

1 **Scaffold-scaffold interactions regulate cell polarity in a bacterium**

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19 **Abstract**

20 The localization of two biochemically distinct signaling hubs at opposite cell poles provides  
21 the foundation for asymmetric cell division in *Caulobacter crescentus*. Here we identify an  
22 interaction between the scaffolds PodJ and PopZ that regulates the assembly of the new cell  
23 pole signaling complex. Time-course imaging of a mCherry-sfGFP-PopZ fluorescent timer  
24 throughout the cell cycle revealed that existing PopZ resides at the old cell pole while newly  
25 translated PopZ accumulates at the new cell pole. Our studies suggest that interactions between  
26 PodJ and PopZ promotes the sequestration of older PopZ and robust accumulation of newl  
27 PopZ at the new cell pole. Elimination of the PodJ-PopZ interaction impacts PopZ client  
28 proteins, leading to chromosome segregation defects in one-third of cells. Additionally, this  
29 PopZ-PodJ interaction is crucial for anchoring PodJ and preventing PodJ extracellular loss at  
30 the old cell pole through unknown mechanism. Therefore, segregation of PopZ protein at the  
31 old pole and recruitment of newly translated PopZ at the new pole via the PodJ scaffold ensures  
32 stringent inheritance and maintenance of the polarity axis within dividing *C. crescentus* cells.

33

34 **Keywords:** *Caulobacter crescentus*; asymmetric cell division; cell polarity; scaffold proteins;  
35 PodJ; PopZ; cell-cycle regulation

36

37 **Introduction**

38 Scaffolding proteins can direct and rewire information flow in cellular signaling  
39 networks<sup>1</sup>. Through the recruitment of signaling proteins into multi-enzymatic complexes,  
40 scaffolding proteins give rise to cellular functions such as cytoskeletal dynamics, cell polarity,  
41 division, and morphogenesis<sup>1,2</sup>. In the bacterium *Caulobacter crescentus*, a set of

42 spatiotemporally distributed scaffolding proteins are essential for the establishment and  
43 maintenance of cell polarity. This underlying asymmetry enables *Caulobacter crescentus* to  
44 divide into a motile swarmer cell and a sessile stalked cell<sup>3-5</sup> (Figure 1).

45 Amongst the client proteins asymmetrically polarized are a set of two-component  
46 signaling systems that collectively regulate the master regulator CtrA<sub>3,6-10</sub>. This intricate  
47 subcellular organization of CtrA regulators leads to selective CtrA phosphorylation at the new  
48 swarmer pole and dephosphorylation CtrA at the old stalked cell pole (Figure 1)<sup>6,11</sup>.  
49 Consequently, not only temporal<sup>12</sup> but also spatial<sup>13</sup> regulation of CtrA phosphorylation  
50 coordinate transcription of more than 90 developmental genes<sup>14</sup>. A scaffolding factor that is  
51 required for cell polarity is the protein PopZ. PopZ self-assembles as a micron-sized  
52 biomolecular condensate at each cell pole<sup>13,15,16</sup>. Single-molecule tracking experiments<sup>13</sup>, FLIP  
53 studies<sup>16</sup>, and *E. coli* reconstitution strategies<sup>2,16,17</sup> have shown that PopZ dynamically recruits  
54 multiple distinct protein clients at each cell poles in pre-divisional cells<sup>18</sup>. However, the  
55 mechanisms that enable a common scaffold to promote the formation of two compositionally  
56 distinct biomolecular condensates remains unclear.

57 The new and old cell pole signaling hubs share some common clients, while others are  
58 selectively recruited to each signaling hub. The PopZ scaffold promotes bipolar accumulation  
59 of the histidine kinase CckA and its modulator DivL<sup>16</sup>. PopZ also serves as an attachment site  
60 for the ParB-*parS* centromere during chromosome segregation<sup>15,18</sup>. On the other hand, the  
61 histidine kinase DivJ specifically resides at the old cell pole, and the scaffolding protein SpmX  
62 mediates this specific recruitment. SpmX bridges the interaction between PopZ and DivJ, and  
63 can even nucleate the formation of new PopZ microdomains at ectopic poles upon  
64 overexpression<sup>2</sup>.

65 At the new cell pole, the scaffold proteins PopZ and PodJ play roles in polar assembly.  
66 Deletion of the PodJ scaffold results in failure to recruit PleC histidine kinase to the new cell  
67 pole<sup>19,20</sup> and less monopolar accumulation of DivL at the new cell pole<sup>21</sup>. Moreover,  $\Delta podJ$   
68 strains exhibited moderate loss of the localization of PopZ's client proteins at the new cell  
69 pole<sup>21</sup>. Downstream, this resulted in the down-regulation of the CtrA signaling pathway<sup>21,22</sup>  
70 and reduced levels of the CtrA-regulated gene PilA<sup>19,21,22</sup>. Therefore, these previous studies  
71 suggest that similar to that of PopZ and SpmX at the old cell pole<sup>2</sup>, there are functional  
72 interactions between the PopZ and PodJ scaffolds at the opposite cell pole. Here we  
73 characterize the physical interactions between PopZ and PodJ within the new cell pole  
74 microdomain, and we demonstrate that PodJ-PopZ interaction coordinates the signaling  
75 transductions between their respective clients to ensure reliable asymmetric cell division.

76

## 77 **Results**

### 78 *Newly translated PopZ accumulates at the new cell pole*

79 A critical step in *C. crescentus* cell-cycle progression is the transition of PopZ from  
80 being localized exclusively at the old cell pole to accumulate at both cell poles. Given that  
81 PopZ scaffolds multiple cell-cycle factors<sup>16,23</sup>, we asked how the cell-pole condensates remain  
82 distinct during this change in localization patterns. One possible model is that PopZ can unbind  
83 its scaffold clients at the old cell pole and self-assemble as a separate matrix at the new cell  
84 pole. Alternatively, the accumulation of PopZ at the new cell pole may originate from the  
85 newly translated PopZ. In support of this second model, an increase in PopZ expression is  
86 observed at the same time as it is found that PopZ accumulates at the new cell pole<sup>24</sup>. We  
87 approached this question with a tandem fluorescent timer by fusing PopZ to one fluorescent

88 protein that matures rapidly (sfGFP) and one that matures substantially more slowly (mCherry)  
89 (Figure 2A)<sup>25</sup>. Protein that exhibits high sfGFP fluorescence and weak mCherry fluorescence  
90 represents a newly translated protein. Protein that exhibits high sfGFP and high mCherry  
91 represents older protein. In past work applying this fluorescent timer approach, we  
92 demonstrated that in newborn swarmer cells, newly translated SpmX-mCherry-sfGFP  
93 accumulates at the old cell pole and ages as cells mature into pre-divisional cells<sup>26</sup>.

