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Regulation of replication initiation: lessons from *Caulobacter crescentus*

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Chromosome replication is a fundamental process in all domains of life. To accurately transmit genetic material to offspring, the initiation of chromosome replication is tightly regulated to ensure that it occurs only once in each cell division cycle. In the model bacterium *Caulobacter crescentus*, the CtrA response regulator inhibits the origin of replication at the pre-replication stage. Inactivation of CtrA permits the universal DnaA initiator to form an initiation complex at the origin, leading to replication initiation. Subsequently, the initiation complex is inactivated to prevent extra initiation. Whereas DNA replication occurs periodically in exponentially growing cells, replication initiation is blocked under various stress conditions to halt cell cycle progression until the normal condition is restored or the cells adapt to the stress. Thus, regulating the initiation complex plays an important role in not only driving cell cycle progression, but also maintaining cell integrity under stress. Multiple regulatory signaling pathways controlling CtrA and DnaA have been identified and recent studies have advanced our knowledge of the underlying mechanistic and molecular processes. This review focuses on how bacterial cells control replication initiation, highlighting the latest findings that have emerged from studies in *C. crescentus*.

Key words: bacteria, cell cycle, CtrA, DnaA, replication origin

CAULOBACTER CRESCENTUS

As an aquatic, gram-negative alphaproteobacterium, *Caulobacter crescentus* is a prime model organism to study the dynamics of bacterial chromosomal replication (Batut et al., 2004; Curtis and Brun, 2010; Tsokos and Laub, 2012; Hallez et al., 2017; Surovtsev and Jacobs-Wagner, 2018). *Caulobacter crescentus* has a bimodal life cycle and undergoes asymmetric cell division to yield two genetically identical but physiologically different progeny cells (Fig. 1). Whereas the stalked progeny is sessile and able to initiate chromosome replication (entry into S phase), the flagellated swarmer progeny remains motile and does not undergo replication initiation, thereby experiencing an extended non-proliferating period termed the G1 phase. Subsequently, the swarmer cell differentiates into a stalked cell via flagellar ejection and stalk biogenesis, accompanied by acquisition of the ability to initiate chromosome replication, thereby allowing the cell to enter S phase.

THE ORIGIN OF REPLICATION IN *C. CRESCENTUS*

Chromosome replication is initiated at the unique origin of replication. In bacteria, the origin contains multiple DnaA boxes, to which the family of ubiquitous AAA+ ATPase DnaA proteins bind to form an initiation complex (Katayama et al., 2010; Kaguni, 2011; Leonard and Grimwade, 2015; Wolanski et al., 2015). The canonical DnaA box consists of a conserved 5'-TTATN-CACA sequence (Messer, 2002; Luo and Gao, 2019) (Fig. 2). The mechanisms underlying formation of the initiation complex have been characterized in *Escherichia coli* (Ozaki and Katayama, 2009; Kaguni, 2011; Leonard and Grimwade, 2015; Katayama et al., 2017); in this organism, the 245-bp minimal origin (*oriC*) contains 11 functional DnaA boxes as well as the binding site (IBS) for integration host factor (IHF), a nucleoid-associated protein that creates sharp kinks in DNA (McGarry et al., 2004; Kawakami et al., 2005; Rozgaja et al., 2011; Shimizu et al., 2016). DnaA boxes at the edges of the DnaA assembly regions have the highest affinity for DnaA with K_d values of 3–9 nM (Ozaki and Katayama, 2009). Along with IHF, ATP-bound DnaA multimers promote unwinding of the AT-rich duplex unwinding element (Ozaki et

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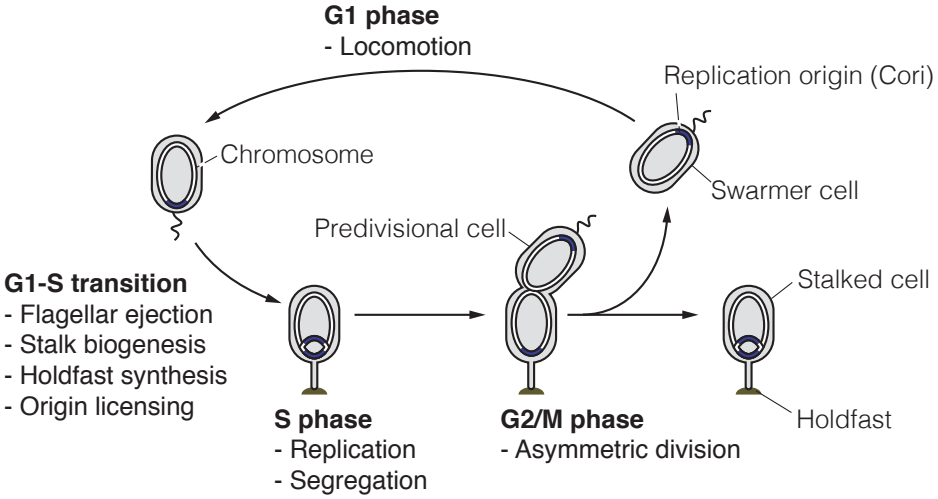


Fig. 1. The cell cycle of *C. crescentus*. Asymmetric cell division produces two distinct progeny cells. The sessile stalked progeny immediately starts the next round of the replication cycle (S phase). By contrast, the motile swarmer progeny is equipped with a unipolar flagellum and does not undergo chromosome replication, but experiences a non-proliferating period termed the G1 phase. Eventually, the swarmer cell undergoes flagellar ejection, stalk biogenesis, and holdfast (adhesin) synthesis to differentiate into a stalked cell. During this transition, the replication origin (Cori) is licensed to initiate chromosome replication.

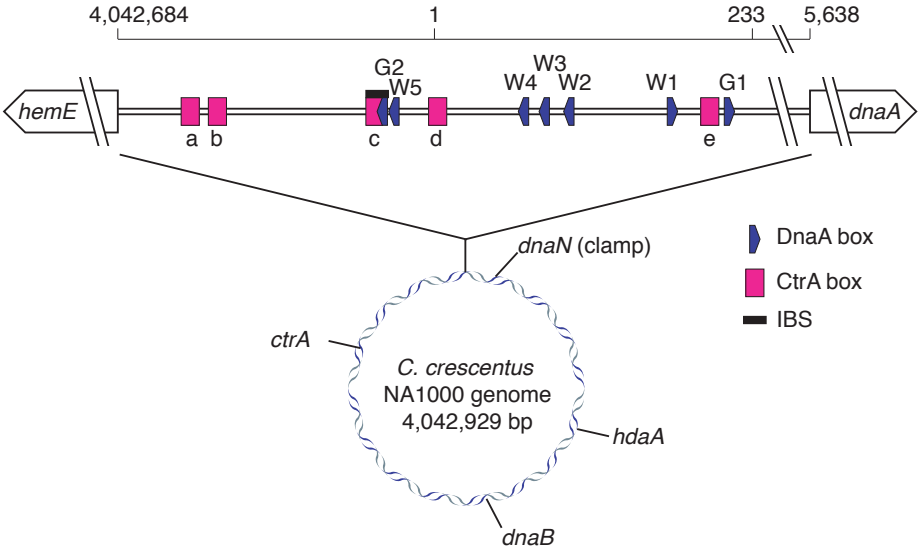
al., 2008; Ozaki and Katayama, 2012; Sakiyama et al., 2017). Subsequently, replicative DnaB helicases are recruited onto the resulting single-stranded DNA regions with the aid of DnaC helicase loaders, thereby triggering the establishment of replisomes (Arias-Palomo et al., 2013; Bell and Kaguni, 2013; Sakiyama et al., 2018). DnaA (CCNA_00005), DnaB (CCNA_01737) and components of the replisomes are well conserved in the wild-type *C. crescentus* NA1000 strain; however, unlike *E. coli*, this strain does not express the DnaC helicase loader (Nierman et al., 2001; Christen et al., 2011) (Fig. 2). Moreover, the origin (Cori) contains at least two moderate-affinity DnaA G boxes (G1 and G2) with the consensus sequence 5'-TGATNNACA (the G replaces a T of the canonical DnaA box sequence), five low-affinity DnaA W boxes (W1–W5; W means weak) with the consensus sequence NNNTCCCCA, and an IBS adjacent to DnaA box G2 (Fig. 2) (Siam et al., 2003; Shaheen et al., 2009; Taylor et al., 2011). These common features suggest that the replication initiation model established from *E. coli* studies is essentially applicable to *C. crescentus*.

