the same subcellular site. Finally, we show that the N-terminal Rec1 domain of PopA interacts with RcdA, arguing that this domain facilitates RcdA recruitment to the cell pole and emphasizing the unorthodox signalling arrangement of this regulatory factor. These data emphasize the versatility of c-di-GMP signalling proteins and demonstrate that the GGDEF domain has adopted a novel role as targeting factor to localize proteins to specific subcellular compartments in response to c-di-GMP binding.

Results

PopA is a structural homologue of the PleD response regulator with different regulatory input

PopA and PleD share a Rec–Rec–GGDEF domain organization but show low overall sequence conservation (22% identity). Searching with the PopA sequence against all profiles of known PDB structures using HHPred yielded PleD as top hit with a highly significant Z-score of $1e^{-63}$. Based on the HHPred alignment (Fig. S1) and the activated PleD dimer structure (2v0n) a homology model of PopA was built (Fig. 1C). Comparison of the two structures revealed only few insertions or deletions that are located primarily in loop regions (Fig. S2A). One exception is the fourth α-helix of the PleD Rec1 domain that appears to be degenerated to a loop in PopA. The energy refined homology model deviates only marginally from the template structure with an rmsd value of 0.3 Å for 828 Cα positions, demonstrating its reliability despite the low overall sequence conservation. In particular, the structural context of key the residues discussed below should be largely correct.

Based on the 3-D homologies and based on similar localization patterns during the cell cycle (Paul, 2004; Duerig *et al*., 2009), we postulated that PleD and PopA might share a common activation and localization mechanism. PleD is activated by phosphorylation of the first receiver domain. PleD D53, the phosphoryl acceptor site crucial for the phosphorylation-mediated conformational change of canonical receiver domains (Gao and Stock, 2009) is conserved in Rec1 of PopA as D55 (Fig. S1). However, D55 is not required for PopA activity and localization (Duerig *et al*., 2009), and F102, the receiver switch of PleD, which transmits phosphorylation-mediated structural rearrangements, is not conserved in PopA (Fig. S1). We conclude that phosphorylation is not involved in PopA regulation and that the conserved D55 has lost its central role in the activation of this response regulator homologue.

Next, we tested if PopA follows an activation-by-dimerization mechanism as observed for PleD (Paul *et al*., 2007). PopA dimer formation was supported by bacterial-two-hybrid analysis (Fig. S3). Unfortunately, biochemical experiments addressing the PopA oligomerization state proved unsuccessful due to poor solubility of the protein. In the PleD dimer, several salt bridges mediate interaction between the Rec1 domain of one protomer and the adjacent Rec2' domain of its partner (Wassmann *et al*., 2007) (Fig. S2A). Similarly, the PopA dimer model shows potential salt bridges involving residues D4, R118, E125, and R129. However, PopA variants with mutations in residues R118, E125, or R129 showed cellular localization and CtrA degradation patterns indistinguishable from wild type (Fig. S2B). From this we conclude that these residues are dispensable for PopA activation and that the PopA activation mechanism is distinct from the Rec1–Rec2 mediated dimerization of PleD.

Contribution of primary and secondary I-site residues to c-di-GMP mediated PopA activation

We next analysed the role of c-di-GMP binding to the C-terminal GGDEF domain for PopA activation. In PleD, conserved residues R359, D362, and R390 co-ordinate a dimer of c-di-GMP and constitute the primary I-site binding pocket (Chan *et al*., 2004; Christen *et al*., 2006) (Fig. 2A). The primary I-site is conserved in PopA. Vari-

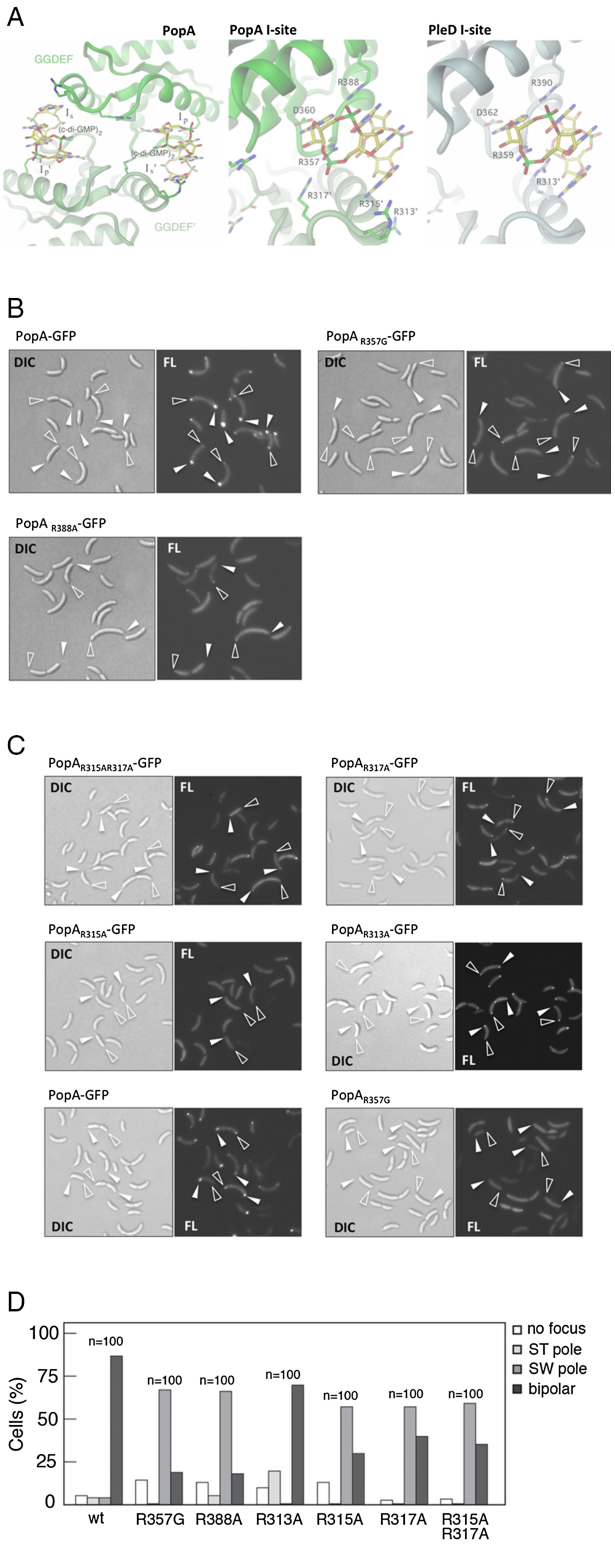


Fig. 2. PopA primary and secondary I-sites are required for correct localization to the stalked pole.
A. Close-up views of the c-di-GMP binding sites of PleD (Wassmann *et al.*, 2007) and modelled PopA. Primary (I_p, I_p') (R357, D360, R388) and secondary I-sites (I_s, I_s') (R313, R315, R317) of the PopA model are indicated on the left. Side-chains of the I-site residues are shown together with surrounding secondary structure elements. The core residues important for c-di-GMP binding (primary I-sites) are shown for PopA (green, middle) and PleD (grey, right). Proposed residues of secondary I-sites are shown on the opposite protomers.
B. Arg residues (R357, R388) of the primary c-di-GMP binding site are required for PopA localization to the stalked pole. GFP fusions of PopA wild type and respective binding mutants were expressed from a low-copy-number plasmid in a Δ*popA* background and analysed by fluorescence microcopy. DIC and fluorescence images (FL) are shown. The identity of cell poles is indicated for individual cells with filled arrows marking stalked poles and open arrows marking poles opposite the stalk.
C. Arg residues of the secondary c-di-GMP binding site (R315, R317) contribute to PopA localization to the stalked pole. GFP fusions of PopA wild type and respective binding mutants were expressed from a low-copy-number plasmid in a Δ*popA* background and analysed by fluorescence microcopy. The identity of cell poles is marked as outlined above.
D. Distribution analysis of localization data from (B) and (C). Only predivisional cells were analysed. The fraction of cells with foci only at the stalked pole (ST), only at the flagellated (SW) pole, with bipolar localization (two foci), or with no focus is indicated.

