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## *Modularity of the Bacterial Cell Cycle Enables Independent Spatial and Temporal Control of DNA Replication*

**Massachusetts Institute of Technology**

# Article

# Modularity of the Bacterial Cell Cycle Enables Independent Spatial and Temporal Control of DNA Replication

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## Summary

**Background:** Complex regulatory circuits in biology are often built of simpler subcircuits or modules. In most cases, the functional consequences and evolutionary origins of modularity remain poorly defined.
**Results:** Here, by combining single-cell microscopy with genetic approaches, we demonstrate that two separable modules independently govern the temporal and spatial control of DNA replication in the asymmetrically dividing bacterium *Caulobacter crescentus*. DNA replication control involves DnaA, which promotes initiation, and CtrA, which silences initiation. We show that oscillations in DnaA activity dictate the periodicity of replication while CtrA governs the asymmetric replicative fates of daughter cells. Importantly, we demonstrate that DnaA activity oscillates independently of CtrA.
**Conclusions:** The genetic separability of spatial and temporal control modules in *Caulobacter* reflects their evolutionary history. DnaA is the central component of an ancient and phylogenetically widespread circuit that governs replication periodicity in *Caulobacter* and most other bacteria. By contrast, CtrA, which is found only in the asymmetrically dividing α-proteobacteria, was integrated later in evolution to enforce replicative asymmetry on daughter cells.

## Introduction

Important cellular processes are often orchestrated by regulatory circuits involving numerous genes and proteins. These complex circuits are often comprised of simpler parts, or modules, that are interconnected but perform separable functions [1, 2]. A modular architecture of biological systems may enhance evolvability because it allows the generation of new functions or network properties by altering the connections between modules, rather than by creating new networks from scratch. Defining the modularity and evolutionary history of regulatory circuits remains a major challenge. Here, we demonstrate an intrinsic modularity to the *Caulobacter crescentus* cell cycle with two interlinked but genetically separable circuits governing the temporal and spatial control of DNA replication. *Caulobacter* is an ideal model for investigating the regulation of DNA replication because cells replicate a single chromosome once and only once per cell cycle [3] (Figure 1A). The absence of multifork replication facilitates the analysis of DNA replication in individual cells via time-lapse fluorescence microscopy [4].

DNA replication initiation in *Caulobacter* requires DnaA [5], which binds and helps melt the origin of replication to promote initiation. DnaA is a member of the AAA[+] family of ATPases, with DnaA-ATP but not DnaA-ADP active for replication initiation [6–8]. DnaA is highly conserved and present in all bacteria, with the exception of some obligate endosymbionts [9]. In *E. coli*, DnaA activity is tightly regulated, peaking immediately before replication initiation and then dropping rapidly after initiation [8]. Inactivation depends largely on Hda, which stimulates ATP hydrolysis by DnaA [10].

In *Caulobacter*, every cell division is asymmetric, resulting in daughter cells with different cell fates [11]. The daughter stalked cell immediately initiates replication, whereas the daughter swarmer cell must first differentiate into a stalked cell before replicating. This cell fate asymmetry and cellular differentiation process depend on CtrA, an essential transcription factor that activates ∼100 genes and also binds to and silences the origin of replication [12–14]. A complex circuit of two-component signaling proteins ensures that swarmer cells phosphorylate and stabilize CtrA to repress replication while stalked cells dephosphorylate and degrade CtrA to permit DNA replication [15–18]. CtrA is again phosphorylated and stabilized in predivisional cells so that it can activate target genes. CtrA is often assumed to also prevent the reinitiation of replication, but mutating CtrA binding sites in the origin does not severely perturb replication control [19].

Although both CtrA and DnaA have been previously studied in *Caulobacter*, the precise roles played by each and the interdependencies of the two regulators remain surprisingly ill-defined. One current model suggests that CtrA and DnaA are connected through a transcriptional circuit, in which *dnaA* transcription is indirectly activated by CtrA and *ctrA* transcription is indirectly activated by DnaA [20, 21]. This circuit, proposed to drive cell cycle progression, implies that the accumulation of DnaA depends on CtrA activity. However, cells lacking active CtrA accumulate multiple chromosomes [12], indicating that DnaA is probably not limiting in these cells. Moreover, cells constitutively transcribing *dnaA* or *ctrA* do not exhibit a severe replication phenotype [16, 22].

It thus remains unresolved how the task of regulating DNA replication in *Caulobacter* is distributed between CtrA and DnaA and how these two regulators are wired together. To address these issues, we have investigated DNA replication in individual living cells via time-lapse fluorescence microscopy. We provide evidence that periodic changes in DnaA activity determine the periodicity of DNA replication and, importantly, that DnaA activity cycles independently of CtrA. By contrast, CtrA establishes replicative asymmetry by silencing replication in daughter swarmer cells, with no major effect on the periodicity of replication in stalked cells. These findings suggest that DnaA lies at the heart of a primordial cell cycle oscillator that continues to dictate replication timing in *Caulobacter*, as it does in most bacteria, while CtrA was recruited later in evolution to coordinate asymmetric replication with cellular differentiation. The modularity of replication control in *Caulobacter* thus reflects its evolution and reveals its relationship to the cell cycles of other bacteria.

