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1 # Integration of cell cycle signals by multi-PAS domain kinases

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20 # Abstract

21 Spatial control of intracellular signaling relies on signaling proteins sensing their
22 subcellular environment. In many cases, a large number of upstream signals are funneled to a
23 master regulator of cellular behavior, but it remains unclear how individual proteins can rapidly
24 integrate a complex array of signals within the appropriate spatial niche within the cell. As a
25 model for how subcellular spatial information can control signaling activity, we have
26 reconstituted the cell pole-specific control of the master regulator kinase/phosphatase CckA from
27 the asymmetrically dividing bacterium *Caulobacter crescentus*. CckA is active as a kinase only
28 when it accumulates within a microdomain at the new cell pole, where it co-localizes with the
29 pseudokinase DivL. Both proteins contain multiple PAS domains, a multifunctional class of
30 sensory domains present across the kingdoms of life. Here, we show that CckA uses its PAS
31 domains to integrate information from DivL and on its own oligomerization state to control the
32 balance of its kinase and phosphatase activities. We reconstituted the DivL-CckA complex on
33 liposomes *in vitro* and found that DivL directly controls the CckA kinase-phosphatase switch,
34 and that stimulation of either CckA catalytic activity depends on the second of its two PAS
35 domains. We further show that CckA oligomerizes through a multi-domain interaction that is
36 critical for stimulation of kinase activity by DivL, while DivL stimulation of CckA phosphatase
37 activity is independent of CckA homo-oligomerization. Our results broadly demonstrate how
38 signaling factors can leverage information from their subcellular niche to drive spatiotemporal
39 control of cell signaling.

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43 # Significance

44 Cells must constantly make decisions involving many pieces of information at a
45 molecular level. Kinases containing multiple PAS sensory domains detect multiple signals to
46 determine their signaling outputs. In the asymmetrically dividing bacterium *Caulobacter*
47 *crescentus*, the multi-sensor proteins DivL and CckA promote different cell types depending
48 upon their subcellular location. We reconstituted the DivL-CckA interaction *in vitro* and showed
49 that specific PAS domains of each protein function to switch CckA between kinase and
50 phosphatase activities, which reflects their functions *in vivo*. Within the context of the cell, our
51 reconstitution illustrates how multi-sensor proteins can use their subcellular location to regulate
52 their signaling functions.
53

54 # Introduction

55 Asymmetric cell division is a fundamental mechanism for generating cell type diversity
56 across the kingdoms of life. Accomplishing an asymmetric division requires coordination
57 between cell cycle-dependent gene expression and the dynamic subcellular localization and
58 function of signaling proteins (1). A well-studied model system exhibiting asymmetric division
59 is the bacterium *Caulobacter crescentus*, which generates two distinct daughter cells every cell
60 division (Fig. 1A). A master regulator of cellular identity is the transcription factor CtrA, which
61 when phosphorylated, directly controls gene expression of over 90 cell cycle regulated promoters
62 while also inhibiting the initiation of DNA replication (2–4). CtrA~P is present and active as a
63 transcription factor in the motile, replication-incompetent swarmer cell. Dephosphorylation and
64 proteolysis of CtrA~P permits differentiation into a sessile stalked cell and the beginning of
65 DNA replication (5). Upon progression into the predivisional stage, CtrA proteolysis ceases and

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66 the transcription of *ctrA* is activated. Concurrently, a set of signaling proteins localize to the new
67 cell pole, opposite the stalk, to promote CtrA phosphorylation and the biogenesis of the
68 flagellum and pili. Spatial and temporal control of CtrA~P thus coordinates cell type identity
69 with DNA replication and cell cycle progression.

70 The bifunctional histidine kinase/phosphatase CckA controls the phosphorylation state of
71 CtrA (6–9). CckA uses sensory PAS domains to change its activity depending upon its
72 subcellular location and the progression of the cell cycle (10, 11). We previously reconstituted
73 CckA on liposomes *in vitro* to show that its kinase activity depends upon CckA surface density,
74 and that this density-dependent response requires the first of its two PAS domains (11). In
75 predivisional cells, CckA is a kinase when it accumulates at high density at the new cell pole,
76 supplying the phosphate for CtrA~P through a phosphorelay protein, ChpT (Fig. 1A) (12).
77 However, in the stalked cell, high levels of the second messenger c-di-GMP (cdG) promote
78 CckA phosphatase activity, which depletes the CtrA pathway of phosphate (Fig. 1B) (11, 13,
79 14).

80 Upstream of CckA, the pseudo-histidine kinase DivL is necessary for CckA localization
81 to the new cell pole and essential for CckA kinase activity in predivisional cells (10, 15). CckA
82 co-immunoprecipitates with DivL, suggesting that CckA regulation requires complex formation
83 with DivL (10). DivL is also essential for effective inhibition of CckA kinase activity when the
84 two proteins are away from the new cell pole. *In vivo*, interaction of DivL and the
85 phosphorylated response protein DivK~P leads to inhibition of CckA kinase activity (16, 17). A
86 tyrosine replaces histidine at the active site of the DivL pseudokinase with no apparent catalytic
87 activity (18). It has been suggested that DivL switches between conformations that promote or
88 inhibit CckA kinase activity when the two are in a complex (17).

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89 In most histidine kinases, extracellular or intracellular signals interact with N-terminal
90 PAS domains leading to a conformational change that in turn regulates the activity of the
91 catalytic domains (19, 20). Kinases can also use this conserved structural link in the reverse
92 direction. DivL (Fig. 1B) uses its kinase-like domain to bind to the phosphorylated response
93 regulator DivK~P (17), predicted to drive a conformational change within DivL's PAS domains.
94 This rearrangement in the PAS domains is then propagated as a regulatory signal to CckA's own
95 PAS domains, regulating CckA catalytic activity (Fig. 1B) (17), and previous studies indicate
96 that the PAS domains of the two proteins are sufficient for communication between the two
97 proteins (10, 21). However, it has remained unclear whether DivL directly regulates CckA
98 catalytic activity, and how a multi-PAS domain protein can pass distinct signals to a target
99 kinase.