ants with mutations in the corresponding amino acids R357 (R359 in PleD) and also R388 (R390 in PleD) (Fig. 2A), although stably expressed in a *popA* deletion strain (data not shown), failed to localize to the stalked cell pole (Fig. 2B and D) and were unable to support CtrA degradation during G1-S transition (Fig. S4A). These data suggested that residues R357 and R388 are strictly required for PopA stalked pole localization and, similar to the situation in PleD, form the core of the primary I-site motif. Substitutions of D360 to alanine or glutamic acid displayed a partial localization defect, while the residues in between the conserved charges of the RXXD motif are dispensable for PopA activation (data not shown).

In PleD, the c-di-GMP ligands bridge to a conserved arginine (R313; referred to as secondary I-site) from an adjacent GGDEF domain, thereby immobilizing the catalytic domains in a non-productive arrangement (Fig. 2A) (Wassmann *et al.*, 2007). Because PopA shows weak self-association we tested if a similar mechanism is involved in PopA activation. Structural modelling exposed several candidate Arg residues in the GGDEF domain adequately positioned to form a secondary I-site (Fig. 2A, R313, R315, R317). Alanine substitutions at positions R315 and R317, but not R313, resulted in a significant reduction of PopA stalked pole localization (Fig. 2C and D). All mutant variants showed *in vivo* stabilities comparable to wild-type PopA (Fig. S4C) arguing against general protein defects. Analysis of CtrA turnover revealed that despite of the localization defect, the PopA secondary I-site mutants R315A and R317A as well as the R315A/R317A double mutant were still able to promote CtrA degradation during G1-S (Fig. S4B). These findings suggested that both the primary and secondary I-sites contribute to PopA subcellular localization. Analogous to the

existence of multiple secondary docking sites for c-di-GMP in PleD (Wassmann *et al*., 2007), the only partial loss of function observed for PopA secondary I-site mutants could be explained by additional, so far unidentified secondary docking sites for c-di-GMP.

PopA localizes to both cell poles via the GGDEF domain

Both Rec1–GFP and Rec1–Rec2–GFP fusion proteins failed to localize to the cell pole (data not shown), arguing that the GGDEF is critical for subcellular positioning of PopA. However, because a GGDEF–GFP fusion was not stable *in vivo*, these experiments failed to demonstrate that the GGDEF is sufficient for PopA localization. To circumvent this limitation and to determine the structural elements necessary for PopA and PleD localization to the cell poles, PopA–PleD hybrid proteins were engineered. The GGDEF domain of PopA was grafted onto the Rec1–Rec2 domain of PleD and vice versa. The resulting hybrid proteins are henceforth named PleD–PopA [$Rec1_{PleD}$–$Rec2_{PleD}$–$GGDEF_{PopA}$] and PopA–PleD [$Rec1_{PopA}$–$Rec2_{PopA}$–$GGDEF_{PleD}$] respectively (for exact co-ordinates see *Experimental procedures*).

The PopA–PleD fusion to GFP was unstable *in vivo* (Fig. S5A) and showed no detectable fluorescence signal (Fig. 3A). In contrast, the PleD–PopA hybrid fusion was stable and produced a robust fluorescence signal (Fig. 3A and B). Although the majority of predivisional cells expressing a PleD–PopA–GFP fusion protein displayed a bipolar localization pattern typical for PopA (42%), the ratio of predivisional cells with no foci (23% for PleD–PopA versus 10% for PopA) or single foci at the stalked pole (35% for PleD–PopA versus 5% for PopA) was increased as compared to PopA. Moreover, the overall signal intensity for the PleD–PopA–GFP spots at the stalked pole was significantly higher as compared to the signal intensities observed for PopA–GFP or PleD–GFP (Fig. 3A). Consistent with this, the PleD–PopA–GFP fusion is present at higher levels than PopA–GFP (Fig. S5A).

To dissect the contribution of the PleD and PopA fragments to polar localization of PleD–PopA, we selectively debilitated each individual activation mechanism. Tyr26 forms a small contact patch between two receiver domains of the active PleD dimer (Wassmann *et al*., 2007). Replacing Tyr26 with Ala abolishes PleD dimerization, activation and polar localization (Paul *et al*., 2007). However, when this mutation was introduced into the PleD–PopA hybrid, bipolar localization was unaffected (35%) (Fig. 3B and C), indicating that the PopA GGDEF domain is sufficient to tag the hybrid to the stalked and flagellated cell poles. Next we analysed the polar distribution pattern of PleD–PopA with a defective I-site (R357G). Although many cells showed mislocalized protein, a relatively large fraction (31%) retained fluorescent foci at the stalked pole, while the number of cells with bipolar, PopA-like distribution, dropped to zero (Fig. 3B and C). This is similar to the localization behaviour of PleD–GFP (Paul, 2004) and indicated that the localization of this mutant fusion protein relies on the PleD portion and that the PleD Rec1–Rec2 stem by itself can tag the hybrid to the stalked pole. In accordance with this view, $PleD_{Y26A}$–$PopA_{R357G}$ containing both mutations failed to localize to the cell poles (Fig. 3B and C). Thus, PleD–PopA contains two individual determinants for stalked pole localization, one derived from PleD and one from PopA. Since all PleD–PopA variants were detected with anti-GFP antibodies, the absence of a polar localization signal is not due to unstable or poorly expressed proteins (Fig. S5B).

To corroborate these findings, PleD–PopA–GFP localization was studied in mutants lacking PleC and DivJ (the upstream components of PleD) (Paul *et al*., 2008) or diguanylate cyclases (the upstream components of PopA) (Abel *et al*., 2013). Consistent with earlier observations, PleD failed to localize in the absence of its upstream regulators PleC and DivJ, while PopA failed to localize to the cell poles in the absence of c-di-GMP (Fig. 3D) (Paul, 2004; Duerig *et al*., 2009). In contrast, PleD–PopA–GFP robustly localized to the poles of a Δ*pleC* Δ*divJ* mutant, indicating that the GGDEF domain of PopA alone is able to drive the protein to the stalked pole. In agreement with this, PleD–$PopA_{R357G}$–GFP, lacking a functional GGDEF I-site, failed to localize in this mutant background (Fig. 3B). Strongly reduced polar signals of PleD–PopA were detected in a strain lacking c-di-GMP (cdG^0) (Fig. 3D), arguing that the PleD-specific polar docking site is absent in this strain. Alternatively, it is possible that DivJ and/or PleC activities depend on the presence of c-di-GMP.