94 Time-course imaging on a synchronized *C. crescentus* population of mCherry-sfGFP-  
95 PopZ revealed that the new cell pole PopZ exhibited high sfGFP but weak mCherry signals at  
96 30-minutes post-synchrony. In contrast, the old cell pole contained PopZ protein displayed  
97 both high sfGFP and mCherry signals (Figure 2A). At later time points in the cell cycle, 120-  
98 minutes post-synchrony, both high levels of sfGFP and mCherry can be observed at the new  
99 cell pole. This experiment indicated that older mCherry-sfGFP-PopZ is sequestered at the old  
100 cell pole, while the new cell pole is populated with newly translated PopZ protein. It is  
101 reasonable to presume that the sequestration of the old-new PopZ scaffolds may play a role in  
102 preventing the homogenization of PopZ and its clients at the new and old cell pole. Since PopZ  
103 subcellular localization abides by DNA occlusion mechanism<sup>27</sup>, a key question that follows is  
104 what promotes the accumulation of the newly translated PopZ at the new cell pole.

105 ***PodJ regulates the amount of PopZ localized at the new cell pole***

106 Previous studies have shown that ZitP<sup>28</sup>, TipN<sup>29</sup>, and ParA<sup>30</sup> play redundant roles in  
107 the accumulation of PopZ at the new cell pole but implicate one or more additional unknown  
108 players. We hypothesized that a PopZ-PodJ scaffold-scaffold interaction may occur since only  
109 PodJ could provide the recruitment capability in these players at the new cell pole<sup>19-22,31</sup>.

110 We observed that sfGFP-PodJ was able to accumulate at the poles in over 90% of cells  
111 in the  $\Delta popZ$  strain (Figure S1A). However, we also observed an increase in cells exhibiting  
112 bipolar localization (Figure S1A). This increase in PodJ bipolar accumulation could be due to  
113 differences in PodJ protein levels or changes levels of PodJ proteolysis. For example, in strains  
114 lacking the PodJ protease PerP, the number of cells that exhibit bipolar accumulation of PodJ  
115 substantially increased (Figure S1B), consistent with past observations<sup>32</sup>. Notably, we did not  
116 observe an increase in diffuse PodJ in the  $\Delta popZ$  strain. Therefore PodJ's ability to accumulate  
117 at the cell poles is independent of the PopZ scaffold.

118 We did, however, observe a 3-fold reduction of PopZ accumulation at the new cell pole  
119 in the  $\Delta podJ$  versus wild-type strain (Figure 3A). Expression of sfGFP-PodJ from the  
120 chromosomal xylose locus recovered the robust PopZ accumulation at the new cell pole  
121 (Figure 3A). These results suggest that PodJ plays a role in regulating the amount of PopZ  
122 accumulation at the new cell pole. We also observed that cells without full-length PodJ also  
123 showed a decrease in total cell mCherry-PopZ intensity (Figure 3C). This suggests that deleting  
124 the native *podJ* gene may alter PopZ transcription levels. Hence, the decreased mcherry-PopZ  
125 accumulation at the new cell pole may be due to reduced expression of mCherry-PopZ or loss  
126 of physical recruitment. We therefore, examined the distribution of PopZ in cells by  
127 constitutive expression of mCherry-PopZ from the vanillate locus. Also, a 4-fold reduction in  
128 the fraction of mCherry-PopZ signal at the new cell pole was observed in  $\Delta podJ$  compared to  
129 the wild-type strain (Figure S2A, S2B). Therefore, higher levels of PopZ expression alone are  
130 not capable of rescuing the loss of PopZ accumulation at the new cell pole.

131 We also performed time-lapse microscopy experiments to examine the mCherry-PopZ  
132 localization throughout the cell cycle starting with a synchronized population of swarmer cells

133 (Figure S2C). Images were acquired every minute, and kymographs were constructed to show  
134 the fluorescence intensity along the cell body over time. In wild-type cells, robust mCherry-  
135 PopZ foci accumulated at the new cell pole approximately 40 minutes post-synchrony (Figure.  
136 S2C, Movie S1). However, in a  $\Delta$ podJ strain, we detected significantly reduced signal at the  
137 new cell pole (Figure S2C, Movie S2). Moreover, a subset of nascent swarmer cells that lacked  
138 any observable PopZ focus were observed (Figure S2D). This loss of PopZ could be  
139 complemented by expressing sfGFP-PodJ (Figure S2D). Amongst these swarmer cells, we  
140 found 91% of cells ultimately accumulated PopZ at the correct, old cell pole (Figure S2E). We  
141 observed that 9% of these cells accumulated PopZ at the new cell pole after inheriting no PopZ  
142 (Figure S2E). Thus, this subpopulation of swarmer cells exhibited an abnormal switching of  
143 the polarity axis.

144 This observed reduction in PopZ new cell pole accumulation mirrors loss other  
145 redundant factors (TipN<sub>33</sub> and ZitP<sub>28</sub>) that play roles in promoting PopZ new cell pole  
146 accumulation. This redundancy in PopZ recruitment likely reflects how deletion of *podJ* does  
147 not result in phenotypes seen in cells with *popZ* deleted<sup>15</sup>. Collectively, these results suggest  
148 that the degree and the time of PopZ accumulation at the new cell pole depends on PodJ, but  
149 PodJ cell pole accumulation is independent of PopZ.

150

151 ***PodJ deletion impacts ParB segregation in a subset of cells.***

152 Past work from Brun and co-workers have shown that the PopZ client CckA exhibits  
153 reduced new cell pole localization when *podJ* is deleted or truncated<sup>21</sup>. Another critical role of  
154 PopZ is to tether the ParB/origin segregation complex at the cell poles<sup>15</sup>. The robust tethering  
155 of ParB to the cell poles involves simultaneous interactions with numerous ParB/*parS*

156 complexes<sup>17,34</sup>. Therefore, we investigated if the reduction of PopZ accumulation at the new  
157 cell pole impacted ParB tethering. Previously, Bowman and co-workers demonstrated that  
158 ParB was tethered more stably at the new cell pole than at the old cell pole after chromosome  
159 segregation<sup>23</sup>. We observed that ParB-CFP was able to readily accumulate at the new cell pole,  
160 while ParB-CFP foci were more mobile at both the swarmer and stalk pole, with the greater  
161 change in mobility at the swarmer cell pole when cells lacking PodJ (Figure 4A). This  
162 observation suggests that a PodJ mediated recruitment of PopZ impacts the dynamics of the  
163 ParB/origin complex at the cell pole. This close association of ParB with the cell poles is likely  
164 due to the lower degree of subcellular accumulation of PopZ at the new cell pole. Alternatively,  
165 it may also suggest that the Pod-PopZ interaction allosterically impacts the PopZ-ParB  
166 interaction.

167 Additionally, we observed that 35% of cells displayed ParB focus detachment  
168 phenotypes in the *podJ* deletion strain at both cell poles. In the most prevalent cases, the ParB  
169 focus would translocate across the cell to the new cell pole before chromosome duplication  
170 (Figure 4B). This premature centromere translocation results in the reversal of the inherited  
171 cell polarity axis. In another case, we observed new and old cell pole ParB foci coalescing into  
172 a single focus at the middle cell, then separating back to the cell poles (Figure 4C). Consistent  
173 with the mobility analysis results (Figure 4A), these phenotypes suggest the PodJ recruitment  
174 of PopZ facilitates robust PopZ-ParB chromosome tethering at the new cell pole.

