Integration of cell cycle signals by multi-PAS domain kinases

Thomas H. Mann[a] and Lucy Shapiro[a,b,1]

[a]Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305; and [b]Chan Zuckerberg Biohub, San Francisco, CA 94158

Contributed by Lucy Shapiro, June 12, 2018 (sent for review May 22, 2018; reviewed by Caroline S. Harwood and Martin Thanbichler)

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

kinase | cell division | *Caulobacter* | cell fate | signaling

Asymmetric cell division is a fundamental mechanism for generating cell type diversity across the kingdoms of life. Accomplishing an asymmetric division requires coordination between cell cycle-dependent gene expression and the dynamic subcellular localization and function of signaling proteins (1). A well-studied model system exhibiting asymmetric division is the bacterium *Caulobacter crescentus*, which generates two distinct daughter cells every cell division (Fig. 1*A*). A master regulator of cellular identity is the transcription factor CtrA, which when phosphorylated, directly controls the activity of over 90 cell cycle-regulated promoters while also inhibiting the initiation of DNA replication (2–4). Phosphorylated CtrA (CtrA~P) is present and active as a transcription factor in the motile, replication-incompetent swarmer cell. Dephosphorylation and proteolysis of CtrA~P permit differentiation into a sessile stalked cell and the beginning of DNA replication (5). Upon progression into the predivisional stage, CtrA proteolysis ceases and the transcription of *ctrA* is activated. Concurrently, a set of signaling proteins localize to the new cell pole, opposite the stalk, to promote CtrA phosphorylation and the biogenesis of the flagellum and pili. Spatial and temporal control of CtrA~P thus coordinates cell type identity with DNA replication and cell cycle progression.

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

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

In most histidine kinases, extracellular or intracellular signals are sensed by N-terminal PAS domains, leading to a conformational change that in turn regulates the activity of the catalytic domains (19, 20). Kinases can also use this conserved structural link in the reverse direction. DivL (Fig. 1*B*) uses its kinase-like domain to bind to the phosphorylated response regulator DivK~P (17), predicted to drive a conformational change within

Significance

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

Author contributions: T.H.M. designed research; T.H.M. performed research; T.H.M. and L.S. analyzed data; and T.H.M. and L.S. wrote the paper.

Reviewers: C.S.H., University of Washington; and M.T., Philipps-Universität.

The authors declare no conflict of interest.

[1]To whom correspondence should be addressed. Email: shapiro@stanford.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1808543115/-/DCSupplemental.

Published online July 9, 2018.