100 Here, we show that DivL directly regulates the kinase-phosphatase switch of CckA *in*
101 *vitro* when the two proteins are reconstituted on proteoliposomes. DivL can inhibit CckA kinase
102 activity in the absence of any ligands, instead stimulating CckA phosphatase activity. We also
103 show that a point mutant in DivL can constitutively activate CckA kinase activity, and that
104 stimulation of either kinase or phosphatase activity requires the PAS domains of DivL and PAS-
105 B of CckA. Additionally, oligomerization of CckA is critical for DivL stimulation of kinase
106 activity but not its stimulation of CckA phosphatase activity. While we must keep in mind that
107 this reconstitution approach represents a simplified system that lacks additional factors present *in*
108 *vivo*, our previous experiments using reconstituted CckA on liposomes have been supported by
109 subsequent work demonstrating agreement that the CckA surface density that is critical for
110 kinase activity in our *in vitro* experiments matches the surface density of CckA present at the
111 new cell pole *in vivo* (22). In this study, we reconstitute an additional aspect of subcellular

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112 regulation of CckA. We propose that DivL integrates information about subcellular localization
113 and cell cycle progression to toggle CckA between its kinase and phosphatase modes in a PAS
114 domain-dependent manner (Fig. 1B).
115

116 Results

117 *DivL directly inhibits CckA kinase activity*

118 We set out to test whether DivL can directly regulate CckA catalytic activity *in vitro*.
119 Membrane tethering is essential for CckA polar localization and kinase activity *in vivo* (6),
120 suggesting that that membrane attachment may be important for productive interaction between
121 CckA and DivL (6). We purified CckA and DivL constructs containing N-terminal His-tags in
122 place of their transmembrane helices, and we tethered the two proteins to Ni-NTA groups on a
123 fluid lipid surface (Fig. 2A) (11). Unless otherwise stated, we present data using a DivL
124 construct lacking its N-terminal domain A, which has distant homology to PAS domains (23,
125 24), due to the increased protein stability of the shortened construct and its indistinguishable
126 behavior from the full length construct in all of our assays. To probe DivL's regulation of CckA
127 kinase activity, we incubated CckA and DivL at equimolar concentrations of 5 μM either in
128 solution or an equivalent amount of protein at 350 molecules of each protein per liposome. CckA
129 kinase activity was measured by auto-phosphorylation in the presence of [γ-P32]-ATP followed
130 by autoradiography. Strikingly, co-loading DivL with CckA on liposomes inhibited CckA
131 kinase activity, whereas co-incubation of the two proteins at 5 μM each in solution, with no
132 liposomes, yielded no change to kinase activity (Fig. 2B), suggesting that membrane tethering is
133 critical for interaction between the two proteins.

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134 Previous studies indicated that the PAS domains of CckA and DivL (Fig. 1B) are
135 important for both proteins' signaling functions (10, 11, 17). To test whether the DivL PAS
136 domains are critical for its inhibition of CckA autophosphorylation, we purified a series of DivL
137 PAS domain deletion mutants, progressively truncating PAS domains from the N-terminus (17).
138 We then incubated these DivL PAS domain truncations with CckA on liposomes to measure
139 their effects on CckA kinase activity (Fig. 2C). While the full-length DivL and a construct
140 lacking PAS domain A inhibited CckA to the same extent, the DivL constructs ΔPAS-AB and
141 ΔPAS-ABCD did not impact CckA kinase activity, demonstrating that the two N-terminal PAS
142 domains of DivL were necessary for a functional interaction on the membrane. This finding is
143 consistent with the prior model suggesting that DivL and CckA communicate through contacts in
144 their PAS domains (17).

145 To determine the potency of DivL as an inhibitor of kinase activity, we titrated DivL at
146 different surface densities against a fixed density of CckA on liposomes. DivL robustly inhibited
147 CckA kinase activity with an IC_{50} of 22 DivL molecules per liposome (95% confidence interval:
148 15-31 DivL/liposome), 15-fold lower than the CckA surface density (Figure 2D). This sub-
149 stoichiometric inhibition implies that DivL inhibition of kinase activity may be due to enhanced
150 phosphatase activity by DivL or CckA towards CckA~P. These *in vitro* data indicate that in the
151 absence of accessory factors, DivL can sub-stoichiometrically inhibit CckA kinase activity (Fig.
152 2D). *In vivo*, DivL binds the accessory factor DivK~P to inhibit CckA kinase activity when
153 these proteins are away from the new cell pole (16), while DivL is critical for stimulation of
154 CckA kinase activity when the two proteins co-localize at the new cell pole (10, 16). Based on
155 these findings, we propose that DivL binds another accessory factor at the new cell pole to
156 facilitate its stimulation of CckA kinase activity.

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157

158 ***DivL stimulates CckA auto-phosphatase activity***

159 Inhibition of kinase activity in many HKs frequently represents a concerted switch to a
160 conformation that has increased phosphatase activity towards the cognate receiver domain (25,
161 26). It was previously shown that binding of the second messenger (cdG) causes CckA to switch
162 from kinase mode to phosphatase mode (11, 13, 27). This finding suggests that DivL's
163 inhibition of CckA kinase activity may, similar to cdG, induce a switch from a kinase-active
164 conformation to a phosphatase-active one. Alternatively, we considered the possibility that DivL
165 may directly perform the phosphatase activity on CckA's receiver domain.

166 We therefore tested whether DivL promotes CckA dephosphorylation *in vitro*,
167 monitoring the rate at which purified CckA~P lost phosphate when incubated with or without
168 DivL on liposomes (Fig. 3A). The presence of DivL on liposomes with CckA stimulated the loss
169 of CckA~P signal, indicating that one of the proteins was performing phosphatase activity (Fig.
170 3B). DivL did not stimulate phosphate decay from the phosphatase-deficient mutant CckA
171 V366P (28), indicating that CckA, and not DivL, is responsible for performing phosphatase
172 activity (Fig. S1A, B). To test if the CckA PAS domains are necessary for this response to DivL
173 (Fig. 1B), we repeated the phosphatase experiment for the CckA variants ΔPAS-A, ΔPAS-B, and
174 ΔPAS-AB. CckA ΔPAS-A rapidly lost phosphate upon co-loading with DivL on liposomes,
175 indicating that CckA PAS-A is not necessary for interaction with DivL or phosphatase activity
176 (Fig. 3C).