175 Given that ParB also directly interacts with the cell division inhibitor protein MipZ<sup>35</sup>,  
176 we examined the impact of the *podJ* deletion upon MipZ and FtsZ. These ParB segregation  
177 defects also resulted in a less robust MipZ localization at the cell poles and a more diffuse FtsZ  
178 Z-ring assembly (Figure S3A, S3B). Overall in the *podJ* deletion strain, cells were viable as

179 chromosome segregation, and division processes remained mostly functional. However, PodJ's  
180 interaction with PopZ seems to fine-tune chromosome segregation such that it avoids polarity  
181 axis inversions.

182 ***PodJ promotes bipolarization of PopZ in *E. coli****

183 To determine if PodJ and PopZ interact directly, we heterologously co-expressed PopZ  
184 and PodJ scaffolds in *E. coli* (Figure 5A, 5B). Notably, the  $\gamma$ -proteobacterium *E. coli* is highly  
185 divergent from the alphaproteobacterium *C. crescentus* and does not contain any *C. crescentus*  
186 polarity protein homologs. *E. coli* has thus been used extensively as an orthologous system for  
187 testing *C. crescentus* protein-protein interactions<sup>15,16,27,28</sup>. A previous screen of PopZ  
188 interaction partners indicates that PopZ and PodJ were only partially co-localized when co-  
189 expressed in *E. coli*<sup>16</sup> despite their co-localization in *C. crescentus*. This previous study utilized  
190 a C-terminal fluorescent protein fusion to PodJ, while previous PodJ studies have used an N-  
191 terminal fluorescent protein fusion of PodJ<sup>32,36</sup>. Therefore, we hypothesized that the C-terminal  
192 fluorescent protein fusion might impact PodJ localization and therefore disturb PodJ-PopZ  
193 binding. To test this idea, we heterologously expressed an N-terminal fluorescent fusion  
194 protein of PodJ in *E. coli*. As shown in Figure 5A, YFP-PodJ exhibited readily bipolar  
195 localization in about 80% of *E. coli* cells (Figure 5A, S4). PopZ accumulates at a single cell  
196 pole in about 75% of cells when expressed alone, as observed in past studies<sup>18,27</sup> (Figure 5A,  
197 S4). However, mCherry-PopZ co-localized in a bipolar pattern when co-expressed with YFP-  
198 PodJ (Figure 5A, 5B). Therefore, these experiments indicated that PodJ could bipolarize PopZ  
199 in *E. coli* (Figure 5, S4). Interestingly, this PodJ-mediated bipolarization of PopZ might be a  
200 general feature of membrane-bound PopZ client proteins as SpmX<sub>2</sub>, ZitP<sub>28</sub>, and DivL<sub>16</sub> all can  
201 bipolarize PopZ in *E. coli*.

202

203 ***PopZ-PodJ interaction is conserved amongst alphaproteobacteria***

204 A subset of alphaproteobacteria encodes both PopZ and PodJ scaffolding proteins.  
205 Notably, in the alphaproteobacteria *Agrobacterium tumefaciens*, past studies have  
206 demonstrated a strong genetic interaction between PodJ and PopZ<sup>37,38</sup>. However, from these  
207 prior studies, it remains unclear if *At*PodJ and *At*PopZ interact directly or indirectly. To test  
208 this idea, we expressed PodJ fusion proteins from select alphaproteobacteria together with their  
209 corresponding PopZ variants in *E. coli* (Figure 5C). Each mCherry-PopZ homolog  
210 accumulated at a single cell pole when expressed alone, similar to *Cc*PopZ (Figure 5C). Each  
211 YFP-PodJ variant accumulated at the cell poles, but compared to *Cc*PodJ, the variants  
212 displayed heterogeneity in their subcellular localization pattern. However, in each case, we  
213 observed that co-expression with PodJ results in bipolarization of PopZ (Figure 5C). These  
214 experiments indicate that the interaction between PopZ and PodJ is direct and conserved  
215 amongst alphaproteobacteria that contain both PopZ and PodJ.

216

217 ***PopZ interacts directly with PodJ's CC4-6 domain***

218 To determine the PopZ binding site within PodJ, we screened the capability of PopZ to  
219 bind to the library of PodJ domain deletion variants through co-expression in *E. coli* (Figure  
220 6A, S4). We considered the following outcomes as an indication of a PopZ interaction with  
221 the PodJ variants: (1) the two proteins are 100% co-localized, and (2) the localization pattern  
222 of either protein is changed after co-expression. We found that the deletion of the C-terminal  
223 periplasmic domain or the intrinsically disordered PSE domain in PodJ did not disrupt the  
224 PodJ-PopZ interaction (Figure 6A, Figure S4). In contrast, the deletion of the CC4-6 domain

225 disrupted PopZ co-localization with PodJ (Figure 6A). We then expressed YFP-CC4-6 alone  
226 and observed that it was diffuse through the cytoplasm in *E. coli*. However, it co-localized with  
227 mCherry-PopZ at the cell pole when co-expressed in *E. coli* (Figure 6A). These data indicate  
228 that coiled-coil 4-6 in PodJ is critical for co-localization with PopZ in *E. coli*.

229 To confirm that this PopZ-PodJ protein-protein interaction is direct, we performed *in*  
230 *vitro* fluorescence polarization assays to detect PopZ-PodJ binding. In these assays, we mixed  
231 16  $\mu$ M PopZ together with 100 nM BODIPY-PodJ CC4-6 or BODIPY-PodJ PSE fluorescently  
232 labeled proteins. As shown in Figure 6B, PopZ bound to PodJ CC4-6 but did not bind to the  
233 PodJ PSE construct. Both the *E. coli* heterologous expression assays and *in vitro* biochemical  
234 assays show that the coiled-coil 4-6 region of PodJ is the site of interaction with PopZ.

235 ***PodJ-PopZ interaction regulates PopZ new pole localization and loss of PodJ from cells***

236 In *C. crescentus*  $\Delta$ podJ, we observed that the expression of sfGFP-PodJ $\Delta$ CC4-6 was  
237 able to localize at the new cell pole (Figure 6C). One notable difference is that sfGFP-  
238 PodJ $\Delta$ CC4-6 exhibited an increased mid-cell accumulation versus sfGFP-PodJ. A second  
239 critical difference is that sfGFP-PodJ $\Delta$ CC4-6 recruited about 2-fold less PopZ to the new cell  
240 pole than the expression of sfGFP-PodJ (Figure 6C, 6D). A comparison of PopZ cell pole  
241 intensity ratio (old/new) in the wild-type strain versus the PodJ $\Delta$ CC4-6 strain and the  $\Delta$ podJ  
242 strain shows the ratio increases in cells lacking PodJ with a functional PopZ binding site  
243 (Figure 6D). Taken together, these results suggest that the PodJ CC4-6 binding site contributes  
244 to PopZ accumulation at the new cell pole.