Whereas DnaA and IHF stimulate replication initiation, CtrA, an essential transcriptional regulator, binds directly to Cori to inhibit initiation (Domian et al., 1997; Quon et al., 1998). CtrA is an OmpR/PhoB family response regulator consisting of an N-terminal phosphoryl receiver (REC) domain and a C-terminal winged helix-turn-helix domain, and has high affinity for a unique binding site termed the CtrA box (5'-TTAA(N7)TTAA) (Siam and Marczynski, 2000). CtrA boxes are found in the promoter regions of various genes. Indeed, microarray and rapid amplification of cDNA ends analyses have suggested that CtrA can directly control the transcription of at least 95 cell cycle-regulated genes (Laub et al., 2002; Zhou et al., 2015), highlighting the role of CtrA as a "master regulator". *Caulobacter crescentus* has five CtrA boxes (a–e) within Cori (Fig. 2). Upon phosphorylation of the Asp51 residue within the REC domain, CtrA dimerizes and its affinity for CtrA boxes increases: the K_d values are > 200 nM for non-phosphorylated CtrA and 3–15 nM for phosphorylated CtrA (Siam and Marczynski, 2000). Because CtrA box c overlaps DnaA box G2, and CtrA box e resides in close proximity to DnaA box G1, DnaA and CtrA may compete with each other at Cori. In support of this idea, *in vitro* DNase I footprint experiments demonstrated that DnaA binding to its binding sites was inhibited when the same molar amount of CtrA was added to the reaction (Taylor et al., 2011).

Regulatory components that coordinate the activities of DnaA and CtrA during the cell cycle play a fundamental role in determining whether Cori initiates replication, which ultimately ensures cell integrity in *C. crescentus* (Fig. 3–5). The following sections describe the regulatory components targeting DnaA and CtrA in more detail.

REGULATION OF DnaA

Feedback regulation of DnaA activity Regulatory inactivation of DnaA (RIDA) plays an essential role in the timely initiation of chromosome replication in *C. crescentus* (Fig. 3). RIDA is the central system that downregulates the activity of DnaA in a replication-coupled manner; this process was originally discovered in *E. coli* and was later found to be conserved in *C.*



Protein	Organism	Motif	Sequence (5'-)
DnaA	*E. coli*	R box	TTATNCACA
	C. crescentus	G box	TGATNNACA
	C. crescentus	W box	NNNTCCCCA
IHF	*E. coli*	IBS	GATCAACAACCTG
	C. crescentus	IBS	GGTTAACGCTCTG

Fig. 2. The origin of replication in *C. crescentus*. Cori (top) and the genome (middle) of the wild-type *C. crescentus* NA1000 strain (NC_011916) are shown schematically. The DnaA boxes, CtrA boxes and IBS within Cori are highlighted. The genomic loci for *dnaN* (clamp), *hdaA*, *dnaB* and *ctrA* are also indicated. DnaA box and IBS sequences in *E. coli* and *C. crescentus* are shown (bottom). Identical residues are highlighted in yellow.

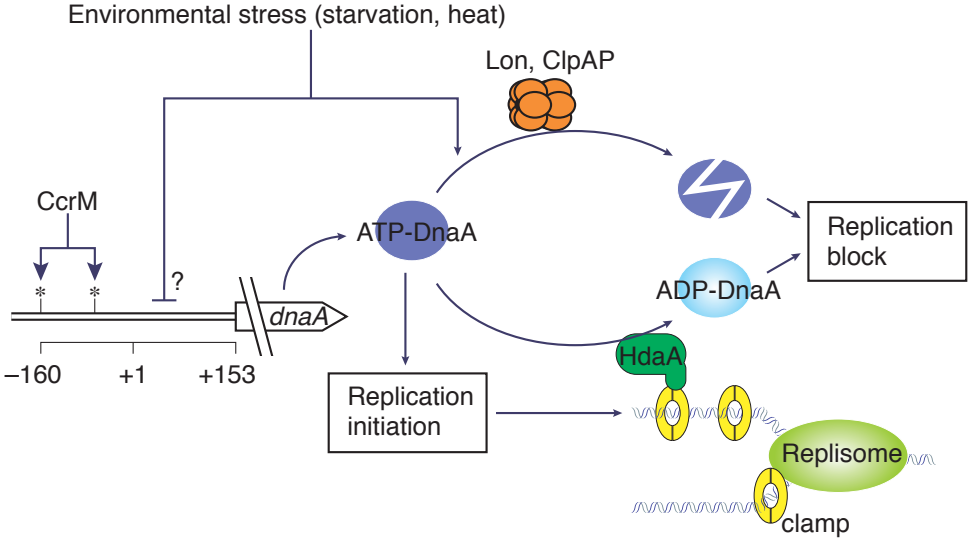


Fig. 3. Regulation of DnaA in *C. crescentus*. Schematic illustration of the regulatory pathways targeting DnaA in *C. crescentus*. In exponentially growing cells, ATP-DnaA most likely initiates chromosome replication at Cori. After initiation, ATP-DnaA is thought to be converted into inactive ADP-DnaA by the DNA-loaded clamp–HdaA complex in a replication-coupled manner, thereby blocking extra initiation events. DnaA levels are also controlled at the transcriptional level. The *dnaA* promoter-proximal region includes two methylation sites (asterisks) that are modified by the CcrM methyltransferase to affect transcription efficiency. Under stress conditions, proteases such as Lon and ClpAP rapidly degrade DnaA to prevent replication initiation. The stress response also inhibits *de novo* DnaA synthesis in a 5'-untranslated region-dependent manner, which ensures rapid clearance of DnaA.