Taken together, these results suggested that the PopA GGDEF domain encodes the information for spatial sequestration to both cell poles, whereas the Rec1–Rec2 dimerization stem is sufficient to target PleD to the stalked cell pole.

Distinct polar receptors recruit PopA and PleD to the stalked cell pole

The experiments described above addressed mechanisms involved in PopA activation and sequestration to the cell poles. However, the nature of the polar receptors required to sequester PopA to the cell poles remains unknown. We showed earlier that the swarmer pole specific protein PodJ is required to localize PopA to the flagellated pole (Duerig *et al*., 2009). PodJ is a membrane-anchored protein with a cytoplasmic N-terminus and a

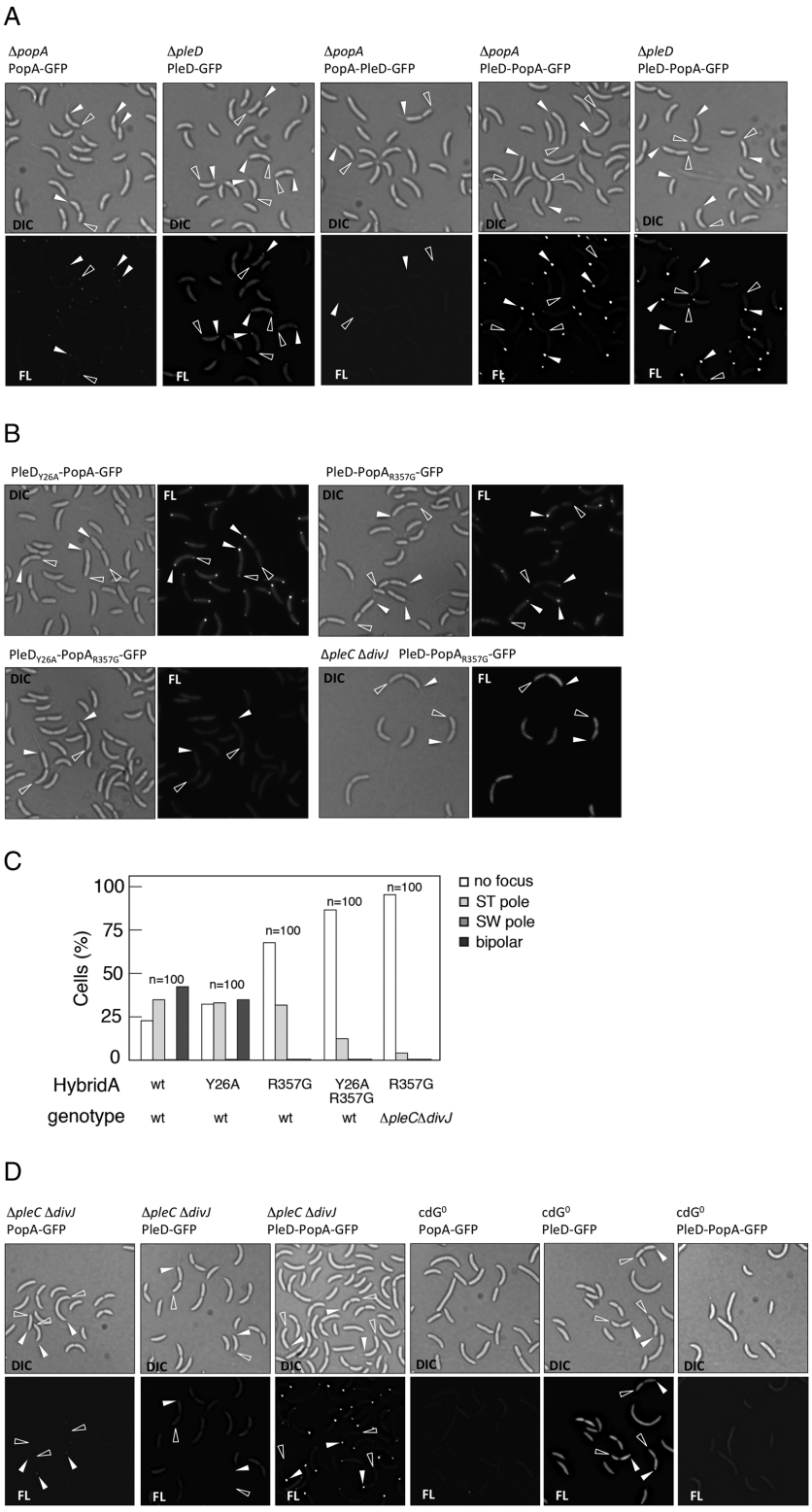


Fig. 3. PopA GGDEF is a polar localization domain.
A. A [Rec1–Rec2]$_{PleD}$–[GGDEF]$_{PopA}$ fusion (PleD–PopA) localizes to the cell poles. The localization of the PleD–PopA and PopA–PleD hybrids ([Rec1–Rec2]$_{PopA}$–[GGDEF]$_{PleD}$) fused to GFP was analysed in Δ*popA* and Δ*pleD* strains by fluorescence microscopy. PopA–GFP and PleD–GFP fusions are shown as controls. DIC and fluorescence images (FL) are shown.
B. PleD–PopA contains two distinct polar addresses. Localization of PleD–PopA–GFP fusions containing mutations affecting PleD Rec-stem dimerization (Y26A) or c-di-GMP binding by PopA GGDEF (R357G). Filled arrows mark stalked cell poles and open arrows mark poles opposite the stalk.
C. Distribution analysis of localization data from (B). Only predivisional cells were analysed.
D. Localization of PleD–PopA–GFP in strains lacking c-di-GMP (cdG^0) or lacking kinases PleC and DivJ. Localization of PopA–GFP and PleD–GFP is shown as control. The identity of cell poles is marked as indicated above. Fusion proteins were expressed from a low-copy-number plasmid in the strains indicated.

periplasmic C-terminal part (Fig. S6). Mutants lacking parts of the periplasmic domain of PodJ showed normal PopA localization (Fig. S6). Similarly, mutants PodJ639 and PodJ660, which are truncated within or immediately following the transmembrane domain showed normal PopA localization. In contrast, cells expressing PodJ589, which maintains most of the cytoplasmic portion except a short stretch proximal to the membrane-spanning region, lost PopA from the flagellated pole (Fig. S6). These results are in agreement with PodJ localization data of Lawler and co-workers who reported that PodJ589 was the only PodJ variant unable to localize to the nascent flagellar pole (Lawler *et al.*, 2006). From this we conclude that, directly or indirectly, PodJ recruits PopA to the flagellated pole via its cytoplasmic portion.