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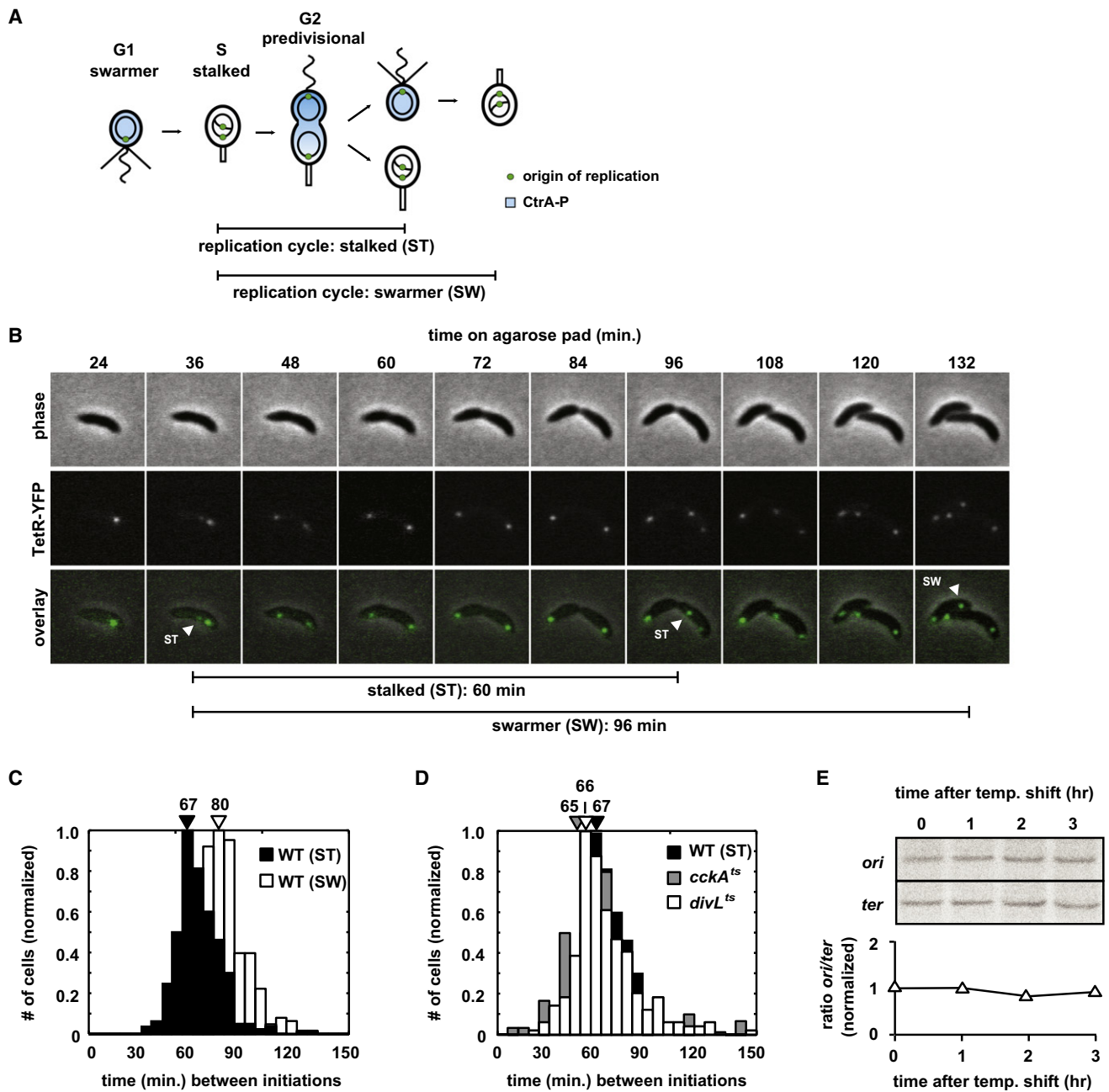
**A**



**B**



**C**



**D**



**E**



Figure 1. A Loss of CtrA Activity Does Not Perturb DNA Replication Periodicity

(A) Schematic of the *Caulobacter* cell cycle showing the stalked (ST) and swarmer (SW) replication cycles.

(B) Representative time-lapse images of wild-type cells with TetR-YFP-labeled origins of replication. Arrowheads depict the appearance of a new origin in either stalked (ST) or swarmer (SW) daughter cell.

(C) Histograms of wild-type stalked (ST) and swarmer (SW) replication cycles for cells grown at 34°C. Mean values for each population are noted. The number of cells (see Table 1) are normalized to 1.0 for visualization.

(D) Histograms showing replication cycle periodicity in *divL*$^{ts}$ and *cckA*$^{ts}$ cells compared to wild-type stalked cells at 34°C.

(E) Southern blots of DNA near the origin (*ori*) and terminus (*ter*) in *divL*$^{ts}$ cells shifted to the restrictive temperature of 37°C at time 0. Band intensities were quantified and ratios normalized to the 0 hr time point.

## Results

### CtrA Dictates Replication Asymmetry but Does Not Influence the Periodicity of Initiation

To probe the temporal regulation of DNA replication in *Caulobacter*, we monitored replication initiation in individual living cells. We used a fluorescent repressor-operator system in which origins of replication are fluorescently marked by TetR-YFP proteins bound to an array of *tet* operator (*tetO*) sites inserted near the origin [4]. Via time-lapse microscopy, we observed that wild-type stalked cells initially harbor a single, polarly localized origin of replication, represented by a single fluorescent focus (Figures 1A and 1B). Shortly after replication initiation, one origin remains at the stalked pole while the other
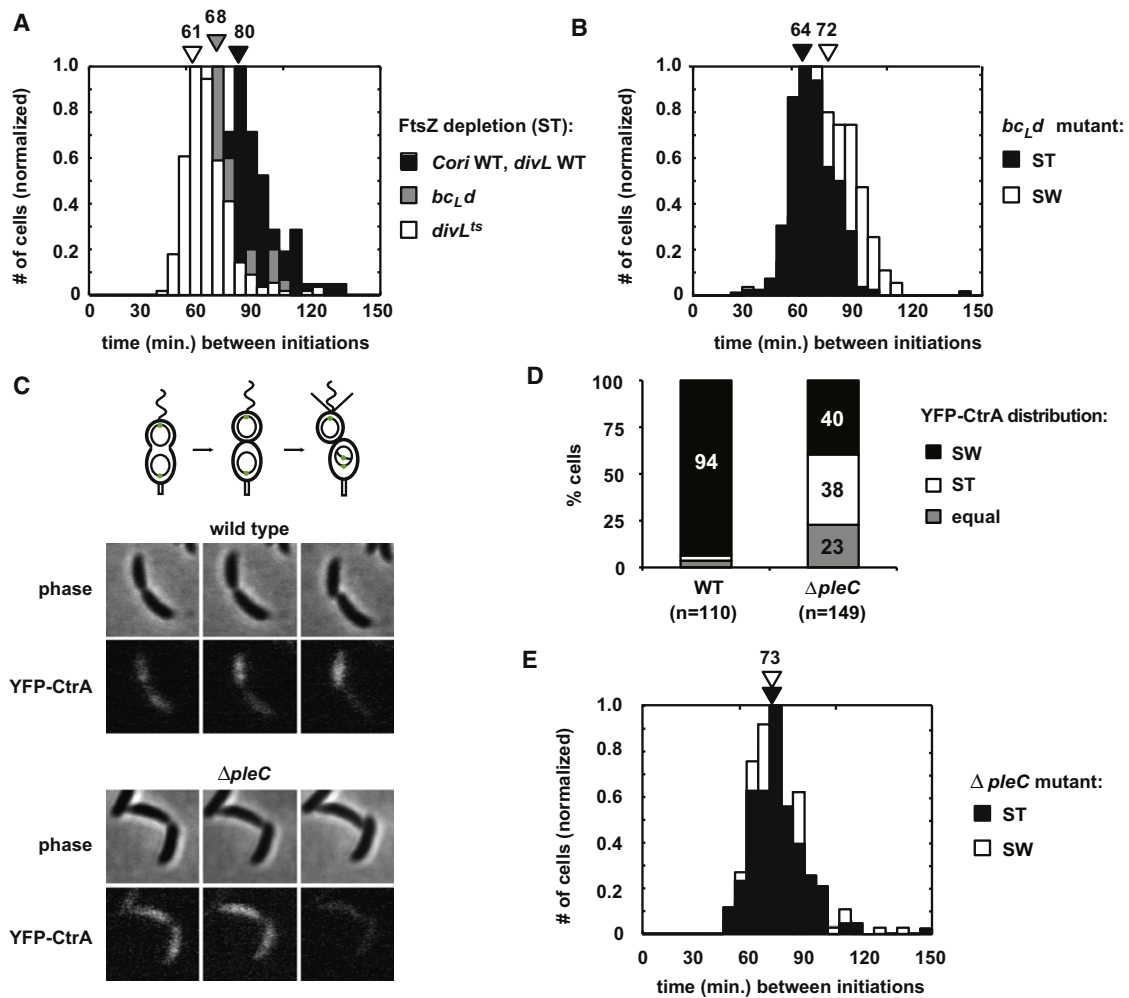
Figure 2. CtrA Dictates Replicative Asymmetry