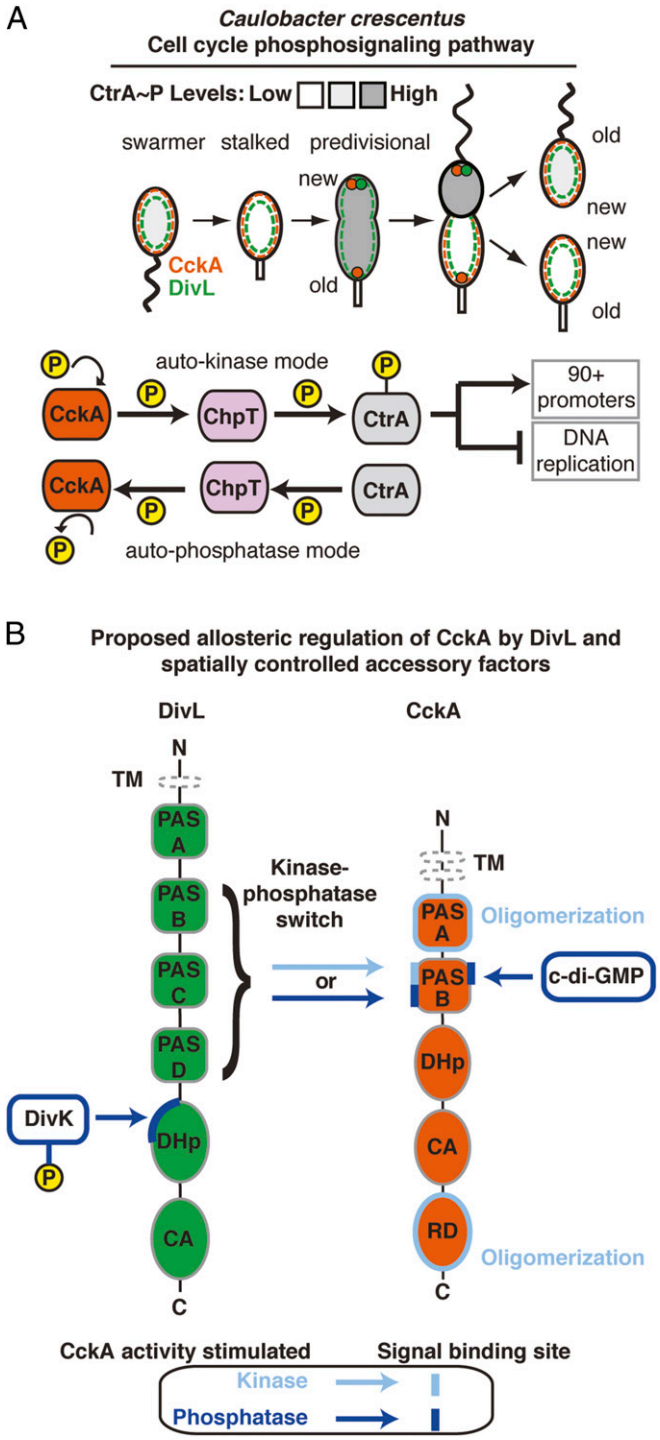

Fig. 1. DivL regulates CckA kinase and phosphatase activities as a function of the cell cycle. (*A*) DivL (green) and CckA (orange) subcellular localizations are dynamic over the *Caulobacter* cell cycle. In the swarmer and stalked cells, both proteins are diffusely deployed around the membrane (dashed lines). In the predivisional cell, DivL accumulates at the new cell pole (green circle), where it is essential for both CckA new pole localization and CckA kinase activity. CckA (orange circles) accumulates at both the old and new cell poles, and both proteins maintain a partial diffuse population outside of the polar niches. CckA kinase and phosphatase activities control the cell cycle-dependent phosphorylation state of the master regulator, CtrA. Phosphorylated CtrA~P (grayscale) inhibits the initiation of DNA replication and activates transcription at over 90 promoters. Phosphate is shuttled between CckA and CtrA via the transfer protein ChpT (lavender). (*B*) CckA and DivL both integrate signals to determine CckA kinase activity. Light blue arrows represent transmission of a CckA kinase-stimulating signal, whereas dark blue arrows represent a phosphatase-stimulating signal. Light and dark bars represent the binding sites for those signals, respectively. DivK~P acts as an accessory factor to DivL, binding in the DHp domain and reconfiguring the DivL PAS domains, signaling to promote CckA phosphatase activity. Stimulation of CckA kinase and phosphatase activities by DivL are both transmitted in a CckA PAS-B–dependent manner, likely through different binding sites. In addition to receiving a signal from DivL, CckA oligomerization and c-di-GMP promote CckA kinase and phosphatase activities, respectively. CckA catalytic output is determined upon integration of all available signals. TM, transmembrane tethers of the two proteins, replaced by His-tags in our in vitro experiments. Unless otherwise noted, the N-terminal domain A of DivL, shown here as PAS-A, due to its distant homology to PAS domains, is truncated to optimize stability of the pure protein.

DivL's PAS domains. This rearrangement in the PAS domains is then propagated as a regulatory signal to CckA's own PAS domains, regulating CckA catalytic activity (Fig. 1*B*) (17), and previous studies indicate that the PAS domains of the two proteins are sufficient for communication between the two proteins (10, 21). However, it has remained unclear whether DivL directly regulates CckA catalytic activity, and how a multi-PAS domain protein can pass distinct signals to a target kinase.

Here, we show that DivL directly regulates the kinase/phosphatase switch of CckA in vitro when the two proteins are reconstituted on proteoliposomes. DivL can inhibit CckA kinase activity in the absence of any ligands, instead stimulating CckA phosphatase activity. We also show that a point mutation in DivL can constitutively activate CckA kinase activity, and that stimulation of either kinase or phosphatase activity requires the PAS domains of DivL and PAS-B of CckA. Additionally, oligomerization of CckA is critical for DivL stimulation of kinase activity but not its stimulation of CckA phosphatase activity. While we must keep in mind that this reconstitution approach represents a simplified system that lacks additional factors present in vivo, our previous experiments using reconstituted CckA on liposomes have been supported by subsequent work demonstrating that the CckA surface density that is critical for kinase activity in our in vitro experiments matches the surface density of CckA present at the new cell pole in vivo (22). In this study, we reconstitute an additional aspect of subcellular regulation of CckA. We propose that DivL integrates information about subcellular localization and cell cycle progression to toggle CckA between its kinase and phosphatase modes in a PAS domain-dependent manner (Fig. 1*B*).

Results

DivL Directly Inhibits CckA Kinase Activity. We set out to test whether DivL can directly regulate CckA catalytic activity in vitro. Membrane tethering is essential for CckA polar localization and kinase activity in vivo (6), suggesting that that membrane attachment may be important for productive interaction between CckA and DivL (6). We purified CckA and DivL constructs containing N-terminal His-tags in place of their transmembrane helices, and we tethered the two proteins to Ni-NTA groups on the fluid lipid surface of large, unilamellar liposomes (Fig. 2*A*) (11, 23–25). Unless otherwise stated, we present data using a DivL construct lacking its N-terminal domain A, which has distant homology to PAS domains (26, 27), due to the increased protein stability of the shortened construct and its indistinguishable behavior from the full-length construct in all of our assays. To probe DivL's regulation of CckA kinase activity, we incubated CckA in the presence of DivL either in solution or on liposomes. In each case, both proteins had a 3D concentration of 5 μM, with addition of the liposomes rendering a 2D concentration of 350 molecules of each protein per liposome. CckA kinase activity was measured by autophosphorylation in the presence of [γ-^{32}P]-ATP followed by autoradiography.

