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**Abstract**

Bacterial cell poles constitute defined subcellular domains where numerous proteins localize, often at specific times, to affect various physiological processes. How pole recognition occurs and what governs the timing of protein localization are often unknown. In this paper, we investigate the mechanisms governing the localization of PopZ, a chromosome-anchoring protein whose unipolar to bipolar localization pattern is critical for cell cycle progression in Caulobacter crescentus. We provide evidence that polar localization of PopZ relied on its self-assembly into a higher-order structure (matrix) and that the unipolar to bipolar transition was coupled to the asymmetric distribution of ParA during the translocation of the origin-proximal ParB–parS partition complex. Collectively, our data suggest a model in which a local increase of ParA concentration promotes the assembly of a PopZ matrix precisely when and where this matrix is needed. Such coupling of protein assembly with a cell...

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Spatiotemporal control of PopZ localization through cell cycle–coupled multimerization

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Bacterial cell poles constitute defined subcellular domains where numerous proteins localize, often at specific times, to affect various physiological processes. How pole recognition occurs and what governs the timing of protein localization are often unknown. In this paper, we investigate the mechanisms governing the localization of PopZ, a chromosome-anchoring protein whose unipolar to bipolar localization pattern is critical for cell cycle progression in Caulobacter crescentus. We provide evidence that polar localization of PopZ relied on its self-assembly into a higher-order structure (matrix) and that the unipolar to bipolar transition was coupled to the asymmetric distribution of ParA during the translocation of the origin-proximal ParB–parS partition complex. Collectively, our data suggest a model in which a local increase of ParA concentration promotes the assembly of a PopZ matrix precisely when and where this matrix is needed. Such coupling of protein assembly with a cell cycle–associated molecular asymmetry may represent a principle of cellular organization for controlling protein localization in both time and space.

Introduction

Despite their relative small size and the lack of membrane-bounded organelles within their cytoplasm, bacterial cells display a remarkable level of spatial organization at the molecular level. In particular, the cell poles constitute a distinct subcellular environment in which a growing number of proteins have been found to localize (Rudner and Losick, 2010; Bowman et al., 2011). The resulting functional confinement is crucial for a broad variety of processes, including motility, chemotaxis, pathogenesis, cellular differentiation, and cell cycle progression. In many cases, a protein localizes at the cell pole through an interaction with an anchoring protein or complex that was already present at the pole, which raises the critical question of how the initial pole recognition is achieved. Geometric cues inherent to the cell poles, such as the degree of membrane curvature, can be “sensed” by some proteins (Lenarcic et al., 2009; Ramamurthi and Losick, 2009; Ramamurthi et al., 2009), but other self-organizing mechanisms likely exist to promote pole accumulation (Rudner and Losick, 2010). Another equally important and perhaps even less understood question regards the temporal dynamics of protein localization. Often, the protein localization pattern changes in time (for example, at a particular stage during the cell cycle). How this temporal regulation occurs remains largely elusive.

To examine these questions, we focused on the multimeric polar scaffold PopZ, whose dynamic localization pattern plays a crucial role during the cell cycle of Caulobacter crescentus (Fig. 1). In “swarmer” (G1 phase) cells, PopZ localizes at the old pole, where it forms a matrix that tethers the origin-proximal parS DNA sequence (and hence the chromosome) through a specific interaction with the parS-binding protein ParB (Bowman et al., 2008; Ebersbach et al., 2008). Initiation of DNA replication, which occurs only once per cell cycle, is quickly followed by the duplication of the parS sequences, resulting in two ParB–parS partition complexes (Mohl and Gober, 1997). Although one complex remains at the old pole, the other rapidly segregates toward the new pole, powered by the retraction of the DNA-bound ParA structure (Ptacin et al., 2010; Schofield et al., 2010; Shebelut et al., 2010). Around the same time, the localization pattern of PopZ becomes bipolar as a result of a new accumulation at the new pole, where PopZ captures the migrating ParB–parS complex (Bowman et al., 2008; Ebersbach et al., 2008). This unipolar to bipolar change in PopZ localization is a...
critical step for coordinating the initiation of chromosome segregation with the formation of the cytokinetic FtsZ ring. This is because the PopZ-dependent anchoring of the ParB–parS complexes at opposite poles stabilizes bipolar gradients of the FtsZ ring inhibitor MipZ, thereby promoting FtsZ ring assembly near the midcell where the MipZ inhibitory activity is the lowest (Thanbichler and Shapiro, 2006; Kiekebusch et al., 2012). Indeed, in △popZ cells, ParB–parS complexes, from which emanate the MipZ gradients, remain unanchored and thereby display considerable motion that affects the timing and location of FtsZ ring assembly (Ebersbach et al., 2008), leading to cell division defects (Bowman et al., 2008; Ebersbach et al., 2008). The dynamic localization pattern of PopZ is also important for other cell cycle–related events, as PopZ is essential for the polar localization of multiple cell cycle regulator proteins (Ebersbach et al., 2008; Bowman et al., 2010).

How PopZ accumulates at the poles and how it reproduces its dynamic localization pattern at every cell cycle remains poorly understood and is the subject of debates (Bowman et al., 2008, 2010; Ebersbach et al., 2008; Curtis and Brun, 2010; Rudner and Losick, 2010). In this work, we address both spatial and temporal aspects of PopZ localization. Our results support a simple model in which the ParA-dependent DNA segregation machinery controls the otherwise stochastic multimerization of PopZ spatially and temporally, such that a PopZ-anchoring matrix assembles at the right pole and at the right time during the cell cycle.

