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A Dynamic Complex of Signaling Proteins Uses Polar Localization to Regulate Cell-Fate Asymmetry in *Caulobacter crescentus*

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SUMMARY

Cellular asymmetry is critical to metazoan development and the life cycle of many microbes. In *Caulobacter*, cell cycle progression and the formation of asymmetric daughter cells depend on the polarly-localized histidine kinase CckA. How CckA is regulated and why activity depends on localization are unknown. Here, we demonstrate that the unorthodox kinase DivL promotes CckA activity and that the phosphorylated regulator DivK inhibits CckA by binding to DivL. Early in the cell cycle, CckA is activated by the dephosphorylation of DivK throughout the cell. However, in later stages, when phosphorylated DivK levels are high, CckA activation relies on polar localization with a DivK phosphatase. Localization thus creates a protected zone for CckA within the cell, without the use of membrane-enclosed compartments. Our results reveal the mechanisms by which CckA is regulated in a cell-type-dependent manner. More generally, our findings reveal how cells exploit subcellular localization to orchestrate sophisticated regulatory processes.

INTRODUCTION

Asymmetric cell divisions are critical to the generation of cellular complexity in both metazoans and many microbes. However, the molecular mechanisms responsible for robustly translating asymmetry into differential cell fates remain incompletely understood. The bacterium *Caulobacter crescentus* represents an excellent model to dissect this process as each cell division is asymmetric. One daughter cell, the stalked cell, is sessile and commits immediately to S phase. The other daughter, the swarmer cell, is motile and locked in G1 until it differentiates into a stalked cell. Strikingly, many of the key regulatory proteins that govern cell cycle progression and cell fate asymmetry in *Caulobacter* are localized to specific sites within the cell (reviewed in Curtis and Brun, 2010). However, the role that localization plays in governing the functions and activities of these regulatory proteins is largely unknown.

Localizing regulatory proteins can serve many different functions. Cells often localize proteins that control morphogenetic processes to their primary site of action (reviewed in Rudner and Losick, 2010). For example, in both eukaryotes and prokaryotes, proteins regulating cell division often localize to the cytokinetic ring at mid-cell. Similarly, bacterial proteins that regulate assembly of a polar flagellum often localize, not surprisingly, to the cell pole. Localization can also facilitate the differential inheritance of proteins by daughter cells, as is the case with Ash1p in *Saccharomyces cerevisiae* that is preferentially retained in daughter cells to prevent mating-type switches (Sil and Herskowitz, 1996). However, the reason for subcellular localization of many proteins is not self-evident. In bacteria, regulatory proteins are frequently localized to the cell poles without having any direct function at those positions and despite regulating factors that freely diffuse.

In *Caulobacter*, the master histidine kinase CckA dynamically localizes to the cell poles, usually first to the nascent swarmer pole and then to both poles before cell division (Angelastro et al., 2010; Chen et al., 2009). CckA is essential for cell cycle progression and the generation of daughter cells positioned at different cell cycle stages (Jacobs et al., 1999). However, why CckA must be polarly localized is mysterious as it ultimately regulates a transcription factor that is dispersed throughout the cell. Moreover, both daughter cells inherit CckA, suggesting that localization does not facilitate asymmetric inheritance.

The primary target of CckA in *Caulobacter* is CtrA, an essential response regulator (Quon et al., 1996) that directly controls the expression of nearly 100 genes (Laub et al., 2002). In G1 swarmer cells, phosphorylated CtrA also binds to the origin of replication to inhibit DNA replication (Quon et al., 1998). As swarmer cells differentiate into stalked cells, CtrA must be dephosphorylated or degraded to permit the initiation of DNA replication (Domian et al., 1997). Once S phase begins, new CtrA is synthesized and phosphorylated allowing it to act as a transcription factor for target genes, many of which are required for cell division.

CckA initiates two phosphorelays that control CtrA (Biondi et al., 2006). One culminates in CtrA phosphorylation whereas the other leads to the phosphorylation of CpdR, which somehow inhibits CtrA proteolysis (Biondi et al., 2006; Iniesta et al., 2006). Activation of CckA as a kinase thus simultaneously drives CtrA phosphorylation and increases CtrA stability. In vivo phosphorylation assays indicate that CckA is active in swarmer cells, inactive in stalked cells, and highly active in predivisional cells (Jacobs et al., 2003). Notably, the peak in activity in predivisional cells correlates with and depends on polar localization (Angelastro et al., 2010; Chen et al., 2009; Jacobs et al., 1999).

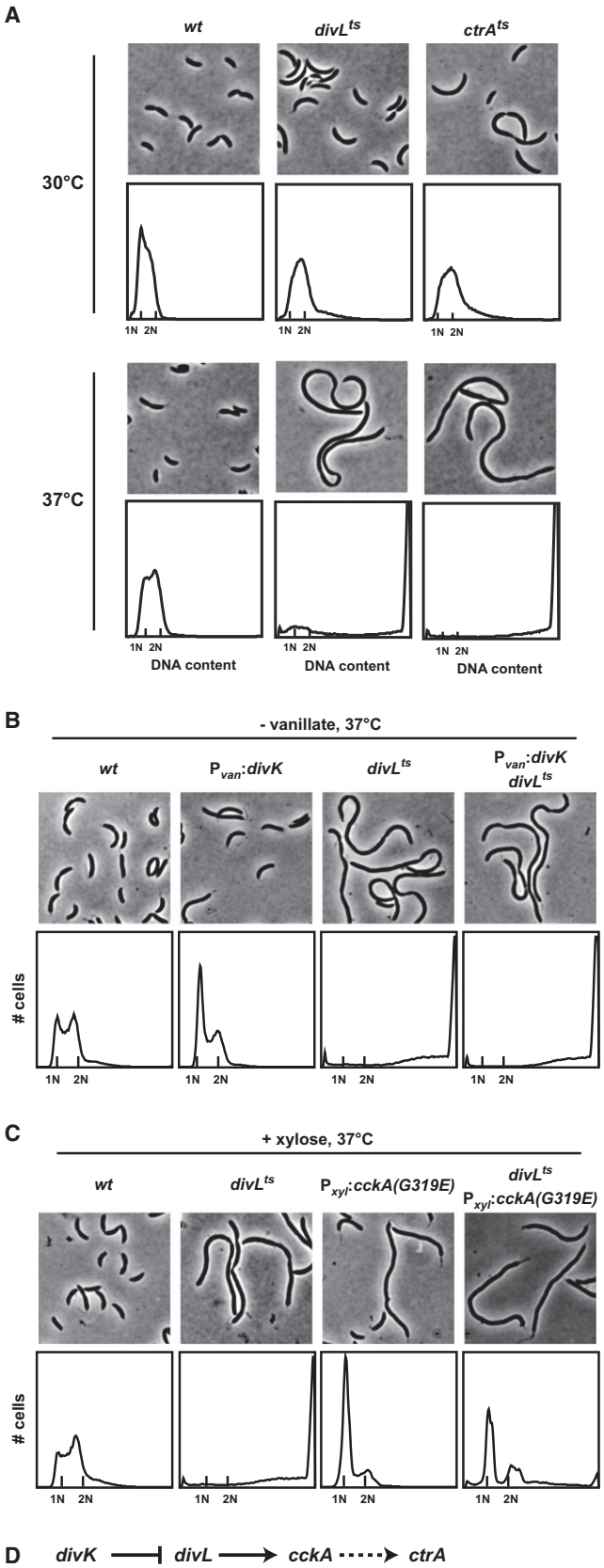

Figure 1. Epistasis Analysis Places *divL* between *divK* and *cckA* in the CtrA Regulatory Pathway

(A) Phase contrast microscopy and flow cytometry analysis of wild type, *divL*^{ts}, and *ctrA*^{ts} grown at the permissive temperature (30°C) and after a shift to the restrictive temperature (37°C) for 4 hr.

(B) Phase contrast images and flow cytometry analysis of wild-type, *divK* depletion, *divL*^{ts}, and double mutant (*divK* depletion and *divL*^{ts}) strains grown without vanillate for 4 hr to deplete *divK* followed by a shift to 37°C for an additional 4 hr.

(C) Phase contrast images and flow cytometry analysis of wild-type, *divL*^{ts}, *cckA*(*G319E*) overexpression, and double mutant (*divL*^{ts} and *cckA*(*G319E*) overexpression) strains grown with xylose for 4 hr to induce *cckA*(*G319E*) and then shifted to 37°C for an additional 4 hr.

(D) Summary of genetic pathway regulating CtrA.

How CckA activity is regulated remains largely undefined, although the essential, single-domain response regulator DivK may play an important role (Hecht et al., 1995). Conspicuously, a *divK* loss-of-function mutant arrests in G1 suggesting that without DivK, CckA may remain active, leading to a maintenance of CtrA activity and a continual silencing of DNA replication (Biondi et al., 2006; Hung and Shapiro, 2002). Consistently, CckA activity is moderately elevated in this *divK* mutant, but it is unclear whether DivK directly inhibits CckA.