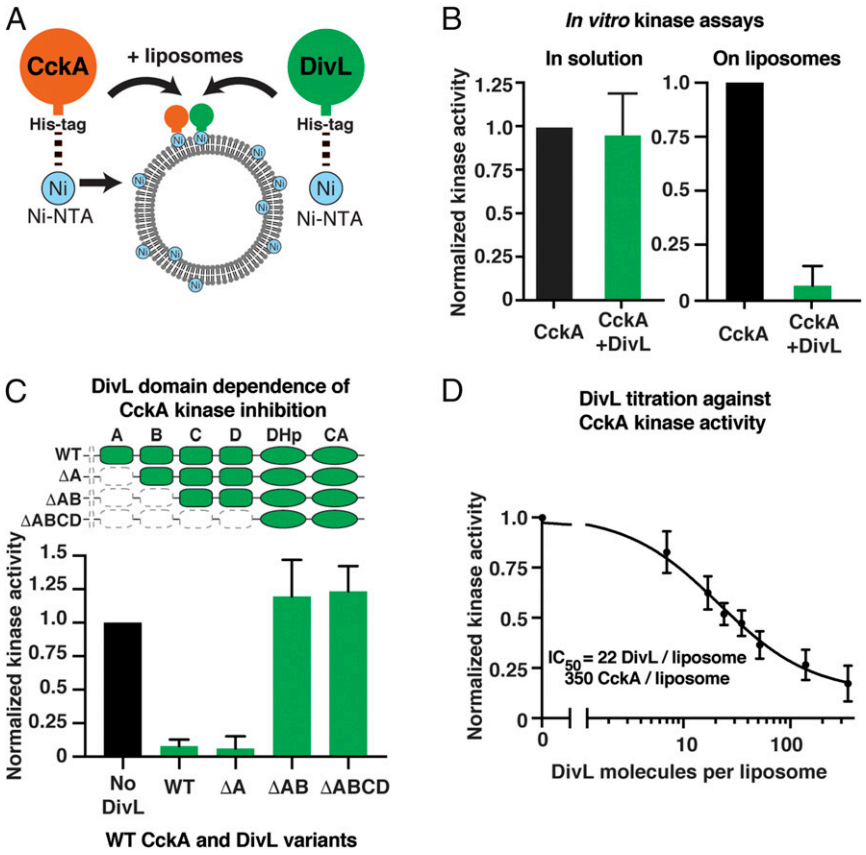

Fig. 2. DivL directly regulates CckA kinase activity in a DivL PAS domain-dependent manner. (*A*) Purified CckA and DivL can be tethered to liposomes to facilitate their interaction. The liposomes are doped with Ni-NTA–containing lipids, and the proteins are attached via N-terminal His-tags, mimicking their membrane tethers in vivo. (*B*) CckA kinase activity was measured by allowing CckA to autophosphorylate for 3 min in the presence of [γ-^{32}P]-ATP. The effect of DivL on CckA kinase activity was measured either free in solution or on liposomes. (*C*) CckA kinase activity was measured in the presence of different DivL PAS domain truncations. For each DivL variant, removal of a domain is represented by the dashed, empty boxes, while presence of the domain is represented by green shading. (*D*) CckA autophosphorylation was measured in the presence of varying surface densities of DivL. Kinase activity is normalized to the no-DivL condition in each panel. All protein densities except for the DivL titration in *D* were 350 molecules per liposome. Error bars represent the SD of at least three experiments for all panels.

Strikingly, coloading DivL with CckA on liposomes inhibited CckA autophosphorylation, whereas coincubation of the two proteins at 5 μM each in solution, with no liposomes, yielded no change to autophosphorylation (Fig. 2*B*), suggesting that membrane tethering facilitates interaction between the two proteins. Incubating CckA in solution with 5- to 10-fold molar excesses of DivL produced similar results on CckA autophosphorylation (*SI Appendix*, Fig. S1), but we pursued the liposome approach further, due to the requirement for CckA to be membrane bound to colocalize with DivL at the cell poles in vivo (6, 10).

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

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

DivL Stimulates CckA Autophosphatase Activity. Inhibition of kinase activity in many HKs frequently represents a concerted switch to a conformation that has increased phosphatase activity toward the cognate receiver domain (28, 29). It was previously shown that binding of the second messenger c-di-GMP (cdG) causes

CckA to switch from kinase mode to phosphatase mode (11, 13, 30). This finding suggests that reduction of CckA autophosphorylation in the presence of DivL may similarly reflect a switch from a kinase-active conformation to a phosphatase-active one, such that autokinase activity is reduced and autophosphatase activity is increased. Alternatively, we considered the possibility that DivL may directly perform the phosphatase activity on CckA's receiver domain.

We therefore tested whether DivL promotes CckA dephosphorylation in vitro, monitoring the rate at which purified CckA~P lost phosphate when incubated with or without DivL on liposomes (Fig. 3*A*). The presence of DivL on liposomes with CckA stimulated the loss of CckA~P signal, indicating that one of the proteins was performing phosphatase activity (Fig. 3*B*). DivL did not stimulate phosphate decay from the phosphatase-deficient variant CckA V366P (31), indicating that CckA, and not DivL, is responsible for performing phosphatase activity (*SI Appendix*, Fig. S2 *A* and *B*). To test whether the CckA PAS domains are necessary for this response to DivL (Fig. 1*B*), we repeated the phosphatase experiment for the CckA variants ΔPAS-A, ΔPAS-B, and ΔPAS-AB. CckA ΔPAS-A rapidly lost phosphate upon coloading with DivL on liposomes, indicating that CckA PAS-A is not necessary for interaction with DivL or phosphatase activity (Fig. 3*C*).

Strikingly, CckA constructs lacking PAS-B did not show a change in autophosphatase activity in the presence of DivL (Fig. 3*D* and *SI Appendix*, Fig. S2*C*). Because their autophosphatase activity was reduced compared with WT CckA, we took advantage of the finding that ADP binding promotes the phosphatase-competent conformation of histidine kinases (28, 30) to show that CckA ΔPAS-B and ΔPAS-AB variants do retain phosphatase function (*SI Appendix*, Fig. S3). Thus, CckA PAS-B is necessary for autophosphatase stimulation by DivL. It is formally possible that CckA constructs lacking PAS-B are conformationally restricted in a manner that prevents interaction between DivL and another part of CckA. It was previously shown that the N-terminal region of CckA comprising its transmembrane region, PAS-A, and PAS-B together are sufficient for a functional interaction with DivL in vivo (10). This result, in combination with our finding that PAS-A is not necessary for DivL to induce CckA autophosphatase activity in the liposome assay