(A) Histograms of replication intervals in FtsZ-depleted cells with or without the *divL*[ts] or *ori(bc_Ld)* mutations grown at 34°C. Mean values for each population are indicated.

(B) Histograms showing the periodicity of stalked (ST) and swarmer (SW) replication cycles in the *ori(bc_Ld)* strain.

(C) Representative time-lapse images at 10 min intervals with phase-contrast and epifluorescence images of wild-type or Δ*pleC* cells expressing YFP-CtrA. The schematic shows the wild-type cell cycle stage for each column.

(D) Quantification of YFP-CtrA partitioning ∼10 min after division in wild-type and Δ*pleC* cells. Pairs of daughter cells were scored for preferentially retaining YFP-CtrA in the swarmer cell, the stalked cell, or neither.

(E) Histograms of replication cycle times for Δ*pleC* daughter cells. Daughter cells were designated "stalked" (ST) or "swarmer" (SW) by following inheritance of the old (stalked) or new (swarmer) poles over two consecutive cell cycles.

rapidly translocates to the opposite pole [4, 23]. The time at which two separate foci are first visible in an individual cell was used as a proxy for the time of initiation. We then tracked each cell through its cell cycle and measured the time at which each daughter cell initiated replication. Daughter stalked cells typically initiated shortly after cell division whereas the daugther swarmer cells had to first differentiate into stalked cells. We refer to the intervals between replication initiation in a stalked cell and its daughter stalked and swarmer cells as the stalked and swarmer replication cycles, respectively (Figure 1A). For wild-type, we observed a stalked replication cycle of ∼67 min and a swarmer cycle of ∼80 min (Figures 1B and 1C), consistent with the known replicative asymmetry of daughter cells.

To investigate whether CtrA influences the periodicity of replication, we analyzed replication in cells harboring either a *divL*[ts] or *cckA*[ts] mutation, such that CtrA activity decreases

after shifting cells to a restrictive growth temperature of 34°C [24–26]. Surprisingly, despite the loss of CtrA activity, and consequently cell division, the *divL*[ts] and *cckA*[ts] mutants accumulated chromosomes with an average time between rounds of DNA replication of 65–66 min, nearly identical to that measured in wild-type stalked cells (Figure 1D; Figure S1A).

This unexpected result suggests that CtrA is not required to maintain the periodicity of DNA replication nor does CtrA prevent reinitiation during an ongoing round of replication. With Southern blotting, we verified that the copy-number ratio of origin- and terminus-proximal DNA (*ori/ter* ratio) was unchanged in the *divL*[ts] strain after a shift to the restrictive temperature (Figure 1E). An increase in the *ori/ter* ratio would have indicated that extra rounds of replication initiated before prior rounds had completed (hereafter referred to as overinitiation). Thus, *divL*[ts] and *cckA*[ts] mutants accumulate multiple chromosomes because cells fire successive rounds of DNA
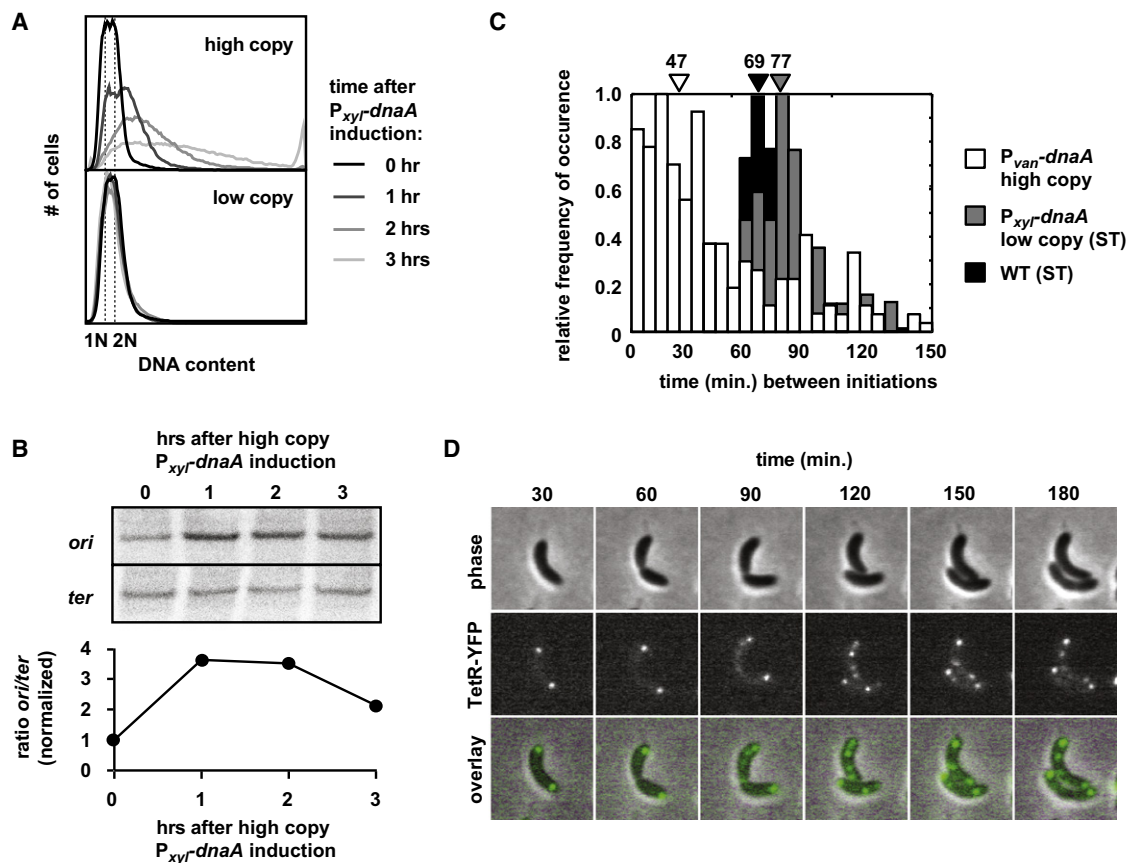
Figure 3. Overproducing DnaA Disrupts Replication Periodicity

(A) Flow cytometry analysis of DNA content in a mixed population of cells overexpressing *dnaA* with a xylose-inducible promoter on a high- or low-copy vector for the times indicated.