Here, we show that (1) the noncanonical histidine kinase DivL promotes CckA kinase activity; and (2) that phosphorylated DivK downregulates CckA by binding directly to DivL. These results demonstrate that transitions in the phosphorylation state of DivK drive cell cycle transitions. When swarmer cells differentiate into stalked cells, a sharp increase in DivK phosphorylation leads to the inhibition of CckA that, in turn, permits the initiation of DNA replication. Paradoxically however, DivK remains highly phosphorylated in predivisional cells when CckA is most active as a kinase. We resolve this apparent conundrum by demonstrating that in predivisional cells CckA is activated by localizing at the swarmer pole with PleC, the primary DivK phosphatase. Our data reveal a rationale for why CckA is polarly localized and how the elaborate spatial arrangement of regulatory proteins in *Caulobacter* enables both cell cycle progression and the establishment of asymmetric daughter cell fates.

RESULTS

divL Acts between *divK* and *cckA* in the CtrA Regulatory Pathway

Previous studies have implicated DivL in the CtrA regulatory pathway, but its precise role has remained unknown (Iniesta et al., 2010; Pierce et al., 2006; Reisinger et al., 2007; Wu et al., 1999). To further characterize DivL we examined cells harboring *divL346*, a temperature-sensitive allele of *divL* (Wu et al., 1999), hereafter referred to as *divL*^{ts}. We found that *divL*^{ts} cells shifted from 30°C to 37°C became extremely filamentous and accumulated multiple chromosomes, phenotypes shared by *ctrA*^{ts} and *cckA*^{ts} mutants that result from continued growth and DNA replication in the absence of cell division (Figure 1A). Using DNA microarrays, we also found that CtrA-dependent gene expression was affected in the *divL*^{ts} mutant in a manner similar to *ctrA*^{ts} and *cckA*^{ts} (see Figure S1 available online). These data confirm that DivL positively regulates CtrA and that *divL346* is a loss-of-function allele at 37°C.

To map the position of *divL* in the regulatory circuitry controlling CtrA, we conducted epistasis experiments, using chromosome content as a readout for CtrA activity. Because CtrA silences the origin of replication, excess CtrA activity results in a G1 arrest, whereas too little CtrA activity results in a disruption of cell division and the accumulation of multiple chromosomes per cell.

First, we sought to establish the relative order of *divK* and *divL* in the CtrA regulatory pathway. DivK inhibits, either directly or indirectly, CtrA activity by decreasing both its phosphorylation (Biondi et al., 2006) and stability (Hung and Shapiro, 2002). Consequently, a loss of *divK* function results in increased CtrA activity and a G1 arrest. By contrast, a loss of *divL* function results in decreased CtrA activity and a consequent accumulation of multiple chromosomes (Figure 1A). We engineered a strain that harbors the *divL*^{ts} mutation and a single copy of *divK* under the control of a vanillate-inducible promoter. When grown in the absence of vanillate to deplete DivK and at 37°C to inactivate DivL, this strain accumulated multiple chromosomes as with the *divL*^{ts} strain (Figure 1B), suggesting that *divL* lies genetically downstream of *divK* and that DivK is a negative regulator of DivL. We corroborated this result by constructing a strain harboring the *divK341* (or *divK*^{cs}) mutation and in which the only copy of *divL* is driven by a xylose-inducible, glucose-repressible promoter (Sciochetti et al., 2005). When grown in the presence of glucose to deplete DivL and at 22°C to eliminate DivK activity, this strain accumulated multiple chromosomes, confirming that *divL* is genetically downstream of *divK* (Figure S2).

Because *divL* is downstream of *divK*, we tested whether *divL* lies between *divK* and *cckA* in the CtrA regulatory pathway. Previously, we identified a mutation in CckA, G319E, that significantly increases its kinase activity and, when expressed from a high-copy plasmid, results in a G1 arrest similar to that seen with *divK*^{cs} (Chen et al., 2009). To test the relationship between *divL* and *cckA*, we constructed a strain carrying a xylose-inducible copy of *cckA*(*G319E*) in a *divL*^{ts} background. Growth in the presence of xylose and at 37°C led to a G1 arrest indicating that CtrA activity remained high and prevented the initiation of DNA replication, despite the loss of DivL function (Figure 1C). The overexpression of *cckAG319E* is thus epistatic to *divL346*. These data are consistent with *divL* lying upstream of *cckA* and with DivL acting as a positive regulator of CckA.

DivL Regulates CtrA by Promoting CckA Activity

Our epistasis analyses suggest that *divK* and *divL* both lie upstream of and regulate CckA (Figure 1D). Formally though, *divK* and *divL* could function in a pathway parallel to and independent of CckA that activates CtrA. To distinguish between these possibilities, we measured CckA activity in vivo in the *divL*^{ts} strain by immunoprecipitating CckA after labeling cells with [γ^{32}P]-ATP (Figures 2A and 2B). At the permissive temperature of 30°C, CckA phosphorylation levels in the *divL*^{ts} strain were slightly elevated relative to wild-type. However, after a shift to the restrictive temperature of 37°C for 15 min, CckA phosphorylation in the *divL*^{ts} strain fell to ~42% that of wild-type at 37°C and ~29% the level in *divL*^{ts} at 30°C. These data are consistent with a recent study showing that *divL* is necessary for full activity of a chimeric CckA-FixL reporter (Iniesta et al., 2010).

If DivL regulates CckA, then DivL should also affect CtrA degradation in vivo as CckA controls the phosphorylation of CpdR through ChpT (Biondi et al., 2006). However, a previous study saw no major changes in CtrA stability in a *divL510* mutant, a different ts-allele of *divL*, after 4 hr at the restrictive temperature (Reisinger et al., 2007). We measured the levels of phosphorylated CpdR and CtrA in our *divL*^{ts} strain after a 15 min shift to the restrictive temperature and found that both were significantly decreased (Figures 2A and 2B). In addition, using pulse-chase analyses, we found that CtrA stability was significantly decreased in *divL*^{ts} (half-life of 8 min) relative to wild-type (half-life of 29 min) at the restrictive temperature (Figure 2C). At the permissive temperature, the half-life of CtrA was nearly identical in wild-type and *divL*^{ts} (34 and 33 min, respectively). These data support the notion that a loss of *divL* function leads to a drop in the phosphorylation of both CpdR and CtrA, further indicating that DivL promotes CtrA activity through CckA.

DivL Is Required to Localize CckA at the Nascent Swarmer Pole

Notably, although CckA usually localizes to both poles of a predivisional cell (Angelastro et al., 2010; Chen et al., 2009), DivL typically localizes only to the nascent swarmer pole (Sciochetti et al., 2005). These observations suggest that CckA is normally most active at the swarmer pole and that DivL may help localize CckA to that pole. We therefore examined the subcellular localization of CckA-GFP in synchronized *divL*^{ts} cells as they progressed through the cell cycle. For cells incubated at the restrictive temperature of 37°C, CckA-GFP localized only to the stalked pole of the predivisional cell; cells did not accumulate a swarmer pole focus of CckA nor did they divide (Figure 2D). By contrast, *divL*^{ts} cells grown at 30°C localized CckA to both poles of predivisional cells and divided, as seen with wild-type cells (Figure 2D). A similar result was obtained on mixed populations, with *divL*^{ts} cells shifted to 37°C for 4 hr rarely showing swarmer pole foci of CckA-GFP (Figure S3), consistent with similar findings in a recent study (Iniesta et al., 2010). To ensure that the lack of swarmer pole localization was not due simply to a loss of CtrA activity or cell filamentation, we examined CckA-GFP localization in a *ctrA*^{ts} strain at 37°C. Unlike *divL*^{ts}, these cells accumulated CckA-GFP foci at both poles and sometimes at intervals throughout the cell (Figure S3). Together, these data demonstrate that DivL is required for CckA to localize to the swarmer pole and that a failure to localize likely prevents the activation of CtrA and, consequently, cell division.

These observations do not, however, reveal why localization is necessary for CckA activity. There are two general possibilities: (1) DivL recruits CckA to the pole where another factor activates it; or (2) localization of DivL and CckA to the swarmer pole sequesters them away from a negative regulator. We favored the latter, given our genetic studies indicating that DivK is an upstream, negative regulator of DivL. We therefore turned our focus to DivK.