**Results**

Multimerization is required for polar localization

PopZ is known to self-assemble into oligomers that further assemble into a matrix (Bowman et al., 2008, 2010; Ebersbach et al., 2008). However, the importance of this assembly process in protein localization is unknown. To examine this question, we first sought to identify the regions within PopZ that are required for localization, oligomerization, and matrix formation inside cells. Because PopZ does not contain any domain of known function, we considered both the secondary structure prediction and the sequence conservation to delineate truncation boundaries. A multiple-sequence alignment of 100 PopZ orthologues highlighted the conservation of the N-terminal and C-terminal regions, which are predicted to form α helices (Fig. 2 A). We defined three main domains over the 177–amino acid sequence of PopZ (Fig. 2, A and B): (1) a conserved N-terminal domain composed mainly of an α helix (H1), (2) a central domain poorly conserved in sequence and size but characterized by a high content of proline residues, and (3) a conserved C-terminal domain, which includes three predicted α helices (H2 and the adjacent H3 and H4). We generated seven variants lacking various parts of the protein (Fig. 2 B) and observed their localization when tagged with YFP and produced from the chromosomal xylose-inducible promoter as a second copy (Fig. 2, C and D; and Fig. S1 A, Western blot). As expected, the full-length PopZ-YFP localized at one or both poles, depending on the cell cycle stage. Interestingly, polar accumulation was abrogated for the PopZ-YFP variants missing the H3H4 domain (Fig. 2, C and D, △H3H4, △C, and △N). Conversely, all variants containing the H3H4 domain (△N, △C, H3H4, and △H2), including the H3H4 domain alone, maintained the proper polar localization of PopZ (Fig. 2, C and D). Thus, the conserved 53–amino acid H3H4 domain is not only required, but also sufficient, for polar accumulation in wild-type cells.

Because wild-type cells also express a native copy of popZ, it was unclear whether the H3H4 domain is directly involved in pole recognition or is required for polar localization through an interaction with the wild-type PopZ already present at the poles. To discriminate between these two possibilities, we expressed the PopZ-YFP variants in a △popZ strain. Not surprisingly, the △H3H4, △C, and △N variants remained diffuse in the △popZ strain (Fig. 3, A and B). The △H2 and △N variants retained their ability to localize at the poles in the absence of the native PopZ (Fig. 3, A and B), although the polar foci of ΔN-YFP were weak, unipolar, and observed in fewer cells (which we discuss later). Polar foci were also observed when ΔN was fused to the small tetracysteine (TC) tag instead of YFP and expressed in △popZ cells (Fig. 3 C), showing that the polar localization pattern was not specific to the tag. These results indicate that neither the N-terminal nor the H2 domain is absolutely required for polar localization. On the other hand, the constructs containing only the C-terminal domain (C) or part of it (H3H4) displayed a diffuse localization in all cells when PopZ was absent (Fig. 3, A and B), in contrast to their localization pattern in popZ cells (Fig. 2, C and D). This localization defect was not caused by a lower protein concentration, as demonstrated by comparing cells with similar concentrations of protein variants (Fig. S1, B and C). We therefore concluded that the polar localization of the H3H4 and C variants in wild-type cells (Fig. 2, C and D) relies on their interaction with the native PopZ. Note that none of the variants, except ΔH2 (the only one with a normal localization pattern), were able to complement the cell length and stalkless phenotypes of △popZ (Fig. 3, A and D).

Our data show that the H3H4 domain does not work as a pole recognition domain per se. Instead, it likely promotes interaction between PopZ molecules. Therefore, we hypothesized that the H3H4 domain enables the oligomerization of PopZ
monomers. In cell extracts, oligomers of PopZ can be detected as high molecular weight species (typically doublets) on native polyacrylamide gels (Bowman et al., 2008). The full-length PopZ-YFP variants in wild-type (popZ+) C. crescentus cells. Synthesis of the PopZ-YFP variants was induced for 5 h in wild-type cells before imaging (phase contrast and YFP fluorescence). The YFP signal has been scaled for display. Bar, 2 µm. Strains are as follows: CJW3693 for full-length; CJW3695 for ΔH3H4; CJW3696 for ΔC; CJW3680 for N; CJW3697 for ΔN; CJW3802 for H3H4; and CJW3816 for ΔH2. (D) YFP spots were detected from images in C. The percentage of cells with at least one focus of PopZ-YFP variant is shown. All foci were polarily localized. Number of cells counted [n]: n = 765 for full-length; n = 701 for ΔH3H4; n = 803 for ΔC; n = 656 for N; n = 568 for ΔN; n = 726 for C; n = 721 for H3H4; and n = 941 for ΔH2. The data shown are from a representative experiment out of three repeats.

Figure 2. The C-terminal H3H4 domain of PopZ is necessary and sufficient for polar localization in wild-type C. crescentus cells. (A) Schematic overview of a multiple sequence alignment of 100 PopZ orthologues highlighting regions of conservation, displayed with Jalview using the Clustal X color scheme (Waterhouse et al., 2009). Proline (Pro) residues are all depicted in light green. The three main domains and the approximate positions of the α helices (H1–4), predicted with Jpred 3 (Cole et al., 2008), are indicated. term, terminal. (B) Schematic representation of the PopZ truncation variants. Domains, predicted α helices, and their position are indicated as well as the size of each variant (in amino acids). All regions are drawn to scale. (C) Localization of PopZ-YFP variants in wild-type (popZ+) C. crescentus. Synthesis of the PopZ-YFP variants was induced for 5 h in wild-type cells before imaging (phase contrast and YFP fluorescence). The YFP signal has been scaled for display. Bar, 2 µm. Strains are as follows: CJW3693 for full-length; CJW3695 for ΔH3H4; CJW3696 for ΔC; CJW3680 for N; CJW3697 for ΔN; CJW3802 for H3H4; and CJW3816 for ΔH2. (D) YFP spots were detected from images in C. The percentage of cells with at least one focus of PopZ-YFP variant is shown. All foci were polarily localized. Number of cells counted [n]: n = 765 for full-length; n = 701 for ΔH3H4; n = 803 for ΔC; n = 656 for N; n = 568 for ΔN; n = 726 for C; n = 721 for H3H4; and n = 941 for ΔH2. The data shown are from a representative experiment out of three repeats.
Although necessary, oligomerization is not sufficient for polar accumulation because the H3H4 domain alone oligomerizes (Fig. 3 E) but does not display polar localization in \( \text{popZ} \) cells (Fig. 3 A), likely because H3H4 oligomers cannot assemble into a matrix. This supposition was supported by overproduction experiments. When full-length PopZ-TC was overproduced, the FlAsH-stained matrix at the pole expanded into the cell interior, creating large fluorescent regions (Fig. S1 D), as shown previously (Ebersbach et al., 2008). In contrast, the TC-tagged H3H4 domain alone failed to form large FlAsH-stained complexes in both \( \text{popZ} \) and \( \text{popZ} \) native samples (Fig. 3 E, \( \Delta \text{N}, \Delta \text{C}, \) and H3H4). Furthermore, PopZ-YFP variants missing H3H4 (Fig. 3 E, \( \Delta \text{H3H4} \) and \( \Delta \text{C} \)) only migrated as monomers regardless of the presence of wild-type PopZ, showing that the H3H4 domain is required for oligomerization and hence interaction with wild-type PopZ. Because these H3H4-lacking variants were unable to localize at the poles, we conclude that the H3H4 domain is necessary for PopZ oligomerization in vivo and that this oligomerization is required for polar localization.

