Computational and Genetic Reduction of a Cell Cycle to Its Simplest, Primordial Components

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Abstract

What are the minimal requirements to sustain an asymmetric cell cycle? Here we use mathematical modelling and forward genetics to reduce an asymmetric cell cycle to its simplest, primordial components. In the Alphaproteobacterium Caulobacter crescentus, cell cycle progression is believed to be controlled by a cyclical genetic circuit comprising four essential master regulators. Unexpectedly, our in silico modelling predicted that one of these regulators, GcrA, is in fact dispensable. We confirmed this experimentally, finding that ∆gcrA cells are viable, but slow-growing and elongated, with the latter mostly due to an insufficiency of a key cell division protein. Furthermore, suppressor analysis showed that another cell cycle regulator, the methyltransferase CcrM, is similarly dispensable with simultaneous gcrA/ccrM disruption ameliorating the cytokinetic and growth defect of ∆gcrA cells. Within the Alphaproteobacteria, gcrA and ccrM are consistently present or absent together, rather than either gene being present alone, suggesting that gcrA/ccrM constitutes an independent, dispensable genetic module. Together our approaches unveil the essential elements of a primordial asymmetric cell cycle that should help illuminate more complex cell cycles.

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Abbreviations: PD, Pre-divisitional; ST, Stalked; SW, Swarmer.

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Introduction

Replatively asymmetric cell cycles exist where the two distinct daughter cells resulting from cell division have distinct abilities to replicate their DNA. This is the case for the many Alphaproteobacteria that reproduce by asymmetric binary fission (e.g., Caulobacter and Brevundimonas species) or budding (e.g., Hyphomonas and Hyphomicrobium species) to produce a motile swarmer cell from a nonmotile stalked mother cell [1] and references therein) [2,3]. Swarmer cells do not replicate their DNA; they must first differentiate into stalked cells. During their motile juvenile phase, swarmer cells expend most of their energy on motility and little on growth [4,5]. Caulobacter crescentus [6], a species that is ubiquitous in water, has for many years been used as a model organism for the study of development and the cell cycle. There are also examples of nonstalked bacteria that exhibit the same asymmetry in replication and motility (e.g., Rhodopseudomonas palustris) [7,8]. Indeed, it has been proposed that morphological and functional asymmetry is much more widespread in the Alphaproteobacteria than previously thought [9]. This makes an understanding of asymmetric cell cycle regulation potentially even more relevant. However, the complexity of cell cycle control has made understanding the basic principles difficult. Here, we address this issue by using a minimal modelling approach to determine the core cell cycle regulatory circuit in Caulobacter crescentus.

Asymmetric division in C. crescentus yields a motile daughter swarmer (SW) cell and a sessile stalked (ST) cell. The ST cell immediately reinitiates replication, while the SW cell must differentiate into a ST cell before it can replicate and divide (Figure 1A). These replicative and morphological asymmetries are, in part, controlled by the essential master regulator CtrA through its ability, when activated by phosphorylation (CtrA~P), to interact with DNA regulatory sequences in the origin of replication (oriC) and with many cell-cycle–regulated promoters [10,11]. A second regulator of oriC, DnaA, ubiquitous in bacteria as an essential replication initiator, also targets many cell-cycle–regulated promoters [11,12]. However, though DnaA levels are reduced in SW cells, they can support plasmid replication [13], indicating that it is likely not the regulator of replication asymmetry. This role is played by CtrA~P: high levels inhibit replication in SW cells, whereas low levels in ST cells allow replication to proceed [14]. Instead, DnaA appears to dictate the underlying frequency of replication [15]. Localization of the activator and stabiliser of CtrA, the essential membrane-bound hybrid histidine kinase CckA [16], specifically at the future SW cell pole, ensures a high level of CtrA~P in postdivisive SW cells and its removal in the ST compartment [17]. As C. crescentus regulates temporally both the abundance and activation of CtrA to control cell cycle progression [14], the cell cycle is very robust [18].
Author Summary

Cell cycle regulation is remarkably complex and the fundamental principles difficult to understand, even in simple cells. The bacterium Caulobacter crescentus is a popular model organism to study cell cycle regulation due to the two different daughter cells resulting from cell division: a mobile “swarmer” cell and a “stalked” cell that adheres to surfaces. Here, we use mathematical modelling and genetic experiments to identify the core components of the asymmetric cell cycle of these bacteria. Using our mathematical model we predicted and confirmed experimentally that the transcription factor and cell cycle regulator, GcrA, hitherto thought to be essential, is in fact dispensable. We also identified another master regulator, the methyltransferase, CcrM as dispensable. Furthermore, simultaneous deletion of both GcrA and CcrM removes the severe cell division defects observed on either single deletion, returning cells to near wild-type morphology. We found that GcrA and CcrM constitute an independent, dispensable, genetic module that regulates transcription of cytokinetic proteins during the cell cycle. Phylogenetically, the module is conserved in Alphaproteobacteria, the class of Caulobacter, but is not present in the tree root of the class, suggesting that we have identified the primordial core of the asymmetric cell cycle regulatory circuit in the Alphaproteobacteria.

It has been proposed [19] that cell cycle progression in C. crescentus is controlled by a cyclical genetic circuit of four essential master cell cycle regulator proteins—DnaA, GcrA, CtrA, and CcrM—that are synthesised and degraded sequentially over the cell cycle. Here, we present a minimal mathematical modelling and experimental approach that challenges this assertion. Our model unexpectedly predicts that the “essential” cell cycle regulator GcrA is dispensable for core cell cycle progression. We experimentally test and verify this prediction. In addition, we experimentally uncover the dispensability of another cell cycle regulator, the methyltransferase, CcrM as dispensable. Furthermore, simultaneous deletion of both GcrA and CcrM removes the severe cell division defects observed on either single deletion, returning cells to near wild-type morphology. We found that GcrA and CcrM constitute an independent, dispensable, genetic module that regulates transcription of cytokinetic proteins during the cell cycle. Phylogenetically, the module is conserved in Alphaproteobacteria, the class of Caulobacter, but is not present in the tree root of the class, suggesting that we have identified the primordial core of the asymmetric cell cycle regulatory circuit in the Alphaproteobacteria.

Results

Mathematical Model

Asymmetric cell cycles are dictated by the spatiotemporally varying concentration of a regulatory protein whose presence inhibits DNA replication initiation in the nonreplicating offspring, whereas its absence in the “mother” cell allows replication (or vice versa). This asymmetry must begin at or before the time of compartmentalisation. Since replication factors are generally cytoplasmic and hence likely diffuse and equipartitioned, this suggests the existence of an additional, localised protein that controls the first’s activation and/or stability. With the above regulatory module, a diffuse regulatory protein, and its localised activator/stabiliser, basic regulatory control of an asymmetric cell cycle should be possible. However, previous mathematical models [21–23] of asymmetric cell cycles (e.g., in C. crescentus) have been much more complex and have not made experimentally verified predictions. Here, we therefore develop a simple, but strongly predictive, mathematical model of an asymmetric cell cycle, as applied to C. crescentus, constructed to include only minimal regulatory elements.