DivK Inhibits the Activation of CckA as a Kinase

To confirm that DivK, like DivL, affects CckA kinase activity in vivo, we measured CckA phosphorylation in the *divK*^{cs} mutant strain (Figure 3A). CckA phosphorylation was previously measured

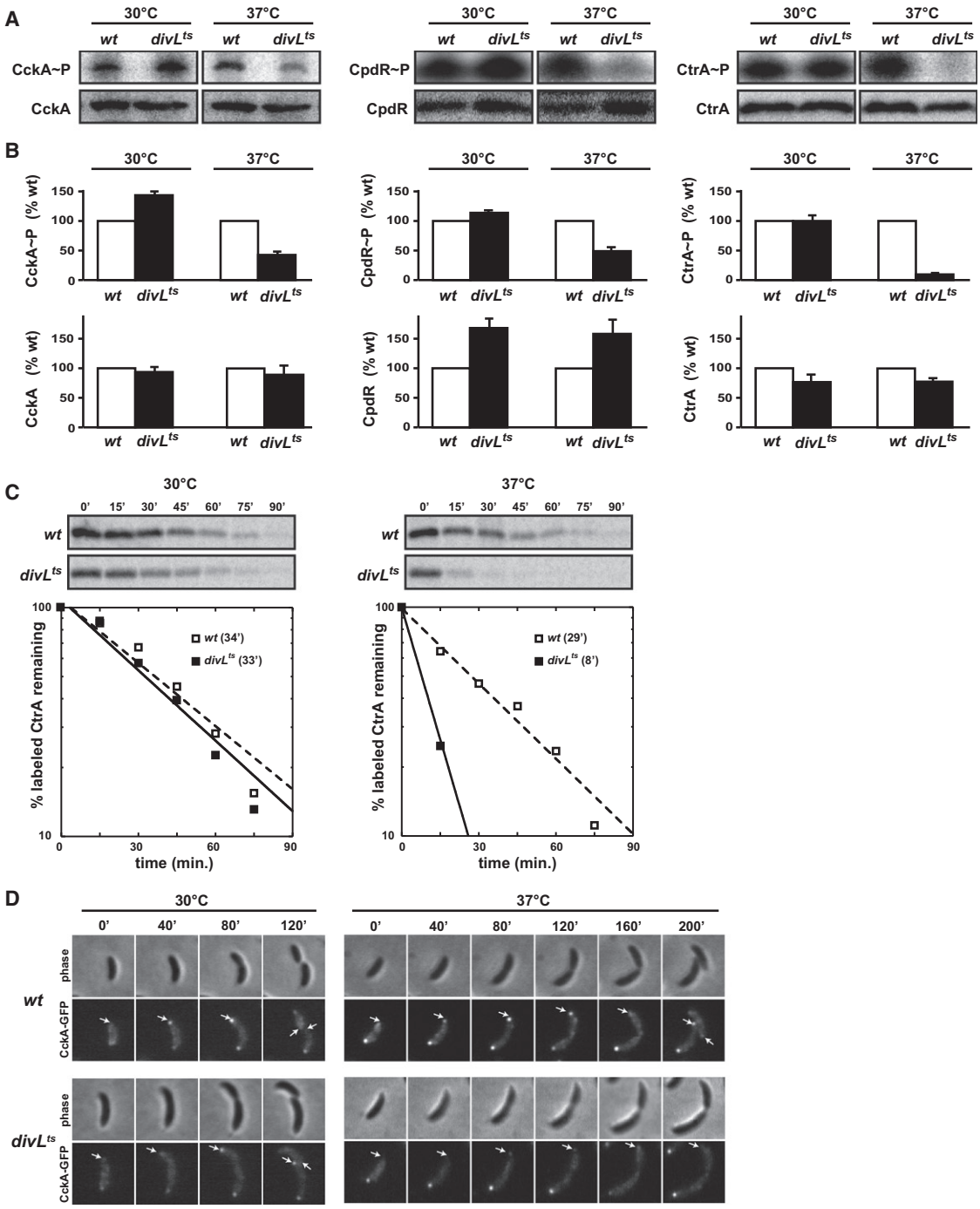

Figure 2. DivL Is Required to Activate CckA as a Kinase In Vivo and to Localize CckA-GFP to the Swarmer Pole of Predivisional Cells

(A) In vivo phosphorylation assays of wild-type and *divL*^{ts} strains grown at the permissive temperature (30°C) or shifted to the restrictive temperature (37°C) for 15 min. Equal optical densities of cells were pulsed with radiolabeled ATP, lysed, and CckA, CtrA, or CpdR immunoprecipitated. Samples from each immunoprecipitation were examined by SDS-PAGE and phosphor imaging (gel images labeled CckA∼P, CpdR∼P, and CtrA∼P). Western blot analysis was performed on samples that were not pulsed (gel images labeled CckA, CpdR, and CtrA).

(B) Quantification of bands from (A). Error bars represent standard deviations from two independent replicates.

(C) Pulse-chase analysis of CtrA. Wild-type and *divL*^{ts} strains were pulsed with radiolabeled L-methionine for 5 min, and then chased with excess unlabeled L-methionine and casamino acids. Cultures were examined at the permissive temperature (30°C) or immediately after a shift to the restrictive temperature (37°C). Each experiment was repeated twice with representative gels and quantifications shown. The half-lives calculated for CtrA are included within each graph.

(D) CckA-GFP localization through the cell cycle in wild type and *divL*^{ts} at the permissive (30°C) and restrictive (37°C) temperatures. Swarmer cells were isolated, placed on agarose pads and followed by time-lapse fluorescence microscopy with minutes post-synchrony indicated above the images. White arrows indicate the new pole that, in predivisional cells, becomes the swarmer pole.

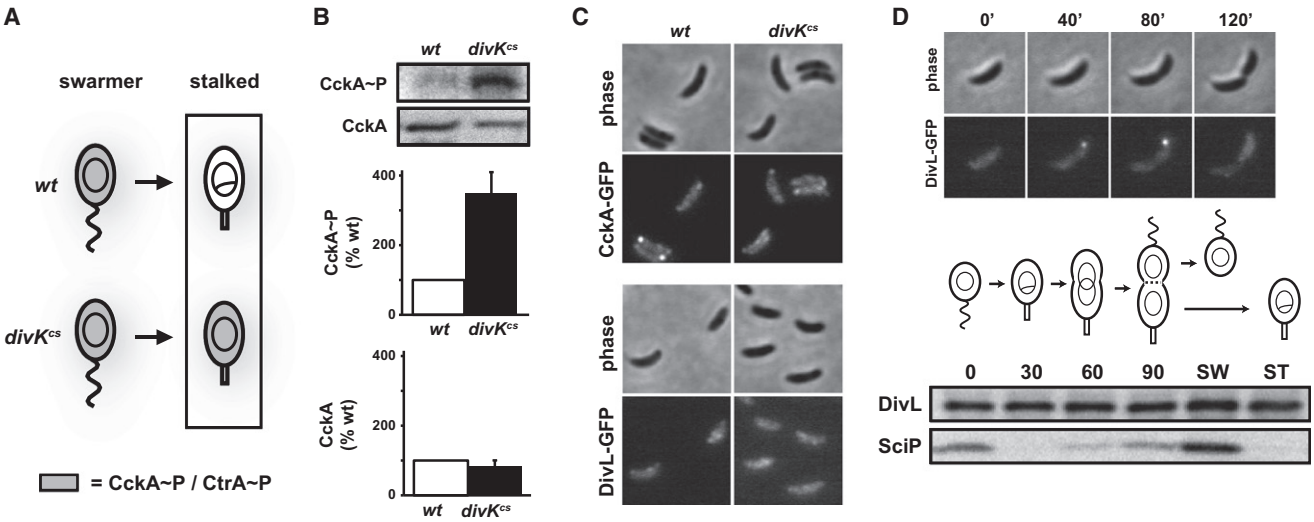

Figure 3. DivK Inhibits CckA Kinase Activity at the G1-S Transition

(A) Diagram of CckA and CtrA activity during the swarmer-to-stalked cell transition in wild-type and *divK*^{cs}.

(B) In vivo phosphorylation measurements of CckA in synchronized stalked cells harboring either *divK* or *divK*^{cs} at the native chromosomal locus. Assays were performed as in Figure 2A, except that stalked cells were isolated by allowing synchronized swarmer cells to differentiate for 50 min at 20°C, the restrictive temperature for *divK*^{cs}. Error bars represent standard deviations from three independent replicates.

(C) Fluorescence microscopy of wild-type and *divK*^{cs} stalked cells expressing CckA-EGFP. Strains were grown and stalked cells isolated exactly as in (B).

(D) Cell cycle localization pattern of DivL-EGFP. Swarmer cells expressing *divL-gfp* were isolated, placed on agarose pads containing M2G^{+} and followed by time-lapse fluorescence microscopy (top). Cell cycle western blot analysis of DivL and SciP (bottom). Swarmer cells were isolated, released into rich media with samples taken for western blot analysis every 30 min. Samples were also taken from swarmer (SW) and stalked (ST) cells collected immediately after cell division.

in a mixed population of *divK*^{cs} cells, revealing a modest increase in CckA∼P levels per cell, but not per protein (Biondi et al., 2006). However, the essential function DivK occurs during a narrow window of time immediately before DNA replication (Hung and Shapiro, 2002). We therefore measured CckA∼P levels in synchronized stalked cells from the wild-type and *divK*^{cs} strains. CckA protein was present at similar levels in stalked cells from the two strains, but CckA∼P levels were significantly higher in *divK*^{cs} cells than in wild-type cells (Figure 3B), on both a per protein and per cell level. These data demonstrate that DivK is normally required to downregulate CckA kinase activity in vivo and that the failure to do so in a *divK*^{cs} strain results in a failure to downregulate CtrA and thus to initiate DNA replication (Figure 1B).