177 Strikingly, CckA constructs lacking PAS-B did not show a change in autophosphatase
178 activity in the presence of DivL (Fig. 3D, Fig. S1C). Because their autophosphatase activity was
179 reduced compared to WT CckA, we took advantage of the finding that ADP binding promotes

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180 the phosphatase-competent conformation of histidine kinases (25, 27) to show that CckA ΔPAS-
181 B and ΔPAS-AB mutants do retain phosphatase function (Fig. S2). Thus, CckA PAS-B is
182 necessary for auto-phosphatase stimulation by DivL. It is formally possible that CckA constructs
183 lacking PAS-B are conformationally restricted in a manner that prevents interaction between
184 DivL and another part of CckA. Given that the N-terminus of CckA comprising its
185 transmembrane region, PAS-A, and PAS-B together are sufficient for response to DivL *in vivo*
186 though (10), and PAS-A is not necessary for response to DivL in this assay (Fig. 3E), we propose
187 that PAS-B is the CckA domain that communicates with DivL.
188 We previously showed that PAS-B of CckA is necessary for binding to cdG, and that it is
189 sufficient for binding when domains involved in dimerization and oligomerization are also
190 present (11). Because our findings indicate that DivL, like cdG, also signals through CckA's
191 PAS domains, we further tested whether cdG cooperatively inhibited CckA in the presence of
192 DivL. While cdG inhibition has been shown to be cooperative with ADP binding (27), we
193 observed additive rather than cooperative inhibition of CckA kinase activity by cdG and DivL
194 inhibition of CckA kinase activity (Fig. S1D).
195
196 ***A DivL point mutant directly stimulates CckA kinase activity***
197 Prior studies indicated that DivL switches between activating or inhibiting CckA kinase
198 activity *in vivo*, depending on the stage of the cell cycle (10, 16), and uses accessory factors such
199 as the phosphorylated response regulator DivK~P to determine its activity towards CckA.
200 Tsokos et al. found that a DivL point mutant, A601L, enhanced CckA kinase activity by fivefold
201 *in vivo* during the stalked cell phase of the cell cycle even when the proteins were localized away
202 from the new cell pole (16). This finding suggests that DivL A601L activates CckA kinase

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203 activity independent of upstream signals. Moreover, structure-function analysis suggested that
204 the A601L mutation changes the global conformation of DivL in a PAS domain-dependent
205 manner (17).

206 To determine if DivL A601L directly stimulates CckA kinase activity through the CckA
207 PAS domains. The CckA PAS domain mutants had different capacities for response to DivL
208 A601L (Fig. 3E). While CckA ΔPAS-B retained kinase activity when tethered to liposomes, its
209 kinase activity did not respond to the presence of DivL A601L. Conversely, CckA variants
210 lacking PAS-A did not demonstrate significant kinase activity on liposomes as we previously
211 observed, and DivL A601L did not rescue their kinase activities. These data indicate that PAS-B
212 is specifically required for a kinase response to DivL A601L, while PAS-A is critical for CckA
213 kinase activity. Because PAS-A is critical for CckA surface density-dependent kinase activity,
214 we tested compared the effects of DivL A601L stimulation of CckA kinase activity at different
215 densities (Fig. 3F). Even at reduced surface density, DivL A601L greatly stimulated CckA
216 kinase activity, albeit to a lower total extent than when the two proteins were incubated at high
217 density. This finding is consistent with the finding that A601L promotes CckA kinase activity
218 even when the proteins are diffusely localized *in vivo* (16). Altogether, these data indicate that
219 offer proof-of-principle that DivL can stimulate CckA kinase activity in a PAS domain specific
220 manner and suggest that additional upstream signals may push DivL into this kinase activity
221 promoting state *in vivo*.
222

223 ***CckA oligomerizes through PAS-A and its receiver domain***

224 The requirement of high surface densities for CckA kinase activity on liposomes suggests
225 that it may need to oligomerize to become active as a kinase (11). While the catalytic core of

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226 CckA, containing the DHp and CA domains, forms a canonical *trans*-phosphorylating dimer
227 (27), we hypothesized that its PAS and receiver domains may drive higher oligomerization.
228 There are a handful of histidine kinases believed to function as tetramers, including the kinase
229 RegB, which loses kinase activity upon tetramerization (29–31). We assayed CckA
230 oligomerization in solution using analytical size exclusion chromatography (SEC) to predict the
231 molecular mass of CckA complexes. Indeed, we found that WT CckA eluted as a single
232 tetrameric peak when injected onto the column at approximately 100 μM in solution (Fig. 4A,
233 Table S1). Knowing that PAS domains frequently mediate oligomerization (19), and that PAS-A
234 is critical for density-dependent kinase activity, we tested the PAS domain dependence of CckA
235 oligomerization. Removal of CckA PAS-A showed a reduction in oligomerization, with a peak
236 center at 3.1 CckA per complex, indicating that PAS-A contributes to higher oligomerization, but
237 that another domain in the protein continues to drive oligomerization in the absence of PAS-A
238 (Fig. 4B). Removal of PAS-B yielded a smaller effect on CckA oligomerization than PAS-A
239 (Supplementary Table S1).
240 Because the DHp domain typically only provides a conserved dimerization interface, we
241 tested whether the receiver domain of CckA might also contribute to tetramerization. CckA
242 ΔRD indeed showed a partial reduction in oligomerization (Fig. 4B), as well as a reduction in
243 kinase activity on liposomes (Fig. S3). Simultaneous deletion of CckA variants lacking both
244 PAS-A and RD (ΔPAS-A ΔRD and ΔPAS-A-B ΔRD), resulted in CckA attaining only a dimeric
245 state. Given that the catalytic core of CckA has been shown to form a canonical dimer (27), it
246 seems apparent that the dimers should not have to be disassembled and reassembled into a
247 fourfold-symmetric tetramer. Similarly, we hypothesize that the elution peak centered at
248 approximately three CckA molecules/complex represents an exchange between states containing

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249 two and four CckA molecules/complex and tetrameric states, rather than forming a distinct
250 trimer. We propose that CckA's PAS-A and the RD mediate the assembly of conserved HK
251 dimers into a dimer of dimers (Fig. 4C).
252