The model incorporates GcrA, CckA, and CtrA, but not DnaA or CcrM (see justifications below). The cell cycle regulator GcrA regulates genes involving DNA replication, division, and polar development [24]. Its synthesis is promoted by DnaA (see below) and repressed by CtrA–P. The ctkA gene has two promoters [25]: P1, activated by GcrA [24] but repressed by CtrA–P and silenced by full DNA methylation [26], and P2, a stronger promoter, activated by CtrA–P in a positive feedback loop. Halving of the P2 methylation state (hemi-methylation), with associated subsequent P1 activation, is due to movement of the DNA replication fork through the ctkA locus. This event is short in duration compared to other cell cycle timescales and is therefore modelled as a discrete event through the parameter S, which is switched from 0 to 1 at this time. We take the time at which CtrA–P levels drop below a low threshold as synchronous with the assembly of the replication machinery at Con and take P1 hemi-methylation to occur a fixed time later (the time required for replication initiation and subsequent movement of the replication fork past P1). The DNA methyltransferase CcrM, which has been reported to be essential for viability, remethylates the P1 promoter at the adenine within its GAnTC target site [27]. This remethylation (and as a result, silencing) occurs in late pre-divisonal (PD) cells when P1 is already repressed and after ccrM has been activated by CtrA–P. Therefore, for the model output, it is not relevant exactly when in late PD cells remethylation occurs, and we take it to be synchronous with compartmentalisation (for the ST compartment) or SW-ST differentiation (for the SW compartment), when the value of S is switched back to 0. The vital primary role of CcrM in resetting methylation-dependent promoters, as described above for the ctkA P1 promoter, is essentially discrete. Hence, although switching of P1 methylation is included, we do not explicitly include CcrM in our minimal model.

CckA initiates two phosphorylases leading to the phosphorylation (activation) and stabilisation of CtrA via a phosphotransferase, ChpT [28]. The active phosphorylated form, CckA–P, is localised primarily to the pole opposite the stalk (Figure 1A) [29]. DivL, an essential noncanonical tyrosine kinase, activates, recruits, and co-localises with CckA [30,31], events for which replication initiation is a prerequisite (handled in the model by a dependence on the parameter S) [32]. Upon compartmentalisation, the phosphotransfer from CckA is cut-off in the ST compartment. As a result, CtrA–P is deactivated and removed from the ST cell progeny, while it remains stable and active in the SW cell (Figure 1A). This distinction is the fundamental origin of the asymmetry between the ST and SW cells.

Diffusive exchange of cytoplasmic molecules is largely unimpeded up until the last moments of constriction [33]. Hence we model compartmentalisation as a discrete event again through the discrete parameter S, which is switched from 1 to 0 at compartmentalisation but only in the ST compartment. This ST compartment-specific switch is the origin of the ST/SW asymmetry in the model. In the SW compartment, S is also eventually switched back to 0, but only at a much later time corresponding to the unknown signals initiating SW to ST differentiation. Furthermore, because high CtrA–P levels activate the essential ftsQA cell division operon [34], we can take a high threshold level of CtrA–P as a proxy for compartmentalisation. In summary, our model consists of the minimal regulatory module (CtA and CckA) suggested above, the cell cycle regulator GcrA, the experimentally described ctkA promoter regulation, and three discrete cell cycle events (replication, compartmentalisation, and SW–ST differentiation).
Figure 1. Minimal model of Caulobacter crescentus cell cycle. (A) Schematic of the cell cycle. Localization of (total) CtrA and phosphorylated CckA proteins indicated. (B) Circuit diagram of the mathematical model, reduced from the biological model shown in Figure 5B. Methylation and compartmentalisation are discrete events affecting ctrA transcription and activity of the CckA phosphorelay, respectively. (C) Mathematical description of model: ordinary differential equations and discrete events. See Text S1 for parameter values and justification.

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It has been proposed [19] that methylation of the dnaA promoter by the CcrM methyltransferase promotes dnaA transcription, thereby restarting the cascade of cell cycle regulators with a surge in DNA synthesis. However, mutation of the putative methylation site in the dnaA promoter does not significantly alter promoter activity [35]. It is therefore unknown what leads to the burst in DNA synthesis prior to replication initiation. It has recently been suggested that DNA controls the initiation frequency rather than the acquisition of replication competence [15]. Due to this uncertainty and the focus of our minimal model on asymmetry, we do not explicitly include DnaA in our minimal model; we instead assume that DNA levels rise as CtrA(−→P) levels drop and take a low threshold in CtrA.

We inactivated gcrA and investigated the phenotypes by generalized transduction of a gcrA in-frame deletion mutant into NA1000 wild-type cells harboring pMT335::gcrA [24]. PCR analysis (Figure S4A) and immunoblotting (Figure 2G) confirmed that transductants had exchanged the gcrA gene with the ΔgcrA::Ω allele. Importantly, the pleiotropic phenotypic defects of ΔgcrA::Ω cells (see below) were corrected by pMT335-gcrA, partially corrected by pMT335-gcrB but not by the empty vector (Figure S4B). Overall, these experiments confirm a key prediction of our model that GcrA is essential for cell cycle progression.

Mathematical Model Predicts and Experiments Confirm That GcrA Is Not Essential

Although the above model is simple and reproduces many features of the C. crescentus cell cycle, it is still more complex than our previous minimal two component module. In particular, we have included GcrA and intricate regulation of CtrA through two promoters. Consequently, we pursued a minimal two component model for C. crescentus by removing GcrA and its regulation of ctcA P1. Previously it was reported that GcrA-depleted cells die [24]. Accordingly, in previous models removal of GcrA led to failed cell cycle control. However, strikingly, our minimal in silico model predicted a functional cell cycle without GcrA, albeit with an extended period (Figure S2C). The model predicts that P2 promoter feedback, combined with the active (forward) CckA phosphorelay, is strong enough to raise CtrA levels without P1.

Prompted by these predictions, we revisited previous experimental approaches used to conclude that GcrA is indispensable. We inactivated gze1 by generalized transduction of a Δgze1::Ω allele (conferring spectinomycin resistance) into the NA1000 wild-type (WT) strain and found that colonies appeared after ~6 d in rich medium (Figure 2C). By contrast, transduction into a WT strain harboring pMT335-gze1 gave clones after ~2 d. This 4-d growth delay may explain why gze1 was first described as essential in complex (PYE) medium [24]. On minimal (M2G) medium this delay may be corrected by pMT335-gze1 (Figure S6A,C).

Consequences of GcrA Deletion

We first focused on the cell doubling time during exponential growth, as determined by the optical density of a liquid culture. This time is dependent on the type of growth medium used. We found that the doubling time of ΔgcrA::Ω cells was 75% longer than for the WT in PYE, and 40% longer in M2G, qualitatively consistent with the model (Figure 2D,E). The cells also exhibited a lengthened lag phase (unpublished data). However, ΔgcrA::Ω cells have an origin-to-terminus ratio similar to WT cells, demonstrating that this lengthened doubling time is not due to a defect in DNA replication initiation (Figure 2F, see below). Consistent with these results, fluorescence-activated cell sorting (FACS, Figure S5) revealed an increase in cell length (Figure S5A) and in chromosome number (Figure S5B) in ΔgcrA::Ω versus WT cells. The increase in chromosome number scales with the increase in cell length, which arises from perturbed cytokinesis (Figure 2D,E, see below). Immunoblotting showed a reduction in levels of the MipZ division regulator and the essential late cell division protein FtsN (Figure 2G). Finally, the difference in cell length in ΔgcrA::Ω versus WT cells was attenuated in M2G (176% of WT) compared to PYE (264% of WT), consistent with the respective increases in doubling time described above.