Finally, we analysed candidate proteins required to direct PopA to the stalked cell pole. Several polar proteins, including TipF, TipN, PopZ and SpmX, are known to serve as anchoring structures for the successive localization of downstream components (Viollier *et al.*, 2002; Huitema *et al.*, 2006; Lam *et al.*, 2006; Radhakrishnan *et al.*, 2008). While, mutants lacking TipF, TipN, or SpmX displayed wild type-like distribution of PopA (data not shown), *popZ* mutants showed a complete loss of bipolar PopA localization (Fig. 4A). In accordance with the existence of two distinct addresses recruiting PopA to opposite cell poles, polar localization was abolished in *popZ podJ* double mutants (Fig. 4A). In contrast, PleD or its constitutive active derivative PleD* (Aldridge *et al.*, 2003) localized normally to the cell pole in the *popZ* mutant (Fig. 4B) and all other mutants tested. Thus, despite of the fact that the paralogous regulators PleD and PopA colocalize to the incipient stalked cell pole during the *Caulobacter* SW-to-ST transition, they recognize distinct structures at this subcellular site.

The Rec1 domain of PopA is involved in RcdA recruitment to the stalked cell pole

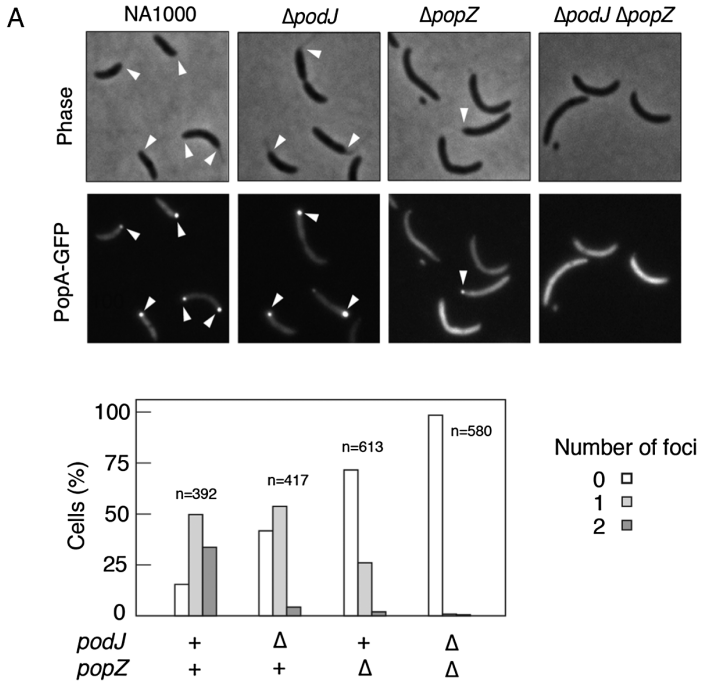
The observation that the PleD–PopA hybrid was able to reach both the polar determinants of PleD and PopA prompted us to analyse the functionality of this hybrid protein. Since PleD is required for flagellar ejection and degradation of the flagellar anchor protein FliF during the SW-to-ST transition (Aldridge *et al.*, 2003) and PopA is important for CtrA degradation during the same cell cycle period, we chose to assay the turnover of these proteins as indicators of PleD–PopA functionality. As shown in Fig. 5, PleD–PopA could neither restore FliF degradation in a *pleD* mutant nor restore CtrA degradation in a *popA* mutant. From this we concluded that PleD–PopA is non-functional. While this is intuitive for its function as a DGC (the PopA GGDEF domain is catalytically inactive), this result was unexpected for PopA and suggested that the Rec1 and/or Rec2 domain(s) might be responsible for PopA output function.

PopA recruits RcdA to the stalked pole via direct protein–protein interaction (Duerig *et al.*, 2009). To determine the requirements for this interaction we analysed if PleD–PopA is able to direct RcdA to the pole. While RcdA–YFP finds the cell pole when coexpressed with PopA, it fails to localize in the presence of PleD–PopA (Fig. 6), indicating that the PopA GGDEF domain, although reaching the stalked pole, is not sufficient to recruit RcdA to this subcellular site. Next, we analysed PopA-RcdA interaction using the bacterial two-hybrid system. Individual PopA domains (Rec1, Rec2, GGDEF) were C-terminally fused to T25 fragment of the adenylate cyclase and assayed for interaction with RcdA fused C- or N-terminally to the T18 fragment. A positive signal was only obtained between RcdA and full-length PopA and PopA–Rec1, whereas PopA–Rec2 and PopA–GGDEF scored negative (Fig. 7). While this result indicates a direct role for the Rec1 domain of PopA in RcdA recruitment, we wanted to test if this domain is sufficient for this function. To this end we constructed a hybrid protein that grafts the Rec2 domain of PleD in between the Rec1 and GGDEF domains of PopA [$Rec1_{PopA}$–$Rec2_{PleD}$–$GGDEF_{PopA}$]. This hybrid protein was able to localize to the cell pole (Fig. 6) again emphasizing the important role of the GGDEF domain for PopA localization. However, the PopA–PleD–PopA hybrid was unable to recruit RcdA to the cell pole. This argues that the Rec1 domain of PopA, despite of interacting with RcdA and being required for its recruitment to the cell pole, is not sufficient for this function.

In summary, these results suggested that PopA recruits RcdA to the cell pole via specific interaction with its receiver stem and that the signal transduction flow in PopA is reversed as compared to classical response regulators like PleD. While the C-terminal GGDEF domain of PopA constitutes the input domain, downstream interactions operate through the N-terminal part of the protein (Fig. 1B).

PopA emerged through gene duplication from its PleD ancestor in alpha-proteobacteria

During evolution DGC and PDE enzyme domains expanded their functional repertoire to become c-di-GMP effectors by co-opting the allosteric I-site or the substrate binding site respectively (Duerig *et al.*, 2009; Petters *et al.*, 2012; Davis *et al.*, 2013). Novel biological functions can arise through gene duplication followed by adaptive evolution (Conant and Wolfe, 2008). Functional expansion of the family of c-di-GMP receptors might thus have occurred through duplication of genes encoding catalytic components followed by co-option of the existing c-di-GMP binding sites, allosteric I-site or substrate binding site, for



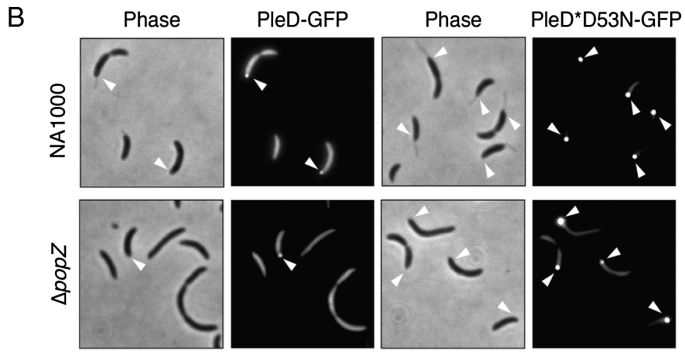


Fig. 4. The stalked pole-specific anchor protein PopZ directs PopA localization.
A. Localization of a PopA–GFP fusion (arrows) in wild type and mutants lacking polar receptors. Phase-contrast and fluorescence images are shown (top) and analysed quantitatively as indicated in the graph below.
B. Localization of a PleD–GFP and PleD*–GFP fusions (arrows) in wild type and in the *popZ* mutant. Phase-contrast and fluorescence images are shown (top) and analysed quantitatively as indicated in the table below. MicrobeTracker (http://microbetracker.org) was used for quantification. All fusion proteins were expressed in the strains indicated from a low-copy-number plasmid.