253 Discussion

254 Cells must constantly integrate information to coordinate cell cycle events. For processes
255 that require spatial control, such as differentiation and asymmetric division, their signaling
256 proteins must additionally be able to recognize and respond to upstream factors found at distinct
257 subcellular locations. Multi-sensor histidine kinases constitute a large set of signaling proteins
258 which have the potential to respond to multiple signals, enabling the processing of information
259 within subcellular niches. In this study, we have shown that the pseudokinase DivL controls the
260 kinase-phosphatase switch of CckA through the PAS domains of the two proteins. DivL
261 stimulation of kinase activity further requires CckA homo-oligomerization, illustrating how
262 subcellular accumulation of CckA at the cell pole can be used as input information for the
263 regulation of cellular asymmetry (Fig. 5).
264 We found that DivL can directly stimulate both CckA kinase and phosphatase activities
265 in a PAS-B dependent manner (Fig. 1B), consistent with our previous finding that PAS-B is
266 critical for new pole localization *in vivo* (11). We also previously showed that cdG binds in
267 PAS-B of CckA, stimulating phosphatase activity (11). While most PAS domains typically
268 sense only one ligand (19, 32), our finding that CckA PAS-B responds to both cdG and DivL
269 adds to a small set of PAS domains that interact with multiple structurally unrelated ligands (19,
270 33, 34). A recent report identified a point mutant within the CckA PAS-B domain that led to
271 increased expression of a subset of early predivisional, CtrA-dependent genes (35), suggesting

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272 that this mutant may specifically affect CckA interaction with DivL, cdG, or another signal, and
273 that this mutant's effect is restricted to the early predivisional cell when CckA first becomes
274 active as a kinase.

275 Consistent with Childers et al. (17), our data suggest that different conformations of the
276 DivL PAS domains can communicate with PAS-B of CckA to regulate catalytic function (Fig.
277 1B). This manner of regulation differs from the more common paradigm of PAS-mediated
278 signaling, in which a given signal simply promotes one conformation of the target kinase (19).
279 Rather, DivL can actively promote both the kinase and phosphatase activities of CckA,
280 presumably through different contacts between CckA PAS-B and some part of the DivL PAS
281 domains. Our truncation analysis of the DivL PAS domains (Fig. 2C) and prior studies indicate
282 that its PAS domains B-D are critical domains involved in CckA regulation, but future
283 investigations will be necessary to determine which domain(s) of DivL directly communicate
284 with CckA and whether DivL also parses multiple input signals to determine its subcellular
285 regulation of CckA activity.

286 Our data indicate that oligomerization is important for CckA kinase but not phosphatase
287 activity, as only kinase activity is surface density dependent (11). The CckA PAS-A domain, a
288 key driver of oligomerization, is necessary for stimulation of kinase but not phosphatase activity
289 by DivL (Fig. 3). In most HKs, a signal in the PAS domain torques the central alpha-helical
290 spine of the protein to regulate downstream catalytic domains (20, 36). The proline-rich linker
291 between PAS-A and PAS-B of CckA is inconsistent with this alpha-helix, but it may provide a
292 semi-rigid connection still capable of torque. Alternatively, oligomerization via PAS-A and RD
293 (Fig. 4) may regulate kinase activity on the conserved principle of stabilizing a rotation of the
294 ATP binding domain relative to the active site histidine by changing CckA-CckA contacts

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295 between higher and lower assembly states. Indeed, structural evidence shows that binding of
296 ATP versus ADP in the CA domain is sufficient to switch an HK between kinase and
297 phosphatase conformations, respectively (25). Broadly, this demonstrates that the molecular
298 impetus for switching between catalytic functions may come from many different parts of the
299 protein, and oligomerization could provide multiple contact points to regulate kinase activity
300 (Fig. 1B).

301 Further, oligomerization offers a straightforward mechanism for linking kinase activity to
302 accumulation of a protein within a subcellular niche. In *Caulobacter*, a specialized collection of
303 signaling and structural proteins at the new cell pole coordinates the stimulation of CckA kinase
304 activity with polar organelle biogenesis (Fig. 5). The polar matrix protein PopZ defines the
305 boundaries of DivL and CckA polar accumulation, and consequently the surface density of
306 CckA, at the new pole (22, 37–39). Thus, the boundaries of the PopZ microdomain give
307 subcellular context to CckA's density-dependent kinase activity.

308 Additional layers of regulation may promote CckA kinase activity at the new cell pole.
309 Tsokos et al. proposed that the new pole constitutes a 'protected zone' in which other factors
310 cannot inhibit CckA kinase activity (16). Moreover, the DivL mutant A601L directly stimulates
311 CckA kinase activity, and suggests that additional factors at the new cell pole, reviewed
312 elsewhere (40), may be necessary to promote kinase activity of the DivL-CckA complex *in vivo*.
313 Our study of the DivL-CckA interaction reveals that DivL can switch between directly
314 stimulating CckA kinase and phosphatase activities, that CckA parses multiple signals through
315 its PAS-B domain, and that CckA oligomerization is critical for its kinase activity. Thus, a
316 multi-sensor domain architecture provides a means for integrating a complex array of input

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317 signals within the different spatial and temporal contexts required for a single master regulator
318 that determines divergent cell fates.
319

320 Materials and Methods

321 Cloning, protein expression, and purification

322 PAS domain cut-offs in CckA and DivL were assigned using the HHpred protein
323 homology web server (23, 24) and described in detail previously (11). New plasmids for this
324 study were created using Gibson assembly as designed through the J5 cloning system(45). Other
325 plasmids were described previously (11, 17, 28, 46, 47). Plasmids were transformed into *E. coli*
326 via heat shock. A complete list of plasmids and strains used in this study is available in
327 Supplementary Table 2. CckA and DivL variants were expressed and purified as described
328 previously (11, 17, 47), and are described at length in the Supplementary Methods.
329

330 Gel filtration chromatography

331 Protein purification beyond Ni-NTA affinity was performed via gel filtration
332 chromatography using a GP-250 gradient programmable chromatographer. Concentrated
333 samples were separated using a Superdex 200 10/300 GL column. Fractions of 0.3 mL were
334 collected, analyzed, and pooled to optimize purity.
335 Studies of CckA oligomerization via analytical gel filtration chromatography were
336 performed using a GE Healthcare Superdex 200 Increase 10/300 GL column and a Bio-Rad
337 NGC chromatography system. Apparent molecular weights of CckA oligomeric complexes were
338 assigned using a standard curve based on elution volumes of a Bio-Rad premixed gel filtration
339 standard (cat #151-1901). Elution was performed at 0.35 mL/min. CckA samples of

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340 approximately 150 μL were loaded at approximately 100 μM and eluted at 350 μL/min. A
341 complete list of SEC experiments is given in Supplementary Table S1.
342