(B) Southern blots of DNA near the origin (*ori*) and terminus (*ter*) in cells overexpressing *dnaA* for 0–3 hr. Band intensities were quantified and *ori/ter* ratios normalized to the 0 hr time point.

(C) Histograms of replication cycle times in the high-copy *dnaA* overexpression strain compared to wild-type and low-copy *dnaA* overexpression stalked cells at 30°C. Mean values of each population are indicated above.

(D) Representative time-lapse images showing accumulation of origins over time in high-copy *dnaA*-overexpression cells grown on agarose pads containing 500 μM vanillate at 30°C.

replication without intervening cell divisions and not because of overinitiation. These data are consistent with a recent study showing that mutations in the CtrA binding sites in the origin do not lead to significant overreplication [19].

## CtrA Delays the Reinitiation of DNA Replication in Division-Inhibited Cells

Because *divL*[ts] and *cckA*[ts] cannot divide at the restrictive temperature, we wanted to compare replication in these strains to an FtsZ depletion strain, which also cannot divide but maintains CtrA activity. By using flow cytometry to measure total DNA as a function of time, we observed that DNA accumulated in the *divL*[ts] mutant faster than in cells depleted of FtsZ (Figure S1B). The retention of active CtrA in FtsZ-depleted cells could explain their slower chromosome accumulation relative to *divL*[ts] cells. To test this hypothesis, we measured DNA replication in the FtsZ depletion strain via fluorescence microscopy. Consistent with our flow cytometry data, the intervals between replication events stemming from the stalked pole averaged 80 min, longer than that of *divL*[ts] and *cckA*[ts] cells (Figure 2A). When the FtsZ depletion was combined with the *divL*[ts] mutation, replication intervals decreased to 61 min at the restrictive temperature. Thus, the presence of active CtrA in division-inhibited cells delays replication initiation.

Previously, we found that division-inhibited cells maintain a gradient of phosphorylated CtrA that preferentially inhibits replication at the swarmer pole [24]. Although replication therefore occurs preferentially at the stalked pole in FtsZ-depleted cells, it was still delayed compared to cells without active CtrA, including *divL*[ts], *cckA*[ts], and wild-type stalked cells (Figures 1D and 2A). If this delay depends on the direct binding of residual CtrA~P to the origin at the stalked pole, it should be possible to shorten the replication period in an FtsZ depletion strain by disrupting CtrA binding sites in the origin. We therefore analyzed replication timing in a strain with mutations in three (bc_Ld) of the five CtrA binding sites in the origin (Figure 2A) [19]. In cells depleted of FtsZ and harboring the bc_Ld mutations, stalked replication cycles were shortened from 80 to 68 min. Thus, disrupting CtrA binding to the origin largely restored the normal timing of replication cycles. We conclude that the compartmentalization of cells during cytokinesis is necessary to completely clear CtrA~P from the stalked cell and to prevent a delay in replication that results from residual CtrA binding to the origin.
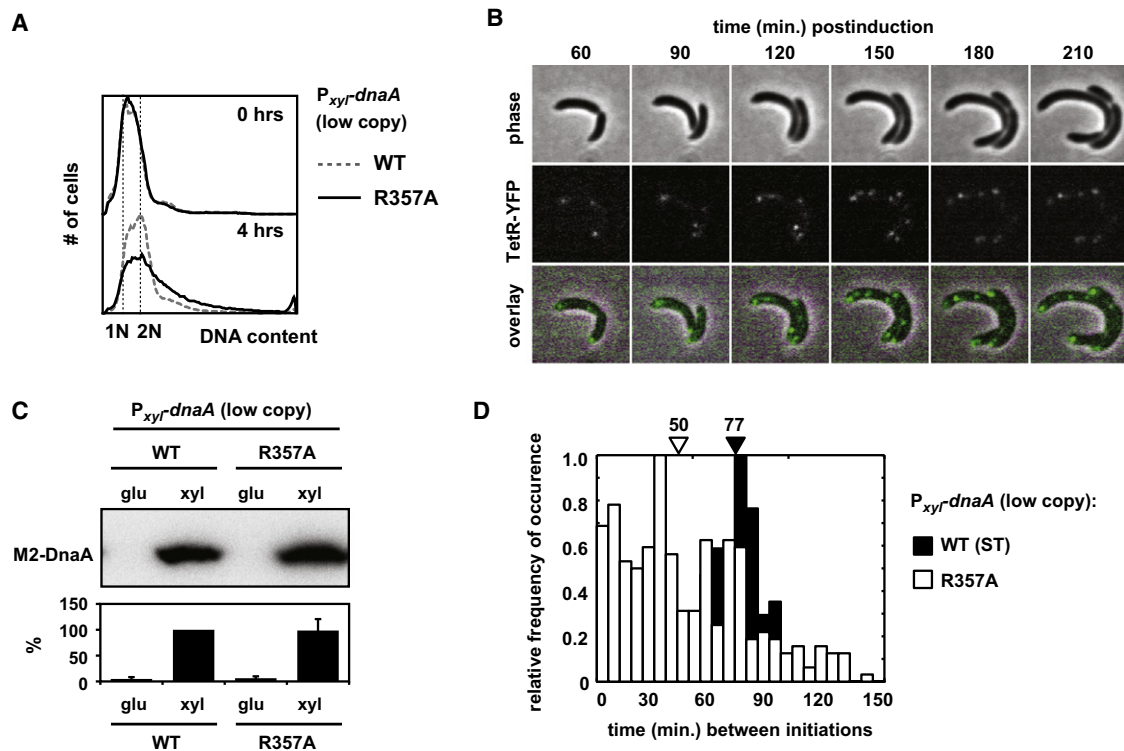
Figure 4. DnaA(R357A) Is Hyperactive and Leads to the Overinitiation of Replication

(A) Flow cytometry profiles showing DNA content in cells expressing wild-type *dnaA* or *dnaA(R357A)* from a xylose-inducible promoter on a low-copy vector for 0 or 4 hr.

(B) Representative time-lapse images of cells expressing *dnaA(R357A)* with TetR-YFP-marked origins in green overlaid onto phase-contrast images.

(C) Western blot (top) and quantification (bottom) showing wild-type M2-DnaA and M2-DnaA(R357A) protein levels after induction with xylose for 4 hr.

(D) Histograms of replication intervals in cells expressing either *dnaA* or *dnaA(R357A)* from a low-copy plasmid in cells grown at 30°C.