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areas when overproduced in wild-type (popZ') cells (Fig. S1 D), consistent with a defect in matrix assembly. Collectively, these experiments suggest that PopZ oligomerization and matrix formation are required for polar localization.

**PopZ stochastically assembles into a polar matrix of selective permeability in *Escherichia coli***

The γ-proteobacterium *E. coli*, which is evolutionary divergent from the α-proteobacterium *C. crescentus*, does not encode homologues of PopZ (or homologues of proteins that are polarly recruited by PopZ in *C. crescentus*). Interestingly, recombinant synthesis of fluorescently labeled *C. crescentus* PopZ in *E. coli* results in polar fluorescent foci (Bowman et al., 2008; Ebersbach et al., 2008). This suggests that PopZ localization does not require an extra protein anchor at the pole, as such protein would be expected to be either absent in *E. coli* or to lack a PopZ-interacting surface because of the absence of selective pressure. An alternative, more trivial explanation for this observation is that the formation of polar PopZ foci in *E. coli* is caused by protein misassembly and amorphous aggregation—as recently demonstrated for fluorescent fusions to other multimeric proteins (Landgraf et al., 2012)—and protein aggregates are known to accumulate at the cell poles (Winkler et al., 2010).

We sought to assess the relevance of the heterologous *E. coli* system by first monitoring the localization of PopZ-YFP variants in *E. coli*. Strikingly, the PopZ truncation variants that were able to form polar foci in ΔpopZ *C. crescentus* cells (i.e., ΔN and ΔH2) also displayed polar accumulations in *E. coli* (Fig. 4 A). Moreover, variants that only displayed a diffuse localization pattern in ΔpopZ *C. crescentus* cells (ΔC, N, and H3H4) also had a diffuse distribution in *E. coli* (Fig. 4 A). The results were confirmed by visualizing the polar PopZ constructs and H3H4 as a control, with the small TC tag and FlAsH staining instead of YFP (Fig. S1 E), showing that the localization pattern is not dependent on a bulky tag. The consistency of localization patterns between *E. coli* and *C. crescentus* provides a first line of validation of the heterologous *E. coli* system.

A second line of validation comes from our finding that not only the pole accumulation of PopZ but also the characteristics was induced for 3 h in CJW3991 cells. Arrows point at poles with a FlAsH-stained PopZ-TC focus. (C) Cells and conditions are the same as in B. Brackets delimit the polar LRI area visualized by DIC microscopy and labeled with FlAsH-stained PopZ-TC. The inset shows a zoomed example of a polar LRI region. (D) PopZ-TC synthesis was induced for 2 h in L1-GFP–producing CJW4673 cells before DAPI staining and imaging. Brackets delimit polar LRI areas visualized by DIC microscopy. (E) PopZ-TC production was induced for 2 h in strain CJW4744. PopZ-TC was labeled with FlAsH. Brackets delimit a polar LRI area visualized by DIC microscopy. (F) CJW3997 cells were treated with cephalixin for 2 h before induction of PopZ-YFP and CFP-ParB synthesis for 1 h. Cells were stained with DAPI before imaging. Overlays of DAPI and PopZ-YFP (top) or CFP-ParB (bottom) with the MicrobeTracker cell outline are shown. Arrowheads indicate polar and non-polar foci. (G) PopZ-YFP synthesis was induced in CJW3997 cells for 1 h before addition of cephalixin for an additional 3 h. CFP-ParB synthesis was induced during the last hour of treatment before DAPI staining and imaging. Overlays are displayed as in F. Arrows delimit the accumulation of PopZ-YFP and CFP-ParB at one pole. Bars: [A–C (main image), D, and E] 2 µm; [C, insets] 1 µm; [F and G] 5 µm.
of the PopZ matrix observed in *C. crescentus* can be recapitu-
lated in *E. coli*. In *C. crescentus*, PopZ assembles into a matrix
that is clearly distinct from amorphous aggregates (Ebersbach
et al., 2008; Bowman et al., 2010). First, the mesh size of the
PopZ matrix is selective by excluding DNA and ribosomes,
while allowing the free diffusion of small proteins such as GFP.
Additionally, when PopZ is overproduced in *C. crescentus*,
the matrix expands from the pole into the cell interior and creates
a region of a low refractive index (LRI) observable by differential
interference contrast (DIC; Ebersbach et al., 2008) and phase-
contrast microscopy (Fig. S2) because of an optical effect likely
related to the ribosome exclusion (Ebersbach et al., 2008).
Although FlAsH-stained PopZ-TC polarly localized in *E. coli*
cells (Fig. 4 B), we observed that the FlAsH signal associated
with PopZ-TC could be quite large in some *E. coli* cells as a
result of PopZ-TC accumulation over multiple generations, as
it expanded into the cell body to form LRI regions visible by
DIC microscopy (Fig. 4 C, brackets). Using a GFP fusion to the
ribosomal protein L1, we found that the polar LRI regions where
PopZ accumulated were devoid of ribosomes (Fig. 4 D, bracket-
s), which contrasts with the polar enrichment of ribosomes
normally observed in *E. coli* cells (Robinow and Kellenberger,
1994). The PopZ-TC LRI areas were always found to be de-
void of DAPI signal (Fig. 4 D). Conversely, free CFP showed an
even distribution in PopZ-TC–producing *E. coli* cells, including
the LRI regions (Fig. 4 E). Thus, the polar structure formed by
PopZ in *E. coli* cells is porous and displays the same differential
permeability as the PopZ matrix in *C. crescentus*. This property
would not be expected from an amorphous aggregate.