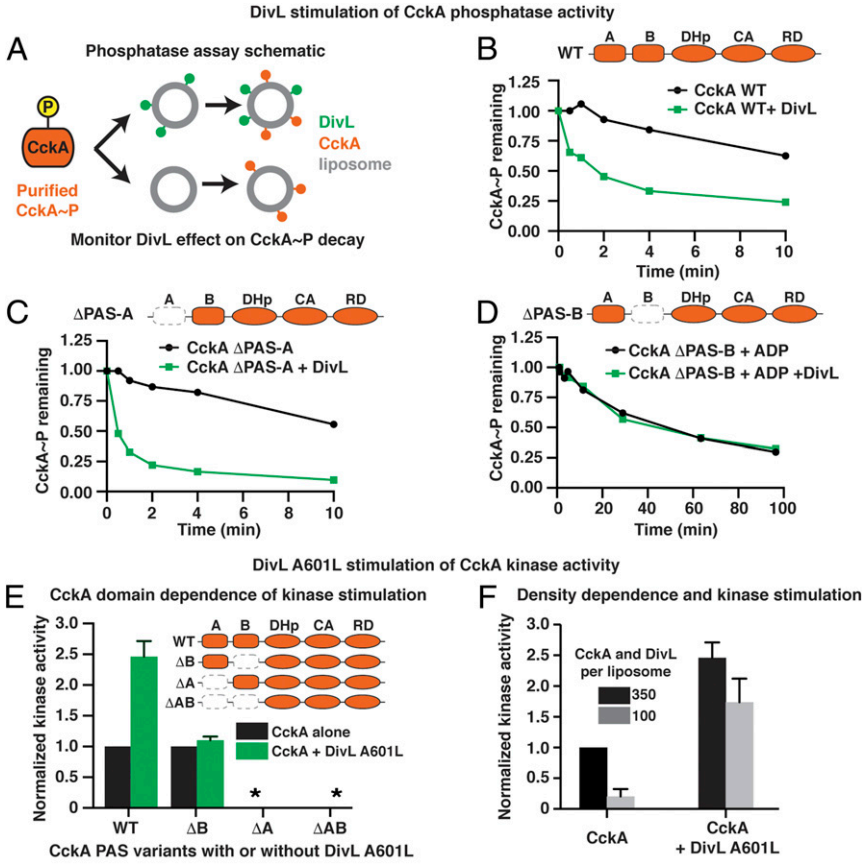

Fig. 3. DivL can stimulate CckA as an autophosphatase or as an autokinase, in a CckA PAS-B–dependent manner. (*A*) Schematic illustrates how to test whether DivL affects CckA phosphatase activity in vitro. More details are available in *Materials and Methods*. (*B*) The loss of CckA~P signal following ATP depletion was monitored by quenching reaction aliquots at 0.5, 1, 2, 4, and 10 min following the mixing of CckA and DivL. (*C* and *D*) The experiment was repeated for the CckA variants CckA ΔPAS-A and ΔPAS-B, respectively. The experiment was modified for CckA ΔPAS-B by omitting the desalting column step, keeping 500 μM ADP in solution following hexokinase treatment, and monitoring CckA~P for ~100 min. PAS domain deletions are represented as empty, dashed boxes. (*E*) The CckA PAS domain dependence for autokinase activation by DivL A601L was tested. The kinase activities of CckA variants ΔPAS-A, ΔPAS-B, and ΔPAS-A-B (orange domain schematics) were tested with DivL A601L (green) or without it (black) at 350 molecules per liposome for each protein. Kinase activity is normalized to the CckA-only condition for each variant. For constructs lacking PAS-A, kinase activity at this density was very low and is represented by an *. (*F*) The density dependence of CckA response to DivL A601L was tested. In each experiment, CckA was present at 5 μM total in solution, with liposomes added to bring CckA to either 350 (black) or 100 (gray) molecules per liposome. DivL A601L was present on liposomes in equimolar quantities to each CckA condition where applicable. For *B–D*, CckA~P decay traces are representative of at least two independent experiments, and for *E* and *F*, error bars represent the range of at least two experiments.

(Fig. 3*C*), suggests that PAS-B is the CckA domain that communicates with DivL.

We previously showed that PAS-B of CckA is necessary for binding to cdG, and that it is sufficient for binding when domains involved in dimerization and oligomerization are also present (11). Because our findings indicate that DivL, like cdG, also signals through CckA's PAS domains, we further tested whether cdG cooperatively inhibited CckA in the presence of DivL. While cdG inhibition has been shown to be cooperative with ADP binding (30), we observed additive rather than cooperative inhibition of CckA kinase activity by cdG and DivL (*SI Appendix*, Fig. S2*D*).

The DivL Variant A601L Directly Stimulates CckA Kinase Activity. Prior studies indicated that DivL switches between activating or inhibiting CckA kinase activity in vivo, depending on the stage of the cell cycle (10, 16) and that it uses accessory factors such as the phosphorylated response regulator DivK~P to determine its activity toward CckA. Tsokos et al. (16) found that a DivL point mutation, A601L, enhanced CckA autophosphorylation by fivefold in vivo during the stalked cell phase of the cell cycle even when the proteins were localized away from the new cell pole. This finding suggests that DivL A601L activates CckA kinase activity independent of upstream signals. Moreover, structure–function analysis suggested that the A601L mutation changes the global conformation of DivL in a PAS domain-dependent manner (17).

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

CckA Oligomerizes Through PAS-A and Its Receiver Domain. The requirement of high surface densities for CckA kinase activity on liposomes suggests that it may need to oligomerize to become active as a kinase (11). While the catalytic core of CckA, containing the DHp and CA domains, forms a canonical *trans*-phosphorylating dimer (30), we hypothesized that its PAS and