We also examined DNA replication in cells harboring the bc$_L$d origin mutations while maintaining native *ftsZ* expression. In this strain, the stalked replication cycle was 64 min, similar to wild-type (Figure 2B). However, the swarmer replication cycle averaged 72 min, 8 min shorter than wild-type swarmer replication cycles (Figure 2B), as bc$_L$d swarmer cells often initiated DNA replication prematurely (defined as an initiation event occurring within one frame of initiation in the sister stalked cell). For swarmer cells that initiated prematurely, DNA replication still continued periodically; consequently, the next round of DNA replication sometimes occurred before cell division, leading to predivisional cells with three origins (Figures S1C–S1E). These cells frequently failed to divide and subsequently became filamentous. Such premature replication, resulting in three origins and cellular filamentation, was never observed in wild-type cells (Figure S1D). Although wild-type swarmer cells occasionally initiated prematurely, these cells did not replicate again before cell division, probably because CtrA can block new rounds of replication, thereby resynchronizing DNA replication with cell division. Taken together, our results suggest that CtrA is important for coordinating DNA replication with cellular differentiation and for enforcing the asymmetric replicative fates of daughter cells but does not significantly affect the intrinsic periodicity of DNA replication.

As an additional test of CtrA's role in replication control, we examined a Δ*pleC* mutant, which produces morphologically symmetric daughter cells [27]. We found that a YFP-CtrA reporter partitions symmetrically in Δ*pleC*, in contrast to the wild-type (Figures 2C and 2D). Consistently, DNA replication in both Δ*pleC* daughter cells occurred with a period of ~73 min, a value intermediate to that of wild-type swarmer (~80 min) and stalked (~67 min) cells (Figure 2E). This finding further supports the notion that the asymmetric accumulation of CtrA in wild-type daughter cells accounts for their differential replication capacities.

**Replication Periodicity Is Governed by DnaA**

If CtrA does not impact the fundamental periodicity of stalked cell replication cycles, another oscillatory control module must exist. We hypothesized that such a module centers on DnaA, an essential, widely conserved positive regulator of replication initiation [8]. To test whether changes in DnaA perturb replication timing, we first examined strains expressing *dnaA* from a xylose-inducible promoter on a high-copy vector. Flow cytometry revealed a substantial accumulation of extra chromosomes per cell within 3 hr of xylose addition (Figure 3A; Figure S2A). Moreover, the *ori/ter* ratio increased ~3.6-fold in this strain within 1 hr after inducing *dnaA*, indicating that new rounds of replication initiated before prior rounds had completed (Figure 3B). Thus, overexpressing *dnaA* in *Caulobacter* causes an overinitiation of DNA replication, as in *E. coli* [28], but in contrast to *divL$^{ts}$* cells (Figure 1D).

We then examined replication timing in a high-copy *dnaA* overexpression strain harboring the TetR-YFP/*tetO* system. Strikingly, DNA replication periodicity was completely lost upon induction (Figure 3C). Many cells initiated multiple rounds of replication within 30 min of a previous initiation
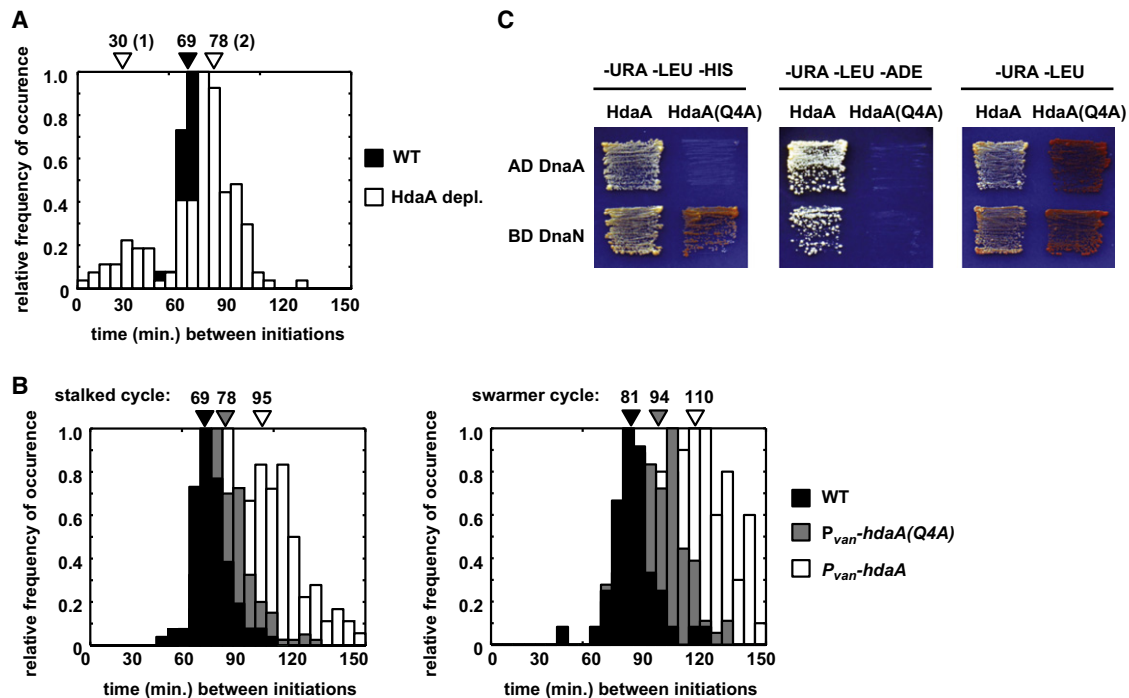
Figure 5. Overproducing HdaA Leads to Slower Replication Cycles

(A) Histograms of stalked replication cycles in the HdaA depletion strain compared to wild-type at 30°C. For the HdaA depletion, replication timing appeared bimodal, so means were calculated separately for cells with replication times shorter or longer than 48 min.

(B) Histograms depicting stalked (left) and swarmer (right) replication cycles in wild-type (black) and in cells in which *hdaA* (white) or *hdaA(Q4A)* (gray) driven by a vanillate-inducible promoter were induced with 2.5 μM vanillate at 30°C.

(C) Yeast two-hybrid assay showing interactions between wild-type and mutant variants of DnaA, HdaA, and DnaN. Yeast containing plasmids encoding the proteins indicated fused to either the activation (AD) or DNA-binding domain (BD) of Gal4 were restreaked on media lacking histidine (−HIS) or adenine (−ADE). Media lacking uracil and leucine (−URA−LEU) selects for plasmid maintenance. Growth and light colony color indicate strong protein interactions, whereas no growth or dark color indicate no or weak interactions.

without an intervening cell division, resulting in the accumulation of 5–6 origins per cell and cellular filamentation (Figure 3D; Figure S2B). In contrast to the high-copy vector, expressing *dnaA* from a xylose-inducible promoter on a low-copy vector did not lead to significant overinitiation (Figures 3A and 3C).