Strain		Number of cells analysed	Number of GFP-foci per cell			Polar localization index[1)]
popZ	PleD-GFP		0	1	> 2	
WT	WT	858	72 %	28 %	0.5 %	82 %
Δ	WT	922	76 %	24 %	0.3 %	87 %
WT	PleD*D53N	1087	36 %	61 %	2.7 %	72 %
Δ	PleD*D53N	533	31 %	65 %	4.9 %	65 %

[1)] % of cells with 1-GFP-focus at a pole which was defined as a cellular position outiside of the 20-80% cell length

novel processes (Jenal and Malone, 2006). To corroborate this idea, we analysed the distribution of PleD and PopA homologues throughout the bacterial kingdom. Orthologous clusters of PopA (CC_1842) and PleD (CC_2462) were initially searched in the SSDB (KEGG) within the bacterial domain using a best-best rule (see *Experimental procedures*). As shown in Fig. 8, PleD orthologues (Rec–Rec–GGDEF) are widespread in bacteria, although with a strong bias towards alpha-proteobacteria. In contrast, PopA orthologues were found only in *Caulobacter* and its closest stalked relatives, suggesting that PopA originated in the common ancestor of this group of organisms through a gene duplication/co-option mechanism possibly as an adaptation to their characteristic dimorphic lifestyle (Brown *et al.*, 2011).

Discussion

Here we investigated the signalling mechanisms of the *C. crescentus* response regulator PleD and its structural homologue, the orphan response regulator PopA. Both proteins are part of the c-di-GMP signalling network con-

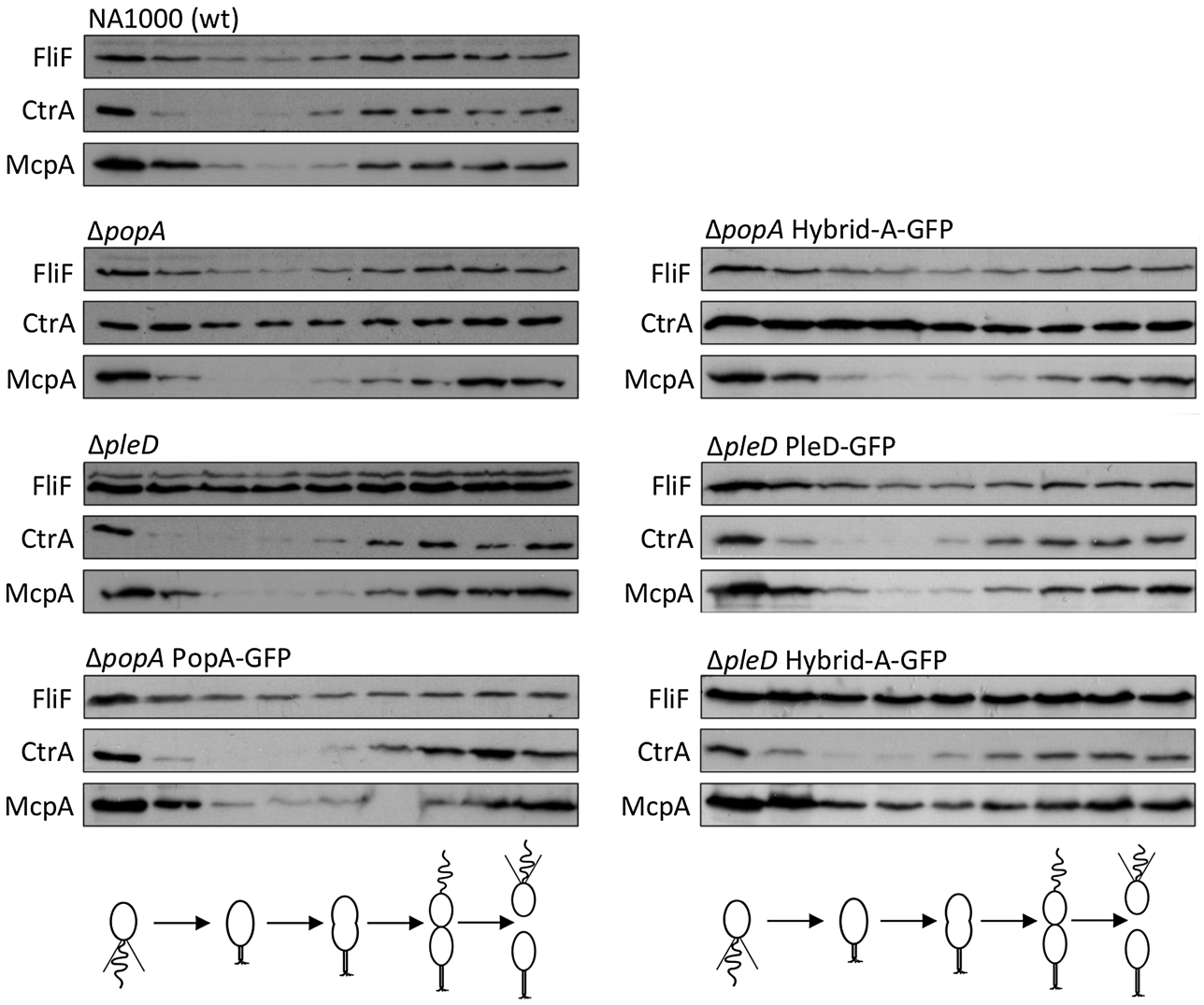


Fig. 5. A PleD–PopA hybrid fails to support cell cycle-dependent protein degradation. Cell cycle-dependent degradation of CtrA and FliF was monitored by immunoblot analysis in synchronized populations of wild-type, *pleD* or *popA* mutant cells. A GFP fusion of PleD, PopA or PleD–PopA was expressed as indicated. Chemoreceptor protein McpA was used as control as its cell cycle-dependent degradation is neither *popA*- nor *pleD*-dependent.

trolling *C. crescentus* cell cycle progression and, upon activation, both dynamically localize to the stalked cell pole prior to S-phase entry. Our studies were motivated by the questions how c-di-GMP binding stimulates PopA activity and how activated PopA reaches the cell pole. Our initial working hypothesis was inspired by PleD and its mechanism of receiver domain-mediated dimerization (Paul *et al.*, 2007; Wassmann *et al.*, 2007). The polar receptor for PleD discriminates between an active dimer and an inactive monomer of PleD. A similar mechanism was proposed for the diguanylate cyclase WspR from *Pseudomonas aeruginosa,* the activity of which is also modulated through oligomerization via its receiver domain (De *et al.*, 2008). In analogy, a c-di-GMP induced conformational change could in principle favour PopA oligomerization via Rec1 and Rec2. However, mutations of the predicted dimerization interface did not affect PopA localization and activity. Moreover, PleD–PopA hybrid experiments strongly suggested that the GGDEF domain is sufficient to localize PopA to the stalked pole in a c-di-GMP-dependent manner and that Rec1 and Rec2 play a subsidiary role in this process. This strongly argued against conserved activation mechanisms for PleD and PopA and suggested that, despite of the same domain organization and evolutionary history, the individual domains of PopA have undergone substantial functional diversification.