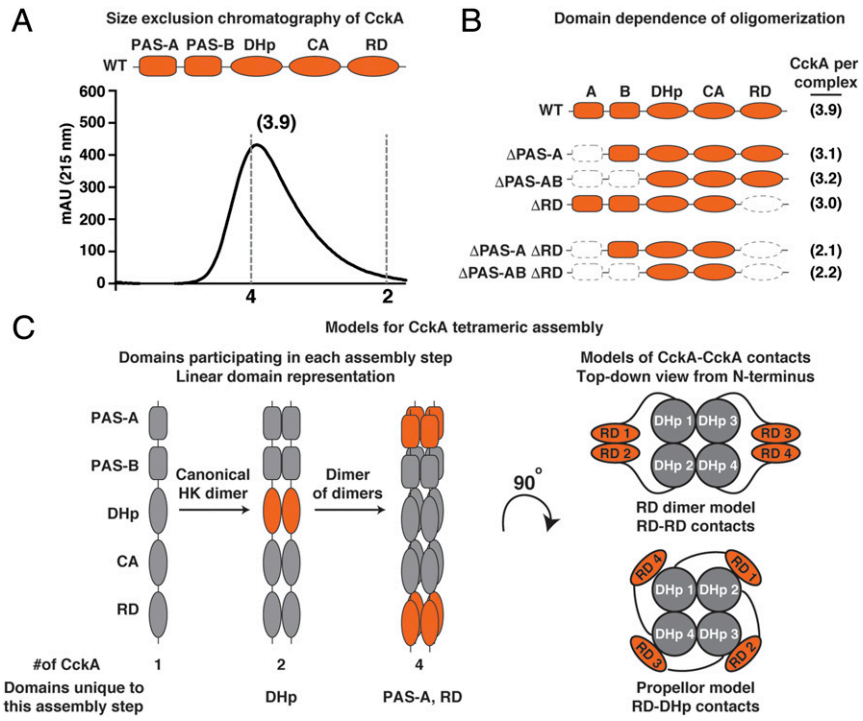

Fig. 4. CckA oligomerizes through a multidomain interaction. (*A*) The oligomerization state of CckA in solution was measured by analytical size exclusion chromatography. The elution trace from the size exclusion column is shown, with dashed vertical lines indicating the predicted elution volumes for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

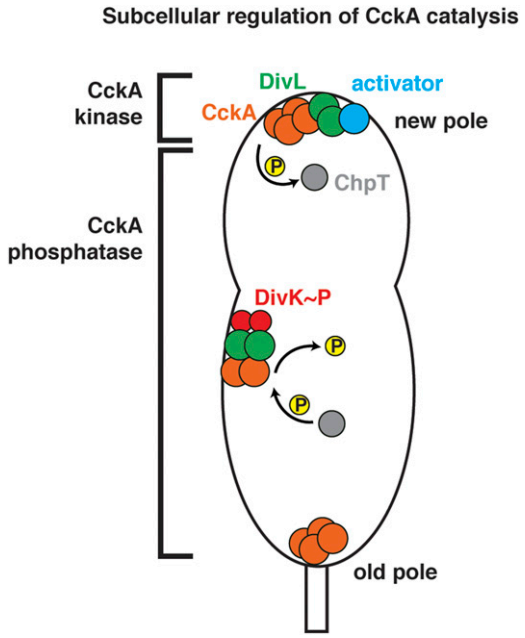

Fig. 5. Multiple signals converge on CckA to regulate its kinase/phosphatase switch depending upon its subcellular niche. A model of spatially controlled kinase/phosphatase signaling. CckA (orange) accumulates at the new cell pole, favoring oligomerization and formation of a kinase-active complex with DivL (green), possibly with the help of an additional kinase-stimulating factor (blue) specifically localized at that pole. Kinase activity leads to phosphorylation of ChpT (gray) and eventually CtrA. Outside of the new pole the DivL/DivK~P (red) complex can promote CckA phosphatase activity regardless of CckA's oligomeric state, siphoning phosphate out of the CtrA pathway.

receiver domains may drive higher oligomerization. There are a handful of histidine kinases believed to function as tetramers, including the kinase RegB, which loses kinase activity upon tetramerization (32–34). We assayed CckA oligomerization in solution using analytical size exclusion chromatography (SEC) to predict the molecular mass of CckA complexes. Indeed, we found that WT CckA eluted as a single peak at a molecular mass equivalent to that of a CckA tetramer when injected onto the column at ∼100 μM in solution (Fig. 4*A* and *SI Appendix*, Table S1). Knowing that PAS domains frequently mediate oligomerization (19), and that PAS-A is critical for density-dependent kinase activity, we tested the PAS domain dependence of CckA oligomerization. Removal of CckA PAS-A led to a reduction in oligomerization, with a peak center at 3.1 CckA per complex, indicating that PAS-A contributes to higher oligomerization, but that another domain in the protein continues to drive oligomerization in the absence of PAS-A (Fig. 4*B*). Removal of PAS-B yielded a smaller effect on CckA oligomerization than PAS-A (*SI Appendix*, Table S1).

Because the DHp domain typically only provides a conserved dimerization interface, we tested whether the receiver domain of CckA might also contribute to tetramerization. CckA ΔRD indeed showed a partial reduction in oligomerization (Fig. 4*B*), as well as a reduction in kinase activity on liposomes (*SI Appendix*, Fig. S4). Simultaneous deletion of both PAS-A and RD (ΔPAS-A ΔRD and ΔPAS-A-B ΔRD) resulted in CckA attaining only a dimeric state. Given that the catalytic core of CckA has been shown to form a canonical dimer (30), it seems apparent that the dimers should not have to be disassembled and reassembled into a fourfold-symmetric tetramer. Similarly, we hypothesize that the elution peak centered at approximately three CckA molecules per complex represents a dynamic equilibrium between the dimeric and tetrameric states rather than a distinct trimer. We propose that CckA's PAS-A and the RD mediate the assembly of conserved HK dimers into a dimer of dimers (Fig. 4*C*).

Discussion

Cells must constantly integrate information to coordinate their cell cycles. For processes that require spatial control, such as differentiation and asymmetric division, their signaling proteins must additionally be able to recognize and respond to upstream signaling factors found at distinct subcellular locations. Multisensor histidine kinases constitute a large but unexplored set of signaling proteins which have the potential to respond to multiple signals, enabling this complex processing of subcellular information. In this study, we have shown that the pseudokinase DivL controls the kinase/phosphatase switch of CckA through the PAS domains of the two proteins. DivL stimulation of kinase activity further requires CckA homooligomerization, illustrating how subcellular accumulation of CckA can be used as input information for its regulation of cellular asymmetry (Fig. 5).

We found that DivL can directly stimulate both CckA kinase and phosphatase activities in a CckA PAS-B–dependent manner (Fig. 1*B*), consistent with our previous finding that PAS-B is critical for new pole localization in vivo (11). We also previously showed that cdG binds in PAS-B of CckA, stimulating phosphatase activity (11). While most PAS domains typically sense only one ligand (19, 35), our finding that CckA PAS-B responds to both cdG and DivL adds to a small set of PAS domains that interact with multiple structurally unrelated ligands (19, 36, 37). A recent report identified a point mutation within the CckA PAS-B domain that led to increased expression of a subset of early predivisional, CtrA-dependent genes (38), suggesting that this mutation may specifically affect CckA interaction with DivL, cdG, or another signal, and that this mutation's effect is restricted to the early predivisional cell when CckA first becomes active as a kinase.