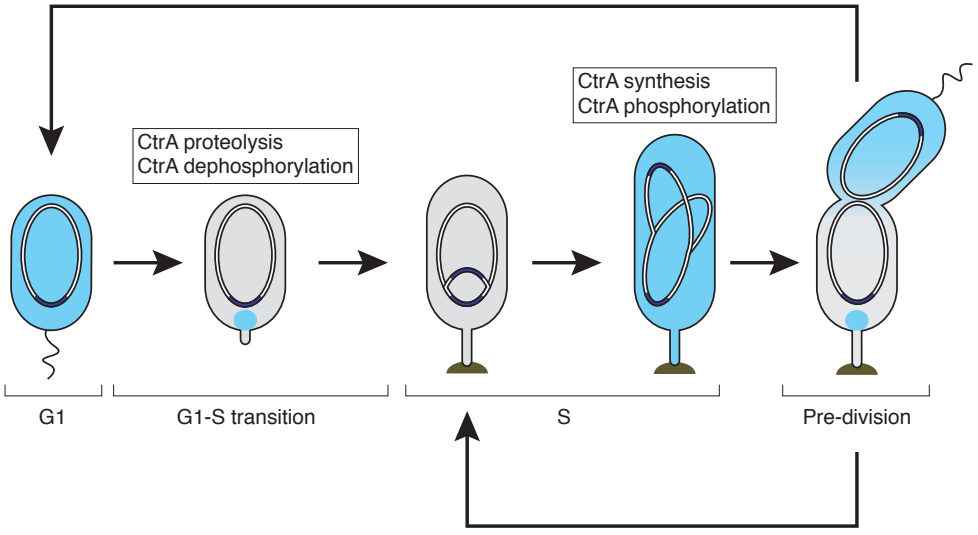


Fig. 4. Subcellular dynamics of CtrA. The cell cycle-regulated CtrA dynamics is shown schematically. In G1 phase, CtrA (blue) is abundant and activated by phosphorylation to repress replication initiation. During the G1-to-S phase transition, CtrA is inactivated through dephosphorylation. In parallel, CtrA is sequestered to the stalked cell pole where ClpXP co-localizes to degrade CtrA. As the cell cycle progresses, CtrA levels are restored by *de novo* synthesis, and CtrA is phosphorylated. In a predivisional cell, a subfraction of CtrA localizes to the stalked cell pole, which reinforces asymmetric segregation of CtrA at division.

crescentus (Katayama et al., 1998; Collier and Shapiro, 2009). In *E. coli*, RIDA is composed of two regulatory components: the clamp (DNA polymerase III subunit-β) and ADP-bound Hda, an AAA+ family protein containing an N-terminal clamp binding motif (Katayama et al., 1998; Kato and Katayama, 2001). Hda forms a complex with the clamp that remains on the nascent DNA strand after completion of Okazaki fragment synthesis. This complex interacts with ATP-DnaA through the arginine finger motif of Hda, thereby catalyzing DnaA-ATP hydrolysis to yield replication-inert ADP-DnaA (Su'etsugu et al., 2005, 2008). The requirement for the DNA-loaded clamp ensures that the RIDA system is activated after the initiation of chromosome replication, thereby forming a negative feedback system.

In *C. crescentus*, the Hda-homologous HdaA protein (65% similarity) operates together with the clamp to prevent extra chromosome replication (Fig. 3) (Collier and Shapiro, 2009; Fernandez-Fernandez et al., 2013; Wargachuk and Marczynski, 2015). As is the case for *E. coli* Hda, the N-terminal domain and conserved arginine finger motif of HdaA are essential for cell growth. Furthermore, fluorescence microscopy analyses demonstrated that HdaA dynamically colocalizes with the ongoing replisome through its N-terminal clamp binding motif (Fernandez-Fernandez et al., 2013). These features of HdaA coincide well with the mechanical basis of the *E. coli* RIDA system, particularly in replication-coupled negative feedback. Notably, a *C. crescentus* strain overexpressing DnaA displays normal chromosomal replication, whereas a strain depleted of HdaA displays over-replication initiation (Collier and Shapiro, 2009; Jonas et al., 2011; Fernandez-Fernandez et al., 2013). Thus, controlling the activity rather than the abundance of the DnaA protein appears to have a predominant role in the cell cycle-dependent initiation of chromosomal replication.

DnaA turnover in the G1 phase In exponentially growing *C. crescentus* cells, the rate of *de novo* DnaA synthesis is reportedly cell cycle-regulated, with a modest peak in the G1 phase (Gorbatyuk and Marczynski, 2005). The rate of DnaA proteolysis in the G1 phase is twice that at other cell cycle stages. Despite these cell cycle-related changes in turnover, DnaA is present throughout the cell cycle with little variation in protein level (Gorbatyuk and Marczynski, 2005; Jonas et al., 2011; Taylor et al., 2011). Although early western blot experiments reported cell cycle-related oscillations in DnaA level (Collier et al., 2006, 2007), those observations may have resulted from the use of impure anti-DnaA antibodies that cross-reacted with a heat shock protein of a similar size to DnaA (Taylor et al., 2011). Given that the intracellular concentrations of ATP are higher than those of ADP, newly synthesized DnaA should predominantly adopt the replication-competent ATP-bound form. Thus, it is plausible that increased DnaA turnover plays a role in the accumulation of ATP-DnaA, such that the level of this protein surpasses a certain threshold level required for replication initiation. However, the exact role and mechanistic basis of cell cycle-regulated DnaA turnover are still to be addressed.