### Cell Cycle-Dependent Regulation of DnaA Is Independent of CtrA

Our finding that *dnaA* overexpression severely disrupted replication periodicity suggests that changes in DnaA activity are critical for temporally regulating replication initiation. A previous model proposed that regulated changes in DnaA transcription and abundance drive cell cycle progression and depend on CtrA; in this model, CtrA promotes DnaA synthesis by driving expression of *ccrM*, a DNA methyltransferase, which methylates the *dnaA* promoter to promote *dnaA* transcription [21]. However, given our observation that replication periodicty is unchanged in cells lacking active CtrA, we expected that the synthesis and hence abundance of DnaA would be independent of CtrA.

To test whether the abundance of DnaA is affected via promoter methylation in *divL^ts* cells, we first measured the methylation status of the chromosome in synchronized populations of cells by Southern blotting. For wild-type, the chromosome alternated between hemimethylated and fully methylated states as the chromosome was replicated and then subsequently methylated de novo (Figure S3A). In *divL^ts* cells, fully methylated DNA disappeared while hemi- and

unmethylated DNA accumulated, confirming that CcrM activity depends on CtrA activity [13] (Figure S3A). However, in contrast to CcrM, DnaA protein levels did not seem to depend strongly on CtrA. Via immunoblotting and quantitative phosphorimaging, we found that DnaA was present at a relatively constant level throughout the cell cycle in *divL^ts* and wild-type cells grown synchronously in rich media at the restrictive temperature of 37°C (Figure S3B). Further, we found that in mixed populations of cells, DnaA levels were comparable in *divL^ts* and wild-type at both the permissive and restrictive temperatures (Figure S3C). The finding that DnaA levels do not depend strongly on CtrA or CcrM is also consistent with a recent study in which the mutation of a putative methylation site in the *dnaA* promoter did not significantly affect expression of a *P_dnaA-lacZ* reporter [29]. Collectively, these data are also consistent with our studies showing that DNA replication continues periodically in cells lacking CtrA activity (Figure 1D).

### Changes in DnaA Activity Govern the Periodicity of DNA Replication

Because DnaA levels do not vary significantly during the cell cycle in rich media (Figure S3B) and because cells constitutively expressing *dnaA* do not overreplicate (Figures 3A and 3C), we hypothesized that oscillations in DnaA activity ultimately determine replication periodicity. In *E. coli*, DnaA switches between an active ATP-bound and an inactive ADP-bound state [6–8]. ATP hydrolysis by DnaA thus prevents

Table 1. Summary of DNA Replication Periodicity Measurements

| Strain | Temp. | Replication Period[a] | n[b] |
|---|---|---|---|
| WT | 30°C | 69 ± 10 (81 ± 13) | 90 (46) |
| WT | 34°C | 67 ± 13 (80 ± 14) | 337 (319) |
| divL[ts] | 34°C | 66 ± 19[c] | 243 |
| cckA[ts] | 34°C | 65 ± 21[c] | 117 |
| ΔpleC | 34°C | 73 ± 14 (73 ± 14) | 178 (175) |
| Cori bc$_L$d | 34°C | 64 ± 17 (72 ± 17) | 395 (331) |
| FtsZ depletion | 34°C | 80 ± 16 | 113 |
| FtsZ depletion, divL[ts] | 34°C | 61 ± 13[c] | 232 |
| FtsZ depletion, Cori bc$_L$d | 34°C | 68 ± 12 | 50 |
| WT + P$_{van}$-dnaA[d] (high copy) | 30°C | 47 ± 35[c] | 228 |
| WT + P$_{xyl}$-M2-dnaA[d] (low copy) | 30°C | 77 ± 15 | 77 |
| WT + P$_{xyl}$-M2-dnaA(R357A)[d] (low copy) | 30°C | 50 ± 33[c] | 280 |
| WT + P$_{van}$-hdaA[d] | 30°C | 95 ± 20 (110 ± 20) | 132 (89) |
| WT + P$_{van}$-hdaA(Q4A)[d] | 30°C | 78 ± 13 (94 ± 15) | 172 (101) |
| HdaA depletion | 30°C | 30 ± 11/78 ± 12[e] | 26/113 |

[a] The mean values with standard deviations are shown for stalked and swarmer (in parentheses) replication periods.

[b] Number of replication cycles counted.

[c] For strains that have lost replicative asymmetry due to a loss of CtrA activity (divL[ts] or cckA[ts]) or that initiate significantly earlier than wild-type, we did not distinguish between stalked and swarmer replication cycles.

[d] For strains harboring plasmids with a xylose- or vanillate-inducible promoter, cells were grown in the presence of 0.3% xylose or 2.5 μM vanillate (for hdaA overexpression) or 500 μM vanillate (for dnaA overexpression), respectively.

[e] Because the distribution for the HdaA depletion strain appeared bimodal, the periods listed are for those cells with replication periods less than or greater than 48 min.

replication reinitiation, and a highly conserved arginine residue, R334, is critical for this hydrolysis [30]. We substituted the corresponding residue, R357, with alanine in *C. crescentus* DnaA (Figure S4) and expressed the resulting mutant from a xylose-inducible promoter on a low-copy plasmid. Unlike cells expressing wild-type *dnaA*, the induction of *dnaA(R357A)* led to an increase in chromosome content per cell and replication overinitiation (Figures 4A and 4B), as in cells overexpressing *dnaA* from a high-copy plasmid. Western blotting indicated that DnaA(R357A) accumulated to similar levels as the wild-type DnaA control after 2 hr of induction (Figure 4C). Quantitative analysis of individual cells confirmed that inducing *dnaA(R357A)* drastically disrupted the periodicity of replication (Figure 4D) with intervals between replication rounds often less than 30 min. These data suggest that the R357A substitution hyperactivates DnaA and that regulated changes in DnaA activity, which are CtrA independent, are important in determining the periodicity of DNA replication.

### HdaA Influences Replication Periodicity, Not Asymmetry, by Interacting Directly with DnaA

ATP hydrolysis by *E. coli* DnaA is stimulated by Hda, a DnaA homolog that also interacts with DnaN, the β-subunit of DNA polymerase [10, 31, 32]. Hda has a homolog in *Caulobacter*, called HdaA, and cells depleted of this protein exhibit a mild accumulation of chromosome content and an increased *ori/ter* ratio [22]. Consistently, when we examined replication in HdaA-depleted cells via fluorescence microscopy, we found that a subpopulation of cells overinitiated, with replication periods less than 48 min (Figure 5A). Taken together, our data suggest that increasing DnaA activity, either by depleting

HdaA or synthesizing DnaA(R357A), leads to shorter intervals between rounds of DNA replication.