343 **Production of Large Unilamellar Liposomes**

344 Liposomes were made as described previously (11), with some adjustments. A mixture
345 of 900 μL of 10 mg/mL 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt)
346 (DOPG: product 840475), was mixed with 1 mL of 1 mg/mL 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-
347 amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (DGS-NTA(Ni): product
348 790404C). Both lipids contain two 18-carbon acyl chains with a *cis*-alkene between carbon
349 atoms 9 and 10 in each chain to membrane fluidity at room temperature. The chloroform
350 solutions were well mixed in a glass scintillation vial, and the solvent was evaporated under a
351 gentle stream of nitrogen for 1 hour to leave a clear film with no clumps. The film was then
352 rehydrated in 500 μL to 20 mg/mL in liposome rehydration buffer (100 mM KCl, 20 mM
353 HEPES-KOH pH 8.0). The buffer was vigorously resuspended with a pipette until all of the
354 lipid was dissolved. The vial containing the lipid mixture was then subjected to 10 freeze/thaw
355 cycles in liquid nitrogen and a 37°C water bath. The freeze-thaw mixture was then extruded for
356 11 passes through 100 nm pores of a polycarbonate filter using the Avanti Mini-Extruder.
357 Following extrusion, the liposomes were diluted in equal volume ultrapure water (to a final
358 concentration of 10 mg/mL) and aliquots were stored under nitrogen in plastic tubes to extend
359 shelf life. Production of large unilamellar vesicles has also been described previously (48–50).
360

361 **Radiolabeling phosphorylation assays**

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362 Radiolabeled auto-phosphorylation assays were similar to previous protocols (11). CckA
363 constructs (5 μM) were incubated in low salt kinase buffer (50 mM KCl, 10 mM HEPES-KOH
364 pH 8.0) with 5 mM $MgCl_2$ and 0.5 mM ATP in 25 μL reaction volumes. Glycerol was removed
365 from protein samples via overnight dialysis prior to reactions to match the solvent conditions
366 within the liposome lumens. Owing to opposite ionic strength preferences for kinase activity as
367 compared to WT CckA, kinase assays for CckA ΔPAS-B and ΔPAS-AB were conducted in high
368 salt buffer (200 mM KCl and 50 mM HEPES-KOH pH 8.0). For Figure 4B, CckA DPAS-B
369 conditions are normalized to the no-DivL comparison in each case. For all LUV-based assays,
370 CckA and DivL were allowed to incubate for 10 minutes with varying amounts of LUVs prior to
371 addition of ATP stocks. Kinase assays on liposomes were conducted at a surface density of 350
372 molecules per liposome of CckA, with 350 molecules per liposome of DivL where appropriate,
373 unless otherwise noted. Maximum density corresponds to 1100 molecules per liposome.
374 Radiolabeled ATP was supplemented at of 2 μCi [γ-^{32}P] ATP per reaction. Reactions
375 were quenched after 3 minutes in 2x Laemmli sample buffer, and the quenched reaction mixtures
376 were loaded onto 4-15% gradient polyacrylamide gels and subjected to electrophoresis.
377 Alternatively, at this step for titration curve experiments, we used a nitrocellulose dot blot assay
378 to separate phosphorylated protein from the reaction mixture, described previously (11). The
379 extent of auto-phosphorylation was measured by exposing a phosphor screen to the gels for at
380 least 3 hours, and the screen was subsequently imaged on a Typhoon storage phosphorimager
381 (Molecular Dynamics). Band intensities were quantified using ImageJ.
382 For phosphatase assays, CckA was allowed to auto-phosphorylate in solution, and
383 CckA~P was subsequently purified away from ATP. Depletion of ATP was accomplished by
384 first rapidly passing the crude reaction mix through a desalting column to remove most of the

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385 nucleotides. Hexokinase (12.5 units) and glucose (10 mM) were then added to convert any
386 remaining ATP to ADP over 10 minutes. CckA~P was then deposited on liposomes that did or
387 did not contain equimolar DivL to test whether DivL impacts CckA dephosphorylation. For
388 phosphatase assays supplemented with ADP, the column purification step was skipped,
389 permitting complete conversion of ATP to ADP by hexokinase.
390

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400

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522
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526 # Figure Legends
527
528 **Figure 1. DivL regulates CckA kinase and phosphatase activities as a function of the cell**
529 **cycle.** A. DivL (green) and CckA (orange) subcellular localizations are dynamic over the
530 *Caulobacter* cell cycle. In the swarmer and stalked cells, both proteins are diffusely deployed
531 around the membrane (dashed lines). In the predivisional cell, DivL accumulates at the new cell
532 pole (green circle), where it is essential for both CckA new pole-localization and CckA kinase
533 activity. CckA (orange circles) accumulates at both the old and new cell poles, and both proteins
534 maintain a partial diffuse population outside of the polar niches. CckA kinase and phosphatase
535 activities control the cell cycle dependent phosphorylation state of the master regulator, CtrA.
536 Phosphorylated CtrA~P (grayscale) inhibits the initiation of DNA replication and activates
537 transcription of over 90 promoters. Phosphate is shuttled between CckA and CtrA via the
538 transfer protein ChpT (lavender). B. CckA and DivL both integrate signals to determine CckA

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539 kinase activity. Light blue arrows represent transmission of a CckA kinase-stimulating signal,
540 whereas dark blue arrows represent a phosphatase-stimulating signal. Light and dark bars
541 represent the binding sites for those signals, respectively. DivK~P acts as an accessory factor to
542 DivL, binding in the DHp domain and reconfiguring the DivL PAS domains signaling to
543 promote CckA phosphatase activity. Stimulation of CckA kinase and phosphatase activities by
544 DivL are both transmitted in a CckA PAS-B dependent manner, likely through different binding
545 sites. In addition to receiving a signal from DivL, CckA oligomerization and c-di-GMP promote
546 CckA kinase and phosphatase activities, respectively. CckA catalytic output is determined upon
547 integration of all available signals. TM represents the transmembrane tethers of the two proteins,
548 replaced by His-tags in our *in vitro* experiments.
549
550 **Figure 2. DivL directly regulates CckA kinase activity in a DivL PAS domain-dependent**
551 **manner.** A. Purified CckA and DivL can be tethered to liposomes to facilitate their interaction.
552 The liposomes are doped with Ni-NTA-containing lipids, and the proteins are attached via N-
553 terminal His-tags, mimicking their membrane tethers *in vivo*. B. CckA kinase activity was
554 measured by allowing CckA to autophosphorylate for 3 minutes in the presence of [γ-P32]-ATP.
555 The effect of DivL on CckA kinase activity was measured either free in solution or on
556 liposomes. C. CckA kinase activity was measured in the presence of different DivL PAS domain
557 truncations. For each DivL variant, removal of a domain is represented by the dashed, empty
558 boxes, while presence of the domain is represented by green shading. D. CckA
559 autophosphorylation was measured in the presence of varying surface densities of DivL. Kinase
560 activity is normalized to the no-DivL condition in each panel. All protein densities except for