We also examined CckA-GFP localization in wild-type and *divK*^{cs} cells grown in the same conditions used to measure CckA phosphorylation. For both strains, CckA-GFP was either dispersed throughout the cell or formed a focus at the stalked pole, with localization to the pole opposite the stalk seen in <2% of cells (Figure 3C). Hence, in a *divK* mutant, CckA is either active at the stalked pole or the delocalized pool of CckA is active. To help distinguish between these possibilities, we examined DivL-GFP localization in the *divK*^{cs} mutant and found that it was consistently delocalized (Figure 3C). Recall that the G1-arrest phenotype of a *divK* depletion strain, and thus CckA activity in these cells, depends on DivL activity (Figure 1B). Taken together, our results indicate that localization of CckA to the swarmer pole is not an obligatory step in its activation. Instead, it appears that the inactivation of DivK is sufficient to activate CckA, regardless of its cellular location, provided that DivL is functional.

If DivK does downregulate CckA kinase activity via DivL to drive the initiation of DNA replication, then stalked cells should harbor DivL. Although DivL is present in stalked cells that result from the differentiation of swarmer cells (Sciochetti et al., 2005), DivL-GFP localizes mainly to the swarmer pole of predivisional cells leaving open the question of whether stalked cells resulting from cell division harbor DivL (Figure 3D). To address this question, we synchronized wild-type cells, allowed them to proceed once through the cell cycle, and then harvested daughter swarmer and stalked cells immediately after cell division. Western blotting revealed that DivL is present at nearly equal levels in the two daughter cells (Figure 3D). As a control, we confirmed that SciP, a swarmer cell-specific factor, was present only in daughter swarmer cells (Gora et al., 2010).

Phosphorylated DivK Directly Binds DivL

DivK was previously found to bind DivL in a yeast two-hybrid system (Ohta and Newton, 2003). To test whether DivK binds directly to DivL in vitro we used Förster resonance energy transfer (FRET). We purified C-terminal fusions of DivK and DivL to CFP and YFP, respectively. For DivL, we used a construct lacking only the putative N-terminal transmembrane domain; for FRET studies we refer to this construct simply as DivL. The FRET ratio measured after mixing DivK-CFP and DivL-YFP was not significantly different from that of free CFP and YFP (Figure S4A), indicating that no significant FRET occurs between DivL and unphosphorylated DivK in our conditions. However,

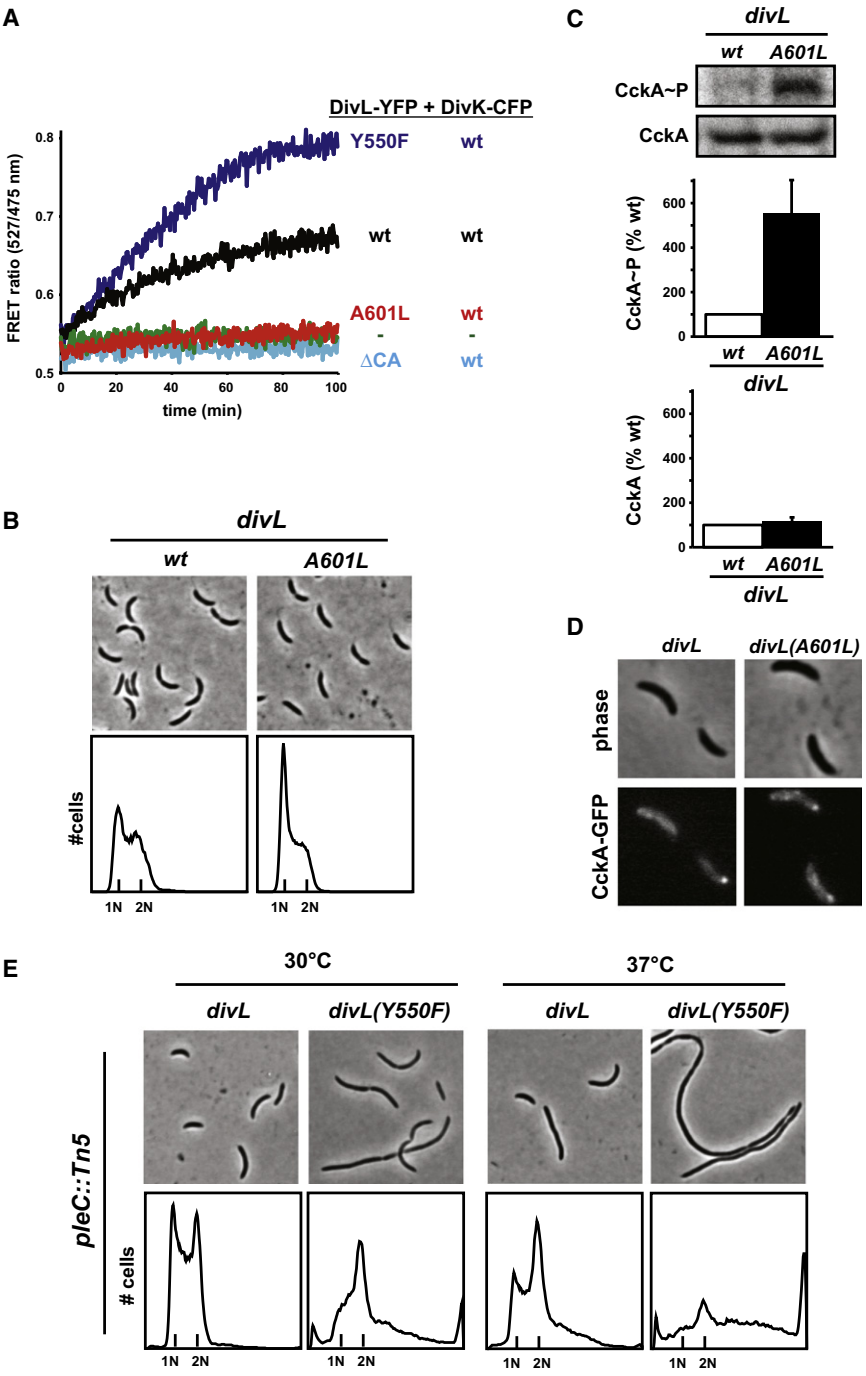

Figure 4. Mutations in DivL that Affect DivK Binding In Vitro Affect CckA Activity In Vivo

(A) In vitro FRET analysis of the DivL-DivK interaction. DivK-CFP and DivL-YFP (each at 2.5 μM) were mixed together with 5 mM $MgCl_2$ and 500 μM ATP. At t = 0, 100 nM DivJ was added and the ratio of the 527 nm to 475 nm emissions (FRET ratio) was measured while exciting the samples at 433 nm. A mixture of free CFP and YFP (denoted with minus signs) each at 2.5 μM was included as a control. DivK-CFP was tested for binding to DivL-YFP and the mutants indicated.

(B) Phase contrast microscopy and flow cytometry analysis of cells expressing either *divL* or *divL* (*A601L*) under the control of a xylose-inducible promoter on a high-copy plasmid. Cells were grown in the presence of glucose; leaky expression from the high-copy plasmid leads to moderate, constitutive levels of expression.

(C) In vivo phosphorylation measurements of CckA in synchronized stalked cells expressing either *divL* or *divL*(*A601L*) as in (B). Assays were performed as in Figure 2A, except that stalked cells were obtained by allowing synchronized swarmer cells to differentiate for 35 min. Error bars represent standard deviations from three independent replicates.

(D) Fluorescence microscopy of CckA-EGFP in stalked cells expressing *divL* or *divL*(*A601L*). Strains were grown and stalked cells harvested exactly as in (C).

(E) Phase contrast microscopy and flow cytometry analysis of strains harboring the *pleC::Tn5* disruption with the chromosomal copy of *divL* deleted and expressing either *divL* or *divL*(*Y550F*) from the native *divL* promoter on a low-copy plasmid grown at 30°C or 37°C for 4 hr.

as a response regulator, DivK activity likely depends on phosphorylation. To test the effect of phosphorylation on binding, we added substoichiometric amounts of untagged DivJ, the cognate kinase for DivK (Ohta et al., 1992), and ATP to a reaction containing DivK-CFP and DivL-YFP. We then observed a rapid and significant increase in FRET efficiency (Figure 4A). A construct containing only the DHp and CA domains of DivL fused to YFP also strongly interacted with DivK-CFP on addition of DivJ and ATP, with a FRET efficiency ~85% that seen with the longer version of DivL (Figure S4B). These experiments demonstrate that the phosphorylation of DivK strongly increases its affinity for DivL.