Our results validate *E. coli* as an in vivo system to investi-
gate the mechanisms underlying PopZ polar localization. They
also support the notion that PopZ has the intrinsic propensity to
assemble into an organized matrix at the cell poles.

An increase in PopZ concentration leads to
the expansion of an existing PopZ matrix
over an initiation of new assemblies

Localization of PopZ is generally unipolar in the relatively fast
growing *E. coli* cells (Bowman et al., 2008; Ebersbach et al.,
2008). The daughter cell that does not inherit the polar PopZ
accumulation builds a new PopZ focus at either the old or new
pole (Ebersbach et al., 2008), consistent with a stochastic pro-
cess. If division in *E. coli* is blocked by cephalaxin treatment,
and PopZ synthesis is induced in the resulting filamentous cells,
PopZ forms foci in any chromosome-free regions, including
internucleoid regions (Ebersbach et al., 2008). Note that be-
cause internucleoid regions do not have the higher degree of
membrane curvature characterizing the cell poles, these results
indicate that PopZ does not localize by “sensing” membrane
curvature, unlike the *Bacillus subtilis* protein DivIVA (Lenarcic
et al., 2009; Ramamurthi and Losick, 2009). Interestingly, we
found that if PopZ-YFP synthesis is already induced before the
cephalexin treatment, PopZ continues to mostly accumulate at
the pole where accumulation had already occurred before drug
treatment, even when more DNA-free spaces become available
within the cell body (Fig. 4 G). In both cases, PopZ accumula-
tions efficiently recruited an exogenously produced *C. crescentus*
CFP-ParB fusion (Fig. 4, F and G), supporting the functional
relevance of these PopZ structures. These results indicate that
PopZ can spontaneously assemble in virtually any low DNA
density regions, unless a PopZ matrix is already present. Col-
lectively, these observations support the notion that PopZ tends
to assemble into a growing matrix—which offers multiple PopZ
self-interaction sites—rather than initiating assemblies else-
where. These results thus argue against the idea that a simple
increase in the cellular concentration of PopZ accounts for the
unipolar to bipolar localization switch observed during the
*C. crescentus* cell cycle (Saberi and Emberly, 2010). Furthermore,
fluorescence intensity measurements indicated that the cellular
concentration of PopZ-YFP does not change during the cell
cycle (Fig. S3 A). Thus, our results suggest the existence of a
mechanism in *C. crescentus* that stimulates (“forces”) the multi-
merization of PopZ at the new pole to yield a bipolar pattern.

The timing of PopZ accumulation at the
new pole is tied to segregation of the
ParB-parS complex in *C. crescentus*

We considered a role for DNA replication because blocking the
initiation of DNA replication in synchronized swarmer cells has
been reported to prevent the accumulation of PopZ at the new
pole by an unidentified mechanism (Bowman et al., 2010). We
confirmed that most cells retained a unipolar PopZ pattern after
depletion of the DNA replication initiator DnaA (unpublished
data). Interestingly, when cells were treated with the gyrase in-
hibitor novobiocin at a concentration that minimize effects on
growth rate, a substantial fraction of cells (up to >60%) dis-
dplayed bipolar localization of PopZ-YFP over time (Fig. 5,
A and B). DNA replication initiation appeared efficiently
blocked by the novobiocin treatment, as indicated by the con-
stant low fraction of cells with two CFP-ParB foci (Fig. 5 C)
and by the diffuse localization of the mCherry-tagged replisome
component DnaN (Fig. 5 D), whose diffuse-to-focus localiza-
tion pattern is used as a proxy for DNA replication initiation
(Collier and Shapiro, 2009). Thus, our results show that a PopZ
focus can appear at the new pole despite a block of DNA repli-
cation, at least in novobiocin-treated cells. Similar trends were
observed when PopZ-YFP was expressed from its native pro-
moter, although a lower fraction of cells with bipolar PopZ was
observed (Fig. 5 D and Fig. S4, A and B). Neither PopZ concen-
tration, nor cell length differences, could explain the ability of
some cells to form a second PopZ focus when DNA replication
is blocked (Fig. S4 C).

How can PopZ accumulate at both poles in novobiocin-
treated cells? It has been previously shown that the unreplicated
parS region often switches from the old pole to the new pole in
novobiocin-treated cells in a ParA-dependent manner (Shebelut
et al., 2010), reminiscent of the translocation of the replicated
parS region that normally occurs after initiation of DNA repli-
cation. We confirmed by time-lapse microscopy that in many
novobiocin-treated cells (61%, n = 314), the unreplicated parS
site labeled with CFP-ParB is translocated, at least once, from
one pole to the other. In all cases in which PopZ-YFP visibly
accumulated at the new pole, it followed a CFP-ParB–parS
translocation to the new pole (Fig. 5 E).
These observations led us to hypothesize that the new pole accumulation of PopZ may depend on DNA segregation, rather than replication. Because, in wild-type cells, the process of parS segregation (that is, translocation of the duplicated parS locus to the new pole) is quickly completed after DNA replication is initiated (Jensen et al., 2001; Viollier et al., 2004), we sought to slow down parS segregation to (a) increase the time resolution at which we can distinguish replication and segregation and, therefore, (b) determine more precisely the time of appearance of PopZ at the new pole relative to both events. For this purpose, we monitored PopZ localization in a TipN-depleted (TipN−) strain, in which replication of the ParB–parS partition complex is normal, but its segregation is slowed down (Patin et al., 2010; Schofield et al., 2010). We predicted that, if the timing of PopZ accumulation at the new pole was dependent on ParB–parS segregation, PopZ should form a focus at the new pole later in TipN− cells compared with wild-type cells. Consistent with this idea, we observed a delay in the appearance of the second PopZ-YFP focus for TipN− cells compared with TipN+ cells (Fig. 6 A). This effect was also seen when using cell length instead of time as a marker of cell cycle progression, which accounts for any cell growth defect (Fig. 6 A). As expected, segregation of ParB–parS (labeled
Next, we asked whether this coupling was also manifest at the single-cell level. This was relevant because cells complete ParB–parS segregation at quite variable times after synchrony (Fig. 6 C, left). Cell-to-cell variability is even wider in the TipN population (Fig. 6 C, left), as ParB–parS translocation is more erratic in this background (Ptacin et al., 2010; Schofield et al., 2010). These broad distributions were not the result of synchronization imperfections or growth rate differences among cells because they were also observed when we considered the