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561 the DivL titration in panel D were 350 molecules per liposome. Error bars represent the standard
562 deviation of at least 3 experiments for all panels.
563
564 **Figure 3. DivL can stimulate CckA as an auto-phosphatase or as an auto-kinase, in a CckA**
565 **PAS-B dependent manner.** A. A schematic illustrates how to test whether DivL affects CckA
566 phosphatase activity *in vitro*. More details are available in the Methods section. B. The loss of
567 CckA~P signal following ATP depletion was monitored by quenching reaction aliquots at 0.5, 1,
568 2, 4, and 10 minutes following mixing of CckA and DivL. (C, D) The experiment was repeated
569 for the CckA variants CckA ΔPAS-A and ΔPAS-B, respectively. The experiment was modified
570 for CckA ΔPAS-B by omitting the desalting column step, keeping 500 μM ADP in solution
571 following hexokinase treatment, and monitoring CckA~P for approximately 100 minutes. PAS
572 domain deletions are represented as empty, dashed boxes. E. The CckA PAS domain dependence
573 for autokinase activation by DivL A601L was tested. The kinase activities of CckA variants
574 ΔPAS-A, ΔPAS-B, and ΔPAS-A-B (orange domain schematics) were tested with DivL A601L
575 (green) or without it (black) at 350 molecules per liposome for each protein. Kinase activity is
576 normalized to the CckA-only condition for each variant. For constructs lacking PAS-A, kinase
577 activity at this density was very low and is represented by an *. F. The density dependence of
578 CckA response to DivL A601L was tested. In each experiment, CckA was present at 5 μM total
579 in solution, with liposomes added to bring CckA to either 350 (black) or 100 (gray) molecules
580 per liposome. DivL A601L was present on liposomes in equimolar quantities to each CckA
581 condition where applicable. For panels B-D, CckA~P decay traces are representative of at least
582 two independent experiments, and for panels E and F, error bars represent the range of at least
583 two experiments.

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584

585 **Figure 4. CckA oligomerizes through a multi-domain interaction.** A. The oligomerization
586 state of CckA in solution was measured by analytical size exclusion chromatography. The
587 elution trace from the size exclusion column is shown, with dashed vertical lines indicating the
588 predicted elution volumes for a tetramer or dimer. Predicted molecular mass of the CckA
589 complex was interpolated using a standard curve of known protein masses. B. The size exclusion
590 experiment was repeated for the CckA variants ΔPAS-A, ΔPAS-A-B, ΔRD, ΔPAS-A-ΔRD, and
591 ΔPAS-A-B-ΔRD. The projected CckA per complex is shown to the right of each variant. A
592 complete list of SEC experiments is given in Supplementary Table S1. In each experiment,
593 CckA was loaded onto the column at a concentration of roughly 100 μM. A complete list of
594 SEC experiments is given in Supplementary Table S1. C. (Left) A two-step model for CckA
595 oligomerization illustrates the domains involved in each oligomerization step, highlighted in
596 orange. The DHp domain promotes a conserved dimerization step. PAS-A and the RD are both
597 critical for assembly into a dimer of dimers. This representation does not reflect the relative
598 positions of the domains in 3D. (Right) Two possible models for how RD contributes to CckA
599 oligomerization are shown as 2D projections looking down the central DHp axis from the N-
600 terminus, one by RD-RD dimerization, common among response regulators, or by RD-DHp
601 contacts that bridge two dimers.

602

603 **Figure 5. Multiple signals converge on CckA to regulate its kinase-phosphatase switch**
604 **depending upon its subcellular niche.** A model of spatially controlled kinase/phosphatase
605 signaling. CckA (orange) accumulates at the new cell pole, favoring oligomerization and
606 formation of a kinase-active complex with DivL (green), possibly with the help of an additional

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607 kinase-stimulating factor (blue) specifically localized at that pole. Kinase activity leads to
608 phosphorylation of ChpT (gray) and eventually CtrA. Outside of the new pole the DivL/DivK~P
609 (red) complex can promote CckA phosphatase activity regardless of CckA's oligomeric state,
610 siphoning phosphate out of the CtrA pathway.
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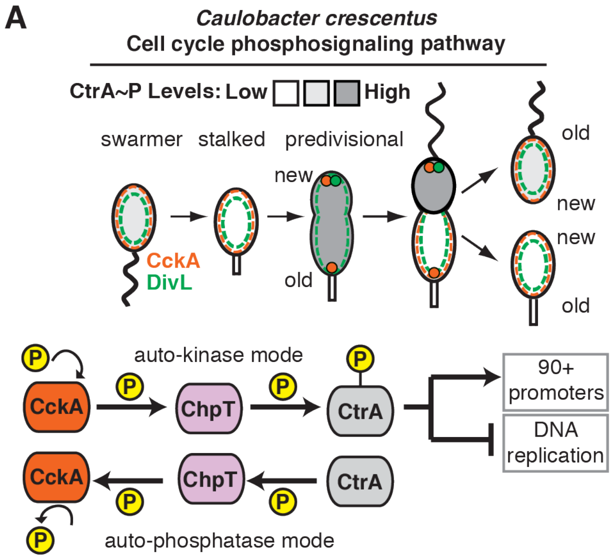
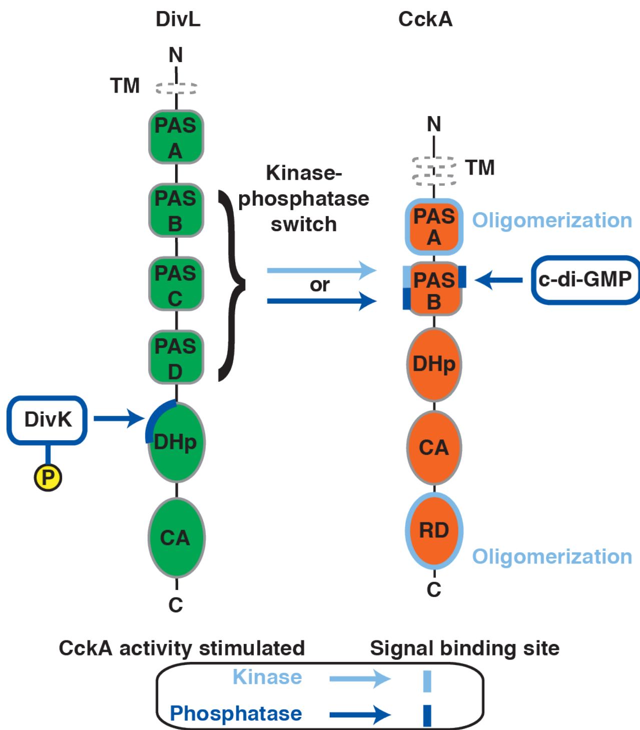
626 # Figures