Mutations in DivL that Affect DivK Binding In Vitro Affect CckA Kinase Activity In Vivo

To bolster the notion that DivK~P binding to DivL is relevant in vivo, we tested whether mutations in *divL* and *divK* that perturb CtrA activity in vivo also affect their interaction in vitro. A transposon insertion in *divL* causing a truncation after amino acid 657 was previously identified in a screen for suppressors of *pleC* (Reisinger et al., 2007). As the loss of *pleC* decreases CtrA activity, suppression requires a compensatory mutation that increases CtrA activity. We hypothesized that the *divL657* mutation may achieve such an increase by disrupting the ability of DivK~P to inhibit DivL and thereby downregulate CckA as a kinase. To test this hypothesis, we purified a construct, DivLΔCA-YFP, that lacks the putative transmembrane domain and the last 112 amino acids of DivL. This construct did not show a significant FRET signal with

DivK, either with or without DivJ (Figure 4A), suggesting that it indeed no longer had the ability to strongly bind DivK.

Next, we wanted to examine a point mutation in DivL that disrupts binding to DivK, as point mutants are less likely to affect folding or tertiary structure. We created a series of DivL point mutants at sites predicted to interface with DivK based on comparison to a cocrystal structure of a histidine kinase-response regulator complex from *T. maritima* (Casino et al., 2009). One mutation, A601L, completely eliminated binding of DivL-YFP to phosphorylated DivK-CFP in vitro (Figure 4A). To test whether this mutation also disrupted binding in vivo, we expressed *divL*(*A601L*) from a xylose-inducible promoter on a plasmid in wild-type cells. Growth in the presence of glucose led to leaky, constitutive expression of *divL*(*A601L*). Using flow cytometry we found that most cells expressing *divL*(*A601L*) contained a single chromosome (Figure 4B), similar to the G1 arrest seen with the $divK^{cs}$ strain. We then synchronized swarmer cells expressing either *divL*(*A601L*) or *divL*, released them into media at 30°C, and allowed them to develop into stalked cells for 35 min. We measured CckA phosphorylation in each population of cells and found that CckA∼P levels were greater than five times higher in the cells expressing *divL*(*A601L*) (Figure 4C). CckA-GFP was also not localized to the swarmer pole in these cells (Figure 4D), again indicating that CckA activation does not require swarmer pole localization if DivK cannot bind and inhibit it via DivL. Collectively, these findings suggest that DivK does not bind DivL(A601L) in vitro or in vivo, thereby preventing the normal downregulation of CckA and CtrA, and so yielding a G1 arrest (Figure 4B). We infer that DivL(A601L) is not simply misfolded as it can still activate CckA; this mutant appears specifically disrupted for binding DivK∼P. Importantly, these results also indicate that DivL is the primary target of DivK in regulating CckA and CtrA, as the *divL*(*A601L*) strain retains wild-type DivK but cannot properly downregulate CckA or CtrA.

We also tested the effect of mutating tyrosine-550 in DivL to phenylalanine. DivL shares extensive homology to histidine kinases but contains a tyrosine in place of the usual phosphorylatable histidine (Wu et al., 1999). DivL(Y550F) does not affect CckA localization (Iniesta et al., 2010), but could affect DivK binding and hence CckA activity. We thus purified DivL(Y550F)-YFP and tested binding to DivK-CFP by measuring FRET. Compared to the wild-type construct, DivL(Y550F) produced a higher FRET signal when mixed with DivK-CFP and substochiometric amounts of DivJ and ATP (Figure 4A). If DivL(Y550F) binds DivK more tightly than wild-type DivL in vivo, introducing this mutation should negatively affect the activity of CckA and CtrA. To test this possibility, we constructed strains in which either *divL* or *divL*(*Y550F*) is carried on a low-copy plasmid as the only copy of *divL*. At 30°C both strains had relatively normal morphology and chromosomal content (Figure S5). However, at 37°C, cells expressing *divL*(*Y550F*) became filamentous and showed a modest accumulation of chromosomes per cell, reflecting a loss of CtrA activity (Figure S5). These phenotypes were significantly exacerbated by introducing a *pleC::Tn5* mutation that, as noted above, sensitizes cells to other mutations that downregulate CtrA (Figure 4E). We conclude that the Y550F mutation renders DivL better at binding DivK∼P in vitro and, consistently, disrupts CtrA activation in vivo.

Mutations in DivK that Affect DivL Binding In Vitro Affect CckA Activity In Vivo

Next, we tested the ability of DivL to bind mutants of DivK. First, we tested DivK(D90G), the mutant encoded by $divK^{cs}$ that prevents downregulation of CckA and CtrA in vivo. DivK(D90G) is phosphorylated in vivo to a similar extent as wild-type DivK suggesting its defect may be an inability to bind and inhibit DivL (Hung and Shapiro, 2002). Indeed, purified DivK(D90G)-CFP produced a significantly weaker FRET ratio when incubated with YFP-DivL along with DivJ and ATP (Figure 5A).

We also examined a mutation in DivK that increases binding. In a screen for point mutants of DivK that affect its interaction with DivL, we found that the substitution Q55A significantly increased binding in our FRET assay (Figure 5A). We predicted that this mutant would hyperactivate DivK in vivo and, consequently, downregulate the CtrA regulatory pathway. To test this possibility, we engineered strains expressing either wild-type *divK* or *divK*(*Q55A*) under the control of a xylose-inducible promoter on a low-copy plasmid. In the presence of glucose, neither strain exhibited major defects in cellular morphology or chromosomal content. However, when grown in xylose for 6 hr, cells expressing *divK*(*Q55A*) became extremely filamentous and accumulated multiple chromosomes, similar to *divL*, *cckA*, and *ctrA* mutants (Figure 5B). The phenotypes for *divK*(*Q55A*) were more severe than for cells overexpressing wild-type *divK*. Using in vivo phosphorylation assays, we verified that overproducing DivK(Q55A) for 2 hr led to a significant decrease in CckA phosphorylation levels, similar to the decrease seen in $divL^{ts}$ cells (Figure 5C). These data lend further support to a model in which phosphorylated DivK antagonizes CckA by binding directly to DivL. Mutations that increased or decreased DivK-DivL binding in vitro led to a corresponding decrease or increase, respectively, of CckA kinase activity in vivo.

Localization to the Swarmer Pole Activates CckA by Localizing It with a DivK Phosphatase

In sum, our findings support a model in which (1) DivK inhibits CckA by binding to DivL; and (2) cell cycle transitions are ultimately driven by changes in the phosphorylation state of DivK. Such a model is consistent with the reciprocal changes in DivK∼P and CckA∼P early in the cell cycle (Jacobs et al., 2003; Lam et al., 2003). In G1 swarmer cells, DivK is predominantly unphosphorylated whereas CckA retains activity and is phosphorylated. In stalked cells, DivK phosphorylation increases whereas CckA phosphorylation drops to its lowest level during the cell cycle. However, in predivisional cells, DivK remains phosphorylated and yet CckA is highly active, in apparent conflict with the model. Conspicuously though, the DivK phosphatase PleC (Ohta et al., 1992) is located at the swarmer pole of predivisional cells (Wheeler and Shapiro, 1999), along with DivL and CckA. Thus, we hypothesized that PleC phosphatase activity may protect DivL and CckA from DivK∼P at the nascent swarmer pole in predivisional cells, thereby allowing the accumulation of high levels of phosphorylated CtrA in this cell type.

If this hypothesis is correct, the phosphorylation levels of CckA and CtrA should decrease in a *pleC* mutant. In a Δ*pleC* mutant we found that CckA∼P levels in vivo dropped to ∼82% of wild-type levels (Figure 6A), and in a *pleC::Tn5* mutant CtrA∼P drops

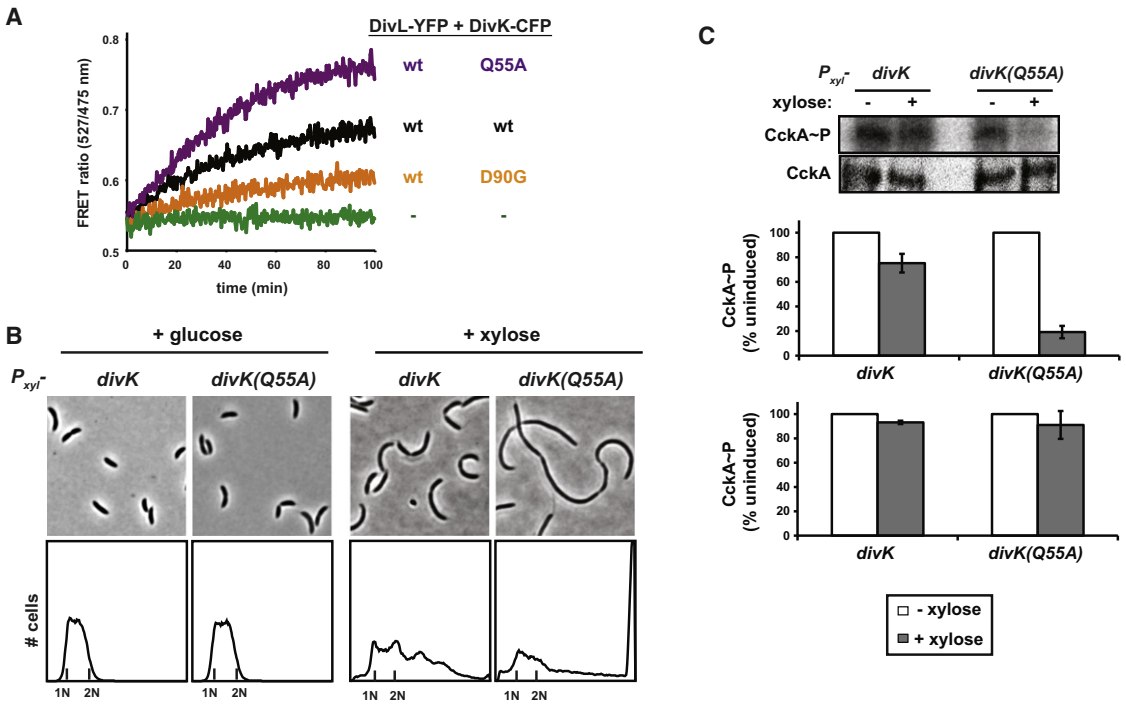

Figure 5. Mutations in DivK that Affect DivL Binding In Vitro Affect CckA Activity In Vivo

(A) In vitro FRET analysis of DivL-YFP binding to wild-type DivK-CFP and the mutants indicated. Assays were performed as in Figure 4A. Wild-type and free CFP/YFP traces are the same experiments as in Figure 4A and are duplicated to facilitate comparison.