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

Our data indicate that oligomerization is important for CckA kinase but not phosphatase activity, as only kinase activity is surface density dependent (11). The CckA PAS-A domain, a key driver of oligomerization, is necessary for stimulation of kinase but not phosphatase activity by DivL (Fig. 3). In most HKs, a signal in the PAS domains torques the central alpha-helical spine of the protein to regulate downstream catalytic domains (20, 39). The proline-rich linker between PAS-A and PAS-B of CckA is inconsistent with this alpha-helix, but it may provide a semirigid connection still capable of torque. Alternatively, oligomerization via PAS-A and RD (Fig. 4) may regulate kinase activity on the conserved principle of stabilizing a rotation of the ATP binding domain relative to the active site histidine by changing CckA–CckA contacts between higher and lower assembly states. Indeed, structural evidence shows that binding of ATP versus ADP in the CA domain is sufficient to switch an HK between kinase and phosphatase conformations, respectively (28). Broadly, this demonstrates that the molecular impetus for switching between catalytic functions may come from many different parts of the

MICROBIOLOGY

protein, and oligomerization could provide multiple contact points to regulate kinase activity (Fig. 1*B*).

Further, oligomerization offers a straightforward mechanism for linking kinase activity to accumulation of a protein within a subcellular niche. In *Caulobacter*, a specialized collection of signaling and structural proteins at the new cell pole coordinates the stimulation of CckA kinase activity with polar organelle biogenesis (Fig. 5). The polar matrix protein PopZ defines the boundaries of DivL and CckA polar accumulation, and consequently the surface density of CckA, at the new pole (22, 40–42). Thus, the boundaries of the PopZ microdomain give subcellular context to CckA's density-dependent kinase activity.

Additional layers of regulation may promote CckA kinase activity at the new cell pole (43). Tsokos et al. (16) proposed that the new pole constitutes a "protected zone" in which other factors cannot inhibit CckA kinase activity. Moreover, the DivL mutation A601L directly stimulates CckA kinase activity, suggesting that an additional factors at the new cell pole, reviewed elsewhere (44), may be necessary to promote kinase activity of the DivL–CckA complex in vivo. Our study of the DivL–CckA interaction reveals that DivL can switch between directly stimulating CckA kinase and phosphatase activities, that CckA parses multiple signals through its PAS-B domain, and that CckA oligomerization is critical for its kinase activity. Thus, a multisensor domain architecture provides a means for integrating a complex array of input signals within the different spatial and temporal contexts required for a single master regulator that determines divergent cell fates.

Materials and Methods

Cloning, Protein Expression, and Purification. PAS domain cutoffs in CckA and DivL were assigned using the HHpred protein homology web server (26, 27) and described in detail previously (11). New plasmids for this study were created using Gibson assembly as designed through the J5 cloning system (45). Other plasmids were described previously (11, 17, 31, 46, 47). Plasmids were transformed into *Escherichia coli* via heat shock. A complete list of plasmids and strains used in this study is available in *SI Appendix*, Table S2. CckA and DivL variants were expressed and purified as described previously (11, 17, 47) and are described at length in *SI Appendix, Supplementary Methods*.

Gel Filtration Chromatography. Protein purification beyond Ni-NTA affinity was performed via gel filtration chromatography using a GP-250 gradient programmable chromatographer. Samples were dialyzed into 200 mM KCl, 50 mM Hepes-KOH pH 8.0, and 10% glycerol (with 1 mM DTT included for DivL) before injection on the column. Concentrated samples were separated in this buffer using a Superdex 200 10/300 GL column. Fractions of 0.3 mL were collected, analyzed, and pooled to optimize purity.

Studies of CckA oligomerization via analytical gel filtration chromatography were performed using a GE Healthcare Superdex 200 Increase 10/300 GL column and a Bio-Rad NGC Chromatography system. Apparent molecular weights of CckA oligomeric complexes were assigned using a standard curve based on elution volumes of a Bio-Rad premixed gel filtration standard (catalog no.151-1901). Elution was performed at 0.35 mL/min. CckA samples of ∼150 μL were loaded at ∼100 μM and eluted at 350 μL/min. A complete list of SEC experiments and buffers used is given in *SI Appendix*, Table S1.

Production of Large Unilamellar Liposomes. Liposomes were made as described previously (11), with some adjustments. Lipids were purchased from Avanti Polar Lipids. A mixture of 900 μL of 10 mg/mL 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (sodium salt) (DOPG) (product no. 840475), was mixed with 1 mL of 1 mg/mL 1,2-dioleoyl-*sn*-glycero-3-[(*N*-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) [DGS-NTA(Ni)] (product no. 790404C). Both lipids contain two 18-carbon acyl chains with a *cis*-alkene between carbon atoms 9 and 10 in each chain to ensure membrane fluidity at room temperature. The chloroform solutions were well mixed in a glass scintillation vial, and the solvent was evaporated under a gentle stream of nitrogen for 1 h to leave a clear film with no clumps. The film was then rehydrated in 500 μL to 20 mg/mL in liposome rehydration buffer (100 mM KCl, 20 mM Hepes-KOH pH 8.0). The buffer was vigorously resuspended with a pipette until all of the lipid was dissolved. The vial containing the lipid mixture was then subjected to 10 freeze/thaw cycles in liquid nitrogen and a 37 °C water bath. The freeze/thaw suspension was then extruded for 11 passes through 100-nm pores of a polycarbonate filter using the Avanti Mini-Extruder. Following extrusion, the liposomes were diluted in equal volume ultrapure water (to a final concentration of 10 mg/mL) and aliquots were stored under nitrogen in plastic tubes to extend shelf life.