We also observed that Δgze1::Ω cells do not undergo the cell-cycle-regulated switch in buoyancy that is exploited to separate swimmer and stalked cells (Figure S4B) and are poorly motile on soft (0.3% PYE) agar (Figure S1C) and in broth (unpublished data). Additionally, Δgze1::Ω cells are resistant to the S-layer-specific bacteriophage ΦC6K and the pilus-specific bacteriophage ΦC8K (Figure S4B) consistent with reduced levels of the pilin subunit PilA, the polarity factor PodJ (both required for pili assembly), and of the S-layer subunit RsaA in Δgze1::Ω versus WT cells (Figure 2G).

The abundance of CtrA, DnaA, and CcrM was also altered in ΔgcrA::Ω cells: while CtrA was diminished (as expected from our model), the abundances of CcrM and DnaA were elevated (Figure 2G). Importantly, immunoblotting revealed identical defects on protein abundance seen in Δgze1::Ω already after 5 h of GcrA depletion using the xylose-inducible promoter (Pxy). These defects were still present 24 h after depletion but were reversed following re-instatement of gze1 expression for 16 h (Figure S6A,C).

Lack of FtsN Is the Major Cause of Defects in ΔgcrA::Ω Cells

To test if one or a combination of these abnormalities impairs growth of Δgze1::Ω cells, we screened for Δgze1 ΔgcrA::Ω cells mutated with an himar1 transposon (Ts) that form colonies faster than the parent. Backcrossing and mapping identified nine
Figure 2. ΔgcrA::Ω mutant cells are viable but have growth and morphological defects. (A) Immunoblots showing GcrA, CtrA, and PilA (reporter for compartmentalization) steady-state levels for WT cells grown in M2G. (B) Simulated protein levels (solid lines) of GcrA and CtrA in WT cells, averaged over two compartments/cells where appropriate and incorporating imperfect cell cycle synchrony (see Text S1). Times of simulated events are indicated by an arrow (DNA replication initiation), an arrowhead (SW to ST differentiation of the SW daughter cell), a dotted line (ctrA P₁, hemi-methylation), and a dashed line (compartmentalisation). CtrA and GcrA relative protein quantifications from immunoblots are the mean of three biological replicates; error bars are data ranges. Both datasets normalized to maximum ST/PD value. See Figure S1 for simulated profiles in ST and SW compartments and for ctrA promoter expression. (C) Time course of colony appearance following ΦCr30-mediated generalized transduction with ΔgcrA::Ω lysates from a ΔgcrA::Ω xyl-Xgal-gcrA donor strain. Error bars are standard deviation from three biological replicates. Transductants scored on PYE (left panel, blue curves) or M2G (right panel, red curves) media supplemented with spectinomycin (30 μg/ml) and streptomycin (5 μg/ml) to select for ΔgcrA::Ω transduction. Table (right) shows conditions or strains used and number key for corresponding curves. Column (far right) shows whether ΔgcrA::Ω transducing lysate was added to cells or media. Total number of SpcR clones obtained after transduction of cells containing the plasmid (7 and 10) reflects the efficiency of transduction of the ΔgcrA::Ω marker. Relative to this value, 33% in PYE and 96% in M2G of NA1000 transduced cells give SpcR clones, confirming that NA1000 ΔgcrA::Ω colonies are not due to suppressors. (D, E) Differential interference contrast (DIC) micrographs of cells grown in PYE (D) or M2G (E). Scale bar represents 2 μm. Value above micrograph shows doubling time (with standard deviation from at least three biological replicates) for each strain. (F) Relative abundance of replication origin versus terminus (Cori/ter ratio) in WT and mutant cells grown in PYE (blue bars) or M2G (red bars). Ratios normalized to WT value. Triplicate measurements from two independent DNA extractions. Error bars are standard deviation. (G) Immunoblots showing steady-state levels of various proteins in WT and mutant cells in M2G. Lowest row shows levels of acid-extracted RsaA protein in M2G detected by Coomassie Brilliant Blue staining.

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distinct Tn insertions, eight of which were in either the 5' end of ftsN or in the 3' end of the upstream gene, CCN_A_02087 (CC_2088), that reads in the same direction as ftsN (Figure 3A). These eight PftsN::Tn insertions attenuate the growth and division defect of ΔgcrA::Ω cells as demonstrated by DIC imaging (cf., Figure 3D with Figure 2D) and by FACS [Figure S5A,B]. Moreover, immunoblotting revealed that these insertions restore FtsN to near WT levels (Figure 3F), presumably because of an outwardly facing promoter of the Tn directing ftsN transcription. As before, the origin-to-terminus ratios were similar to the WT (Figure 3E). We also confirmed that transduction of ΔgcrA::Ω into WT cells expressing FtsN from Pfms on pMT335 (pMT335-ftsN) or from Pmaz at the chromosomal yulX locus (ΔftsN yulX::Pmaz-ftsN) gave colonies after ~3 d on rich medium (unpublished data), compared to ~6 d for the WT expressing FtsN from the endogenous promoter. Consistent with functional interaction of ftsN and gcrA, accumulation of ftsN mRNA was shown to be GcrA-dependent [24]. Using a lacZ-based transcriptional reporter plasmid, we confirmed that PftsN indeed requires GcrA for full activity. After 5 h and 24 h of GcrA depletion, PftsN-lacZ is reduced
to 52% and 46% of WT activity, respectively (Figure 3G).

Chromatin immunoprecipitation using antibodies to GcrA followed by deep-sequencing (ChiP-Seq) revealed that GcrA binds the ftsN promoter (PftsN in vvo) (Figure 3C), suggesting that ftsN is a direct target of GcrA. In sum, activation of PftsN by GcrA is critical for efficient growth and division, and reduced FtsN levels cause growth defects in ΔgcrA::Ω cells.

ccrM Is Dispensable for Viability

Surprisingly, one Tn of the nine insertions was found in the middle of the ccrM gene (ccrM::Tn, Figure 4A). We performed complementation experiments and found that WT cells expressing ccrM from PccrM on pMT335 (pMT335-RBS-ccrM) formed colonies on PYE ~3 d after transduction of ccrM::Tn compared to ~5 d for WT cells harbouring the empty vector (unpublished data). We also found that ccrM::Tn could be transduced into WT cells on PYE and that genomic DNA extracted from the ccrM::Tn mutant is susceptible to cleavage by the methylation-sensitive restriction enzyme HinFI, as is the case for genomic DNA extracted from ∆ccrM::Ω cells (Figure S7A) [39]. Immunoblotting with antibodies to CcrM provided further confirmation that ccrM::Tn is a null allele (Figure 4A). Therefore, ccrM, like gcrA, is dispensable for viability, consistent with the recent report by Gonzalez and Collier [40]. However, ccrM::Tn colonies take 4 d to form on PYE, similar to ΔcrrM::Ω, and present a lengthened doubling time (Figure 4B). This slow growth rate may explain why ccrM was previously reported to be essential [27]. As before, neither the ΔgcrA::Ω mutation nor the ccrM::Ω mutation affect the origin-to-terminus ratio compared to the WT (Figure 4C).