(B) Phase contrast microscopy and flow cytometry analysis of strains expressing either *divK* or *divK*(*Q55A*) from a low-copy plasmid under the control of a xylose-inducible promoter. Cells were grown in glucose or in the presence of xylose for 6 hr.

(C) In vivo phosphorylation measurements of CckA in a mixed population of cells expressing *divK* or *divK*(*Q55A*). Assays were performed as in Figure 2A, except strains were induced with xylose for 2 hr and compared to identically treated, but uninduced cultures. Error bars represent standard deviations from three independent replicates.

to ~10% of wild-type levels (Biondi et al., 2006). Consistently, *pleC* mutants are highly sensitive to other mutations that decrease CtrA activity, often with synthetic, nearly lethal phenotypes (Chen et al., 2009). Conversely, *pleC* null mutants are suppressed by mutations in genes that promote CtrA activity (Sommer and Newton, 1991). Nevertheless, for cells harboring only a *pleC* null mutation, the consequent decrease in CckA~P and CtrA~P does not lead to a severe cell cycle phenotype or major changes in CtrA-dependent gene expression (Figure 6B), as with *divL* and *cckA* mutants. Either another DivK phosphatase exists or cells compensate for the loss of *pleC*; the latter possibility is suggested by previous observations that *pleC* null strains exhibit alternative patterns of localization for many key regulatory proteins (Reisinger et al., 2007; Wheeler and Shapiro, 1999).

To better address the consequence of losing PleC phosphatase activity, we measured CckA~P levels in a $pleC^{ts}$ strain 15 min after shifting to the restrictive temperature. In this case, we found that CckA~P levels dropped to ~18% of wild-type, similar to the decrease measured in $divL^{ts}$ cells, and with virtually no change in CckA protein level (Figure 6C). Moreover, DNA microarray analysis revealed that in $pleC^{ts}$ cells grown at 37°C for 1 hr, CtrA regulated genes were downregulated much more significantly than in *ΔpleC*, and comparable to that seen in $divL^{ts}$ (Figure 6B). These data demonstrate that PleC is, in fact, critical to maintaining the activity of CckA in predivisional cells.

Based on these findings, we conclude that in swarmer cells, PleC maintains a low level of DivK~P allowing DivL to associate with and promote CckA activity. In stalked cells, DivJ replaces PleC at the old pole and drives a surge in DivK phosphorylation, resulting in the downregulation of CckA. In predivisional cells, DivJ continues to phosphorylate DivK, but the localization of CckA and DivL to the swarmer pole along with PleC enables CckA to function again as a kinase and drive CtrA phosphorylation.

This model further suggests that the mutant DivK(Q55A) may downregulate CckA as a kinase by binding more tightly to DivL at the swarmer pole and thus overcoming the effects of PleC. To test this prediction, we examined the localization of a DivK (Q55A)-CFP fusion expressed from a low-copy plasmid in an otherwise wild-type background. Most cells expressing DivK (Q55A)-CFP showed clear, significant polar foci as well as irregular foci within filamentous cells at pinched sites that likely represent nascent poles (Figure 6D). In cells producing DivK(Q55A), we also found that DivL-GFP and CckA-GFP formed foci at the cell poles and at highly pinched, nascent poles within the cell, similar to the pattern seen with DivK(Q55A)-CFP (Figure 6E). Collectively, our data indicate that DivK(Q55A), by virtue of its tighter binding to DivL, can effectively overcome the PleC phosphatase, infiltrate the swarmer pole, and downregulate CckA, without disrupting the polar localization of DivL or CckA. We

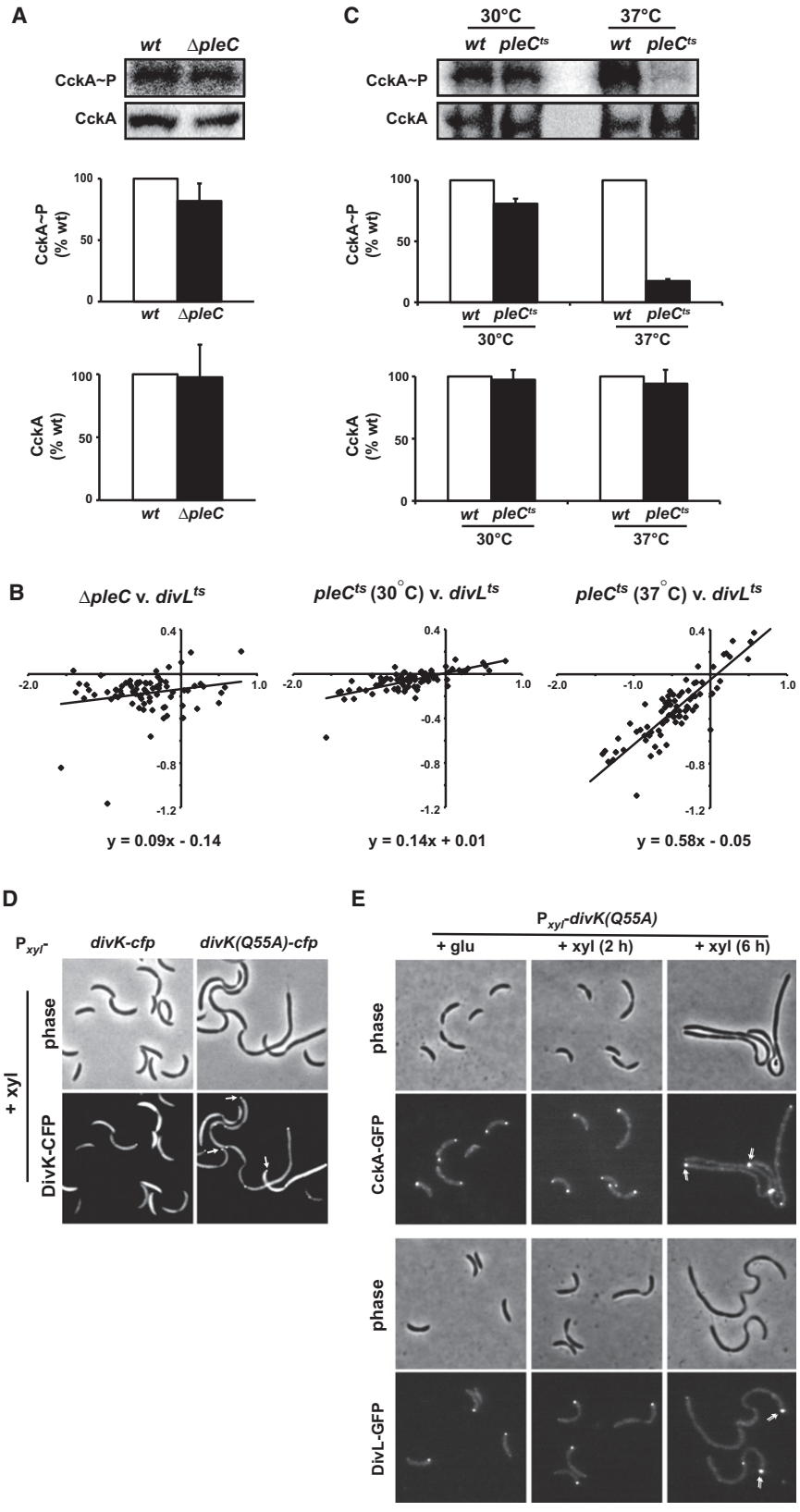

Figure 6. CckA and DivL Both Localize at the Swarmer Pole with PleC to Avoid Downregulation by DivK

(A) In vivo phosphorylation measurements of CckA in wild-type and *ΔpleC*.