245 To our surprise, we observed that sfGFP-PodJ $\Delta$ CC4-6 foci outside of the cell,  
246 specifically at the old cell pole (Figure 6E). We also observed a similar phenomenon when  
247 expressing sfGFP-PodJ in *popZ* deletion strain (Figure 6F). One possible explanation is the

248 formation of minicells, which have been described in previous studies of PopZ<sup>27</sup> and SpmX<sup>39</sup>  
249 mutant strains, and SpmX overproducing cells<sup>2</sup>. Previous work from Thanbichler et al.  
250 demonstrated that mini-cell formation is commonly the result of chromosome detachment  
251 errors, as observed in MipZ mutant strains<sup>35</sup>. This is partially consistent with our observation  
252 of increased ParB mobility at the cell poles and abnormal ParB translocation events (Figure  
253 4B, 4C). However, given the role of the PopZ-PodJ interaction at the cell poles, we would  
254 expect mini-cell formation to occur equally at both poles especially at the new cell pole.  
255 Ebersbach et al. previously showed that minicells produced in the *popZ* deletion strain occur  
256 exclusively at the new cell pole<sup>27</sup>. In contrast, in the *popZ* deletion strain we observed  
257 extracellular PodJ-rich foci exclusively at the old cell pole (Figure 6D). In addition, these foci  
258 were significantly smaller than mini-cells and not observable by phase in most cases. Another  
259 possibility for the observed extracellular PodJ is that PodJ or a complex, including PodJ, is  
260 secreted from the cell body. This could occur via the CpaC outer membrane secretion channel,  
261 which remains assembled at the old cell pole after facilitating the secretion of the PilA pilin  
262 protein at the new cell pole early in the cell cycle<sup>19,40</sup>. Notably, a second factor that plays a  
263 role in pilus assembly, CpaE, is recruited to the cell pole by the PodJ scaffolding protein and  
264 is required for CpaC localization<sup>19,40</sup>. Investigation of this process and its relevance to cell-  
265 cycle regulation will require further genetic studies. Regardless of the mechanism of PodJ loss,  
266 these results suggest that PopZ-PodJ interaction is critical for robust tethering of the  
267 chromosome at the cell poles (Figure 4) and prevention of loss of PodJ from the cell body  
268 (Figure 6).

269 **Discussion**

270 Recently, biomolecular condensation has emerged as an organizing principle of the  
271 bacterial cytoplasm<sup>13,41-44</sup>. Moreover, it has been shown that the scaffolding protein PopZ play  
272 an essential role in the formation of two biomolecular condensates at each cell pole<sup>13,16</sup>. Here  
273 we have discovered a direct and conserved interaction between the PopZ and PodJ scaffolds  
274 (Figure 6B, S5) influences the composition and the size of biomolecular condensates at the  
275 new cell pole (Figure 3, S2)<sup>13</sup>. In the absence of PodJ, we observed a 3 to 4-fold reduction in  
276 the amount of PopZ that localized to the new cell pole (Figure 3, S5). This reduction in new  
277 cell pole localized PopZ also had an impact upon tethering of ParB to the cell poles. We  
278 observed erroneous ParB translocations from the old cell pole to the new cell pole before  
279 chromosome duplication in the *podJ* deletion strain (Figure 4B, S5). Therefore, PodJ plays a  
280 role in ensuring cells inherit and maintain their polarity axis. Overall, the observed segregation  
281 and division phenotypes were mild, indicating that PopZ has the ability to self-assemble at the  
282 new cell pole as other redundant proteins play a role in PopZ new-pole promotion (Figure  
283 S5)<sup>28,30</sup>.

284 A key event in *C. crescentus* asymmetric division is the formation of a signaling hub  
285 at the new cell pole that is compositionally distinct from the old cell pole (Figure S5). Previous  
286 fluorescence recovery after photobleaching (FRAP) experiments<sup>13,16</sup> and single-molecule  
287 tracking experiments<sup>15</sup> collectively indicate that PopZ is sequestered at the old poles for long  
288 periods of time. From these past experiments, we hypothesized that PopZ accumulation at the  
289 new cell pole primarily occurs through the assembly of newly translated PopZ. To distinguish  
290 newly translated from older PopZ, we applied a fluorescent-timer approach. These fluorescent-  
291 timer protein fusions demonstrated that newly translated protein was enriched at the new cell  
292 pole (Figure 2), while old PopZ protein was sequestered mainly at the old cell pole. Thus the

293 combination of single-molecule tracking (< 1 min)<sup>15</sup>, FRAP (0-10 min)<sup>13,16</sup>, and fluorescent  
294 timer data (>10 min) (Figure 1) allow tracking of protein over a range of timescales, and each  
295 of these methods suggests that sequestration of static PopZ assemblies play a role in preventing  
296 the scrambling of contents at the cell poles.

297 Super-resolution imaging of the cell poles suggests that the molecular organization is  
298 well mixed at the spatial resolution of approximately 20 nm<sup>45</sup>. In the absence of protein-protein  
299 interaction information, the PopZ-CckA-DivL and PodJ-PleC complexes could either be  
300 interacting and well mixed or non-interacting and phase-separated into discrete clusters at the  
301 new cell pole. Our observation of a direct-scaffold interaction between PodJ and PopZ (Figure  
302 3, 6, S2) likely mediates placement of PleC, CckA, DivL as a well-defined signaling complex  
303 in alphaproteobacteria (Figure 5). This proximity would support previously proposed models  
304 in which PleC's dephosphorylation of DivK~P may generate localized zones of  
305 unphosphorylated DivK~P<sup>11,19</sup>. In contrast, simple co-localization of signaling proteins at the  
306 cell poles as heterogeneous clusters and without direct interactions may not overcome the rapid  
307 DivK diffusion rates that generate shallow DivK~P gradients across the cell<sup>46</sup>.

308 More broadly, recent work has identified an array of scaffolds that promote the  
309 organization of bacterial cytoplasm from signaling biochemistry<sup>16,45</sup> to RNA biochemistry<sup>41</sup>  
310 through self-assembly as biomolecular condensates. Key questions remain as to the factors that  
311 promote co-assembly, phase separation, and compositional control of these bacterial  
312 biomolecular condensates. Future studies will be needed to determine if PodJ can self-assemble  
313 and whether it is homogenously integrated at the membrane-PopZ microdomain surface. In  
314 contrast, the absence of these scaffold-scaffold interactions, and other yet to be learned  
315 mechanisms, may facilitate phase separation of distinct biomolecular condensates. For

316 example, *C. crescentus* contains three known spatially resolved biomolecular condensates:  
317 BR-bodies involved in mRNA decay dispersed in the cell-body<sup>41</sup>, and two PopZ-mediated  
318 assemblies at opposite cell poles<sup>16</sup>. System-level understanding of the bacterial cytoplasm  
319 organization within these biomolecular condensates will center on understanding the breadth  
320 of scaffold-scaffold interactions.

321

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325

326 **Methods**

327

328 **Bacterial Strains**

329 All experiments were performed using *Caulobacter crescentus* NA1000 (also known as  
330 CB15N) and *Escherichia coli* BL21. *E. coli* BL21 was purchased from Promega. *C. crescentus*  
331 NA1000 was a kind gift from Dr. Lucy Shapiro (Stanford University School of Medicine).

332 More strains and expression plasmids used in this study are listed in Table S1. All relevant  
333 primers are given in detail in Table S2. Plasmid and strain construction are described in the  
334 supplemental information. Transformations and phage transductions were carried out as  
335 described<sup>47</sup>.