The finding that polar recruitment of PopA depends on the C-terminal GGDEF domain and its ability to bind c-di-GMP raised the question of how this domain contributes to PopA activation. Inspired by a mechanism postulated for

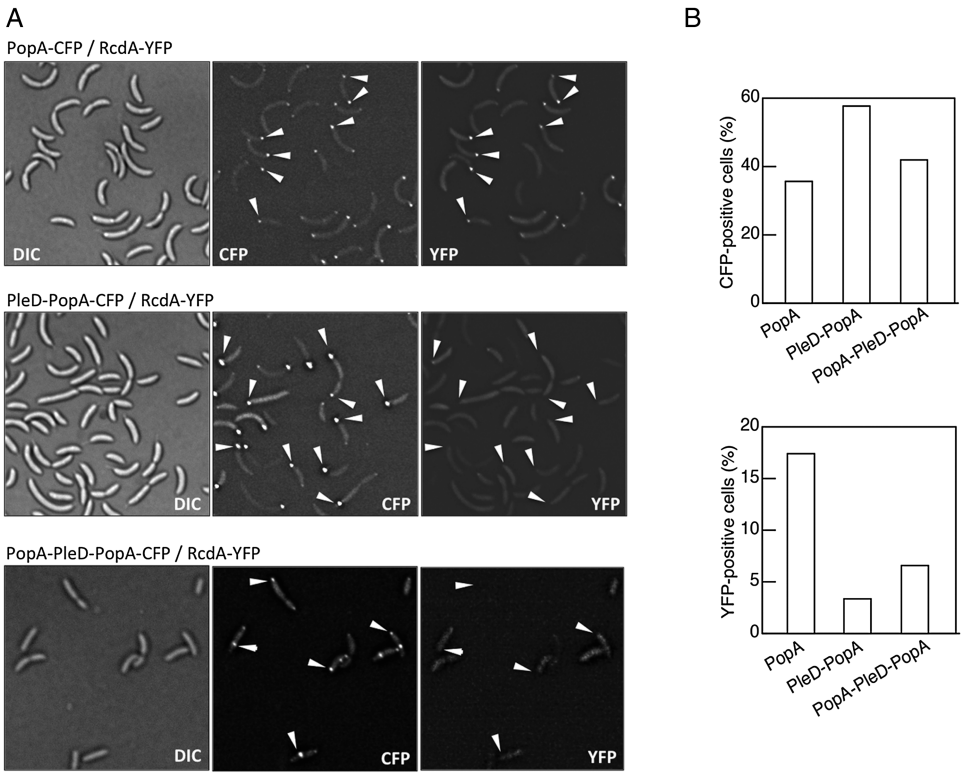


Fig. 6. The PopA receiver domains are required for RcdA recruitment to the stalked cell pole. Colocalization analysis of PopA–PleD hybrid proteins and RcdA. Cells of a Δ*popA* mutant expressing RcdA–YFP and either PopA–CFP (control), PleD–PopA–CFP, or PopA–PleD–PopA–CFP ($[Rec1]_{PopA}$–$[Rec2]_{PleD}$–$[GGDEF]_{PopA}$) from plasmids were analysed by fluorescence microscopy. RcdA–YFP was expressed from its native promoter cloned on a high-copy-number plasmid and CFP fusions were induced by 1 mM vanillate for ~ 2 h. DIC, YFP fluorescence, and CFP fluorescence are shown. Arrows indicate the positions of selected foci. In each strain, more than 200 cells were analysed to determine fractions of CFP- and YFP-positive cells.

the allosteric control of PleD, we tested if PopA activation and localization could depend on c-di-GMP mediated oligomerization of its GGDEF domain. Interference of two GGDEF domains would leave a large surface area provided by the two pseudo-receiver domains to interact with partner proteins that need to be recruited to the cell poles either because they have to be delivered to the ClpXP protease or for other functional reasons (Fig. 1B) (Duerig *et al*., 2009; Radhakrishnan *et al*., 2010). In the available PleD crystals GGDEF domains are cross-linked to neighbouring domains by two c-di-GMP molecules via primary and secondary binding sites in a non-productive manner, arranging the substrate binding sites away from each other (Chan *et al*., 2004; Wassmann *et al*., 2007). Mutations of Arg residues of the secondary binding sites abolished product inhibition of PleD indicating that this conformation is physiologically relevant (Paul *et al*., 2007). Importantly, PleD feedback control was only abolished when several secondary I-site residues positioned on the surface of either the GGDEF or the Rec2 domain were mutated sequentially, indicating that a c-di-GMP dimer bound to the primary I-site has several possible docking sites on neighbouring domains (Chan *et al*., 2004; Wassmann *et al*., 2007). In analogy, it is possible that several secondary docking sites can be utilized to cross-link individual GGDEF domains of PopA. This would explain why PopA mutants with single Arg substitutions representing potential secondary binding sites show only partial loss-of-function. Although our genetic data are consistent with a model in which PopA uses a flexible GGDEF-mediated dimerization interface, more solid evidence in favour of such a mechanism is currently missing because biochemical analyses of PopA were hampered by its poor *in vitro* solubility.

A subset of GGDEF and EAL domains have given up on their original catalytic properties and have co-opted a novel role as c-di-GMP effectors (Jenal and Malone, 2006). While EAL domains exploited their substrate-binding pocket for this purpose (Navarro *et al*., 2011), it was proposed earlier that GGDEF domains employ an ancestral allosteric site to probe cellular c-di-GMP levels and transduce this information into a specific molecular readout (Jenal and Malone, 2006). Based on our findings with PleD and PopA we postulate that c-di-GMP-dependent activa-