with MipZ-CFP, which binds ParB; Thanbichler and Shapiro, 2006) was slowed down in TipN+ cells compared with TipN cells, as indicated by mean kymographs of the MipZ-CFP signal (Fig. 6 B, left) for each cell population. The delay in MipZ-CFP segregation to the new pole in TipN+ cells was accompanied by a comparable delay in PopZ-YFP accumulation at that pole (Fig. 6 B, right, arrowheads). Thus, at least at the population level, our data suggested a temporal coupling between the segregation of ParB–parS and the localization of PopZ at the new pole.
cell length (instead of the time) at which ParB–parS segregation is completed (Fig. 6 C, right). Strikingly, the variability of PopZ focus formation at the new pole in time and cell length mirrored that of parS segregation, for both TipN+ and TipN− populations (Fig. 6 D). Furthermore, we found a strong positive correlation between the cell length—used to follow cell cycle progression—at which PopZ appears at the new pole and the cell length when segregation ends in individual cells (Fig. 6 E). On the other hand, the correlation was much poorer between DNA replication initiation and the new pole localization of PopZ in TipN− cells (Fig. S4 D), in which replication initiation and segregation are well separated in time.

Moreover, the reported effect of MreB inhibition on the new pole accumulation of PopZ (Bowman et al., 2008) could be fully explained by a delay in chromosome segregation, which is caused by a general slowdown in growth and cell cycle progression (Fig. S4, E–H; Takacs et al., 2010; Sliusarenko et al., 2011). There again, the dynamics of PopZ localization and ParB–parS translocation were very well correlated at the single-cell level (Fig. S4 H).

A PopZ variant lacking the N-terminal domain has a unipolar localization pattern and is deficient in the polar recruitment of ParA and ParB

How could the translocation of ParB–parS to the new pole lead to PopZ accumulation at that location? This DNA translocation process involves two proteins, ParB and ParA (Mohl and Gober, 1997; Ptacin et al., 2010; Schofield et al., 2010; Shebelut et al., 2010). The presence of the ParB focus (associated with parS) at the new pole correlates with the appearance of a PopZ focus at the new pole (Fig. 6 E). Similarly, ParA, whose concentration gradient shifts toward the new pole as ParB–parS segregation proceeds (Schofield et al., 2010), increases its local concentration in the vicinity of the new pole at the time when a second PopZ focus starts building at that location (Fig. 6 F). Thus, the unipolar to bipolar transition of PopZ correlates with both the translocation of ParB–parS and the new pole accumulation of ParA. PopZ is known to interact with both ParB (Bowman et al., 2008; Ebersbach et al., 2008) and ParA (Schofield et al., 2010), although the reason for the PopZ–ParA interaction was not clear. It is important to note that not all PopZ molecules localize at the poles; there is a diffusing cytoplasmic pool of PopZ (Bowman et al., 2008), which we estimate to be ~40% of the total PopZ proteins per cell based on our fluorescence intensity measurements (Fig. S3 B). Therefore, we speculated that a high concentration of either ParB or ParA (or both) may, through interaction with PopZ, increase the local concentration of diffusing PopZ to a level sufficient to nucleate PopZ assembly into a matrix in the low DNA density region of the new pole.

This hypothesis predicts that a PopZ mutant unable to interact with ParA and/or ParB in C. crescentus would be unable to display bipolar localization; instead, it would preferentially accumulate at one pole only through stochastic multimerization, as observed in E. coli. Interestingly, the foci of the TC or YFP-tagged ΔN variant were always unipolar, not only in E. coli (Fig. 4 A) but also in C. crescentus in the absence of native PopZ (Fig. 3, A and C). Using three different assays, we found that the N-terminal domain of PopZ (missing in the ΔN variant) is required for in vivo interaction of PopZ with ParA and ParB, consistent with the hypothesis that ParB and/or ParA are involved in bipolar localization of PopZ.

In our first assay, we took advantage of a DNA binding–deficient variant of ParA, ParA_{R195E}, which localizes predominantly at the poles in wild-type C. crescentus cells (Ptacin et al., 2010; Schofield et al., 2010) but is evenly distributed in the cytoplasm of ΔpopZ cells, allowing for a straightforward monitoring of ParA–PopZ interaction (Schofield et al., 2010). As expected, polar ParA_{R195E–CFP} foci were observed in a large fraction (58.4%) of ΔpopZ cells producing full-length PopZ-YFP (Fig. 7 A), and virtually all of these foci (99.8%) colocalized with a polar PopZ-YFP focus. However, the PopZ variant lacking the N-terminal domain failed to recruit ParA_{R195E–CFP} as ParA_{R195E–CFP} only displayed a diffuse pattern when ΔN-YFP was produced (Fig. 7 A). Because the polar ΔN-YFP foci are relatively weak in ΔpopZ cells (Fig. 3 A), our second interaction assay examined the localization of ParA_{R195E–CFP} or CFP-ParB in ΔpopZ cells overproducing ΔN-TC, in which case clear polar accumulations of ΔN-TC can be visualized by FlAsH staining (Fig. 7, B and C; and Fig. S5, A and B). ParA_{R195E–CFP} was evenly distributed throughout ΔN-TC–producing cells, without the clear enrichment normally seen in PopZ-TC–rich regions (Fig. 7 B). Similarly, the ΔN-TC–rich regions failed to recruit CFP-ParB, unlike the wild-type PopZ–TC–rich regions (Fig. 7 C). The unipolar localization pattern of ΔN or its loss of interaction with ParA and ParB is not caused by a major self-assembly defect, as this ΔN variant can assemble into a porous matrix upon overproduction (Fig. S5, A and B). Our third interaction assay was based on the observation that PopZ can recruit C. crescentus ParB and ParA_{R195E} at the cell pole in E. coli, whereas these proteins display a diffuse localization pattern without PopZ (Bowman et al., 2008; Ebersbach et al., 2008; Schofield et al., 2010). In contrast to the full-length PopZ-YFP, YFP–ΔN foci were unable to recruit ParA_{R195E–CFP} (Fig. 7 D) and CFP-ParB (Fig. 7 E). Thus, all three tests show that the N-terminal domain of PopZ is required for interaction with both ParA and ParB. The inability of the ΔN variant to accumulate at both poles (Fig. 3, A and C) supports the hypothesis that an interaction with ParA, ParB, or both is important for bipolar localization of PopZ.