The methylation state of the *dnaA* promoter can also

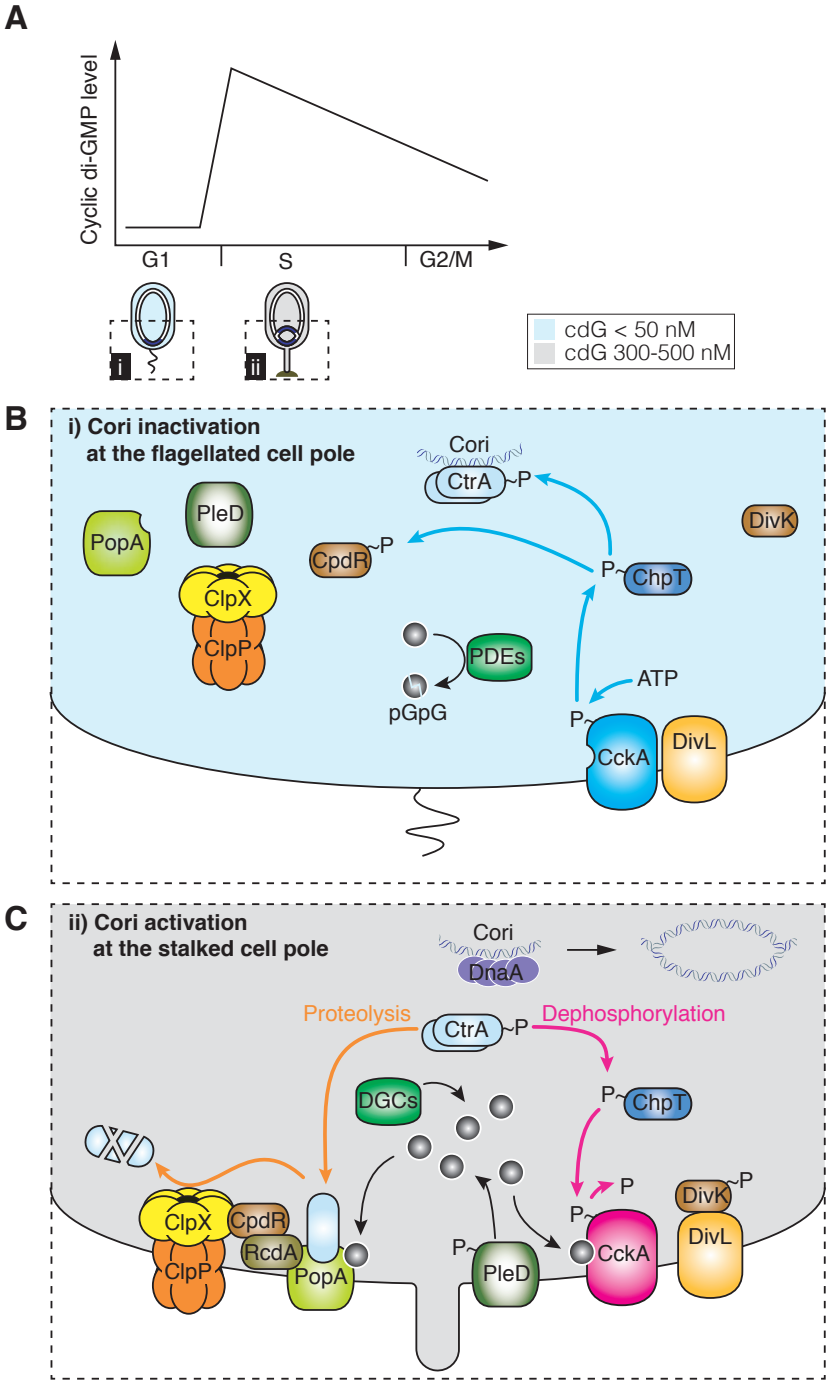


Fig. 5. Regulation of CtrA in *C. crescentus*. (A) Cell cycle-related changes in the level of the second messenger cyclic di-GMP (cdG). The subcellular level of cdG is low in the G1 phase (i) and reaches a peak upon entry into the S phase (ii). (B) Cori inactivation at the flagellated cell pole is shown schematically. In the G1 phase, CtrA is activated by phosphorylation and stabilization to block Cori. Phosphorylation of CtrA is driven by CckA kinase via the ChpT phosphotransfer protein. DivL functionally interacts with CckA to stimulate the kinase activity. In addition, cyclic di-GMP, which inhibits CckA kinase, is maintained at low levels resulting from the inactive PleD diguanylate cyclase and the active phosphodiesterases (PDEs). Conversely, CtrA is stabilized by phosphorylated CpdR and cyclic di-GMP-unbound PopA, both of which are incompetent to form a proteolytic ClpXP–adaptor complex. (C) Cori activation at the stalked cell pole is shown schematically. During the G1-to-S phase transition, Cori is licensed for DnaA-dependent initiation through dephosphorylation and degradation of CtrA. To dephosphorylate CtrA, CckA is switched from kinase to phosphatase by its direct binding to cyclic di-GMP. An upshift of subcellular cyclic di-GMP levels is ensured by phosphorylated PleD and possibly other diguanylate cyclases (DGCs). In addition, phosphorylated DivK converges in induction of the CckA phosphatase through its direct interaction with DivL. Conversely, CtrA degradation is driven by the proteolytic ClpXP–adaptor complex consisting of ClpXP, CpdR, RcdA, PopA and cyclic di-GMP. This complex is specifically localized at the stalked cell pole.

contribute to the accumulation of ATP-DnaA in the G1 phase. Whereas chromosomes are fully methylated prior to replication initiation, newly replicated chromosomal regions are hemimethylated until they are remethylated by DNA methyltransferases, which can epigenetically alter the transcriptional activities of certain promoters containing methylation sites (Campbell and Kleckner, 1990). In *C. crescentus*, the essential CcrM methyltransferase methylates adenosines at a 5'-GANTC consensus sequence and the *dnaA* promoter has two CcrM methylation sites (Fig. 3). Collier et al. (2007) suggested that the *dnaA* gene is preferentially transcribed from a fully methylated promoter, which is consistent with the idea that the methylation state of the *dnaA* promoter affects the rate of *de novo* DnaA synthesis. A mutant strain of *C. crescentus* that constitutively transcribes the *dnaA* gene displays normal chromosome replication and cell cycle progression; hence, it is plausible that CcrM-mediated transcriptional control plays a subsidiary role in chromosome replication under experimental growth conditions.

In *E. coli*, DnaA reactivating sequences (DARSs) are responsible for reactivation of replication-inert ADP-DnaA (Fujimitsu et al., 2009; Kasho et al., 2014; Frimodt-Møller et al., 2016; Inoue et al., 2016). DARSs are non-coding DNA regions containing three DnaA boxes that are uniquely arranged on the chromosome. When *E. coli* ADP-DnaA proteins form a complex on a DARS, dissociation of ADP from ADP-DnaA is promoted to yield nucleotide-free DnaA, which then binds ATP to regenerate ATP-DnaA (Fujimitsu et al., 2009; Kasho et al., 2014). DnaA reactivation is likely energetically less costly than protein turnover. However, it is unknown whether *C. crescentus*, as well as other bacteria, use DARS-like mechanisms to modulate ATP-DnaA levels. Because DnaA box clusters are often seen in intergenic regions in diverse bacterial species (Fujimitsu et al., 2009), some of these clusters may possess a DARS-like function.

Regulatory tuning of DnaA abundance under stress conditions Whereas synthesis and degradation of DnaA are well balanced to maintain nearly constant DnaA levels in logarithmically growing *C. crescentus* cells, entry into the stationary phase reduces the rate of *de novo* DnaA synthesis, leading to rapid DnaA removal (Gorbatyuk and Marczynski, 2005). This process is reasonable because active replication cycles are no longer required in stationary-phase cells. Indeed, early stationary-phase cells effectively constrain replication initiation and increase the population of G1 swarmer cells. In addition, a substantial portion of CtrA is retained in stationary-phase cells, which likely reinforces replication block.

Nutrient availability has been implicated in the control of DnaA clearance (Gorbatyuk and Marczynski, 2005; Leslie et al., 2015) (Fig. 3). Intracellular DnaA levels are reduced upon nitrogen or carbon starvation. By contrast, the DnaA level is restored when cells growing in a limited-nutrients synthetic medium are supplemented with peptone and yeast extract. These observations are consistent with the idea that regulatory tuning of DnaA abundance is directly coupled to the signaling pathways that control adaptation to environmental changes. However, the mechanisms underlying this process remain to be elucidated. Leslie et al. (2015) demonstrated that the 5'-untranslated region of the *dnaA* transcript is indispensable for rapid DnaA clearance during the stationary phase, suggesting that control of DnaA abundance occurs predominantly at the post-transcriptional level. It is reasonable to assume that one or more regulatory factors, such as small RNAs or metabolites, interact with the 5'-untranslated region of the *dnaA* transcript to control the efficiency of translation. The specific regulatory factor(s) and their links to nutrient-dependent signaling pathways should be identified in the future.