Tn-Insertions in ccrM Are Greatly Overrepresented in ΔgcrA::Ω Cells as Compared to WT

To quantitatively evaluate the relationship between gcrA and ccrM, we determined the relative frequency of Tn insertions in WT and ΔgcrA::Ω mutant cells by Tn-Seq following himar1 Tn mutagenesis. Tn insertions were hugely overrepresented along the ccrM coding sequence for ΔgcrA::Ω cells compared to the WT (Figure S7B), being ~115 times more abundant than the average of insertions over other coding sequences (Figure 4D). By contrast, Tn insertions in secAB and fisE, both with promoters bound by GcrA in vvo based on ChiP-Seq (Table S1), are underrepresented in ΔgcrA::Ω compared to the WT (Figure 4E). Insertions in the region upstream of fisN were also found to be greatly overrepresented in ΔgcrA::Ω cells compared to the WT (Figure S7C) and were even more frequent than in the ccrM sequence (Figure S7B) consistent with the number and location of the nine insertions found in the Tn suppressor screen (Figures 3A and 4A). We also observed an increased bias in insertions in the gcrA promoter region (Table S3),
confirming the previously observed partial complementation of ΔgcrA:Ω.

**ccrM::Tn Ameliorates the Defects of ΔgcrB ΔgcrA::V Cells**

Returning to the ccrM::Tn insertion found in the screen, we discovered that it greatly improves the cytokinetic defect of ΔgcrB ΔgcrA::V cells (Figure 4B). This was quantified with FACS analyses (Figure S5), which showed a substantial reduction in cell length and chromosome number, and with a variance much closer to WT than that of ΔgcrB ΔgcrA::V PftsN::Tn2 cells. We also observed a reduced lag phase (unpublished data), though there was little or no improvement in the doubling time. The presence of stalked cells

Figure 4. Dispensability and genetic interactions of gcrA-ccrM regulatory module. (A) Immunoblots showing abundance of various proteins in WT and mutant strains in PYE. FtsN relative protein quantifications were normalized to the NA1000 sample value and represent the average and standard deviation of three independent experiments. Schematic shows Tn position in ccrM::Tn allele. (B) Representative DIC micrographs of gcrA and ccrM mutant cells grown in PYE. Leftmost figure reproduced from Figure 2D. Scale bar represents 2 μm. Arrowheads indicate stalks. Values above micrograph show doubling time for each strain (with standard deviation from at least three biological replicates) and, in brackets, the number of days after which the majority of colonies are visible. (C) Relative Ctr/ter ratio in mutant strains grown in PYE (blue bars) or M2G (red bars). Ratios normalized to WT value. Triplicate measurements from two independent DNA extractions. Error bars are standard deviation. (D) Tn insertion bias in coding sequences (CDS) of AgcrA::Ω cells relative to WT cells as determined by Tn-seq. Abscissa shows position as function of genome position, and ordinate gives insertion ratio. Peaks show CDSs with the highest number of Tn insertions. Noncoding sequences are not included (see Figure S7). (E) Inverse ratio shown compared to ratio in (D), with peaks indicating CDSs receiving fewest insertions in ΔgcrA::V cells relative to WT cells. (F) Simulated protein levels of CtrA in ΔgcrA (solid line) and ΔgcrA ΔccrM (dashed line) averaged as in Figure 2B. Both curves normalised to maximum PD value.

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in DIC images indicated that morphological asymmetry is at least partially maintained in ΔgcrB ΔgcrA ΔccrM ΔftsN::Tn cells (Figure 1B). To investigate this further, we examined the localisation pattern of the stalked pole-specific marker SpmX [41] in ΔgcrA ΔccrM ΔftsN::Tn and found a unipolar focus of SpmX-mCherry at the same site as the stalk (Figure S8A), confirming that morphological asymmetry is maintained. Consistent with elevated steady-state levels of P1A (Figure 4A), we also observed that the resistance of ΔgcrA ΔftsN::Tn cells to φCs30 and φChK is diminished by the ΔccrM ΔftsN mutation (Figure S4B). Lastly, we addressed replicative asymmetry by studying the localisation pattern of the centromere-binding protein ParB, which binds to the parS site near the origin of replication. Localisation of GFP-ParB [42] revealed an uneven number of foci in ΔgcrA ΔccrM ΔftsN::Tn cells, consistent with the presence of replicative asymmetry (Figure S8B).

Most importantly, time-lapse imaging of GFP-ParB in ΔgcrA ΔccrM ΔftsN::Tn cells showed asymmetric duplication and segregation of GFP-ParB (Figure S8C), strongly suggesting that replicative asymmetry is maintained in these cells.

ftsN Is Regulated by CcrM Methylation

We next explored the regulatory basis for the Tn insertion enrichment. The results of the suppressor screen and Tn::seq suggest that lack of FtsN is a significant limiting growth factor in ΔgcrB ΔgcrA ΔftsN::Tn cells. Therefore, we reasoned that the recovery due to the ΔccrM ΔftsN::Tn insertion might, at least partially, be mediated through ftsN. Accordingly we noted that PxyN also harbors a GanaTc methylation site. We found in ChiP-seq experiments that a polyclonal antibody to N6-methyladenosine (m6A) [39] precipitated PxyN efficiently from WT but not from ΔgcrA ΔccrM::Tn cells (Figure 3B), indicating that PxyN carries a CcrM-dependent m6A mark overlapping the GcrA target site. To evaluate the importance of this methylation site, we mutated the GanaTc site to GTnTC and found in lacZ-promoter probe assays (Figure 3H) that the mutant (PxyN::Tn) promoter fires only at 14% of the WT rate in PYE (25% in M2G), consistent with reduced FtsN levels when ccrM is disrupted (Figure 4A, lanes 1 and 6). Moreover, PxyN activity doubles by 24 h after depletion of GcrA from ΔgcrB ΔftsN::Tn cells (Figure 3G), consistent with increased FtsN levels in the ΔgcrB ΔgcrA ΔftsN::Tn mutant compared to ΔccrM ΔftsN::Tn (Figure 4A, lanes 5 and 6), while, as noted earlier, the activity of WT PxyN responds in the opposite fashion (Figure 3G). However, the activities, in absolute units, of PxyN and PxyN::Tn 24 h after depletion of GcrA in the ΔgcrB background are very similar (637 ± 6 and 608 ± 6 Miller units, respectively). This would suggest that the methylation state of PxyN has an effect only in the presence of GcrA and that therefore the recovery in FtsN levels observed in ΔgcrB ΔgcrA ΔftsN::Tn cells compared to ΔgcrB ΔgcrA ΔftsN::Tn (Figure 4A, lanes 4 and 5) is probably not mediated through the ftsN promoter (assuming the mutation does not have unwanted side effects on the firing of the core promoter), at least on a low-copy lacZ-reporter plasmid. Because methylation is transient, the net effect on PxyN may result in the same measured activity as PxyN, but the timing of methylation could be important or the results may be skewed due to a low level of GcrA expression being maintained from PxyN even under repressive conditions that is absent in the ΔgcrB ΔgcrA ΔftsN::Tn mutant. However, taken together, these data suggest that ftsN is positively regulated by GcrA in the presence of methylation but negatively regulated in its absence.

FtsN abundance in ΔgcrB ΔgcrA ΔccrM ΔftsN::Tn is still considerably lower than in WT or ΔgcrB ΔgcrA ΔftsN::Tn PxyN::Tn cells, even though the aberrant division of ΔgcrB ΔgcrA ΔftsN::Tn cells is largely repaired. The improved variance in cell length and chromosome number and reduced lag in growth of ΔgcrB ΔgcrA ΔftsN::Tn cells carrying the ΔccrM ΔftsN::Tn mutation (see above) is likely due to the pleiotropic nature of the mutation, elevating the expression of many division genes to some extent. This is consistent with the somewhat raised steady state levels of CtrA and MipZ in ΔgcrB ΔgcrA ΔccrM ΔftsN::Tn compared to ΔgcrB ΔgcrA ΔftsN::Tn (Figure 4A, lanes 4 and 5).