627 Figure 1

628





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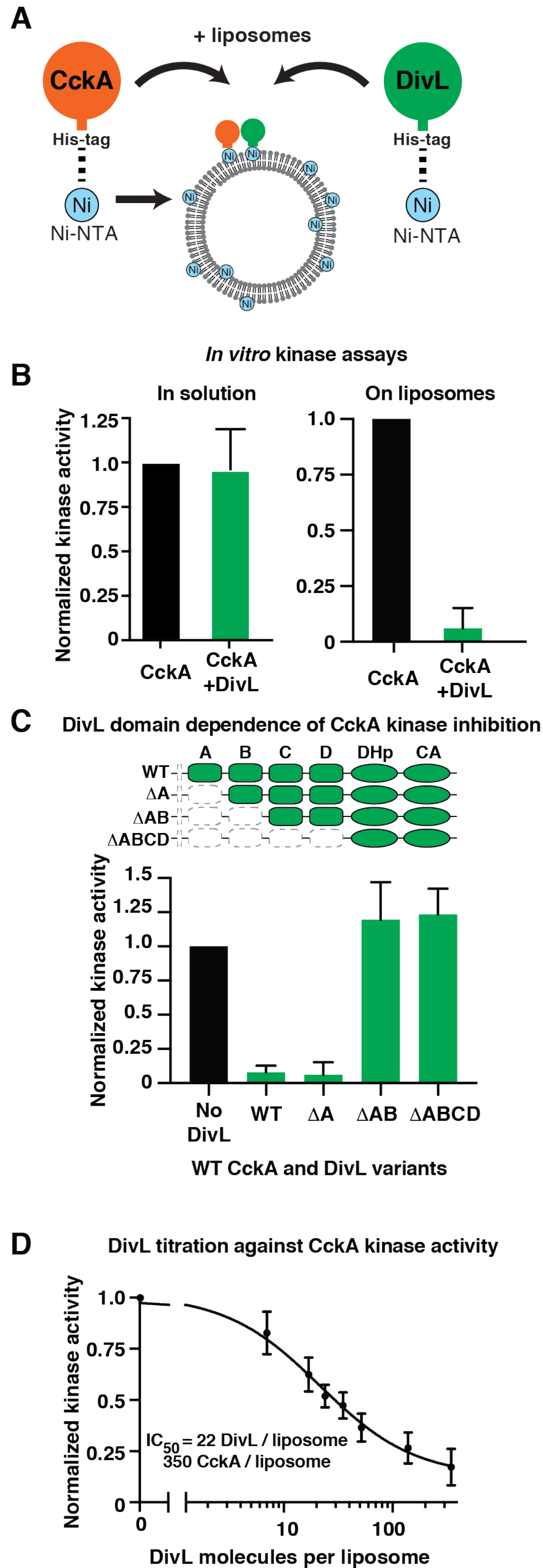
634 Figure 2



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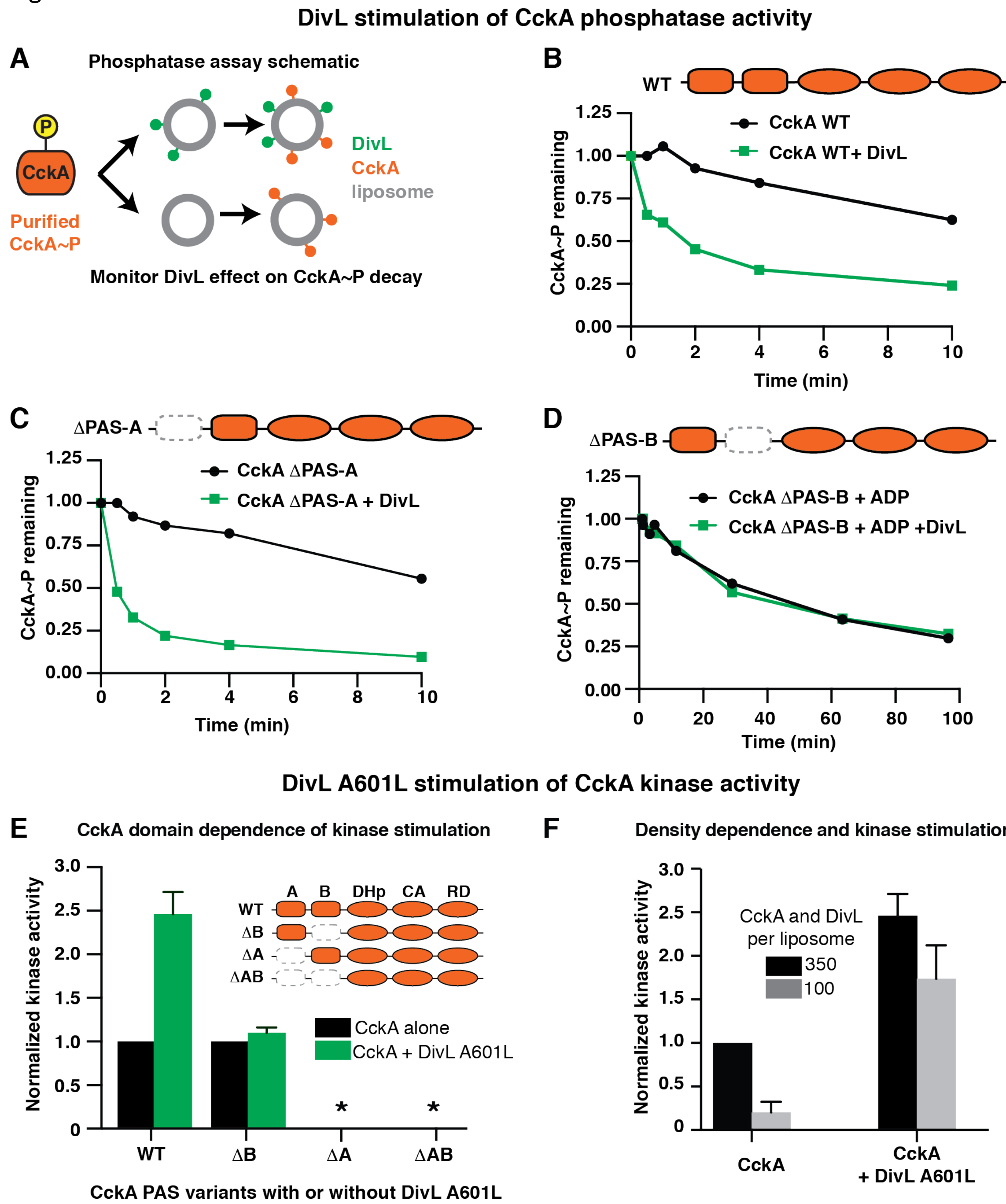
636 Figure 3