Rapid DnaA clearance is also induced by the heat shock response, which is mediated by the AAA+ Lon protease (Jonas et al., 2013) (Fig. 3). The *lon* gene is a member of the sigma 32 regulon that is repressed under normal conditions via destabilization by the Hsp70 family DnaK chaperone (Sauer and Baker, 2011; Joshi and Chien, 2016; Mayer and Gierasch, 2018). As the temperature increases, DnaK is proposed to be occupied by misfolded protein substrates, which in turn liberates sigma 32 from DnaK and activates Lon expression (Rodriguez et al., 2008; Jonas et al., 2013). Consequently, the increased abundance of Lon promotes rapid DnaA degradation, thereby arresting the cell cycle in the G1 phase. Notably, a Δ*lon* mutant strain is incapable of not only heat-dependent DnaA clearance, but also stationary phase-dependent DnaA clearance (Leslie et al., 2015), suggesting that Lon plays a predominant role in stress-induced DnaA proteolysis. Recently, *E. coli* DnaA was also shown to undergo Lon-dependent proteolysis (Puri and Karzai, 2017). Thus, regulated DnaA proteolysis by Lon may be widespread in diverse bacterial species. Mechanistically, *C. crescentus* Lon binds specifically to the AAA+ domain of *C. crescentus* DnaA *in vitro* (Liu et al., 2019). Moreover, the *C. crescentus* DnaA N-terminal domain facilitates DnaA degradation by Lon *in vitro*. In the *E. coli* system, the heat-inducible HspQ protein acts as an adaptor to markedly enhance DnaA proteolysis by Lon (Shimuta et al., 2004; Puri and Karzai, 2017). Notably, HspQ is conserved in *C. crescentus*. Therefore, these observations suggest that a dedicated mechanism, rather than non-specific protein recognition, operates for Lon-dependent DnaA clearance.

In addition, the ClpAP complex reportedly acts as an auxiliary protease for DnaA degradation (Liu et al., 2016). Intriguingly, the *C. crescentus* HdaA protein

has also been implicated in the proteolysis of DnaA (Wargachuk and Marczynski, 2015). The existence of multiple redundant regulatory pathways highlights the importance of timely DnaA removal for survival in *C. crescentus*.

REGULATION OF CtrA

Two distinct regulatory signaling pathways for CtrA As mentioned above, CtrA plays a central role in the regulation of replication initiation. In the G1 phase, CtrA is activated by phosphorylation and prevents the assembly of DnaA and the initiation complex at Cori (Fig. 4, 5A and 5B). To initiate chromosome replication, *C. crescentus* needs to inactivate CtrA during the G1-to-S phase transition (Fig. 5A and 5C). This timely inactivation of CtrA is maintained through two mechanisms: regulated proteolysis and dephosphorylation (Domian et al., 1997). Mutant *C. crescentus* strains expressing a non-degradable *ctrA* allele (ctrAΔ3M2) or a phospho-mimetic *ctrA* allele (ctrA D51E) are viable and retain normal DNA replication activity. However, a combination of these two alleles results in severe growth defects accompanied by cell cycle arrest in the G1 phase, suggesting that regulation of both CtrA proteolysis and dephosphorylation ensures timely chromosomal replication. The following sections describe the regulatory signaling pathways for CtrA in more detail.

Regulated CtrA proteolysis Cell cycle-regulated CtrA proteolysis is coupled to dynamic localization of the protein (Fig. 4). Whereas CtrA is evenly distributed throughout the cytosol in the G1 phase, it is recruited to the incipient stalked cell pole during the G1-to-S phase transition, followed by removal from the cell (Domian et al., 1997). This polar sequestration occurs independently of CtrA phosphorylation. As the cell cycle progresses, the levels of CtrA are elevated via *de novo* synthesis; subsequently, a fraction of CtrA localizes to the stalked cell pole of the late predivisional cell. This asymmetric distribution of CtrA leads to differential fates of the protein in progeny cells: a progeny stalked cell immediately degrades polar CtrA, while a progeny swarmer cell stabilizes CtrA until it enters S phase.

CtrA has a signal peptide sequence at the C-terminus that is directly recognized by ClpXP, a ubiquitous ATP-dependent protease (Domian et al., 1997). ClpXP consists of the hexameric AAA+ ATPase ClpX and the tetradecameric peptidase ClpP (Sauer and Baker, 2011; Joshi and Chien, 2016). Mutant cells depleted of either component stabilize CtrA, indicating that ClpXP plays a central role in the cell cycle-dependent proteolysis of CtrA (Jenal and Fuchs, 1998).

Biochemical characterization revealed that ClpXP alone slowly catalyzes CtrA proteolysis, and this process is markedly accelerated in the presence of accessory factors (Smith et al., 2014; Joshi et al., 2015). To date, three adaptor proteins, CpdR, RcdA and PopA, and the bacterial second messenger cyclic di-GMP have been reported as the minimal components required to reconstitute the ClpXP-dependent CtrA degradation system *in vitro* (Smith et al., 2014; Joshi et al., 2015). Consistent with this, a lack of any of these factors results in failure of cell cycle-regulated CtrA proteolysis (Iniesta et al., 2006; McGrath et al., 2006; Duerig et al., 2009; Ozaki et al., 2014). Furthermore, accumulating evidence indicates that each accessory factor plays a distinct role in the CtrA degradation system. The following sections describe the molecular and cellular principles underlying the functions of these individual components.

ClpXP. Localized CtrA degradation coincides well with the cell cycle-dependent dynamics of ClpXP (McGrath et al., 2006). In a newborn G1 cell in which CtrA is stabilized, ClpX and ClpP are dispersed in the cytosol (Fig. 5B). The transition from the G1 to the S phase drives ClpX and ClpP to the incipient stalked cell pole, to which CtrA is recruited concomitantly (Fig. 5C). After CtrA removal, ClpXP is delocalized from the stalked cell pole to the cytosol. Finally, ClpXP relocates to the stalked cell pole of the pre-divisional cell. Consequently, cell division generates two asymmetric progeny cells with differential ClpXP distribution: a G1 phase progeny with dispersed ClpXP and an S phase progeny with polar ClpXP proteases. Because the subcellular levels of ClpX and ClpP do not fluctuate throughout the cell cycle (Jenal and Fuchs, 1998), it seems that cell cycle-dependent assembly of the CtrA–ClpXP complexes plays a crucial role in sanctioning CtrA removal in specific cell types.

Although dynamic localization of ClpXP is evidently interwoven with the cell cycle-dependent proteolysis of CtrA, mutant strains that are unable to recruit ClpXP to the stalked cell pole largely retain normal growth and cell behavior (McGrath et al., 2006). Therefore, spatial control of CtrA proteolysis alone does not fully account for the essentiality of the ClpXP genes for normal progression of the *C. cresentus* cell cycle. Notably, recent studies have suggested that the DnaX DNA polymerase subunit and the SocB toxin are substrates for ClpXP and that their proteolytic processing is crucial to sustain normal cell growth (Aakre et al., 2013; Vass et al., 2017). Thus, regulated proteolysis by ClpXP is applied to multiple target substrates to orchestrate cell cycle progression.