336

337 **Growth Conditions and Inducer Concentrations**

338 *C. crescentus* strains were grown at 28°C in PYE (peptone yeast extract) or M2G (minimal  
339 medium supplemented with glucose)<sup>47</sup>. When needed, *C. crescentus* cells were synchronized  
340 as described<sup>48</sup>, and swarmer cells were harvested by Percoll density-gradient centrifugation. *E.*  
341 *coli* strains used for protein purifications and microscopy experiments were grown at 37 °C in  
342 LB medium unless otherwise stated. When required, protein expression was induced by adding  
343 0.002-0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) or 0.5-10 mM arabinose in *E.*  
344 *coli*, and 0.003%–0.3% xylose or 0.05-0.5 mM vanillic acid in *C. crescentus* unless otherwise  
345 stated. The induction time for microscopy experiments is 2 hours in *E. coli* and 3 hours in *C.*  
346 *crescentus*. Generalized CR30 phage transduction was performed as described<sup>47</sup>.

347

348 **Phase Contrast, DIC, and Epifluorescence Microscopy**

349 Cells were imaged after being immobilized on a 1.5% agarose pad containing corresponding  
350 inducers when required. Phase microscopy was performed by using a Nikon Eclipse Ti-E  
351 inverted microscope equipped with an Andor Ixon Ultra DU897 EMCCD camera and a Nikon  
352 CFI Plan-Apochromat 100X/1.45 Oil objective. DIC (differential interference contrast)  
353 microscopy was performed using the same microscope and camera but with a Nikon CFI Plan-  
354 Apochromat 100X/1.45 Oil DIC objective with a Nikon DIC polarizer and slider in place. The  
355 excitation source was a Lumencor SpectraX light engine. Chroma filter cube  
356 CFP/YFP/MCHRY MTD TI was used to image ECFP (465/25M), EYFP (545/30M), and  
357 mCherry (630/60M). Chroma filter cube DAPI/GFP/TRITC was used to image EGFP, sfGFP,  
358 and mNeonGreen (515/30M). Images were collected and processed with Nikon NIS-Elements  
359 AR software.

360

361 **Time-lapse Microscopy**

362 sfGFP-PodJ, mCherry-PopZ, or SpmX-mCherry were tracked using phase and fluorescence  
363 microscopy. During time-lapse experiments, phase and fluorescence images were taken in 1  
364 min intervals for sfGFP-PodJ, mCherry-PopZ, and SpmX-mCherry for 1-2 cell divisions (~ 4  
365 h). ParB-CFP fast time-lapses images were recorded every 4 minutes over 20 minutes. Long  
366 ParB-CFP time-lapses were recorded every 15 minutes for 3-4 hours. The imaging system used  
367 was the Nikon Eclipse Ti-E microscope equipped with an Andor Ixon Ultra DU897 EMCCD  
368 camera and NIS-Elements software. *C. crescentus* cells with corresponding expression gene  
369 were grown to the early-log phase in M2G or PYE medium ( $OD_{600} = 0.2$ ), and then induced

370 by xylose or vanillic acid for 2 hours before synchronization. Swarmer cells were isolated from  
371 the culture by centrifugation (20 mins at 11,000 rpm, 4°C) after mixture with 1 volume of  
372 Percoll (GE Healthcare). The synchronized swarmer cells were pipetted onto an agarose (2%)  
373 pad containing medium with inducers and sealed with wax. NIS-Elements software was used  
374 to align time-lapse images post-acquisition.

375

### 376 **ParB-CFP tracking analysis**

377 MicrobeJ<sub>49</sub> was used to track ParB-CFP foci during fast time-lapse experiments. Predivisional  
378 cells that had already segregated a ParB-CFP focus to the new cell pole were at t=0 were  
379 analyzed. Maxima were tracked, and the raw distance changes for each 4-minute difference  
380 were averaged for new and old cell pole ParB-CFP foci. Averages for two separate experiments  
381 were pooled and plotted. A student's t-test was used to determine statistical significance.

382

### 383 **Fluorescence Intensity Profile Analysis**

384 sfGFP-PodJ variants expressing mCherry-PopZ from the native PopZ promoter were imaged  
385 using the above methods. After imaging, predivisional cells expressing sfGFP-PodJ variants  
386 were oriented by visualization of the stalk. The average fluorescence intensity profile using  
387 normalized cell length was generated using MicrobeJ<sub>49</sub> with the new pole at 0.0 and old pole  
388 at 1.0. mCherry-PopZ was made in the same way in the same strains. MipZ and FtsZ analysis  
389 were performed in the same way.

390

391 **Purification of PodJ and PopZ**

392 Protein expression of all PodJ variants followed the same protocol and is described in detail  
393 below for PodJ (1-635). To purify the cytoplasmic portion of PodJ(1-635), Rosetta (DE3)  
394 containing plasmid pwz091 was grown in 6 liters LB medium (20 µg/ml chloramphenicol and  
395 100 µg/ml ampicillin) at 37°C. The culture was then induced at an OD<sub>600</sub> of 0.4–0.6 with 0.5  
396 mM IPTG overnight at 18°C. The cells were harvested, resuspended in the lysis buffer (50 mM  
397 Tris-HCl, 700 mM KCl, 20 mM Imidazole, 0.05% dextran sulfate, pH 8.0), in the presence of  
398 protease inhibitor cocktail tablets without EDTA (Roche).

399 The cell suspension was lysed with three passes through an EmulsiFlex-C5 cell disruptor  
400 (AVESTIN, Inc., Ottawa, Canada), and the supernatant was collected by centrifuging at 13000  
401 g for 30 min at 4°C. Also, the insoluble cell debris was resuspended by the recovery buffer (50  
402 mM Tris-HCl, 1000 mM KCl, 20 mM Imidazole, 0.05% dextran sulfate, pH 8.0) and its  
403 supernatant was collected as well as the previous centrifugation. The combined supernatants  
404 were loaded onto a 5 ml HisTrap<sup>TM</sup> HP column (GE Healthcare) and purified with the ÄKTA<sup>TM</sup>  
405 FPLC System. After washing with 10 volumes of wash buffer (50 mM Tris-HCl, 300 mM KCl,  
406 and 25 mM imidazole, pH 8.0), the protein was collected by elution from the system with  
407 elution buffer (50 mM Tris-HCl, 300 mM KCl, and 500 mM imidazole, pH 8.0), and  
408 concentrated to a 3 ml volume using Amicon Centrifugal Filter Units, resulting in > 95%  
409 purity. All PodJ variants were dialyzed with a buffer containing 50 mM Tris-HCl (pH 8.0),  
410 300 mM KCl, and then aliquoted to a small volume (100 µl) and kept frozen at -80°C until  
411 use.