Because ccrM mutants also exhibit a reduction in FtsN abundance compared to WT cells (Figure 4A), we tested if transduction of ΔgcrA ΔftsN::Tn into ΔgcrB ΔftsN::Tn yielded colonies on PYE (with 0.3% xylose) similar to the ΔgcrA ΔftsN::Tn mutation. Transduced ΔgcrB ΔftsN::Tn colonies appeared after ~4 d, whereas transduced WT colonies expressing FtsN from the endogenous promoter only appeared after 5-6 d (unpublished data). Thus, the growth defect of ccrM mutants can be improved by expression of extra FtsN (in addition to extra FtsZ [40]).

The Mathematical Model Provides an Explanation for the Lack of Recovery in Doubling Time

Disruption of ccrM ameliorates the cytokinetic and morphological defects of ΔgcrB ΔgcrA ΔftsN::Tn but not the doubling time (Figures 2D and 4B). Returning to our model, we asked if these experimental findings could be recapitulated. We found in simulations that maintaining cdt P1 in the unmethylated state mimicking the loss of ccrM in ΔgcrA ΔftsN::Tn cells caused only a slight change in cell cycle timing compared to the loss of GcrA alone (Figure 4F), with a ~3% decrease in the swarmer cell cycle period, consistent with the experimentally observed trend (Figure 4B).

Without methylation to suppress early activation, our model suggests that basal cdt P1 transcription in ΔgcrA ΔftsN::Tn cells results in premature synthesis of CtrA and so has a negative effect on cell cycle progression, which cancels the positive effect during CtrA re-accumulation. The same neutral effect on doubling time was also seen in a WT background (Figure S2B) consistent with previous results (Text S1) [26]. Our model therefore provides a possible explanation for why the decrease in cell doubling time is small, even though ΔgcrB ΔgcrA ΔftsN::Tn cells otherwise show substantial phenotypic recovery versus ΔgcrB ΔgcrA ΔftsN::Tn cells.

Discussion

Our combined minimal modelling and forward genetics approach in C. crescentus has unexpectedly uncovered gcrA and ccrM as a dispensable genetic module. Strikingly, the core C. crescentus asymmetric cell cycle network can therefore function without two of the four “master” regulators, revealing a high level of robustness. Though both gcrA and ccrM are highly conserved, it is much more common for both to be present or absent in the alphabacterial lineages rather than either gene being present alone (Figure 5A) [13], which supports our findings. Furthermore, gcrA and ccrM are not present in the tree root of the Alphaproteobacteria, whereas cdtA and cckA are [43], suggesting that our minimal model describes the cell cycle of the primordial Alphaproteobacterium. Within our minimal cell cycle network, we find that asymmetry can be controlled with just two fundamental components: an inhibitor of DNA replication initiation (CtrA) and a localised activator that controls the former’s cell-type–specific activation (CckA). In Figure 5B we present a schematic of the current biological model of cell cycle regulation in C. crescentus with the dispensable GcrA/CcrM module highlighted. Several elements have yet to be fully understood (indicated by question marks): What triggers the pulse in DnaA concentration at the beginning of the cycle? Is this mediated by the Lon protease [44] and/or transcriptional control of dnaA [19]? How are CckA localisation and activation dependent on replication initiation? What are the mechanisms underlying SW cell differentiation?

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**A**

Reducing acell cycle to its primordial components:

- **A** - Diagram showing the relationship between various bacterial species and their genetic elements. The diagram includes a phylogenetic tree highlighting key bacterial genera such as Rhizobiales, Caulobacteriales, Rhodobacteriales, Rhodospirillales, Sphingomonadales, and Rickettsiales. The tree is annotated with numerous genetic elements, including transcription factors and regulatory proteins.

**B**

Transcriptional regulation and phosphorylation/dephosphorylation pathways:

- **B** - Diagram illustrating transcriptional regulation and phosphorylation/dephosphorylation pathways. The diagram features a network of protein interactions, highlighting the roles of transcription factors such as GcrA and CcrM. The pathways are color-coded to distinguish between transcription (black), transpositional regulation (red), phosphorylation/dephosphorylation (green), other interactions (blue), conditional events (dotted lines), methylated sites (*), and degradation (black triangle). The network includes annotated processes such as replication initiation, cell differentiation, and compartmentalization.

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**Key Abbreviations:**
- **ccrM**
- **gcrA**
- **cckA**
- **ctrA**
- **dnaA**
- **clpP**
- **chpT**
- **divL**
- **P1**
- **P2**
- **CcrM**
- **GcrA**
- **DnaA**
- **CtrA**
- **CcrA**
- **Stalked Compartment**
- **Replication Initiation**
- **Swarmer to Stalked Cell Differentiation**

**Genetic Elements:**
- **Transcription**
- **Transpositional Regulation**
- **Phosphorylation/Dephosphorylation**
- **Other Interactions**
- **Conditional Events**
- **Methylation Site**
- **Degradation**
The co-evolution of GcrA and CcrM and our results on 
ftsN regulation are consistent with the recent discovery that promoter 
binding and transcriptional activation by GcrA is methylation-
dependent [39]. Connecting transcriptional activation to chromo-
some position and cell cycle timing (via the hemi-methylation 
cau sed by the passing of the replication fork) potentially 
contributes greatly to cell cycle robustness, an important property 
especially for oligotrophic bacteria. From the point of view of 
the circuit shown in Figure 5B the GcrA/CcrM module could 
contribute to robustness in two ways: (1) Methylation-regulated 
transcription of 
ccrM 
P1 controls the timing and prevents the early 
accumulation of CcrA, which would hinder cell cycle progression; 
(2) GcrA provides an additional connection between DnaA and 
CcrA, resulting in more reliable accumulation and activation of 
CcrA after DNA replication initiation, in addition to the link 
between DNA replication initiation and CckA localisation/ 
activation.

The potential role of the GcrA/CcrM module in the robustness 
of the timing of cell cycle events may also explain why its absence 
is primarily observed in obligate endosymbionts and pathogens, as 
are found in the 
Rickettsiales. These bacteria have a long generation 
time as an adaption to the slower growth of the host cell. For 
example, 
Rickettsia prowazekii 
has a doubling time of about 10 h. 

The success of our approach in reducing an asymmetric cell 
cycle to its simplest, primordial components illustrates the 
potential to define the core regulation of sophisticated cell cycles 
using this strategy. This methodology would be especially useful 
for eukaryotic cell cycles. The complexity of their regulatory 
networks has made understanding the basic principles involved a 
challenging task. Mathematical models of eukaryotic cell cycles, 
such as those of fission and budding yeast [45–47], have made 
limited progress in simplifying the regulatory networks or 
identifying essential, core components. Indeed, progress on 
identifying redundancy in the cell cycle circuitry has been led by 
experiments rather than by modelling [20,48]. We therefore 
expect that our minimal modelling approach could play an 
important role in dissecting these more complex cell cycles.

Materials and Methods

Growth Conditions


caulobacter crescentus NA1000 [49] and derivatives were cultivated 
at 30 °C in peptone yeast extract (PYE) rich medium or in M2 
minimal salts plus 0.2% glucose (M2G) supplemented by 0.4% 
liquid PYE. 