CpdR. As a member of the single-domain response regulator family, CpdR harbors a REC domain but lacks a dedicated output domain (Jenal, 2009). The phosphorylation state of this protein is cell cycle controlled by cell cycle kinase A (CckA)-dependent phosphor-signaling (see section 4.3.1). Specifically, CpdR is phosphorylated

during the G1 phase and dephosphorylated during the G1-to-S phase transition (Iniesta et al., 2006; Iniesta and Shapiro, 2008) (Fig. 5B and 5C). Strikingly, dephosphorylated CpdR localizes to the incipient stalked cell pole and recruits the ClpXP proteases to the same place via a direct interaction with ClpX (Iniesta et al., 2006; Duerig et al., 2009) (Fig. 5C). Thus, the phosphor-signaling pathway involving CpdR is key to controlling the activity of ClpXP in time and space. Notably, CtrA sustains its polar localization irrespective of the presence or absence of CpdR, suggesting that localization of CtrA is regulated independently to that of ClpXP.

PopA and cyclic di-GMP. ClpXP-independent control of CtrA localization is mediated by Paralog of *pleD* (PopA), a prerequisite for polar sequestration of CtrA (Duerig et al., 2009; Ozaki et al., 2014). PopA was originally identified as a structural homolog of the PleD diguanylate cyclase (Duerig et al., 2009); despite low overall sequence conservation, both proteins share the same domain architecture comprising two REC domains fused to a GGDEF domain. Whereas PleD catalyzes synthesis of the second messenger cyclic di-GMP (Wassmann et al., 2007; Jenal et al., 2017), PopA lacks catalytic activity but acts as a cyclic di-GMP effector protein by utilizing its allosteric I-site within the GGDEF domain as a high-affinity binding site for cyclic di-GMP. The PopA–cyclic di-GMP complex localizes to the incipient stalked cell pole in a manner dependent on PopZ, a polar scaffold protein (Bowman et al., 2008; Ebersbach et al., 2008; Ozaki et al., 2014) (Fig. 5C). Because localization of PopA to the stalked cell pole is not affected in mutant cells with depleted levels of ClpX or those lacking the *cpdR* gene, the mechanism of PopA localization is basically independent of the regulatory pathway for CpdR–ClpXP localization. Conversely, a mutant strain lacking the *popA* gene displays disrupted CtrA proteolysis, accompanied by failure of CtrA to localize to the stalked cell pole (Duerig et al., 2009). These observations strongly suggest a distinct role for PopA in controlling the polar sequestration of CtrA. In agreement with this proposal, *in vitro* pulldown experiments using recombinant proteins revealed that PopA physically interacts with CtrA (Smith et al., 2014). Strikingly, the interaction between PopA and CtrA is tightly dependent on cyclic di-GMP. Thus, a cyclic di-GMP-induced conformational change in PopA is key to the timely localization of PopA to the stalked cell pole, as well as its binding to CtrA.

In addition to its localization at the stalked cell pole, PopA is recruited to the opposite, flagellated cell pole during the S phase. However, unlike the case at the stalked cell pole, PopA requires neither PopZ nor cyclic di-GMP for its flagellated cell pole localization, but utilizes PodJ as a receptor at the flagellated cell pole (Duerig et al., 2009; Ozaki et al., 2014). Thus, *C. crescentus* cells use two distinct mechanisms to recruit PopA to both cell poles. However, the physiological role of flagellated polar PopA remains unknown.

Formation of the PopA–cyclic di-GMP complex is promoted within a limited time window during the G1-to-S phase transition. This timing is controlled by the cell cycle oscillation of the intracellular cyclic di-GMP levels in *C. crescentus*: low in the G1 phase, high during the G1-to-S phase transition, and intermediate in the G2/M phase (Paul et al., 2008; Christen et al., 2010; Abel et al., 2013) (Fig. 5A). The *C. crescentus* genome encodes 12 genes potentially involved in cyclic di-GMP synthesis and/or degradation. Of these, the PleD diguanylate cyclase is the main driver for the cyclic di-GMP oscillation (Paul et al., 2008; Abel et al., 2013). While PleD is inactivated by dephosphorylation in the G1 phase, the DivJ kinase activates PleD through phosphorylation of the N-terminal REC domain during the G1-to-S phase transition (Wassmann et al., 2007; Paul et al., 2008). In addition, cyclic di-GMP-specific phosphodiesterases contribute to dynamic changes in cyclic di-GMP levels. In particular, the PdeA phosphodiesterase, which is abundant in the G1 phase, cleaves cyclic di-GMP into pGpG to ensure the low cyclic di-GMP levels (Abel et al., 2011). As the cell enters S phase, PdeA is degraded by the ClpXP protease. Therefore, the timely inactivation of PdeA in combination with the activated PleD leads to a steep increase of cyclic di-GMP at the G1-to-S phase transition. Intriguingly, although PleD and PdeA are sufficient to generate the cell-cycle oscillation of the cyclic di-GMP levels, other diguanylate cyclases and phosphodiesterases are proposed to bear specific roles in controlling the cyclic di-GMP levels. For instance, the DgcB diguanylate cyclase is activated by surface sensing in order to coordinate cell cycle progression with the environment (Hug et al., 2017).

RcdA. PopA does not interact directly with CpdR or ClpXP (Duerig et al., 2009; Ozaki et al., 2014). RcdA has been identified as the molecule linking CpdR-guided ClpXP and PopA-guided CtrA at the stalked cell pole. The *rcdA* gene was originally identified as a conserved gene that is targeted directly by CtrA (McGrath et al., 2006). The promoter region of *rcdA* contains a CtrA box, and binding of CtrA to this promoter is thought to stimulate *rcdA* expression (Laub et al., 2002). As is the case for other components involved in regulated CtrA degradation, RcdA transiently localizes to the incipient stalked cell pole during the G1-to-S phase transition (McGrath et al., 2006) (Fig. 5C). This polar localization of RcdA is dependent on both PopA and ClpX (McGrath et al., 2006; Duerig et al., 2009). By contrast, neither PopA nor ClpX require RcdA for their stalked cell polar localization (McGrath et al., 2006; Duerig et al., 2009; Taylor et al., 2009). These findings are consistent with the idea

that RcdA acts as a molecular bridge that connects the CpdR–ClpXP complex to the PopA–CtrA complex. In agreement with this proposal, protein–protein interaction analyses revealed that RcdA physically interacts with PopA through its N-terminal Rec1 domain (Ozaki et al., 2014; Smith et al., 2014). In addition, the C-terminal tail of RcdA was reported to directly bind CpdR *in vitro* (Joshi et al., 2015).

CpdR, RcdA, PopA and cyclic di-GMP form an adaptor complex that promotes CtrA degradation by ClpXP in an *in vitro* reconstituted system (Smith et al., 2014). Compared with that in the reaction with ClpXP alone, the K_m of CtrA proteolysis in the reconstituted system is almost 10-fold lower, indicating a higher affinity of ClpXP for CtrA in the presence of the adaptor complex. Consistent with *in vivo* observations, omitting any of the adaptor components from the *in vitro* system results in inefficient CtrA proteolysis. Thus, an elaborate cell cycle program that controls CtrA abundance in time and space is sufficiently explained by these accessory factors. However, it is possible that additional factors also play a part in modulating CtrA stability under stress conditions (Heinrich et al., 2016).