To test whether decreasing DnaA activity extends replication intervals, we overexpressed *hdaA* from a vanillate-inducible promoter on a high-copy plasmid. At a vanillate concentration of 2.5 μM, HdaA protein levels were moderately elevated (Figure S5A) and replication intervals were extended to ~95 min in stalked cells and ~110 min in swarmer cells (Figure 5B). These data indicate that overproducing HdaA changes replication periodicity, but, importantly, not asymmetry. We verified that this mild overexpression of *hdaA* did not substantially change DnaA protein levels (Figure S5B), suggesting that the elevated levels of HdaA impact DnaA activity.

To test whether HdaA's effect on replication depends on a direct interaction with DnaA, we searched for a mutation in HdaA that specifically disrupts interaction with DnaA. In *E. coli*, Hda(Q6A) is deficient in binding DnaA and in stimulating ATP hydrolysis, the latter probably stemming from a failure to also interact with DnaN [32, 33]. We engineered a mutant variant of *Caulobacter* HdaA harboring the corresponding substitution, Q4A (Figure S5C). Yeast two-hybrid analysis indicated that HdaA(Q4A), but not wild-type HdaA, was indeed impaired in binding *Caulobacter* DnaA and DnaN (Figure 5C). We then measured the timing of DNA replication initiations in a strain mildly overexpressing HdaA(Q4A) and observed significantly shorter interreplication times compared to isogenic cells overexpressing wild-type HdaA (Figure 5B). Immunoblots indicated that HdaA(Q4A) accumulated with a similar rate and to similar levels as the wild-type HdaA protein (Figure S5A). We conclude that excess HdaA slows down replication cycles by directly modulating DnaA activity, but without affecting the replicative asymmetry of daughter cells.

### Discussion

We found that DNA replication initiation in *Caulobacter* is regulated by two genetically separable modules. One module centers on the essential initiation factor DnaA, which promotes replication initiation. Mutations affecting DnaA activity severely perturbed the timing of replication initiation, with increases and decreases in activity leading to shorter and longer intervals between rounds of replication. The other module involves the essential response regulator CtrA, which silences DNA replication in a cell type-specific manner, ensuring that swarmer cells cannot initiate replication until after differentiating into stalked cells. In stark contrast to DnaA, mutations that reduced or eliminated CtrA activity had almost no effect on the periodicity of DNA replication during the stalked cell cycle of *Caulobacter* (for a summary of all replication periods measured, see Table 1). We conclude that CtrA's role in replication control is mainly to enforce the asymmetry of daughter cells while DnaA serves as the fundamental pacemaker of DNA replication.

The functional separability of these two modules was revealed by examining DNA replication in individual cells lacking active CtrA. In *divL[ts]* and *cckA[ts]* strains, which harbor very little phosphorylated CtrA [24–26], the periodicity of replication was virtually indistinguishable from that of wild-type stalked cells, indicating that DnaA activity oscillates independent of CtrA. However, CtrA activity does not oscillate completely independent of DnaA. Cells depleted of DnaA stop replicating and also fail to activate CtrA through an unknown mechanism
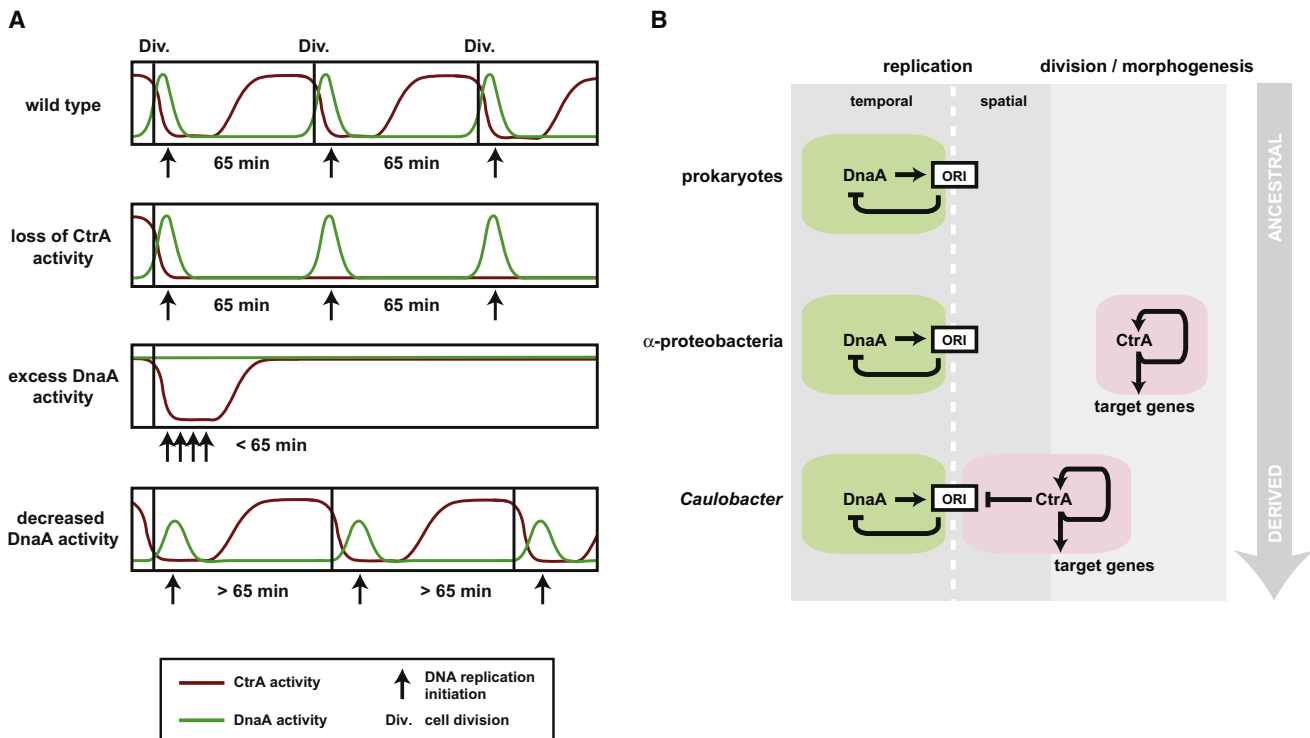
Figure 6. Modularity of Replication Control in *Caulobacter crescentus* Reflects the Evolution of the Bacterial Cell Cycle

(A) Schematic summary of CtrA and DnaA activities during the stalked cell cycle in various strains. For a comparison to the swarmer cell cycle, see Figure S6A.

(B) Evolution of replication control in *Caulobacter.* DnaA is part of an ancestral control module that sets the periodicity of replication in nearly all bacteria. In α-proteobacteria the CtrA module arose to transcriptionally regulate division and polar morphogenesis. In a subgroup of α-proteobacteria including *Caulobacter*, CtrA evolved to bind to the origin, thereby enforcing replicative asymmetry of daughter cells. Although DnaA and CtrA each influence the origin (ORI), each factor is regulated largely independently.