Escherichus coli S17-1 [51] and EC100D (Epicentre 
Technologies, Madison, WI) were cultivated at 37 °C in 
Luria Broth (LB) rich medium. We added 1.5% agar into M2G or PYE 
plates, and motility was assayed on PYE plates containing 0.3% 
agar. Antibiotic concentrations used for 
C. crescentus 
include kanamycin (solid, 20 µg/ml), liquid, 5 µg/ml), tetracycline (1 µg/ml), spectino-
mycin (liquid, 25 µg/ml), spectinomycin/streptomycin (solid, 30 and 
5 µg/ml, respectively), gentamicin (1 µg/ml), and nalidixic acid 
(20 µg/ml). When needed, D-xylose or sucrose was added at 0.3% 
final concentration.

Bacterial Strains, Plasmids, and Oligonucleotides

Bacterial strains, plasmids, and oligonucleotides used in this 
study are listed and described in tables below.

Plasmid Constructions

pNPTS138-Δicators-B-KO. The plasmid construct used for 

gcrB 
(CCNA_01269 or CC_1211) deletion was made by PCR amplifi-
cation of two fragments. The first, a 633 bp fragment flanked by an 
EcoRI site at the 3′ end and a BamHI site at the 3′ end (amplified 
using primers delgcrB_1-BamHI and delgcrB_1-BamHI), encum-
passes the upstream region of 
gcrB 
and extends to 15 bp 
downstream of the predicted start codon. The second fragment, 
flanked by a BamHI site at the 3′ end and a HindIII site at the 3′ end (amplified 
using primers delgcrB_2-BamHI and delgcrB_2-HindIII), harbors the last 6 bp of the 
gcrB 
coding sequence and 
extends 629 bp downstream of the gene. These two fragments 
were first digested with appropriate restriction enzymes and then 
triple ligated into pNPTS138 [M.R.K. Alley, unpublished] that 
had been previously restricted with EcoRI and HindIII.

pNPTS138-Δrsaa. The plasmid construct used for 

rssA 
(CCNA_01059 or CC_1007) disruption was made by PCR 
amplification of a 691 bp fragment that overlaps the 
rssA 
coding sequence (+1,500 to +2,191 relative to the 
Tryptophilia start codon, amplified using primers delrssA-EcoRI and delrssA-HindIII). This 
fragment is flanked by a 
HindIII site at the 5′ end and an EcoRI at the 3′ end, and then cloned into corresponding sites of the 
pNPTS138 after hydrolysis with the appropriate restriction 
enzymes.

pMT335gcrA. The 
gcrA 
coding sequence was PCR amplified from the 
ΔgcrA::P 
PsylX::gcrA 
strain [24] using the 
PsylX and 
gcrA-EcoRI primers. This fragment was digested with 
NdeI/EcoRI and cloned into pMT335 [54].

pMT335gcrB. The 
gcrB 
(CCNA_01269 or CC_1211) coding sequence was PCR amplified from NA1000 using gcrB-NdeI and 
gcrB-EcoRI and cloned into pMT335 using 
NdeI-EcoRI.

pMT335-RBS-ccrM. The 
ccrM 
coding sequence (CCNA_00382 or 
CC_0378) was amplified using ccrM-RBS-EcoRI and 
ccrM-XbaI and cloned into pMT335 using 
EcoRI and 
XbaI. The 
ccrM-RBS-EcoRI primer encoded an optimized RBS to optimize 
CcrM translation.

pMT335-ftsN. The 
ftsN 
(CCNA_02086 or 
CC_0207) coding sequence was PCR amplified from NA1000 using ftsN-NdeI and 
ftsN-EcoRI and cloned into pMT335 using 
NdeI and 
EcoRI.

placZ290-ptsN. The 
ftsN 
promoter region (~469 to +228 relative to the ATG) was PCR-amplified using 
ptsN-EcoRI and 
ptsN-XbaI primers using 
NA1000 chromosomal DNA as a 
template. This fragment was digested by appropriate enzymes and 
cloned into a 
EcoRI-XbaI-digested 
placZ290 promoter vector.

placZ290-ptsN*. Same as previous, using the same 
ptsN-EcoRI and 
ptsN-XbaI primers and synthesized 
ptsN* DNA 
fragment (DNA2.0 Inc, Menlo Park, CA) as a template where the
A position (~52 relative to the ATG) of the G\textsubscript{ATTC} site is replaced by a T.

Strain Constructions
NA1000 \textit{AgerB}. pNPTS138-\textit{AgerB}-KO was first introduced into NA1000 (WT) by intergeneric conjugation and then plated on PYE harboring kanamycin (to select for recombinants) and nalidixic acid to counter select \textit{E. coli} donor cells [50]. A single homologous recombination event at the \textit{CCNA_01269} locus of kanamycin-resistant colonies was verified by PCR. The resulting strain was grown to stationary phase in PYE medium lacking kanamycin. Cells were then plated on PYE supplemented with 3% sucrose and incubated at 30°C. Single colonies were picked and transferred in parallel onto plain PYE plates and PYE plates containing kanamycin. Kanamycin-sensitive cells, which had lost the integrated plasmid due to a second recombination event, were then identified for disruption of the \textit{gcrB} locus by PCR.

NA1000 \textit{Arsad}:pNPTS138. As above, the pNPTS138-\textit{Arsad} plasmid was introduced into NA1000 by intergeneric conjugation. Kanamycin-resistant ex-conjugants having undergone a single homologous recombination event were isolated and the integration was verified by PCR.

NA1000 \textit{AgerB AgerA}:\textit{gcrB} xylX:Psyl-\textit{gcrA}. The \textit{xylX:Psyl-\textit{gcrA}} (\textit{Kan}^\text{R}) construction from LS3707 [24] was first transduced into NA1000 \textit{AgerB} using \textit{gCr30}. Next, the \textit{AgerA}:\textit{gcrB} (\textit{Spc}^\text{R}) allele was then transduced into NA1000 \textit{AgerB} \textit{xylX:Psyl-\textit{gcrA}} and plated on solid PYE containing spectinomycin/streptomycin antibiotics and 0.3% xylose.

NA1000 \textit{ccrM}:\textit{Tn}. The \textit{ccrM}:\textit{Tn} (399278\textsuperscript{R}) insertion (\textit{Kan}^\text{R}) from LT419 was transduced into NA1000 using \textit{gCr30} and plated on solid PYE containing kanamycin.

NA1000 \textit{spmX-mCherry AgerA}:\textit{gcrB} ccrM:\textit{Tn}. The \textit{AgerA}:\textit{gcrB} (\textit{Spc}^\text{R}) allele from LS3707 and \textit{ccrM}:\textit{Tn} (399278\textsuperscript{R}) insertion (\textit{Kan}^\text{R}) from LT419 were successively transduced using \textit{gCr30} into NA1000 \textit{spmX-mCherry} [41].

NA1000 \textit{egfp-parB AgerA}:\textit{gcrB} ccrM:\textit{Tn}. The \textit{AgerA}:\textit{gcrB} (\textit{Spc}^\text{R}) allele from LS3707 and \textit{ccrM}:\textit{Tn} (399278\textsuperscript{R}) insertion (\textit{Kan}^\text{R}) from LT419 were successively transduced using \textit{gCr30} into NA1000 \textit{egfp-parB} [42].