412 His-PopZ was expressed and purified the same as described <sup>17</sup>.

413

414 **Fluorescence Polarization Assay**

415 To label PodJ\_PSE (471-635) and PodJ\_CC4-6 (250-430), we cloned a cysteine just after the  
416 6X-His-tag proteins at the N-terminal of each protein. PodJ\_PSE (Cys) and PodJ\_CC4-6 (Cys)  
417 expression and purification followed the same protocol as PodJ mentioned above. These two  
418 proteins were labeled at the cysteine using thiol-reactive BODIPY™ FL N-(2-Aminoethyl)  
419 Maleimide (Thermo Fisher). The proteins were mixed with 10-fold excess BODIPY™ FL N-  
420 (2-Aminoethyl) Maleimide and allowed to react for 2 hours at room temperature, and the  
421 unreacted dye was quenched with mercaptoethanol (5% final concentration). The labeled  
422 proteins were purified via dialysis to remove unreacted fluorescent dye (5 times, 500 ml buffer,  
423 and 30 mins each).

424 Fluorescence polarization binding assays were performed by mixing 100 nM labeled proteins  
425 with 0, 0.25, 0.5, 1, 2, 4, 8, 16  $\mu$ M partner protein (PopZ or BSA) for 45 minutes to reach  
426 binding equilibrium at the room temperature. Fluorescent proteins were excited at 470 nm, and  
427 emission polarization was measured at 530 nm in a UV-vis Evol 600  
428 spectrophotometer (Thermo). Fluorescent polarization measurements were performed in  
429 triplicates, and three independent trials were averaged with error bars representing the standard  
430 deviation.

431 **Quantification and Statistical Analyses**

432 FIJI/ImageJ<sup>50, 51</sup>, and MicrobeJ<sup>49</sup> were used for image analysis. The number of replicates and  
433 the number of cells analyzed per replicate is specified in corresponding legends. All

434 experiments were replicated at least 2 times, and statistical comparisons were carried out using  
435 GraphPad Prism with two-tailed Student's t-tests. Differences were considered to be significant  
436 when  $p$  values were below 0.05. In all figures, measurements are shown as mean  $\pm$  standard  
437 deviations (s.d.).

438

439 **Kymograph Analyses**

440 Kymographs of fluorescence intensity was obtained by using the built-in kymograph function  
441 of MicrobeJ<sup>49</sup>. The background signal was subtracted before the kymograph analysis, and the  
442 observation of stalk at the pole of *C. crescentus* cell was defined as the old pole. The  
443 predivisional cell was selected as the start point in Figure 1C and Figure 3C. In Figure 1C,  
444 another round of kymograph analysis was performed after the first cell division. The new pole  
445 **b** became the old pole after cell division and another two new poles (**c** and **d**) were formed.

446

447 **Calculation of Subcellular Co-Localization with PodJ variants**

448 To interpret the co-localization ratio in Figure 4C and Figure S2, we used strict criteria to  
449 calculate how the proteins interact with the PodJ variants, *i.e.*, (I), the localization patterns of  
450 the interaction proteins are changed after co-expression. (II), the two proteins are 100% co-  
451 localized at the pole (binding) or drive each other apart from the pole (dispersion). Failure to  
452 meet either of these two criteria means the interaction of the two proteins is undetermined.  
453 About 200 cells were analyzed for each interaction set.

454

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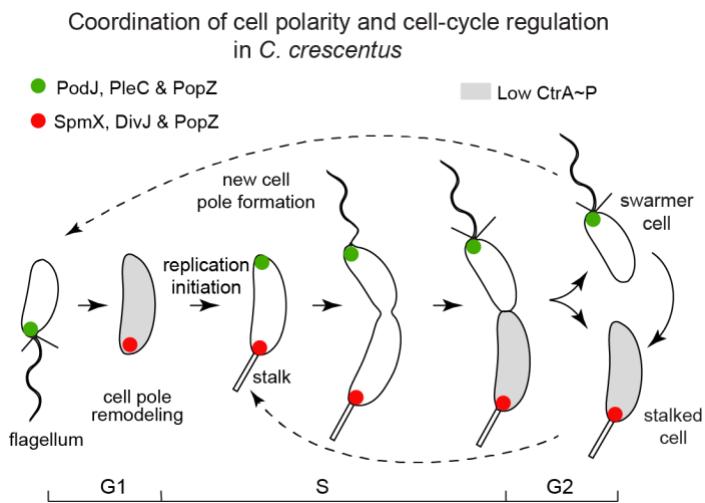
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629 **Figure Legends**

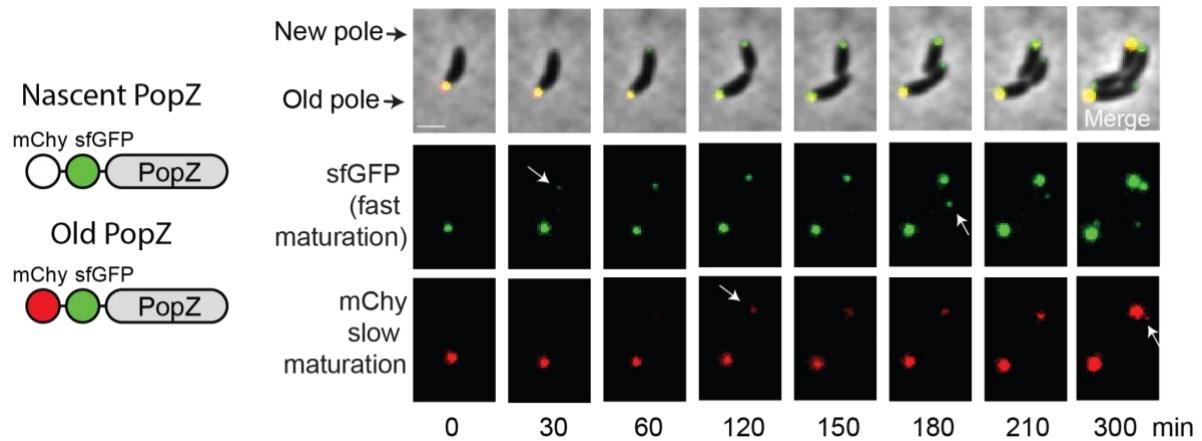
630



631 **Figure 1: The PopZ and PodJ scaffold proteins are involved in the asymmetric**  
632 **accumulation of signaling proteins at the new cell pole. Swarmer cells of *Caulobacter***  
633 ***crescentus* differentiate into stalked cells, which is associated with cell pole remodeling of a**  
634 **PodJ-rich signaling hub (green) into a SpmX-rich signaling hub (red). At the new pole of the**  
635 **stalked cells, a PodJ-rich signaling hub with scaffolding protein PopZ accumulates gradually**  
636 **upon initiation of replication. Cell division results in daughter cells that involved unequal**  
637 **inheritance of a PodJ-rich signaling hub in swarmer cell and a SpmX-rich signaling hub in**  
638 **stalked cell.**