(B) CtrA-dependent gene expression in *pleC* mutants. Oligonucleotide microarrays were used to measure global gene expression patterns in *ΔpleC* and *pleC*^{ts} relative to wild-type at 30°C and in *pleC*^{ts} relative to wild-type at 37°C for 1 hr. The log ratio for each CtrA-regulated gene was compared to the log ratio of expression in *divL*^{ts} relative to wild-type, each grown at 37°C for 4 hr (see Figure S1). The best fit line and equation are shown on each plot.

(C) In vivo phosphorylation measurements of CckA in wild-type and *pleC*^{ts} at the permissive temperature (30°C) and after shift to the restrictive temperature (37°C) for 15 min. Error bars represent standard deviations from three independent replicates.

(D) *divK* and *divK*(*Q55A*) were each fused to *cfp* and expressed from a low-copy plasmid under the control of a xylose-inducible promoter. Subcellular localization was examined by epifluorescence microscopy after growth in xylose for 6 hr. For cells expressing *divK*(*Q55A*)-*cfp*, white arrows indicate swarmer pole foci, where swarmer poles were identified as those opposite stalked poles.

(E) CckA-EGFP and DivL-EGFP localization in cells harboring P$_{xyl}$-*divK*(*Q55A*) on a low-copy plasmid and grown in the presence of glucose or in xylose for 2 or 6 hr. At the 6-hr time point, white arrows indicate foci of CckA-GFP or DivL-GFP at the putative swarmer pole, identified as the pole opposite the stalked pole.

speculate that this ability to bypass PleC may be due in part to competition between DivL and PleC for DivK∼P binding; enhanced binding to DivL may thus protect DivK∼P from PleC. Taken together with our analyses of *pleC* mutants, these data strongly support a model in which the joint localization of PleC, DivL, and CckA at the swarmer pole normally enables CckA to avoid downregulation by DivK∼P.

DISCUSSION

Throughout biology, developmental processes rely heavily on the subcellular localization of key regulatory proteins. For many proteins, localization enables the regulation of a morphogenetic or structural process that is itself localized, such as the cytokinetic ring, DNA replication, and flagellar assembly. For other proteins, localization may promote asymmetric inheritance after cell division, as with Ash1p in *S. cerevisiae* (Sil and Herskowitz, 1996) and with DivJ and PleC in *Caulobacter* (Wheeler and Shapiro, 1999). Localization can also directly stimulate the activity of some regulatory proteins. For instance, the polar localization of chemotaxis proteins in *Escherichia coli* (Maddock and Shapiro, 1993) facilitates the assembly of a supramolecular cluster that enables signal adaptation and exquisite sensitivity, properties critical to chemotaxis (Hansen et al., 2010). Finally, localization can act to sequester regulatory proteins from their targets, as with the nucleolar localization of the phosphatase Cdc14 in *S. cerevisiae* (Visintin et al., 1999).

Why CckA localizes to the poles of *Caulobacter* predivisional cells had previously been unclear. CckA does not directly regulate a morphogenetic process nor is it asymmetrically inherited. A major clue came from our observation that in certain mutants, the activity of CckA is no longer dependent on localization to the swarmer cell pole (Figures 3B, 3C, 4C, and 4D). Conversely, in cells producing hyperactive DivK, CckA remains localized to the swarmer pole but is not active (Figures 5C and 6E). These results highlight another reason for subcellular localization: to create a microenvironment within the cell where CckA can avoid downregulation by its inhibitor, DivK∼P. In predivisional cells, bulk measurements indicate that DivK∼P levels are high (Jacobs et al., 2001). Although this DivK∼P can diffuse throughout the cell, our data suggest that the enforced proximity of CckA and PleC, a DivK phosphatase, at the pole promotes CckA kinase activity. Consistently, the immediate consequence of losing PleC activity is a downregulation of CckA and CtrA (Figure 6C). It is then the transition from a delocalized to localized state that triggers CckA kinase activity and, in turn, drives the late stages of cell cycle progression.

DivK Dictates Cell Cycle Progression and Cellular Asymmetry by Regulating CckA

Our results underscore DivK as a key regulator of the *Caulobacter* cell cycle and the establishment of cellular asymmetry. Although DivK was first identified almost 20 years ago (Hecht et al., 1995; Sommer and Newton, 1991), it has been unknown precisely how it regulates development and the cellular asymmetry of *Caulobacter*. DivK is a single-domain response regulator and hence was presumed not to directly affect transcription. Indeed, our results indicate that the primary cell cycle role of DivK is the regulation of CckA through a direct, phosphorylation-dependent interaction with the essential, noncanonical kinase DivL.

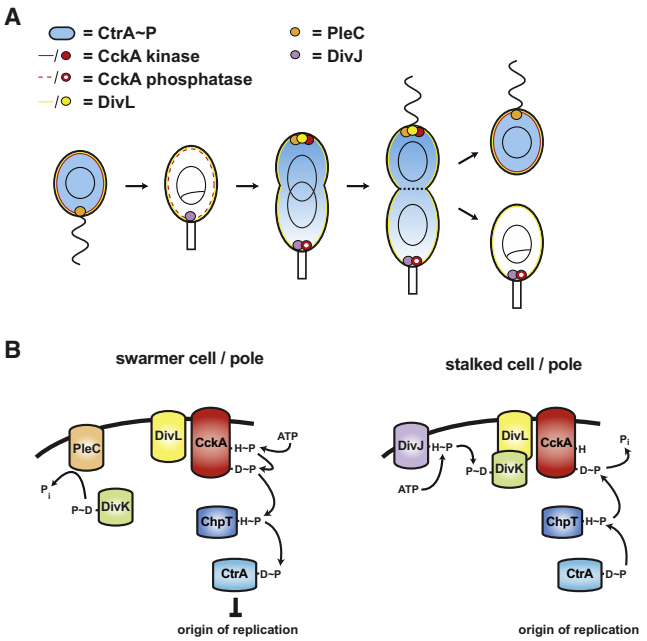

Figure 7. Model of Regulatory Circuitry Controlling CtrA, Cell Cycle Transitions, and Cell Fate Asymmetry in *Caulobacter crescentus*

(A) Localization of CtrA regulatory factors and CtrA activity throughout the cell cycle. PleC and DivJ are localized to the swarmer and stalked poles, respectively. After DNA replication initiates in stalked cells, DivL, CckA, and PleC are recruited to the nascent swarmer pole.

(B) Model of protein-protein interactions regulating CckA in swarmer and stalked cells and at the poles of predivisional cells. In swarmer cells, DivK is dephosphorylated by PleC allowing DivL to promote CckA kinase activity and, consequently, phosphorylation of CtrA. In stalked cells, DivJ phosphorylates DivK that then binds to DivL, inhibiting CckA kinase activity and ultimately driving the dephosphorylation of CtrA. In predivisional cells, CckA localizes with DivL and PleC at the swarmer pole, enabling CckA to escape downregulation by DivK∼P. CckA is also frequently found at the stalked pole of stalked and predivisional cells. However, DivL is either absent from the stalked pole (not shown) or present but inhibited by phosphorylated DivK (shown); in either case, CckA remains in a phosphatase state.

Synthesis of our results with those published previously yields a molecular-level model for the regulation of *Caulobacter* cell cycle progression and cell fate asymmetry (Figure 7). In swarmer cells, polarly localized PleC actively dephosphorylates DivK to permit a productive interaction between DivL and CckA and, consequently, to maintain the phosphorylation of CtrA and a G1 state. During the swarmer-to-stalked cell transition, PleC is replaced by DivJ at the stalked pole, resulting in the rise of DivK phosphorylation and, consequently, the downregulation of CckA kinase activity via DivL. The inhibition of CckA and consequent loss of CtrA binding to the origin permits DNA replication to initiate. As the stalked cell develops into a predivisional cell, CckA, DivL, and PleC are recruited to the nascent swarmer pole. PleC phosphatase activity shields CckA from DivK∼P and thus drives the phosphorylation of CtrA, enabling the late stages of cell cycle progression and morphogenesis. CckA is also found at the stalked pole of predivisional cells.

DivL is usually absent from this pole, but even when present, it would be inhibited by DivK∼P. Like most histidine kinases, CckA is bifunctional such that when not stimulated as a kinase, it functions as a phosphatase (Chen et al., 2009). Predivisional cells thus have CckA in the kinase and phosphatase states at opposing poles, resulting in a gradient of phosphorylated CtrA across the cell (Chen et al., 2011). After cell division, the daughter swarmer cell retains PleC and hence dephosphorylates DivK to maintain CckA and CtrA activity. The daughter stalked cell inherits DivJ, leading to DivK phosphorylation, which prevents DivL from stimulating CckA kinase activity, thereby facilitating the onset of DNA replication in this cell type.

Protein-Protein Interactions Underlying the Control of CckA Activity

At the heart of our model is a dynamic protein-protein interaction system comprising DivK, DivL, and CckA. Our results indicate that a complex of DivL and CckA is active with respect to CckA autophosphorylation and phosphotransfer, and that the binding of DivK∼P to DivL inhibits CckA. Toggling the phosphorylation state of DivK thus inversely toggles the phosphorylation state of CckA and, consequently, CtrA. Whether DivL and CckA directly interact is not yet clear, although both proteins localize to the swarmer pole and were suggested to coimmunoprecipitate (Iniesta et al., 2010).