Radiolabeling Autophosphorylation Assays. Radiolabeled autophosphorylation assays were similar to previous protocols (11). CckA constructs (5 μM) were incubated in low salt kinase buffer (50 mM KCl, 10 mM Hepes-KOH pH 8.0) with 5 mM $MgCl_2$ and 0.5 mM ATP in 25-μL reaction volumes. Glycerol was removed from protein samples via overnight dialysis before reactions to match the solvent conditions within the liposome lumens. Owing to opposite ionic strength preferences for kinase activity compared with WT CckA, kinase assays for CckA ΔPAS-B and ΔPAS-AB were conducted in high salt buffer (200 mM KCl and 50 mM Hepes-KOH pH 8.0). For Fig. 4*B*, CckA ΔPAS-B conditions are normalized to the no-DivL comparison in each case. For all liposome-based assays, CckA and DivL were allowed to incubate for 10 min with varying amounts of liposomes before addition of ATP stocks. Kinase assays on liposomes were conducted at a surface density of 350 molecules per liposome of CckA, with 350 molecules per liposome of DivL where appropriate, unless otherwise noted. Maximum density corresponds to 1,100 molecules per liposome. Liposome loading calibration experiments were performed to determine that at least 90% of the protein mass stably attached to liposomes at the protein surface densities used in these experiments.

Radiolabeled ATP was supplemented at of 2 μCi [γ-^{32}P]-ATP per reaction. Reactions were quenched after 3 min in 2× Laemmli sample buffer, and the quenched reaction mixtures were loaded onto 4–15% gradient polyacrylamide gels and subjected to electrophoresis. Alternatively, at this step for titration curve experiments, we used a nitrocellulose dot blot assay to separate phosphorylated protein from the reaction mixture, described previously (11). The extent of autophosphorylation was measured by exposing a phosphor screen to the gels for at least 3 h, and the screen was subsequently imaged on a Typhoon storage phosphorimager (Molecular Dynamics). Band intensities were quantified using ImageJ.

For phosphatase assays, CckA was allowed to autophosphorylate in solution, and CckA∼P was subsequently purified away from ATP. Depletion of ATP was accomplished by first rapidly passing the crude reaction mix through a desalting column to remove most of the nucleotides. Hexokinase (12.5 units) and glucose (10 mM) were then added to convert any remaining ATP to ADP over 10 min. CckA∼P was then deposited on liposomes that did or did not contain equimolar DivL to test whether DivL impacts CckA dephosphorylation. For phosphatase assays supplemented with ADP, the column purification step was skipped, permitting complete conversion of ATP to ADP by hexokinase.

ACKNOWLEDGMENTS. We thank Seth Childers, Keith Moffat, Kim Kowallis, Sam Duvall, Michael Collins, and Keren Lasker for helpful discussion and critical review of the manuscript; Saumya Saurabh for helpful improvements in the liposome preparation protocol; and Jonathan Herrmann for sharing access to chromatography equipment. Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health (NIH) under Award T32 GM007276 (to T.H.M.) as well as NIH Awards R01 GM032506 and R35-GM118071 (to L.S.). The content is solely the responsibility of the authors and does not necessarily represent the official view of the NIH. L.S. is a Chan Zuckerberg Biohub investigator.

1. Shapiro L, McAdams HH, Losick R (2009) Why and how bacteria localize proteins. *Science* 326:1225–1228.
2. Quon KC, Marczynski GT, Shapiro L (1996) Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell* 84:83–93.
3. Laub MT, Chen SL, Shapiro L, McAdams HH (2002) Genes directly controlled by CtrA, a master regulator of the *Caulobacter* cell cycle. *Proc Natl Acad Sci USA* 99:4632–4637.
4. Quon KC, Yang B, Domian IJ, Shapiro L, Marczynski GT (1998) Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin. *Proc Natl Acad Sci USA* 95:120–125.
5. Domian IJ, Quon KC, Shapiro L (1997) Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1-to-S transition in a bacterial cell cycle. *Cell* 90:415–424.
6. Jacobs C, Domian IJ, Maddock JR, Shapiro L (1999) Cell cycle-dependent polar localization of an essential bacterial histidine kinase that controls DNA replication and cell division. *Cell* 97:111–120.
7. Jacobs C, Ausmees N, Cordwell SJ, Shapiro L, Laub MT (2003) Functions of the CckA histidine kinase in *Caulobacter* cell cycle control. *Mol Microbiol* 47:1279–1290.
8. Heinrich K, Sobetzko P, Jonas K (2016) A kinase-phosphatase switch transduces environmental information into a bacterial cell cycle circuit. *PLoS Genet* 12:e1006522.