\textit{gCr30} Transduction of the \textit{AgerA} Mutation

\textit{AgerA}:\textit{gcrB} (\textit{Spc}^\text{R}) transducing phage stock is a \textit{gCr30} lysate of LS3707 [24]. Overnight cultures of NA1000 and NA1000 \textit{AgerB} strains harboring or not \textit{pMT335}, \textit{pMT335}\textit{gcrA}, or \textit{pMT335}\textit{gcrB} plasmids were first washed with fresh liquid medium (PYE or M2G) and resuspended at a concentration of 10\textsuperscript{9} cfu/ml. We infected 0.5 ml of cells with 5 \textmu l of \textit{gCr30} phage stock (~10\textsuperscript{10} pfu/ml), incubated them for 2 h at room temperature, and then plated them on solid PYE or M2G containing spectinomycin/streptomycin antibiotics. Plates were incubated at 30°C, and visible colonies were counted each day. Experimental values represent the average of 3 independent DNA extractions. Real-time PCR was performed using the Step-One Real-Time PCR system (Applied Biosystems) at different DNA dilutions (5 \textmu l, with 12.5 \textmu l of SYBR green PCR master mix (Quanta Biosciences)). 0.5 \mu l of each primer (10 \mu M), and 6.5 \mu l of water per reaction. PCR assay parameters were one cycle at 95°C for 5 min followed by 40 cycles at 95°C for 15 s, 55°C for 20 s, and 60°C for 20 s. Dilutions of NA1000 extracted DNA were used to generate \textit{Cori} and \textit{ter} standard curves, involving all \textit{Cori}/\textit{ter} ratios being normalized to the WT value. Average values are from triplicate measurements from two independent DNA extractions.

\beta-Galactosidase Assays
\beta-Galactosidase assays were performed at 30°C as described previously [52,56]. We lysed 50 \mu l of washed cells at OD\textsubscript{600 nm}=0.1–0.6 with chloroform and mixed them with 750 \mu l of \textit{Z buffer} (60 mM \textit{NaHPO}\textsubscript{4}, 40 mM \textit{NaH}_2\textit{PO}_4, 10 mM KCl, and 1 mM MgSO\textsubscript{4}·heptahydrate). We added 200 \mu l of ONPG (4 mg/ml o-nitrophenyl-\beta-D-galactopyranoside in 0.1 M KPO\textsubscript{4}, pH 7.0), and the reaction was timed. When a medium-yellow color developed, the reaction was stopped with 400 \mu l of 1 M Na\textsubscript{2}CO\textsubscript{3}. The OD\textsubscript{420 nm} of the supernatant was determined and the units were calculated with the equation: U = (OD\textsubscript{420 nm} * 1000)/[OD\textsubscript{600 nm} * time (in min) * volume of culture (in ml)]. For \textit{GcrA} depletion experiments in M2G using strain NA1000 \textit{AgerB AgerA}:\textit{gcrB} \textit{gcrA} \textit{xylX}:Psyl-\textit{gcrA}, M2G supplemented with 0.3% xylose overnight cultures were harvested and washed 3 times with M2 minimal salt solution, and then resuspended in appropriate M2G or M2GX medium for 5 or 24 h at 30°C. For the 24 h time point, culture dilutions were done to maintain cells in exponential growth phase. Experimental values represent the averages of four independent experiments.

GcrA Depletion Experiment
For \textit{GcrA} depletion experiments, overnight cultures of strain NA1000 \textit{AgerB AgerA}:\textit{gcrB} \textit{xylX}:Psyl-\textit{gcrA} grown in M2G supplemented with 0.3% xylose were harvested and washed 3 times with M2 minimal salt solution, and then resuspended in M2G (\textit{GcrA} depletion) or M2GX (\textit{GcrA} expression) medium for 2, 5, or 24 h at 30°C (Figure S6A). Then, the 24 h M2G culture was supplemented with 0.3% xylose and incubated with the 24 h M2GX culture for an additional 16 h at 30°C. For the 24 h and 24 h time points, culture dilutions were done to maintain cells in exponential growth throughout the experiment.

Immunoblot Analysis
Protein samples were separated by SDS-PAGE and blotted on PVDF (polyvinylidene fluoride) membranes (Merek Millipore).
Membranes were blocked for 1 h with phosphate buffered saline (PBS), 0.05% Tween 20, and 5% dry milk and then incubated for an additional 1 h with the primary antibodies diluted in PBS, 0.05% Tween 20, 5% dry milk. The different antisera were used at the following dilutions: anti-DnaA (1:20,000) [30], anti-GcrA (1:5,000) [24], anti-FtsZ (1:20,000) [57], anti-MipZ (1:5,000) [42], anti-FodJ (NTD) (1:10,000) [58], anti-FtsN (1:10,000) [59], anti-CtrA (1:10,000) [14], anti-CrM (1:10,000) [27], anti-P1a (1:10,000) [60], and anti-Fik (1:20,000) [61]. The membranes were washed 4 times for 5 min in PBS and incubated for 1 h with the secondary antibody diluted in PBS, 0.05% Tween 20, and 5% dry milk. The membranes were finally washed again 4 times for 5 min in PBS and revealed with Immobilon Western Blotting Chemoluminescence HRP substrate (Merck Millipore) and Super RX-film (Fujifilm). For CtrA and GcrA relative protein quantifications during the cell cycle (Figure 2B), membranes were scanned using the LAS-4000 digital imaging system (Fujifilm) and analyzed with the Multi Gauge V3.0 software. Each protein quantification value was normalized to the ODNA1000 am. Both datasets were normalized to their maximum value. Data represent the averages of three independent synchrony experiments. Each membrane was used for only one antibody detection to avoid any cross-reaction. For FtsN relative protein quantifications (of unsynchronized cultures), datasets were normalized to the NA1000 sample value and represent the average of three independent experiments.

RsA Acid Extraction

Cultures of 5 ml were grown to exponential phase in M2G medium and then harvested. Cells were washed twice with 5 ml of 100 mM HEPES (pH 7.2) and then resuspended in 200 μl of 100 mM HEPES (pH 2). After 10 min of incubation at room temperature, cells were pelleted and removed. The supernatant pH was neutralized by adding 3 μl of 5N NaOH solution. Samples were separated on 7.5% acrylamide SDS-PAGE gel followed by Coomassie Blue staining.

Microscopy

PYE or M2G cultivated cells in exponential growth phase were immobilized using a thin layer of 1% agarose. For time-lapse experiments, LT494 cells in exponential growth phase were immobilized using a thin layer of PYE or M2G cultivated cells in exponential growth phase to allow the comparison of different conditions and to isolate regions of interest. To this end, the genome was subdivided into 50 bp probes, and for every probe an associated value was calculated, a value that derives from the pattern of reads that occurs within the probe region used for the quantitation using the Red Count Quantitation option. To discern between background signal (modeled with a Poisson or negative binomial distribution) and candidate peaks, we calculated the ratio of reads per probe as contents, respectively. Relative chromosome number was directly estimated from the FL-I-A value of NA1000 cells treated with 20 μg/ml Rifampicin for 3 h at 30°C. Rifampicin treatment of cells blocks the initiation of chromosomal replication, but allows ongoing rounds of replication to finish.

Cell Generation Time Determination

Cell growth in PYE or M2G medium was done in an incubator at 30°C under agitation (190 rpm) and monitored at ODNA1000 am. Generation time values were extracted from the curves using the Doubling Time application (http://www.doubling-time.com). Values represent the averages of at least three independent clones.