[5, 34]. Thus, the DnaA module entrains the CtrA module, but not vice versa. This coupling of DnaA and CtrA presumably helps cells coordinate DNA replication with the cellular functions controlled transcriptionally by CtrA, such as polar morphogenesis and cell division. Indeed, our results suggest that a failure to coordinate replication initiation with other cell cycle events can have lethal consequences. Mutations that disrupted CtrA binding to the origin often resulted in replication occurring out of phase with other cell cycle events (Figure 2B; Figures S1C–S1E), leading to cell division defects, chromosome accumulation, and a failure to proliferate. Under stressful conditions, the failure to coordinate replication control with cellular development might result in a particularly severe fitness disadvantage [19].

In sum, our data lead to a new model for DNA replication control during the *Caulobacter* cell cycle in rich media (Figure 6A; Figure S6A). In a swarmer cell, CtrA is abundant and phosphorylated such that it can inhibit the initiation of DNA replication. Upon differentiating into a stalked cell, CtrA activity is eliminated, thereby enabling active DnaA-ATP to drive the initiation of DNA replication and promote the activation of newly synthesized CtrA. DnaA is inactivated immediately after replication initiation, probably by the combined action of HdaA and other factors that promote ATP hydrolysis. DnaA activity then reaccumulates approximately 60 min later, through new synthesis and the reloading of DnaA with ATP. If DnaA activity accumulates to high levels before cell division, the abundance of CtrA at this stage will prevent premature

initiation, thereby ensuring that replication occurs once and only once per cell cycle. After cell division, both daughter cells probably inherit active DnaA. In stalked cells, CtrA is rapidly eliminated, enabling DnaA to immediately drive a new round of replication. By contrast, swarmer cells maintain active CtrA, thereby delaying DNA replication until after they differentiate into stalked cells and eliminate CtrA activity.

## Regulated Changes in DnaA Activity Are Important for Replication Periodicity

Our results indicate that changes in DnaA activity are the primary means by which DNA replication timing is controlled during the *Caulobacter* cell cycle. Although the transcription of *dnaA* peaks in G1 [35, 36] and de novo synthesis is probably required to replenish DnaA-ATP levels after initiation occurs, cells constitutively expressing *dnaA* initiated replication with a periodicity similar to wild-type (Figures 3A and 3C). Thus, while the transcription of *dnaA* is necessary for replication, it appears that regulated transcription alone cannot account for the periodicity of initiation. By contrast, mutations that affect the regulation of DnaA activity, but not its abundance, significantly disrupted the timing of replication initiation (Table 1). In *E. coli*, the primary mechanism for inhibiting DnaA, termed RIDA (regulatory inactivation of DnaA), involves direct stimulation of DnaA ATPase activity by Hda [10]. In *B. subtilis*, DnaA activity is regulated by direct interactions with the Soj (ParA), SirA, and YabA [37–40]. Thus, it appears that, like these other bacteria, *Caulobacter* primarily regulates the timing of

replication by modulating DnaA activity, at least in rich medium, the growth condition used here. Regulated changes in DnaA abundance may, however, be important in stressful and nutrient-limited conditions [41].

### Modularity of the Bacterial Cell Cycle

Recognizing the intrinsically modular design of replication control during the *Caulobacter* cell cycle has important implications for understanding the evolution of the bacterial cell cycle. We propose that DnaA lies at the heart of a primordial cell cycle oscillator that has been conserved in virtually all bacteria and that sets the fundamental periodicity of DNA replication in each (Figure 6B; Figure S6B), whereas CtrA was recruited later, in a subset of α-proteobacteria, to establish replicative asymmetry in daughter cells (Figure 6B). CtrA is highly conserved throughout the α-proteobacteria (Figure S6B), where it transcriptionally regulates a range of genes, often involved in polar morphogenesis and cell division [14, 42, 43]. Many, and perhaps most, α-proteobacteria divide asymmetrically and there is evidence that CtrA is often differentially regulated in daughter cells [43–45]. Hence, the evolution of replicative asymmetry in *Caulobacter* and closely related species probably required only the evolution of CtrA binding sites within the origin. The plausibility of this parsimonious scenario is supported by the fact that CtrA binding sites within an origin are thought to have evolved independently at least twice within the lineage leading to the *Caulobacterales* [46]. Also, in the more distantly related α-proteobacteria *Rickettsia*, functional binding sites for the CtrA homolog CzcR were identified in the chromosomal origin of replication [47]. However, whether the binding of CzcR to these sites silences replication is difficult to address because *Rickettsia* grow slowly and only as intracellular parasites of eukaryotic cells.

In sum, we propose that CtrA and the regulatory circuit that controls it arose early within the α-proteobacteria lineage; this CtrA module was subsequently recruited to spatially control DNA replication, without significantly impacting the DnaA-based temporal control of replication (Figure 6B). The net result is a system with two genetically separable modules, as observed here. Our results thus help to provide a unifying view of cell cycle regulation in bacteria with a conserved cell cycle engine built around DnaA. Other modules and components, such as CtrA, have then been integrated with, and are often entrained by, DnaA, but are otherwise autonomous and hence genetically separable. Notably, a modular circuit was also recently proposed to control the cell cycle in *S. cerevisiae*, where multiple regulatory modules, each capable of oscillating on their own, were phase-locked to a central pacemaker [48]. More generally, our findings emphasize the notion that regulatory circuits are not monolithic entities of irreducible complexity but instead are built over time by the relatively straightforward, sequential integration of discrete modules [1, 2, 48].

### Experimental Procedures

Strains, plasmids, and primers used are listed in Table S1. Strain construction and additional experimental procedures are provided as Supplemental Information.

### Time-Lapse Microscopy

Time-lapse movies were performed as previously described [24]. In brief, cells were immobilized on agarose pads, containing 1%–1.5% agarose in PYE. Imaging was performed at 30°C or 34°C with a Zeiss Axiovert 200 microscope with a 63× phase objective fitted with an objective heater (Bioptechs)

and a culture dish heater (Warner Instruments). Images were acquired with the phase and YFP emission/excitation filters in 6 min intervals over at least 4 hr. ImageJ software was used to analyze and process the images (http://rsbweb.nih.gov/ij/). To analyze DNA replication timing, we measured the interval between consecutive DNA replication initiations by using the TetR-YFP/*tetO* system as diagrammed in Figure 1A. The time between replication initiation in a stalked cell and its daughter stalked and swarmer cells are referred to as the stalked and swarmer replication cycles, respectively. In division-inhibited cells, stalked and swarmer cycles were examined by measuring the time interval between replication in a stalked cell and at the stalked and swarmer poles, respectively, of the filamenting cell. For all timing measurements, Matlab (Mathworks) was used to generate histograms of replication times and to calculate the mean values of replication periods.

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