Anti-adaptor model. Whereas the whole adaptor complex is required for ClpXP-dependent proteolysis of CtrA, proteolysis of some ClpXP substrate proteins, such as the cyclic di-GMP-specific phosphodiesterase PdeA, depends only on CpdR, and proteolysis of others, such as the transcriptional regulator TacA, requires only CpdR and RcdA (Abel et al., 2011; Joshi et al., 2015). Therefore, an obvious question is how *C. crescentus* cells coordinate these adaptors for regulated proteolysis. A recent study provided evidence that the adaptors are recruited in a hierarchical manner, which ensures selective protein degradation depending on the degree of adaptor assembly (Joshi et al., 2015). The authors suggested that adaptors operating at a higher level of the hierarchy serve as anti-adaptors for substrates operating at a lower level. For instance, PopA can act as an anti-adaptor for RcdA, thereby preventing ClpXP-mediated TacA degradation. In other words, PopA can increase the availability of ClpXP for CtrA removal by blocking degradation of other ClpXP substrates recruited by RcdA.

Intriguingly, in contrast to the high conservation of CpdR and RcdA throughout Alphaproteobacteria, PopA is found only in *Caulobacter* and some of its closest relatives (Brilli et al., 2010; Ozaki et al., 2014). Given that cyclic di-GMP is a global regulator coordinating bacterial development, it is likely that *Caulobacter* species have integrated cyclic di-GMP–PopA signaling into regulated CtrA proteolysis to strictly couple cellular developmental programs with cell cycle progression.

Phosphor-signaling regulation of CtrA The levels of phosphorylated CtrA oscillate throughout the cell cycle and are lowest during the G1-to-S phase transition (Domian et al., 1997). This oscillation is maintained in a mutant strain expressing stabilized CtrA, suggesting that the system controlling CtrA phosphorylation is independent of that controlling its degradation.

Phosphorylation of CtrA is governed by a phosphorylation cascade originating from the CckA kinase (Jacobs et al., 1999; Biondi et al., 2006). In the G1 phase, CckA exhibits an autokinase activity and donates its phosphoryl moiety to the N-terminal REC domain of CtrA via the ChpT phosphotransfer protein (Biondi et al., 2006; Blair et al., 2013; Dubey et al., 2016) (Fig. 5B). Upon entry into the S phase, CckA switches from a kinase mode to a phosphatase mode to reverse the phosphate flux through the CckA–ChpT–CtrA cascade, thereby dephosphorylating CtrA (Chen et al., 2009) (Fig. 5C). Because CckA is central to the phosphorylation cascade, multiple regulatory pathways, involving DivK, DivL and cyclic di-GMP, converge to regulate CckA activity in time and space. The following sections describe the molecular mechanisms of CckA regulation in more detail.

CckA. As a member of the hybrid histidine kinase family, CckA contains three characteristic functional domains: a dimerization histidine phosphotransfer (DHp) domain where the conserved histidine phosphoryl acceptor resides, a catalytic ATPase (CA) domain that hydrolyzes ATP to transfer its gamma phosphate to the DHp domain, and a C-terminal REC domain that accepts the phosphoryl group from the His residue on the DHp domain (Jacobs et al., 1999; Angelastro et al., 2010; Dubey et al., 2016) (Fig. 6). In addition, CckA contains an N-terminal transmembrane domain and two Per-Arnt-Sim domains (PAS-1 and PAS-2) (Lori et al., 2015; Dubey et al., 2016; Mann et al., 2016). The two transmembrane helices within the transmembrane domain tether the N-terminus of CckA to the inner membrane, thereby exposing the other domains to the cytosolic fraction. Because the transmembrane domain is dispensable for CckA activity (Chen et al., 2009; Lori et al., 2015; Dubey et al., 2016), it likely imposes a spatial constraint on CckA function *in vivo*. PAS domains are widely conserved signaling modules that are often associated with sensory functions (Möglich et al., 2009; Rojas-Pirela et al., 2018). In CckA, PAS-2 is crucial for the kinase/phosphatase functional switch, but the role of PAS-1 remains elusive.

Cyclic di-GMP-mediated molecular switch. Recent studies have identified cyclic di-GMP as a key molecule that controls CckA activity (Lori et al., 2015; Dubey et al., 2016; Mann et al., 2016) (Fig. 5 and Fig. 6). Cyclic di-GMP binds directly to CckA and induces its phosphatase activity, which can effectively dephosphorylate CtrA in an *in vitro* reconstituted CckA–ChpT–CtrA phosphorelay

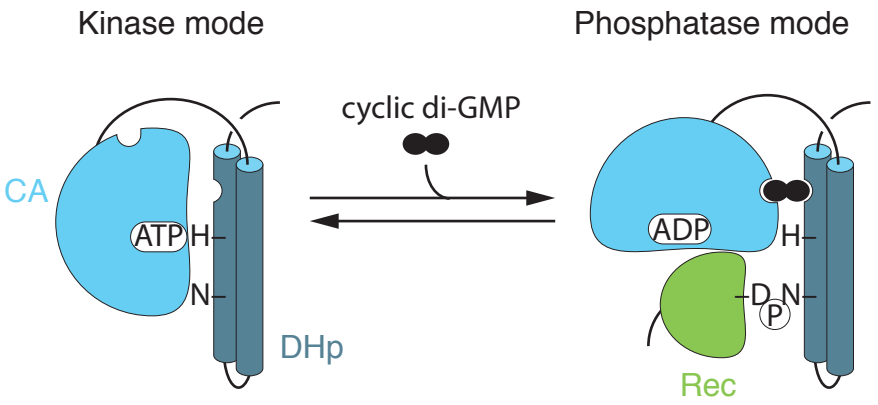


Fig. 6. Cyclic di-GMP-mediated functional switch of CckA. Schematic illustration of the kinase and phosphatase modes of CckA. Only the CA, DHp and REC domains are shown for simplicity. In the kinase mode, ATP bound to the CA domain resides in close proximity to the His322 (H) residue on the DHp domain, to which the gamma phosphate of ATP is transferred. Subsequently, the phosphoryl group is transferred onto the Asp623 (D) residue within the REC domain, which facilitates CtrA phosphorylation via ChpT. By contrast, the phosphatase mode requires the open conformation that enables interaction between the phosphorylated Asp623 residue and the conserved Thr/Asn residue (N) within the DHp domain. Cyclic di-GMP is proposed to impose the open conformation on CckA.

system (Lori et al., 2015). Because cyclic di-GMP levels oscillate throughout the cell cycle (Fig. 5A), the increased levels that occur during the G1-to-S phase transition permit timely induction of CckA phosphatase activity, leading to inactivation of CtrA and subsequent initiation of chromosome replication. The involvement of an oscillating molecule in regulating the cell cycle kinase is reminiscent of the eukaryotic cyclin–CDK systems (Hochegger et al., 2008; Hydbring et al., 2016).

In addition to its role in the G1-to-S phase transition, the cyclic di-GMP-mediated CckA switch has been implicated in asymmetric distribution of differential CckA activities at opposite poles during the G2/M phase (Lori et al., 2015). In the proposed model, most CckA molecules bind to the cellular pool of cyclic di-GMP and display phosphatase activity; however, a sub-population displays strong kinase activity at the swarmer cell pole as a consequence of a microenvironment with low levels of cyclic di-GMP. Lori et al. (2015) speculated that specific localization of diguanylate cyclases and/or cyclic di-GMP-specific phosphodiesterases is involved in establishment of the microenvironment. Although the existence of a local cyclic di-GMP pool has not yet been proven, further studies will help to address this issue.