639

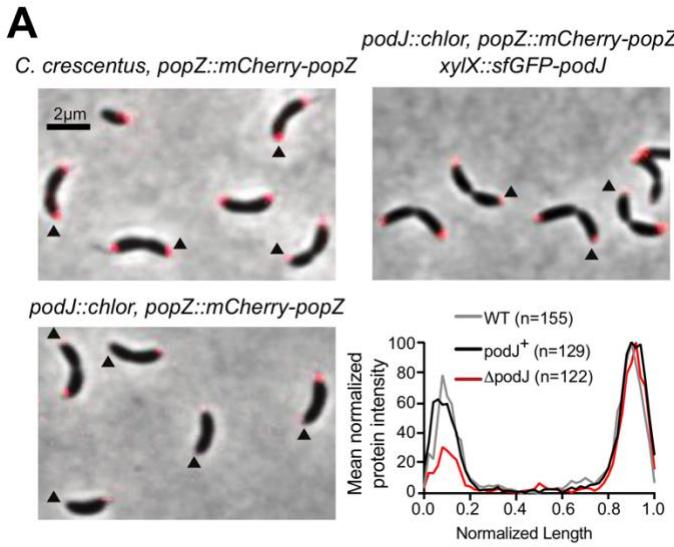
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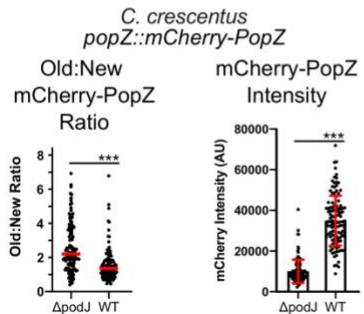
641 **Figure 2: Newly translated PopZ localizes to the new cell pole in developing cells.**

642 mCherry-sfGFP-PopZ is expressed under the xylose promoter in NA1000 cells. mCherry ( $t_{50}$   
643 maturation time of 45 min at 32°C) and sfGFP ( $t_{50}$  maturation time of 19 minutes at 32°C)  
644 chromophores mature at different times so newly synthesized PopZ will appear green and older  
645 synthesized PopZ appears as yellow. At time 0 min, the old pole shows both green and red  
646 indicating it is older yellow PopZ. At times 30-60 min a green PopZ focus appears at the  
647 opposite pole. At time 120 min the new foci contain both green and red fluorescence, indicating  
648 the subsequent maturation of the mCherry chromophore. Subsequently, in the second round of  
649 cell division, a green PopZ focus appears at the new cell pole of the divided cell at time 180  
650 min as the newly translated PopZ appears at the new cell pole.

651

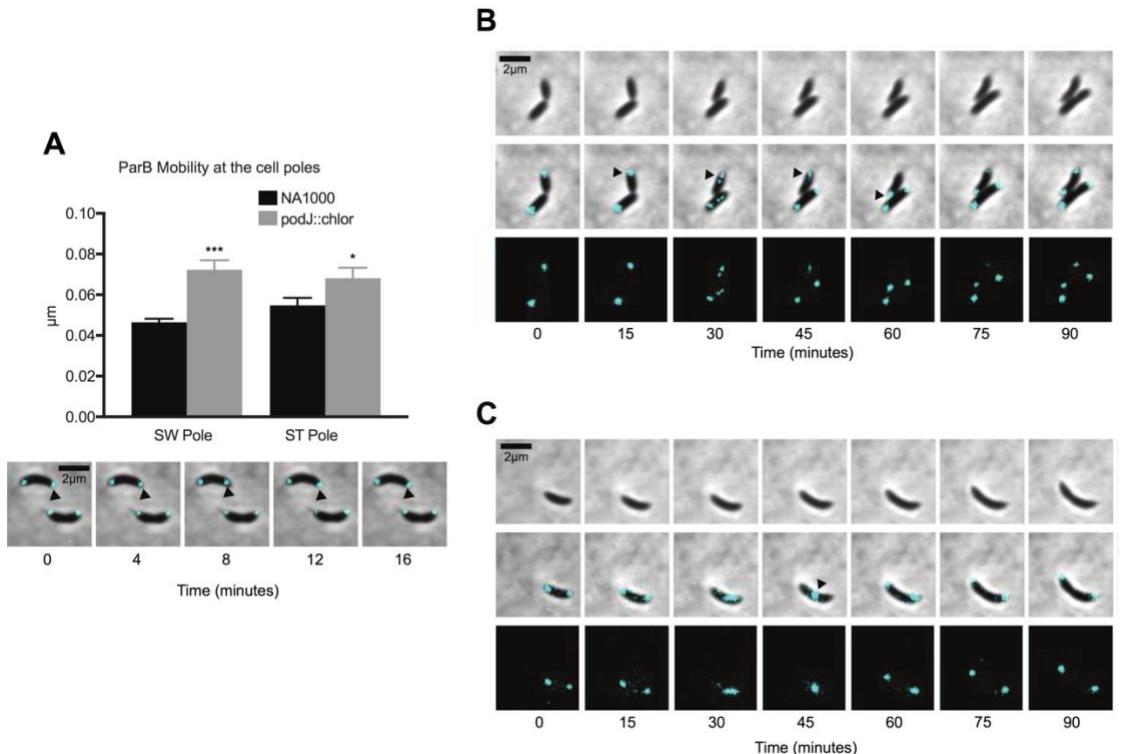


**B**



652

653 **Figure 3: PodJ regulates PopZ assembly at the new cell pole.** Analysis of the impact of  
654 the  $\Delta podJ$  upon mCherry-PopZ's localization pattern in *C. crescentus*. The expression of the  
655 sole copy *popZ* was induced from PopZ's native promoter in the chromosome. (A) mCherry-  
656 PopZ localization in predivisional cells in the wild-type (bipolar) versus the *podJ* deletion *C.*  
657 *crescentus* (monopolar). The quantitative analysis reveals a substantial reduction of PopZ  
658 abundance at the new cell pole of  $\Delta podJ$  predivisional cells. Bars, 2  $\mu$ m. (B) Comparison of  
659 the percentage of cells displaying bipolar PopZ in wild-type and  $\Delta podJ$ . Analysis of  
660 Old/New cell pole ratio and total cell intensity of mCherry-PopZ in different PodJ  
661 backgrounds. \*\*\* indicates  $p < 0.0001$ . Red line indicates mean. Red bars indicated mean  $\pm$   
662 standard deviation. Statistical analysis done using student's t-test.



663

664 **Figure 4: *C. crescentus* strains lacking PodJ exhibit chromosome segregation defects (A)**