The accumulation of PopZ at the new pole is associated with the condensation of the ParA concentration gradient at the new pole region

Both ParA and ParB are essential for viability in C. crescentus (Mohl and Gober, 1997), and their depletion would result in cell filamentation (Mohl et al., 2001), which presents challenges in experiment design and interpretation for studying whether ParA or ParB affects PopZ localization pattern. Therefore, we took advantage of a dominant-negative variant of ParA, ParA_{K20R}, whose production in wild-type (parA^{+}) cells results in the stall- ing of the ParB–parS complex roughly halfway across the cell (Toro et al., 2008; Shebelut et al., 2010), whereas a ParA accumulation still occurred at the new pole (Fig. 7, G and H).
Figure 7. ParA mediates spatiotemporal control of PopZ localization. (A) The synthesis of PopZ-YFP variants and ParA_{R195E}-CFP was induced for 5 and 2 h, respectively. The bar graph shows the fraction of cells with ParA_{R195E}-CFP spots in C. crescentus cells producing PopZ-YFP (CJW4769) or ΔN-YFP (CJW4770). Cell counts (n) are as follows: 456 out of 478 CJW4769 cells and 137 out of 817 CJW4770 cells had at least one PopZ-YFP focus. The data.
We found that PopZ-YFP retains the ability to form a second focus under these conditions (Fig. 7 F), indicating that the presence of a ParB focus at the new pole is not required for the accumulation of PopZ at that location. In some ParA<sup>K20R</sup>-producing cells, CFP-ParB-<i>parS</i> eventually reached the new pole, but even then, the new pole accumulation of PopZ-YFP was temporally uncoupled from the completion of CFP-ParB-<i>parS</i> segregation (Fig. S5 C). In contrast, the new pole accumulation of PopZ-CFP under the same ParA<sup>K20R</sup>-producing conditions was still correlated with an accumulation of Par<sub>AWT</sub>-YFP in the new pole region at both the single-cell (Fig. 7 G) and the population levels (Fig. 7 H). Thus, although the new pole localization of PopZ-YFP can occur independently of the presence of ParB-<i>parS</i> at the new pole, it is concomitant with ParA accumulation in that region of the cell.

These results, together with the observation that a loss of ParA interaction is associated with a loss of bipolar localization (Fig. 3, A and C; and Fig. 7, A–E), suggest that the cell cycle–regulated accumulation of PopZ at the new pole is promoted by ParA accumulation during ParB-<i>parS</i> segregation. In other words, the ParA accumulation in the new pole region would contribute to locally increase PopZ concentration in this area, which, in turn, would raise the probability of PopZ to self-interact and to nucleate its polymerization into a matrix.

**Discussion**

Although many proteins that localize are known to multimerize (Rudner and Losick, 2010), the importance of multimerization in protein localization had not been examined. Collectively, our data suggest that not only protein multimerization can result in polar localization (Fig. 8 A) but also that this otherwise
stochastic multimerization can be controlled by a cell cycle event (Fig. 8 B) to obtain both spatial and temporal control of protein localization. In this model, the two conserved domains of PopZ play distinct roles. Through its C-terminal H3H4 domain, PopZ promotes and sustains assembly into a higher-order structure (PopZ matrix) at one pole. (B) Spatial and temporal regulation exploits a molecular asymmetry that is inherently associated with a cell cycle event to modulate the local concentration and time to anchor the sister chromosome.

In this model, the mechanistic principle is simple; the cell exploits a molecular asymmetry that is inherently associated with a cell cycle event to modulate the local concentration and thereby assembly of a protein. This organizing principle may represent a widespread strategy to produce precise localization patterns in time and space.