9. Vo CD, et al. (2017) Repurposing Hsp90 inhibitors as antibiotics targeting histidine kinases. *Bioorg Med Chem Lett* 27:5235–5244.
10. Iniesta AA, Hillson NJ, Shapiro L (2010) Cell pole-specific activation of a critical bacterial cell cycle kinase. *Proc Natl Acad Sci USA* 107:7012–7017.
11. Mann TH, Seth Childers W, Blair JA, Eckart MR, Shapiro L (2016) A cell cycle kinase with tandem sensory PAS domains integrates cell fate cues. *Nat Commun* 7:11454.
12. Biondi EG, et al. (2006) Regulation of the bacterial cell cycle by an integrated genetic circuit. *Nature* 444:899–904.
13. Lori C, et al. (2015) Cyclic di-GMP acts as a cell cycle oscillator to drive chromosome replication. *Nature* 523:236–239.
14. Christen M, et al. (2010) Asymmetrical distribution of the second messenger c-di-GMP upon bacterial cell division. *Science* 328:1295–1297.
15. Wu J, Ohta N, Zhao J-L, Newton A (1999) A novel bacterial tyrosine kinase essential for cell division and differentiation. *Proc Natl Acad Sci USA* 96:13068–13073.
16. Tsokos CG, Perchuk BS, Laub MT (2011) A dynamic complex of signaling proteins uses polar localization to regulate cell-fate asymmetry in *Caulobacter crescentus*. *Dev Cell* 20:329–341.
17. Childers WS, et al. (2014) Cell fate regulation governed by a repurposed bacterial histidine kinase. *PLoS Biol* 12:e1001979.
18. Reisinger SJ, Huntwork S, Viollier PH, Ryan KR (2007) DivL performs critical cell cycle functions in *Caulobacter crescentus* independent of kinase activity. *J Bacteriol* 189: 8308–8320.
19. Möglich A, Ayers RA, Moffat K (2009) Structure and signaling mechanism of Per-ARNT-Sim domains. *Structure* 17:1282–1294.
20. Diensthuber RP, Bommer M, Gleichmann T, Möglich A (2013) Full-length structure of a sensor histidine kinase pinpoints coaxial coiled coils as signal transducers and modulators. *Structure* 21:1127–1136.
21. Westbye AB, et al. (2018) The protease ClpXP and the PAS-domain protein DivL regulate CtrA and gene transfer agent production in *Rhodobacter capsulatus*. *Appl Environ Microbiol* 84:e00275-18.
22. Lasker K, et al. (2017) Phospho-signal flow from a pole-localized microdomain spatially patterns transcription factor activity. bioRxiv:10.1101/220293. Preprint, updated version posted February 21, 2018.
23. Hope MJ, Bally MB, Webb G, Cullis PR (1985) Production of large unilamellar vesicles by a rapid extrusion procedure: Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim Biophys Acta* 812:55–65.
24. Mayer LD, Hope MJ, Cullis PR (1986) Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim Biophys Acta* 858:161–168.
25. Kim J, Blackshear PJ, Johnson JD, McLaughlin S (1994) Phosphorylation reverses the membrane association of peptides that correspond to the basic domains of MARCKS and neuromodulin. *Biophys J* 67:227–237.
26. Söding J, Biegert A, Lupas AN (2005) The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Res* 33:W244–W248.
27. Zimmermann L, et al. (2017) A completely reimplemented MPI bioinformatics toolkit with a new HHpred server at its core. *J Mol Biol* S0022-2836(17)30587-9.
28. Casino P, Miguel-Romero L, Marina A (2014) Visualizing autophosphorylation in histidine kinases. *Nat Commun* 5:3258.
29. Huynh TN, Stewart V (2011) Negative control in two-component signal transduction by transmitter phosphatase activity. *Mol Microbiol* 82:275–286.
30. Dubey BN, et al. (2016) Cyclic di-GMP mediates a histidine kinase/phosphatase switch by noncovalent domain cross-linking. *Sci Adv* 2:e1600823.
31. Chen YE, Tsokos CG, Biondi EG, Perchuk BS, Laub MT (2009) Dynamics of two Phosphorelays controlling cell cycle progression in *Caulobacter crescentus*. *J Bacteriol* 191: 7417–7429.
32. Little R, Martinez-Argudo I, Perry S, Dixon R (2007) Role of the H domain of the histidine kinase-like protein NifL in signal transmission. *J Biol Chem* 282:13429–13437.
33. Eswaramoorthy P, Guo T, Fujita M (2009) *In vivo* domain-based functional analysis of the major sporulation sensor kinase, KinA, in *Bacillus subtilis*. *J Bacteriol* 191: 5358–5368.
34. Willett JW, Crosson S (2017) Atypical modes of bacterial histidine kinase signaling. *Mol Microbiol* 103:197–202.
35. Henry JT, Crosson S (2011) Ligand-binding PAS domains in a genomic, cellular, and structural context. *Annu Rev Microbiol* 65:261–286.
36. Scheuermann TH, et al. (2009) Artificial ligand binding within the HIF2α PAS-B domain of the HIF2 transcription factor. *Proc Natl Acad Sci USA* 106:450–455.
37. Hoffman EC, et al. (1991) Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science* 252:954–958.
38. Narayanan S, Kumar L, Radhakrishnan SK (2018) Sensory domain of the cell cycle kinase CckA in Caulobacter crescentus regulates the differential DNA binding activity of the master regulator CtrA. bioRxiv:10.1101/300178. Preprint, posted April 14, 2018.
39. Möglich A, Ayers RA, Moffat K (2010) Addition at the molecular level: Signal integration in designed per-ARNT-Sim receptor proteins. *J Mol Biol* 400:477–486.
40. Ebersbach G, Briegel A, Jensen GJ, Jacobs-Wagner C (2008) A self-associating protein critical for chromosome attachment, division, and polar organization in *caulobacter*. *Cell* 134:956–968.
41. Bowman GR, et al. (2008) A polymeric protein anchors the chromosomal origin/ParB complex at a bacterial cell pole. *Cell* 134:945–955.
42. Holmes JA, et al. (2016) *Caulobacter* PopZ forms an intrinsically disordered hub in organizing bacterial cell poles. *Proc Natl Acad Sci USA* 113:12490–12495.
43. Chen YE, et al. (2011) Spatial gradient of protein phosphorylation underlies replicative asymmetry in a bacterium. *Proc Natl Acad Sci USA* 108:1052–1057.
44. Lasker K, Mann TH, Shapiro L (2016) An intracellular compass spatially coordinates cell cycle modules in *Caulobacter crescentus*. *Curr Opin Microbiol* 33:131–139.
45. Hillson NJ, Rosengarten RD, Keasling JD (2012) j5 DNA assembly design automation software. *ACS Synth Biol* 1:14–21.
46. Rocco CJ, Dennison KL, Klenchin VA, Rayment I, Escalante-Semerena JC (2008) Construction and use of new cloning vectors for the rapid isolation of recombinant proteins from *Escherichia coli*. *Plasmid* 59:231–237.
47. Blair JA, et al. (2013) Branched signal wiring of an essential bacterial cell-cycle phosphotransfer protein. *Structure* 21:1590–1601.