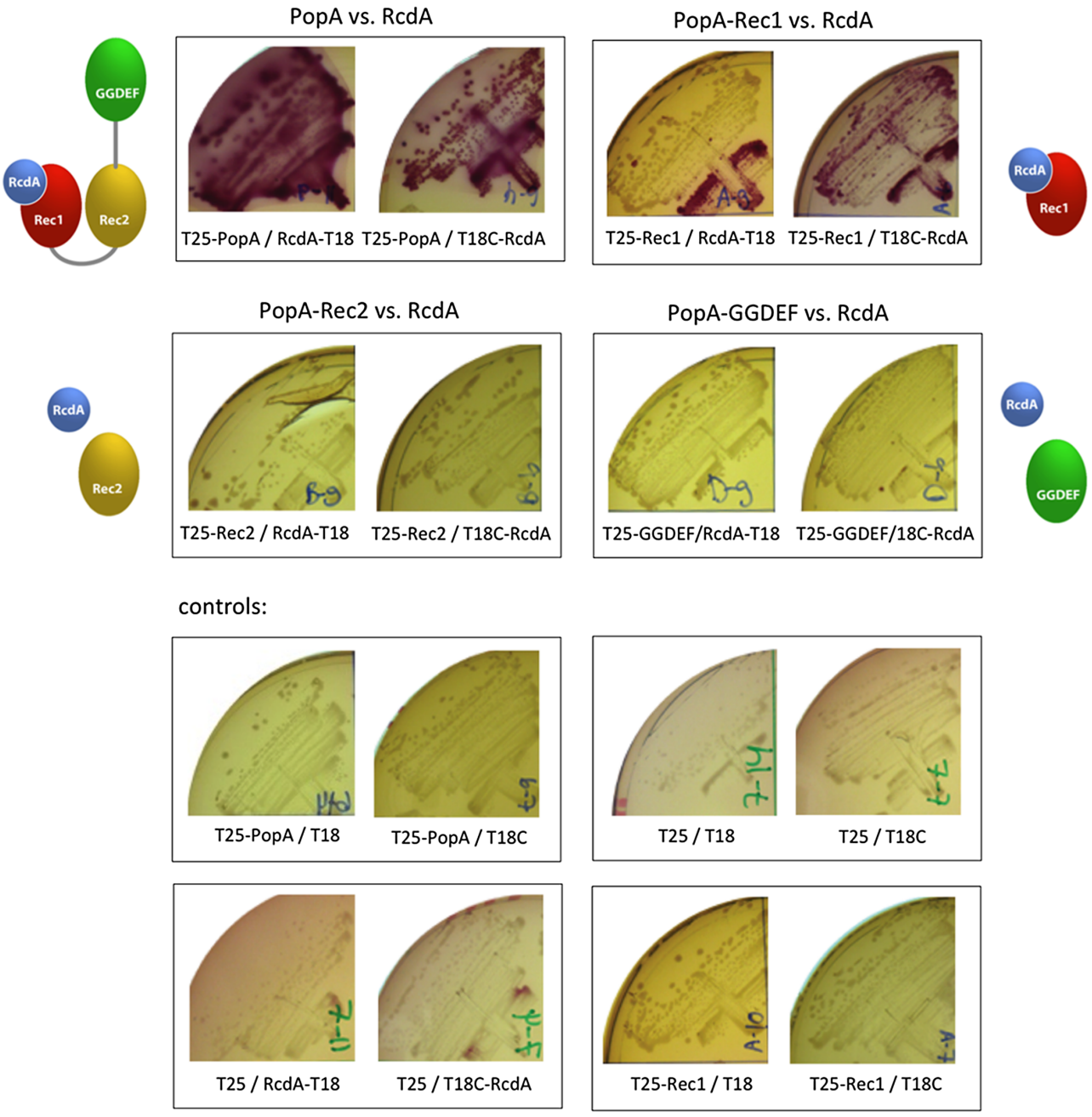


Fig. 7. The first receiver domain (Rec1) of PopA directly interacts with RcdA. Full-length PopA and individual PopA domains (Rec1, Rec2, GGDEF) were fused to the T18 and T25 fragments of *B. pertussis* adenylate cyclase. Both N- and C-terminal fusions to T18 were analysed. *E. coli* strains expressing the respective fusion pairs were examined on McConkey agar plates. Interactions of the individual domains are indicated schematically.

tion of non-catalytic GGDEF domains involves ligand-induced oligomerization via one or several possible secondary binding site. Alternatively, primary I-sites might bridge to heterologous partner proteins via bound ligand. Several reports have recently indicated that c-di-GMP can activate enzyme complexes or transcription factors by mediating specific protein–protein interaction (Wassmann *et al.*, 2007; De *et al.*, 2008; Krasteva *et al.*, 2010; Steiner *et al.*, 2013). It is interesting to note that, with the exception of EAL domain-based effectors, which have likely emerged from PDEs and therefore by default bind a monomer of c-di-GMP, effector proteins generally bind a twofold symmetric dimer of the ligand with intercalated bases (Chan *et al.*, 2004; Wassmann *et al.*, 2007; De *et al.*, 2008;

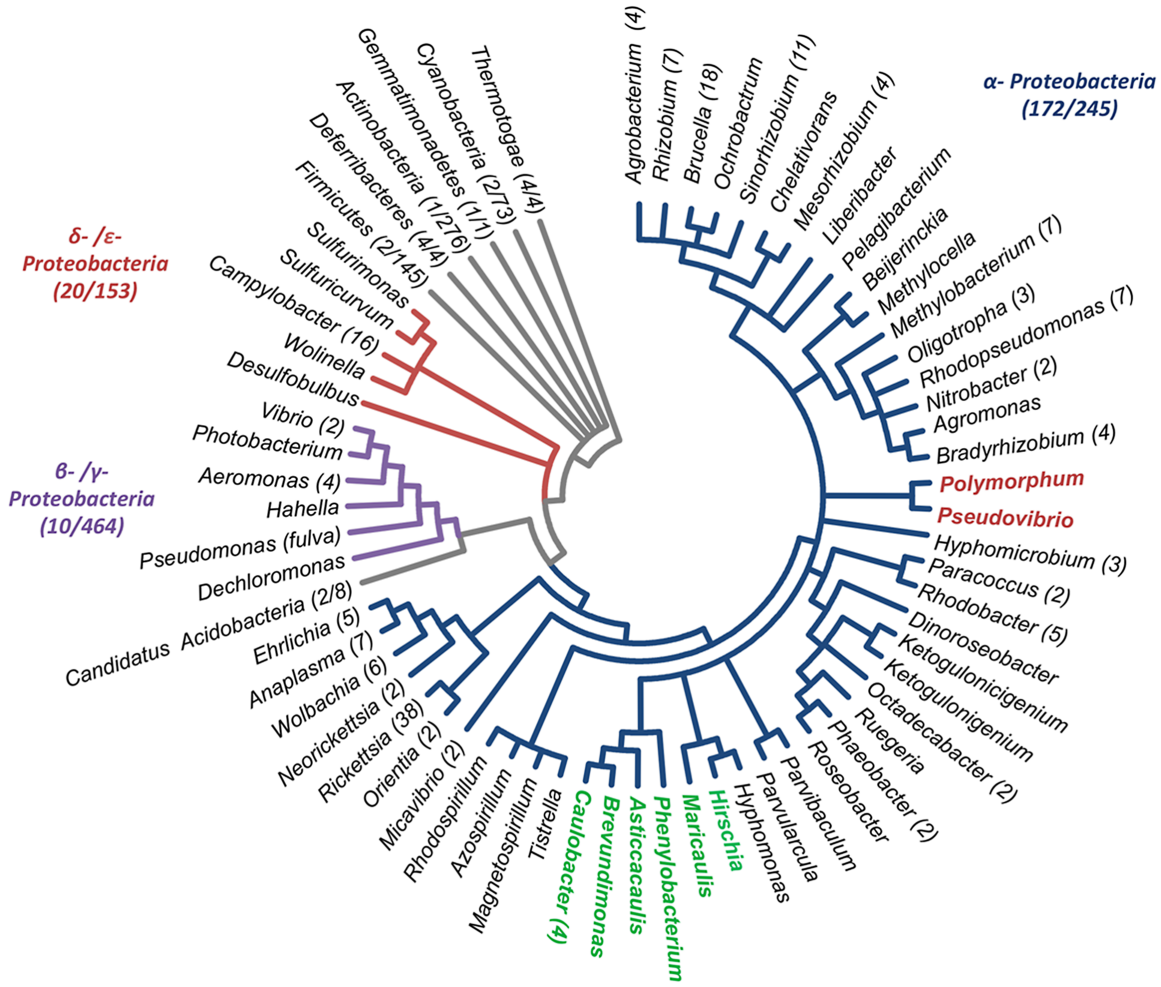


Fig. 8. Phylogenetic distribution of PleD and PopA orthologues. A 16S rRNA phylogenetic tree is shown based on the Neighbor-Joining method. The analysis involved 74 nucleotide sequences corresponding to taxa that harboured PleD orthologues only (black), PopA orthologues only (2, red) and both PleD and PopA orthologues (6, green). Numbers in brackets indicate multiple genomes encoding for a PleD orthologue within the same taxon. A second number after a slash indicates the total number of genomes screened within the corresponding taxon.