Materials and methods

Culture conditions

Synchrony, conjugation, transformation, and transduction with the bacteriophage fC30 were performed as previously described (Ely, 1991). For all experiments, cells were harvested from exponentially growing cultures. E. coli strains were grown at 30°C in the defined minimal M2G medium [0.87 g/liter Na₂HPO₄, 0.54 g/liter KH₂PO₄, 0.50 g/liter NH₄Cl, 0.02% [wt/vol] glucose, 0.5 mM MgSO₄, 0.5 mM CaCl₂, and 0.01 mM FeSO₄] unless otherwise stated or in rich PYE medium [2 g/liter broth peptone, 1 g/liter yeast extract, 1 mM MgSO₄, and 0.5 mM CaCl₂] when indicated. Unless otherwise stated, E. coli strains were grown at 37°C in the defined minimal M9 medium [12 g/liter Na₂HPO₄, 7H₂O, 6 g/liter KH₂PO₄, 1 g/liter NaCl, 2 g NH₄Cl, 1 mM MgSO₄, 0.067% casamino acids, 0.2% glycerol, and 1 mg/liter thiamine] containing 0.2% of glycerol or 0.2% glucose (when indicated) for optimal repression of ParA. E. coli cells were grown in Luria Bertani (LB)–rich medium [10 g/liter NaCl, 5 g/liter yeast extract, 10 g/liter tryptone, and 10 g/liter casamino acids] when indicated. When appropriate, media were supplemented with antibiotics at the following concentrations (µg/ml in liquid/solid medium for E. coli strains): ampicillin (10/50; 100/200), chloramphenicol (2/5; 20/30), kanamycin (5/20; 50/50), nalidixic acid (15/20; 15/20), gentamicin (2/5; 15/20), spectinomycin (25/100; 50/50), and streptomycin (5/5; 30/30). Cephalexin was used at a final concentration of 10 µg/ml. Novobiocin solutions were always prepared fresh and used at the indicated concentrations in M2G medium or M2G agarose pads. When applicable, xylene was added to the culture medium of C. crescentus strains at a final concentration of 0.03% to induce expression from the P_xyl, or 0.3% for overexpression from the psI14-based vectors. Vanillic acid was used at 0.5 mM for induction of expression from the Pvan. For TipN depletion, cells were grown without xylene in the medium, starting from the preculture. To induce the synthesis of ParA_{Rho} fusions, cells were grown in the presence of xylene 0.03% for 1–1.75 h before synchrony. For induction of expression from the pBAD33 vectors in E. coli, arabinose was used to a final concentration of 0.02%. 1 mM IPTG was used to induce expression from the Plac in E. coli.
Plasmids construction

Plasmids pCFPC-1, pCFPC-4, pCHY1C, pXYFPC-2, pXCHY2C, pXYFPN-2, and pCFPC-4 are described in Thambichler et al. (2007).

pXYFPC-2-based plasmids. The following popZ variants were amplified from a CB15N colony using the primers indicated between parentheses: full-length (CJW173 + CJW1734); ΔH3H4 (CJW173 + CJW1741); ΔC (CJW173 + CJW1739); N (CJW173 + CJW1742); ΔN (CJW1740 + CJW1734); C (CJW1743 + CJW1734); and H3H4 (CJW1744 + CJW1734). The sequence coding the ΔH2 variant was generated by joint PCR. A CB15N colony was used as a template for the amplification of two initial fragments using primers CJW1733 + CJW1745 and CJW1746 + CJW1734. These fragments were then mixed and used as template for a joint PCR using primers CJW1733 + CJW1734 to generate the popZΔH2 product. PCR products were digested with KpnI and EcoRI and ligated with pXYFPC-2 cut with the same enzymes. Thus, three popZ variants have C-terminal deletions: ΔH3H4 and ΔC variants lack portions of the C terminus starting from the H3 helix (residue 135) or the beginning of the C-terminal domain (residue 107), respectively, whereas the N variant is limited to the N-terminal domain (residues 1–26). N-terminal truncations affect three other variants (a start codon adds a methionine at the N terminus of each variant). ΔN lacks the first 21 residues including the H1 helix, C is missing the N-terminal and the proline-rich domains, thus leaving the C-terminal domain only, whereas H3H4 only retains part of the C-terminal domain starting at residue 126, thus containing the H3H4 helices but excluding the H2 helix. ΔH2 is missing a part of the C terminus between residues 110 and 125 that covers the helix 2.

pXYTCYC-2-popZΔN, popZΔN was amplified from pXYFPC-2-popZΔN using primers CJW1740 and CJW1742, digested with KpnI and EcoRI, and ligated with pxylΔC cut with the same enzymes. The digested pxylΔC was cut out of pXYFPC-2 using NdeI and HindIII.

pXYFPN-2 was amplified from pXYFPC-2 using primers CJW1816 and CJW1818 (containing the K20R mutation). A second fragment was amplified from a CB15N colony using primers CJW1819 (complementary to CJW1818) and CJW1820. Both fragments were mixed and used as a template for joint PCR using primers CJW1817 and CJW1820. The resulting PCR product was blunt ligated into a pBluescript II KS+ linearized with EcoRV, giving pBluescriptIIKS+(+)parAΔR195E. To construct pXCHYC-2-parAΔR195E, parAΔR195E was amplified from pBluescriptIIKS+(+)parAΔR195E using primers CJW1821 and CJW1820, digested with NdeI and EcoRI, and ligated into pXCHYC-2 cut with the same enzymes.

pBAD18kan plasmids. popZ-yfp or popZ-TC was amplified from the pBAD33-popZ-yfp or pBAD33-popZ-TC plasmid using primers CJW1892 and CJW1897 or CJW1897 and CJW1898, respectively. For popZ-yfp and popZ-TC, the PCR product was blunt ligated into a pBluescript II KS+ linearized with EcoRV, giving pBluescriptIIKS+(+)parAΔR195E. To construct pXCHYC-2-parAΔR195E, parAΔR195E was amplified from pBluescriptIIKS+(+)parAΔR195E using primers CJW1821 and CJW1820, digested with NdeI and EcoRI, and ligated into pXCHYC-2 cut with the same enzymes.

pBD18kan plasmids. popZ-yfp or popZ-TC was amplified from the pBAD33-popZ-yfp or pBAD33-popZ-TC plasmid using primers CJW1892 and CJW1897 or CJW1897 and CJW1898, respectively. For popZ-yfp and popZ-TC, the PCR product was blunt ligated into a pBluescript II KS+ linearized with EcoRV, giving pBluescriptIIKS+(+)parAΔR195E. To construct pXCHYC-2-parAΔR195E, parAΔR195E was amplified from pBluescriptIIKS+(+)parAΔR195E using primers CJW1821 and CJW1820, digested with NdeI and EcoRI, and ligated into pXCHYC-2 cut with the same enzymes.

Vectors for integration of tagged dnaN at the dnaN locus in C. crescentus. A 3′ fragment of dnaN was amplified from a CB15N colony using primers CJW1822 and CJW1823. The PCR product was digested with NdeI and KpnI and ligated into pCHYC-1 and pCFPC-1, both digested with the same enzymes, to construct pCHYC-1–dnaN and pCFPC-1–dnaN, respectively. Plasmids were integrated into the dnaN locus of CB15N genome, and the resultant strain was used as a donor for phase transduction to obtain strains harboring the dnaN::pCFPC-1–dnaN or dnaN::pCHYC-1–dnaN construct.