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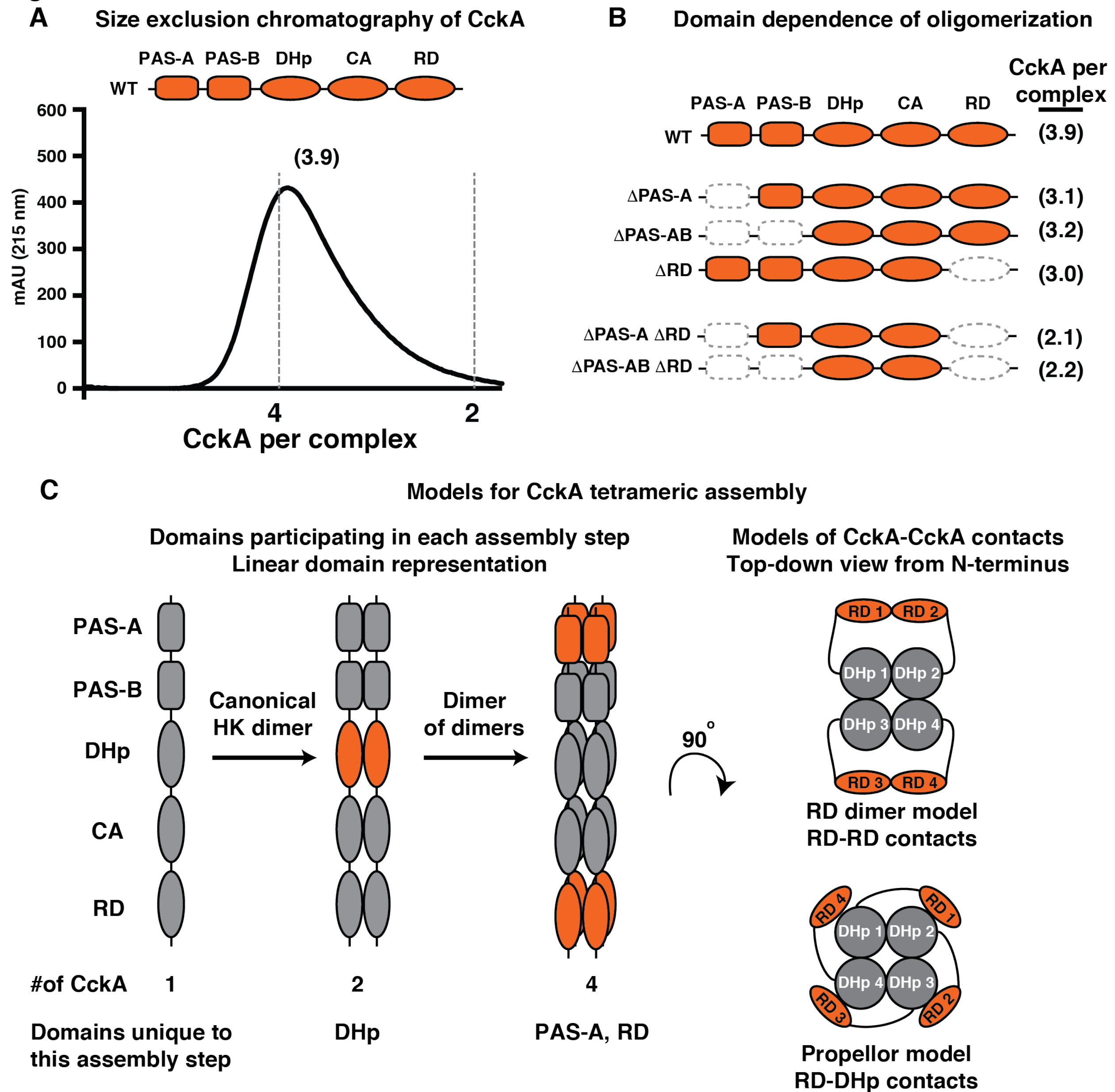
646 Figure 4



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659 Figure 5

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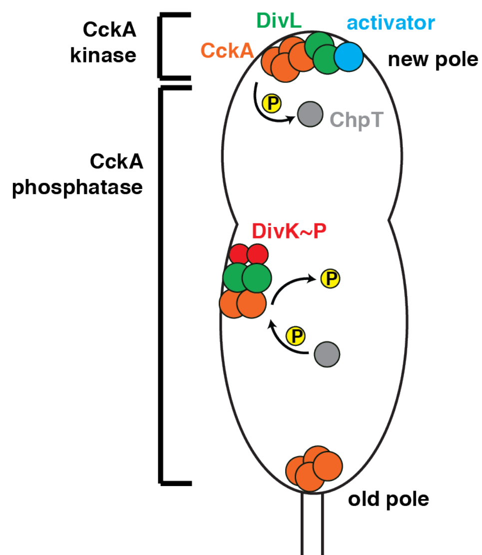

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