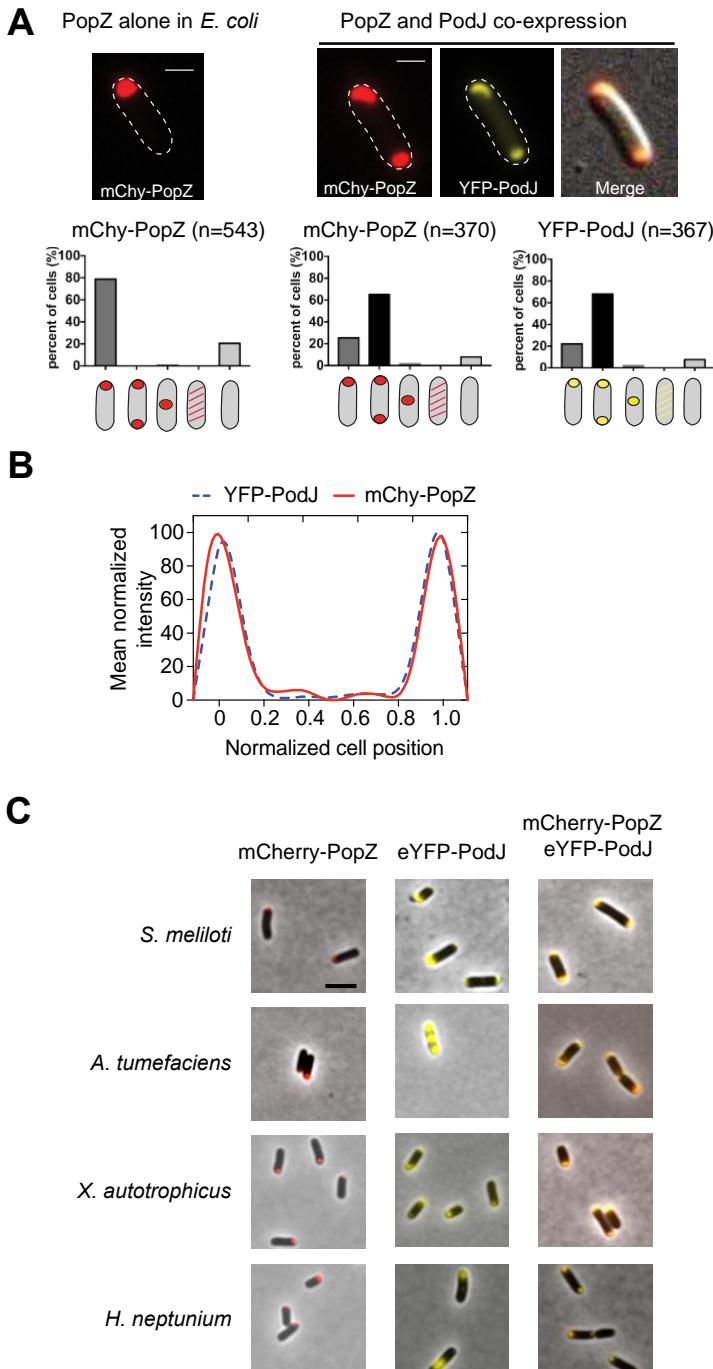
665 Analysis of ParB foci mobility at the cell poles in wild-type versus  $\Delta podJ$  strains. Cells shown

666 are  $\Delta podJ$  background. \*\*\* indicates  $p < 0.0001$  and \* indicates  $p < 0.05$ . Student's t-test used

667 for statistical significance. (B and C) Observed chromosome translocation defects in the  $\Delta podJ$

668 strain.

669



670

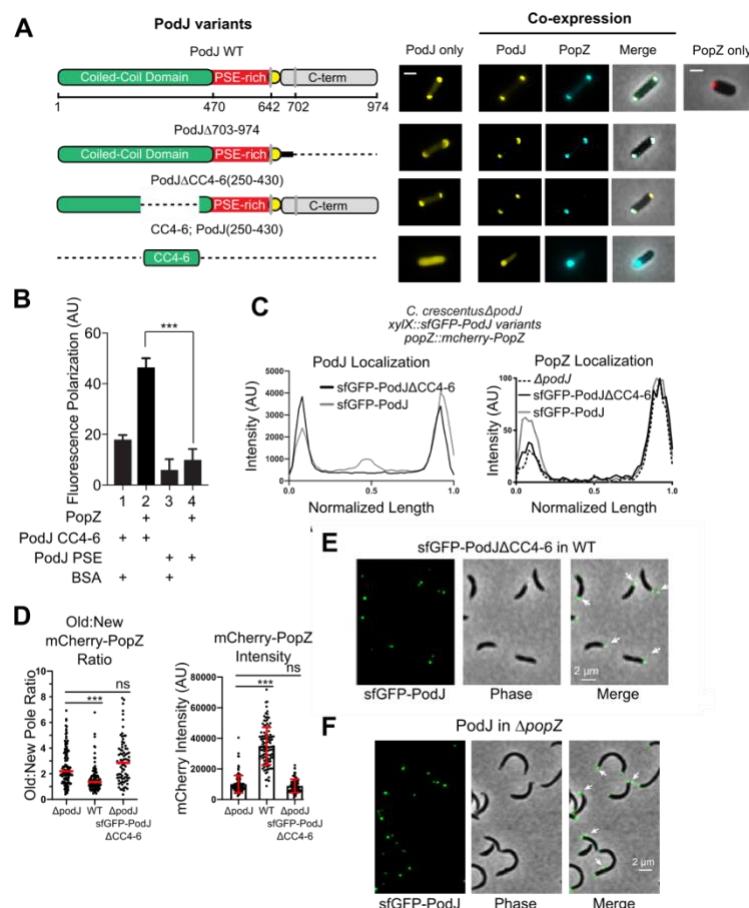
671

672 **Figure 5: PodJ bipolarizes PopZ when expressed in *E. coli*, via an interaction conserved**  
673 **across alphaproteobacteria. (A)** Heterologous expression of YFP-PodJ and mCherry-PopZ  
674 in *E. coli*. Co-expression with PodJ causes bipolar PopZ accumulation in *E. coli*. (B) Mean

675 protein intensity of YFP-PodJ and mCherry-PopZ versus cell length (n = 370). The signal  
676 intensity was normalized with the highest value as 100% in each strain. **(C)** Co-expression of  
677 PopZ-PodJ scaffold pairs from *Sinhorhizobium meliloti*, *Agrobacterium tumefaciens*,  
678 *Xanthobacter autotrophicus*, and *Hyphomonas neptunium*. All PopZ homologs accumulate  
679 specifically at one cell pole when expressed alone. Co-expression of PopZ together with PodJ  
680 results in co-localized PopZ-PodJ bipolar localization.

681

682



683 **Figure 6: PopZ binds directly to the coiled-coil 4-6 region of PodJ. (A)** Co-expression of  
 684 PodJ variants together with PopZ in *E. coli* reveals that the coiled-coil 4-6 region in PodJ is  
 685 necessary for the interaction with PopZ (please refer to Figure S4 for more details). (B)  
 686 Fluorescence polarization binding assay of the BODIPY dye-labeled PodJ\_PSE or  
 687 PodJΔCC4-6 mixed with 10 μM PopZ, using BSA as a negative control. PopZ binds  
 688 specifically to the CC4-6 domain of PodJ. However, PopZ does not bind to its PSE-rich  
 689 domain. (C) Fluorescent plots normalized by cell length where 0.0 is the new cell pole, 1.0 is  
 690 the old cell pole with the expression of sfGFP-PodJ variants from the xylose promoter in *C.*  
 691 *crescentus*. These Δ*podJ* cells are also expressing mCherry-PopZ from the *popZ* promoter.  
 692 (D) Analysis of Old/New cell pole ratio and total cell intensity of mCherry-PopZ in different

693 PodJ backgrounds. \*\*\* indicates  $p < 0.0001$ . Red line indicates mean. Red bars indicated  
694 mean  $\pm$  standard deviation. Statistical analysis done using student's t-test. (E) Loss of PodJ-  
695 PopZ interaction results in stalk-pole specific foci that contain PodJ $\Delta$ CC4-6 protein. (F)  
696 sfGFP-PodJ in  $\Delta$ popZ cells. Arrows indicate sfGFP-PodJ found outside of the cell or in non-  
697 polar regions of the cell.