Krasteva *et al.*, 2010; Habazettl *et al.*, 2011). Such an arrangement might offer larger surfaces to facilitate complex c-di-GMP mediated protein–protein interaction and might thus represent a general mechanistic principle for how c-di-GMP activates specific cellular responses.

PleD has likely evolved through duplication of the receiver domain in the ancestral WspR-like Rec–GGDEF protein family. It is tempting to speculate that the selective pressure for this evolutionary process came from a need for increased versatility in signalling and/or protein interaction. For example, duplication of the Rec domain might have led to a larger and more robust dimerization interface and interaction surface, a key innovation to introduce spatial control of PleD through specific contacts to a polar receptor. In contrast, PopA has evolved from PleD relatively recently through gene duplication, as it is found only in *Caulobacter* and some of its closest relatives. Co-opting PopA into a c-di-GMP effector by exploiting the existing c-di-GMP binding site, converted the GGDEF domain from a classical output domain into the actual input of the protein, consequently superseding the need for phosphorylation control. Similar phosphorylation-independent response regulators were described before and can be divided into two categories with respect to the function of their receiver domains. In some cases receiver domains are still involved in activating a C-terminal output domain but use different input mechanisms. For example, VpsT from *Vibrio cholerae* directly binds c-di-GMP to an extension of its receiver domain, thereby inducing dimerization and DNA binding of this transcription regulator (Krasteva *et al.*, 2010). Members of the second group of atypical receiver domains have given up on acting as true signal input domains and instead have adopted novel roles by exploiting their inherent protein–protein interaction abilities

(Fraser *et al*., 2007; Mignot *et al*., 2007; Xu *et al*., 2012). Similarly, the large surface provided by the Rec1–Rec2 part of PopA might not only be used for RcdA recruitment but also to 'communicate', directly or indirectly, with different polar receptors like PodJ and PopZ.

Experimental procedures

Growth conditions

Caulobacter crescentus strains were grown in peptone yeast extract (PYE) or minimal media supplemented with 0.2% glucose or 0.3% D-xylose (M2G or M2X) (Ely, 1991) at 30°C with constant shaking (150 r.p.m.). When selection was required antibiotics in the following concentrations were added: (solid/liquid media in μg ml^{-1}): gentamicin (5/0.5), kanamycin (20/5), nalidixic acid (20/not used) and oxytetracycline (5/2.5). For inducible gene expression the medium was supplemented with 1 mM vanillate, 0.3% xylose or 1 mM IPTG. For synchronization experiments newborn swarmer cells were isolated by Ludox gradient centrifugation (Jenal and Shapiro, 1996) and released into fresh minimal medium. *Escherichia coli* strains were grown in Luria Broth (LB) at 37°C. The following antibiotic concentrations were used for selection: (solid/liquid media in μg ml^{-1}) ampicillin (100/50), gentamicin (20/15), kanamycin (50/30) and oxytetracycline (12.5/12.5).

Strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table S1. Plasmids were constructed and propagated in *E. coli* DH10B and transferred by conjugation (Ely, 1991) into *C. crescentus* strains. The Strain Arctic^{R} BL21 (DE3) (Stratagene, USA) was used for protein overexpression and strain MM337 (Karimova *et al*., 1998) for bacterial two-hybrid assays. Detailed protocols of strain and plasmid constructions are available on request. PleD and PopA hybrid proteins were constructed by fusing amino acids 1–286 of PleD to amino acids 284–441 of PopA to generate the PleD–PopA hybrid; and amino acids 1–283 of PopA to amino acids 287–454 of PleD to generate PopA–PleD hybrid, and by replacing amino acids 140–286 of PleD with amino acids 137–284 of PopA to generate hybrid PopA–PleD–PopA.

Microscopy

DIC and fluorescence microscopy were performed on a DeltaVision Core (Applied Precision, USA)/Olympus IX71 microscope equipped with an UPlanSApo 100×/1.40 Oil objective (Olympus, Japan) and a coolSNAP HQ-2 (Photometrics, USA) CCD camera. Cells were placed on a patch consisting of 1% agarose (Sigma, USA) in water (Sigma, USA). Images were processed with softWoRx version 5.0.0 (Applied Precision, USA) and Photoshop CS3/CS5 (Adobe, USA) software and Image J (NIH, USA).

Bacterial two-hybrid analysis

Bacterial two-hybrid analysis was performed as described (Karimova *et al*., 1998). Proteins of interest were fused N or C-terminally to the T18 or T25 fragment of the *Bordetella pertussis* adenylate cyclase. Two microlitres of the MM337 culture containing pUT18 and pKT25 derivatives were spotted on McConkey indicator agar supplemented with 0.1% maltose, kanamycin and ampicillin and grown at 30°C.

Immunoblots

Log-phase cells were analysed using antibodies against PopA, CtrA, McpA, FliF and GFP, which were diluted as reported earlier (Domian *et al*., 1997; Duerig *et al*., 2009). Primary antibodies were detected by HPR-conjugated swine anti-rabbit secondary antibodies (Dako Cytomation, Denmark). Immunoblots were developed with ECL detection reagents (Western Lightning, Perkin Elmer, USA). When synchronized *C. crescentus* cultures were used, samples for immunoblot analysis were taken at 20 min intervals from synchronized *C. crescentus* cultures.

Phylogenetic analyses

Orthologous clusters of KEGG:ccr:CC_1842 (PopA) and KEGG:ccr:CC_2462 (PleD) were initially searched in the SSDB (KEGG) within the bacterial domain and with a best-best rule. Only hits above a threshold of 150 that showed over 66% of size conservation were further considered. In addition, PleD orthologues that displayed more than a single aligning region (hsp) when blasted against PleD were discarded from the analysis. Seventy-four consensual 16S rRNA sequences (derived from 2 to 3899 individual sequences from the Ribosomal Database Project website) (Cole *et al*., 2009) from the genera identified here above were used to determine a phylogeny. All positions with less than 98% site coverage were eliminated. There were a total of 1093 positions in the final data set. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Bootstrapping cut-off was set to 50%. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011).

Structural modelling of PopA

The structure of PopA was modelled by Swiss Model (Arnold *et al*., 2006) on the basis of a sequence alignment between PleD and PopA, which was obtained by matching their sequence profiles using HHpred (Hildebrand *et al*., 2009). As template structure the dimeric, c-di-GMP complexed conformation of PleD (PDB code 2V0N) was used. All protein visualizations were made with the program DINO (http://www.dino3d.org).

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Supporting information

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