Structural and biochemical analyses suggested that CckA has two cyclic di-GMP binding sites (Lori et al., 2015; Dubey et al., 2016) (Fig. 6). The primary binding site resides within the CA domain, which coordinates one of the two guanine moieties using specific residues located distal to the ATP/ADP binding pocket. The other guanine moiety is thought to interact with the secondary CckA binding site within the DHp domain. Therefore, binding of cyclic di-GMP to CckA can noncovalently crosslink two functional domains, and thereby restrict the conformation of CckA to that of the phosphatase mode (Dubey et al., 2016). A recent study found that a mutant CckA lacking the PAS2 domain displays an impaired response to cyclic di-GMP *in vitro* (Mann et al., 2016).

DivK and DivL. DivK and DivL form a distinct regulatory branch of the control of CckA activity that operates independently of the cyclic di-GMP-mediated CckA switch. DivL is an essential pseudo-histidine kinase that lacks the conserved phosphor-acceptor His residue (Reisinger et al., 2007; Tsokos et al., 2011; Childers et al., 2014). DivK is a single-domain response regulator that is phosphorylated by the histidine kinase DivJ and dephosphorylated by the histidine kinase PleC (Matroule et al., 2004; Paul et al., 2008). Upon phosphorylation, DivK binds directly to the DHp domain of DivL (Wheeler and Shapiro, 1999; Tsokos et al., 2011; Childers et al., 2014) (Fig. 5C). Although a direct role for the DivK–DivL complex in controlling the enzymatic activity of CckA remains unclear, a series of genetic experiments suggested that DivL acts as a positive regulator of CtrA, and that this activity is antagonized through its physical interaction with DivK (Tsokos et al., 2011). Notably, a mutant strain expressing a loss-of-function *divK* gene displays increased levels of phosphorylated CckA, accompanied by G1 cell cycle arrest. By contrast, inactivation of *divL* reduces the level of phosphorylated CckA, resulting in over-replication of chromosomes. Moreover, a mutant strain in which *divK* and *divL* are inactivated phenocopies a *divL* single mutant strain, indicating that *divL* lies genetically downstream of *divK*. These observations are consistent with the idea that DivL activates CtrA through stimulation of CckA kinase activity and/or inhibition of CckA phosphatase activity, and that DivK inactivates

CtrA by modulating the functioning of DivL. However, the molecular mechanism by which the DivK–DivL complex interferes with the CckA switch remains unknown.

Previous work highlighted the role of DivL as a localization factor for CckA (Tsokos et al., 2011). The subcellular localization of CckA is dynamically regulated throughout the cell cycle: CckA is dispersed in the cytoplasm during the G1 phase but assembles at the pole opposite to the incipient stalked cell pole during the G1-to-S phase transition, and remains localized until cell division (Jacobs et al., 1999; Angelastro et al., 2010). In addition, CckA can localize to the stalked cell pole during the S and G2/M phases. Strikingly, a *divL* temperature-sensitive mutant strain fails to sequester CckA to the pole opposite to the incipient stalked cell pole at non-permissive temperatures (Tsokos et al., 2011), suggesting the formation of a complex between CckA and DivL. In agreement with this proposal, an interaction between CckA and DivL was demonstrated using a co-immunoprecipitation assay (Iniesta et al., 2010). Nonetheless, it remains unclear whether the interaction between CckA and DivL is direct. More importantly, the mechanism by which DivL modulates CckA activity in time and space is a key question to be addressed.

CONCLUDING REMARKS

Decades of research in *C. crescentus* have identified a number of key regulatory molecules involved in cell cycle progression and have identified the molecular and cellular mechanisms by which bacteria coordinate chromosome replication in time and space. However, the process that kick-starts chromosome replication during the G1-to-S phase transition remains elusive. Given that inactivation of CtrA does not affect the periodicity of replication initiation, removal of CtrA is unlikely to be a direct trigger for initiation. Rather, it is plausible that an increase in the level of active ATP-DnaA ultimately determines entry into S phase. In this context, it is crucial to address how *C. crescentus* cells accumulate ATP-DnaA molecules in a timely manner. In *E. coli*, DARS regenerates ATP-DnaA from inert ADP-DnaA; thus, it is important to examine whether the same mechanism also operates in *C. crescentus*. Other questions of general importance include how the ATP-DnaA–Cori complex unwinds duplex DNA and how DnaB helicases are recruited. Recent work has implicated a novel *dciA* gene in modulation of DnaB helicase activity in *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* (Brézellec et al., 2016; Mann et al., 2017). Because the *C. crescentus* genome does not encode *dnaC*, it is possible that a *dciA*-like gene is co-opted by *C. crescentus* to facilitate the helicase function. Ultimately, it remains elusive how DnaB loading is regulated *in vivo*. These questions should be investigated in future studies.

Adapting the chromosome replication cycle to environmental conditions is crucial for cell survival in nature. Whereas cells accelerate the replication cycle to achieve rapid growth and exploit available nutrients, stalling the replication cycle is important to maintain chromosome integrity under stringent conditions. In *C. crescentus*, environmental changes can trigger DnaA clearance, thereby restricting replication initiation. Intriguingly, nutrient availability has also been implicated in the direct or indirect control of DnaA activity in *E. coli*. Thus, DnaA may be a universal regulatory point that connects environmental signals to the replication cycle in the bacterial kingdom. Nonetheless, the underlying molecular basis is poorly characterized in *C. crescentus* and *E. coli*, and additional analyses of how DnaA is regulated in response to environmental changes are crucial to address how bacteria sense and adapt to their environment.

Recent studies indicate that cell cycle-related oscillation of the bacterial second messenger cyclic di-GMP acts as a cyclin-like signaling process to coordinate cell cycle progression in *C. crescentus*. To date, several effector proteins have been shown to bind cyclic di-GMP, thereby controlling diverse cellular activities including chromosome replication, flagellar morphogenesis, motility and surface attachment. However, given the pleiotropic defects in a mutant strain devoid of cyclic di-GMP, the identity of effector proteins that are crucial for cell cycle regulation awaits elucidation. Characterization of cyclic di-GMP effectors will provide novel insights into cell cycle regulation. Moreover, it is unclear how far the role of cyclic di-GMP signaling in cell cycle progression has been conserved during bacterial evolution. Given that cyclic di-GMP is a ubiquitous signaling molecule, it seems reasonable to assume that *C. crescentus* is not the only bacterium that has integrated cyclic di-GMP as a regulator of the cell cycle. Additional analyses of cyclic di-GMP signaling in diverse model organisms will reveal the commonalities and differences in cell cycle regulation between bacterial species.

The class Alphaproteobacteria to which *C. crescentus* belongs includes symbiotic bacteria as well as key plant, animal and human pathogens. These bacterial species share a number of important cell cycle regulators with *C. crescentus*, such as CtrA, CckA and DivL. Thus, the identification and in-depth characterization of novel cell cycle genes in *C. crescentus* will not only expose the molecular and cellular basis of bacterial cell cycle control, but will also expand our understanding of the physiology of pathogens and commensals in the Alphaproteobacteria.

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