Vectors for integration of tagged parAΔR195E at the pxyl locus in C. crescentus. A joint PCR strategy was used to amplify parAΔR195E. A first fragment was amplified from a CB15N colony using primers CJW1817 and CJW1818 (containing the K20R mutation). A second fragment was amplified from a CB15N colony using primers CJW1819 (complementary to CJW1818) and CJW1820. Both fragments were mixed and used as a template for joint PCR using primers CJW1817 and CJW1820. The resulting PCR product was blunt ligated into a pBluescript II KS+ linearized with EcoRV, giving pBluescriptIIKS+(+)parAΔR195E. To construct parAΔR195E–parAΔR195E, parAΔR195E was amplified from pBluescriptIIKS+(+)parAΔR195E using primers CJW1821 and CJW1820, digested with NdeI and EcoRI, and ligated into pXCHYC-2 cut with the same enzymes.

Multiple alignment

100 sequences of the 116 BLASTP (Protein Basic Local Alignment Search Tool) hits (highest e value of 2 × 10⁻⁴) obtained using popZ as a query were aligned by COBALT (NCBI). The alignment shown on Fig. 2 A was displayed with Jalview (Waterhouse et al., 2009) using the Clustal default color scheme (Larkin et al., 2007).

Native PAGE, SDS-PAGE, and Western blot

For the preparation of native samples, exponentially growing cells were harvested when OD560 = 0.2–0.3, after 3 h of induction of PopZ-YFP variants synthesis with xylose (0.03%). The pellet was resuspended in lysis buffer (50 mM Tris, 100 mM NaCl, and 0.5 mM EDTA, pH 7.5, at 4°C, fully supplemented with 1 mM DTT, 1 mg/ml lysozyme, and one tablet of EDTA-free protease inhibitor cocktail [Complete; Roche]) per ml). This suspension was spun down for 10 min at 8,000 g at 4°C, and the pellet was resuspended in lysis buffer before sonication in a water-ice bath. Sonicated samples were spun down for 15 min at 8,000 g at 4°C, and the supernatant was diluted in 2× native PAGE loading buffer (0.16 M Tris, pH 6.8, 20% glycerol, 10% β-mercaptoethanol, and 0.4 mg/ml bromophenol blue) or 2× SDS-PAGE loading buffer (Laemmli, 1970) as indicated. All samples were kept on ice before loading on a 4–20% Mini-PROTEAN TGX Precast Gel (Bio-Rad Laboratories) for electrophoresis at 4°C in Tris-glycine native PAGE running buffer (0.32 g/liter Tris base and 14.4 g/liter glycine). For SDS-PAGE sample preparation, exponentially growing cells at OD560 of 0.2–0.3 were spun down, and the pellet was resuspended and boiled in 2× SDS-PAGE loading buffer. Resolved proteins were electrotransferred to polyvinylidene difluoride membranes, which were probed with an α-GFP antibody (1:2,000; Living Colors JL-8, #632381; BD). We used Precision Plus Protein All Blue Standards (Bio-Rad Laboratories) and NativeMark Unstained Protein Standards as molecular weight markers for SDS-PAGE and native PAGE, respectively. The polyvinylidene difluoride membrane was briefly stained with Ponceau (Sigma-Aldrich) after electrotransfer to visualize the NativeMark Unstained Protein Standards and to mark all approximate positions on the membrane for reference.

Microscopy image acquisition and analysis

For all microscopy observations, cells were spotted on 1% agarose pads (containing M2G or M9-based medium for C. crescentus or E. coli strains, respectively).
respectively) between a glass slide and a coverslip. When applicable, cells were stained with 0.025 mg/ml DAPI and/or FTAâH (30-min incubation with 5 μM FTAâH and 20 μM 1,2-ethanediol in the appropriate cell culture medium at growth temperature). Live-cell imaging was performed using either an Eclipse 80i microscope (Nikon) equipped with a camera (Orca-ER; Hamamatsu Photonics; DIC objective Plan Apochromat VC 100×/1.40 NA [Nikon] or phase-contrast objective Plan Apochromat 100×/1.40 NA, optivar 1.5x [Nikon] when appropriate) or an Eclipse Ti-U microscope (Nikon) with a liquid crystal display camera (Orca-ER; Hamamatsu Photonics; phase-contrast objective Plan Apochromat 100×/1.40 NA [Nikon]) at room temperature except for time-lapse experiments (30°C). Images were acquired and processed with MetaMorph software (Molecular Devices), and cell meshes were obtained using the open source, MATLAB-based software MicrobeTracker (Sliusarenko et al., 2011). Spots were automatically detected on fluorescence images using the SpotFinderZ tool from MicrobeTracker, with parameters trained for each set of images, or manually recorded with SpotFinderM. Further quantitative analysis from cell meshes was performed with MATLAB (MathWorks, Inc.). Kymographs of fluorescence intensity (normalized by cell area and corrected for photobleaching) were obtained as described for the built-in kymograph function of MicrobeTracker. To obtain mean kymographs for a cell population, the fluorescence profiles obtained for each cell (as for single kymographs) were averaged for each time point. For Fig. 6 (C and E) and Fig. S4 H, we considered that PzpZ-CPF or PopZ-YFP reached the new pole when the fluorescence intensity at the new pole (calculated as the new pole-proximal fifth portion of the cell) was at least equal to 20% of the total fluorescence intensity in the cell (based on empirical measurements). To determine cell polarity, cell meshes were oriented using the SpotPopZ-YFP fluorescence signal profile at the first time point of the time lapse (when cells are in the swarmer stage); the half of the cell that contained the most PopZ-YFP intensity was defined as the old pole proximal.

Online supplemental material
Fig. S1 provides supporting data on the PopZ variants. Fig. S2 shows that the selective PzP matrix can be detected by phase-contrast microscopy. Fig. S3 shows the cellular concentration and the diffusing fraction of PopZ during the cell cycle. Fig. S4 shows data supporting the experiments on ParA.

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