Our results do, however, demonstrate that the interaction between DivK∼P and DivL is direct and several lines of evidence indicate that binding is similar to canonical two-component signaling interactions, but without phosphotransfer occurring. First, binding requires only the DHp and CA domains of DivL, the same domains used in canonical HK-RR interactions. Also, the substitutions Y550F and A601L in DivL that affect binding are at sites likely to mediate canonical two-component protein interactions. In the cocrystal structure of HK853 and RR468 from *Thermotoga maritima* (Casino et al., 2009), the residues in HK853 corresponding to Y550 and A601 directly contact RR468. Similarly, for DivK, the substitution D90G decreases binding to DivL (Figure 5A) and the corresponding residue in RR468 is in contact with HK853. Notably, aspartate-90 resides at the N terminus of α-helix 4 in DivK (Guillet et al., 2002). For most response regulators, the α4-β5-α5 face changes conformation in a phosphorylation-dependent manner to effect an output (Gao et al., 2007), often by modulating protein-protein interactions. We propose that the phosphorylation of DivK induces a conformational change that enables tighter binding to DivL.

Although binding occurs, DivL and DivK likely do not participate in phosphotransfer reactions. DivL does not harbor significant autokinase or DivK∼P phosphatase activity in vitro (CGT and MTL, unpublished) and a previous report found that the ATPase domain of DivL is not required to support viability (Reisinger et al., 2007). Nevertheless, we cannot rule out that tyrosine phosphorylation of DivL plays a regulatory role.

Finally, our data suggest that DivL is the primary output for phosphorylated DivK during cell cycle progression. DivK was suggested to independently control CpdR (Iniesta and Shapiro, 2008). However, the fact that *divL*(*A601L*) led to an increase in CckA activity and a G1 arrest indicates that DivK acts primarily through DivL to downregulate CpdR and CtrA.

Noncanonical Topologies and Activities for Two-Component Signaling Proteins

The connectivity of the two-component signaling proteins that regulate the *Caulobacter* cell cycle includes both canonical and noncanonical features. The phosphorylation and dephosphorylation of DivK by DivJ and PleC, respectively, and the multistep phosphorelays initiated by CckA exemplify the two most common topologies for two-component proteins. These pathways are, however, connected in a highly unconventional manner, with the response regulator DivK∼P binding the noncanonical kinase DivL to, in turn, modulate the activity of another histidine kinase, CckA. There are very few examples of other two-component proteins wired together in such unorthodox ways. In *P. aeruginosa*, the histidine kinase RetS directly modulates the activity of another histidine kinase, GacS (Goodman et al., 2009), although in that case, the two kinases have nearly identical DHp domains and probably heterodimerize.

Most histidine kinases mediate adaptive responses to environmental signals by binding small molecule inducers or ligands. However, CckA may not respond to anything other than DivK and DivL. Although DivL and CckA are transmembrane proteins, neither has a substantial periplasmic domain. The transmembrane domains thus may serve mainly to facilitate polar localization. Each kinase does have several intracellular PAS domains, and although these domains sometimes modulate response to environmental or metabolic signals, they are also often involved in protein-protein interactions (Lee et al., 2008). Although CckA and DivL may not directly integrate environmental signals, PleC and DivJ may.

The regulation of DivL and CckA by DivK also highlights the expanding role of single-domain response regulators in bacteria. Although the majority of response regulators control transcription, single-domain regulators are relatively common and modulate a wide range of physiological processes through protein-protein interaction (Jenal and Galperin, 2009).

Molecular Mechanisms for Producing and Maintaining Cellular Asymmetry

The identification of DivL as an intermediary between DivK and CckA fills a major gap in our understanding of the regulatory circuit governing the *Caulobacter* cell cycle. Central to this circuit is the response regulator DivK, which ultimately dictates cell cycle progression and replicative asymmetry via DivL. Our work further suggests that the subcellular localization of regulatory proteins is crucial to the development and cell cycle of *Caulobacter* for at least two reasons. First, as noted, the localization of factors such as DivJ and PleC likely promotes their asymmetric inheritance, helping to enforce the asymmetry of daughter cells. Second, we now find that the localization of CckA, DivL, and PleC to a single pole of the predivisional cell effectively partitions the cytoplasm but without the use of membrane-enclosed compartments or other physical barriers. Our findings reveal a remarkable mechanism through which bacterial cells can create and exploit a heterogeneous cytoplasm to activate a master kinase and to produce cell fate asymmetry.

EXPERIMENTAL PROCEDURES

Growth Conditions

C. crescentus strains were grown in PYE (rich medium), M2G (minimal medium), M2G$^+$ (M2G + 1% PYE), or M5G (low phosphate medium) supplemented when necessary with oxytetracycline (1 μg/ml), kanamycin (25 μg/ml), chloramphenicol (2 μg/ml), gentamycin (0.6 μg/ml), novobiocin (100 μg/ml), 0.2% glucose, or 0.3% xylose. Cultures were grown at 30°C unless otherwise noted and diluted when necessary to maintain exponential growth. *E. coli* strains were grown at 37°C in LB supplemented when necessary with carbenicillin (100 μg/ml), oxytetracycline (12 μg/ml), kanamycin (50 μg/ml), chloramphenicol (30 μg/ml), or gentamycin (15 μg/ml). Synchronies were performed as described previously (Jones et al., 2001).

Protein Expression, Purification, and Antibody Production

Protein expression and purification were performed as described (Skerker et al., 2005) except with modified expression conditions. After reaching mid exponential phase, cultures were induced with 0.5 mM IPTG for 16 hr at 18°C. Fluorescent fusion protein concentrations were determined using absorbances at 433 nm for CFP fusions (molar extinction coefficient 32,500 $M^{-1}cm^{-1}$) or 514 nm for YFP fusions (molar extinction coefficient 83,400 $M^{-1}cm^{-1}$). Nonfluorescent protein concentrations were determined by measuring absorbance at 280 nm and using extinction coefficients calculated with the Protparam tool (http://ca.expasy.org/tools/protparam.html). Purified His_6-DivL, expressed from pHIS-*divL* and lacking only the putative N-terminal transmembrane domain, was used to generate rabbit polyclonal antiserum (Covance). Crude antisera were used at a 1:5000 dilution.

In Vivo Phosphorylation Measurements

In vivo phosphorylation measurements were carried out as described previously (Domian et al., 1997) with the following modifications. One colony was inoculated into M5G medium and grown overnight at 30°C until the optical density at 660 nm was between 0.2 to 0.4. Cultures were normalized by optical density to the least dense culture in the batch and 1 ml of cells from each culture pulsed with 1 μM [γ^{32}P]-ATP having a specific activity of 30 Ci/mmol (Perkin-Elmer) for 5 min. Labeling was carried out at the temperatures indicated. Immunoprecipitations were performed using Protein A agarose beads (Roche). In synchrony experiments, swarmer cells were isolated from cultures at OD_{660} ~0.2 and resuspended in the original media, which was filter sterilized, to avoid replenishing phosphate in the culture. Cells were grown at the temperatures and for the times indicated to isolate synchronized stalked cells.

In Vivo CtrA Stability Measurements

CtrA pulse-chase experiments were performed as described previously (Gora et al., 2010) with the exception that Protein A agarose beads from Roche were used.

Band Quantification

Quantification of bands on SDS-PAGE gels were done using the Gel Analyzer function in ImageJ (http://rsbweb.nih.gov/ij).

FRET

FRET was performed at 30°C, reading 70 μl reactions from 96-well polystyrene plates (Corning) using a Varioskan Flash fluorescence plate reader (Thermo-Fisher Scientific). Samples were excited at 433 nm and emission measured at 525 nm and 475 nm.

DNA Microarrays

Gene expression profiles were obtained as described previously (Gora et al., 2010) using custom Agilent arrays. RNA was collected from *divL^{ts}* cells grown to mid-exponential phase in rich media at 30°C and compared to RNA from cells shifted to 37°C for 2 or 4 hr.

Flow Cytometry

DNA content per cell was determined as described previously (Chen et al., 2009) except cells were not treated with rifampicin.

Microscopy

Both live and fixed cells were mounted onto M2G$^+$ 1.5% agarose pads (supplemented with xylose when applicable) and imaged using a Axiovert 200 microscope (Zeiss) with a 63×/1.4 NA objective (Zeiss) with 1.6× Optivar and an Orca II camera (Hamatsu) controlled using software from Metamorph (Universal Imaging, PA). Fluorescent images were obtained using an EXFO X-cite 120 light source and CFP or GFP filters (Chroma). Fluorescence images were taken on live cells transferred from culture to agarose pads and kept at the temperatures indicated using an objective heater (Bioptechs) during the imaging process. Cells examined were in mid-exponential phase.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at doi:10.1016/j.devcel.2011.01.007.

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