Chromatin Immunoprecipitation Coupled to Deep Sequencing (ChIP-Seq)

Midlog phase cells cultivated in PYE were cross-linked in 10 mM sodium phosphate (pH 7.6) and 1% formaldehyde at room temperature for 10 min and thereafter on ice for 30 min, then washed three times in PBS, and lysed in a Ready-Lyse lysosome solution (Epigenetix Biotechnologies, Madison, WI) according to the manufacturer’s instructions. Lysates were sonicated (Soniifier Cell Disruptor B-30; Branson Sonic Power Co., www.branson.com) on ice using 10 bursts of 20 s at output level 4.5 to shear DNA fragments to an average length of 0.3–0.5 kbp and cleared by centrifugation at 14,000 rpm for 2 min at 4°C. Lysates were then diluted to 1 ml using ChIP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl plus protease inhibitors (Roche, www.roche.com) and precleared with 80 μl of protein-A agarose (Roche, www.roche.com) and 100 μg BSA. Polyclonal antibodies to GcrA [24] were added to the remains of the supernatant (1:1,000 dilution), incubated overnight at 4°C with 50 μl of protein-A agarose beads pre-saturated with BSA, washed once with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl), and LiCl buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)) and twice with TE buffer (10 mM Tris-HCl (pH 8.1) and 1 mM EDTA). The protein-DNA complexes were eluted in 500 μl freshly prepared elution buffer (1% SDS, 0.1 M NaHCO3), supplemented with NaCl to a final concentration of 300 mM and incubated overnight at 65°C to reverse the crosslinks. The samples were treated with 2 μg of Proteinase K for 2 h at 45°C in 40 mM EDTA and 40 mM Tris-HCl (pH 6.5). DNA was extracted using phenolchloroform:isoamyl alcohol (25:24:1), ethanol-precipitated using 20 μg of glycogen as a carrier, and resuspended in 100 μl of water.

HiSeq 2000 runs of barcoded ChIP-Seq libraries yielded several million reads that were mapped to Caulobacter crescentus NA1000 (NC_011916) according to the ELAND alignment algorithm (services provided by Fasteris SA, Switzerland). The standard genomic positioning format files (BAM) were imported into SeqMonk (Braham http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/, version 0.21.0) to build sequence read profiles. The initial quantification of the sequencing data was done in SeqMonk to allow the comparison of different conditions and to isolate regions of interest. This ended, the genome was subdivided into 50 bp probes, and for every probe an associated value was calculated, a value that derives from the pattern of reads that occurs within the probe region used for the quantitation using the Red Count Quantitation option. To discern between background signal (modeled with a Poisson or negative binomial distribution) and candidate peaks, we calculated the ratio of reads per probe as

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a function of the total number of reads. The overall average read count (for all probes) plus twice the standard deviation was used to establish the lower cut-off that separates the background from candidate peaks. Analyzed data are provided in Table S1 with selected peak values highlighted in yellow. Figure 3C focuses on the \( \text{fs} \)N and \( \text{CCNA}_01269 \) region (2,235,000 to 2,239,000 bp on the \textit{Caulobacter crescentus} genome). Figure 3B used global m6A ChIP-Seq analysis data obtained in the laboratory [39].

Transposon Suppressor Screen and Mapping

An overnight \( \text{Age}B \) \( \text{Age}A::\Omega \) cell culture was grown in PYE and mutagenized with a \( \text{himar}1 \) transposon (\( \text{Tn} \)) [62]. To create the \( \text{himar}1 \) strain collection, transposition was induced by mobilizing the \( \text{himar}1 \) transposon (\( \text{Kan}^R \)) from plasmid pHV414 in \textit{Escherichia coli} (strain S17-1) to the NA1000 \( \text{Age}B \) \( \text{Age}A::\Omega \) strain and selecting for kanamycin-nalidixic acid-resistant \textit{Caulobacter} clones that form colonies faster than the parent at 30°C (\( \sim 5/6 \) d) on PYE. Nine distinct \( \text{Tn} \) clones appearing after \( -3 \) d were selected and already showed, under the microscope, less filamentous defects than the parent strain. Using \( \varphi \text{Cr}30 \)-mediated generalized transduction, the \( \text{Tn} \) insertions were backcrossed in NA1000 \( \text{Age}B \) followed by transduction of the \( \text{Age}A::\Omega \) strain to verify the phenotypes. Chromosomal DNA of the nine selected suppressors was extracted and partially digested for 4 min with \( \text{Hin}P1 \) restriction. Digested DNA was recircularized by T4 DNA ligase (Roche) treatment and then electroporated in \( \text{E. coli} \) EC100D promoter 116 (Epigenic Biotechnologies). Plasmids of kanamycin-resistant clones were extracted and mapped using the himar-Seq2 primer that allowed binning the DNA of all CDS-\( \text{Tn} \) insertions normalized to the gene size was calculated. An average value of all CDS-\( \text{Tn} \) insertion values of 0 and excludes also from the analyses CDS that do not share sufficient statistical \( \text{Tn} \) insertions. This file was used to generate Figure 4D and 4E.

Supporting Information

Figure S1 Simulated \( \text{GcrA}/\text{CtrA} \) profiles. (A, B) Simulated concentrations of \( \text{GcrA} \) and (total) \( \text{CtrA} \) and tracked into the SW (\( \text{A} \)) and ST (\( \text{B} \)) compartments at the single cell level. Concentrations have the same normalisation factor as in Figure 2B. (C) Simulated \( \text{CtrA} \) synthesis from the \( \text{ctrA} \) \( \text{P}_1 \) and \( \text{P}_2 \) promoters (solid lines) is in good qualitative agreement with published plasmid-monitoring expression profiles (points and dashed lines) [26]. Curves are normalised to peak \( \text{ctrA} \) \( \text{P}_2 \) expression. (A–C) Times of simulated events are indicated by arrows (DNA replication initiation), an arrowhead (SW to ST differentiation of the SW daughter cell), dotted lines (\( \text{ctrA} \) \( \text{P}_1 \) hemi-methylated), and dashed lines (compartimentalisation).

Figure S2 Predicted \( \text{GcrA}/\text{CtrA} \) profiles. (A) Simulated concentrations from Figure 2B reproduced for comparison. (B) Simulated \( \text{GcrA} \) and \( \text{CtrA} \) concentrations of synchronised cells with \( \text{ctrA} \) \( \text{P}_1 \) promoter maintained in its hemi-methylated state. The SW cell cycle period is very similar to the WT consistent with [26]. (C) Simulated \( \text{CtrA} \) concentration of synchronised \( \text{Age}A \) cells. The SW cell cycle period is 13% longer than the WT. (A–C) Times of simulated events are indicated as in Figure S1.

Figure S3 Identification of \( \text{gcrA} \) paralog. The \( \text{gcrA} \) coding sequence (Holtzendorff et al., 2004) [24] was blasted (Sbjct) against the \textit{Caulobacter crescentus} genome (\( \text{NC}_011916 \)) using the NCBI online blastx application (http://blast.ncbi.nlm.nih.gov/). The typical result of this query is presented. This analysis allows identification of the \( \text{CCNA}_01269 \) (Query) as a putative GcrA protein paralog, sharing 44% sequence identity and henceforth denoted \( \text{gcrB} \).

Figure S4 Confirmation and phage sensitivity of \( \text{Age}A::\Omega \) cells. (A) Verification of the \( \text{Age}A::\Omega \) deletion after transduction into NA1000 (WT) cells. Colonies appearing after 5–6 d on PYE (upper panel) or M2G (lower panel) plates (supplemented with spectinomycin 30 \( \mu \)g/ml and streptomycin 5 \( \mu \)g/ml) were screened by PCR using primers \( \text{Pro-gcrA} \) and \( \text{gcrA-EcoRI} \), revealing that the majority of the 17 tested colonies had exchanged endogenous \( \text{gcrA} \) with \( \text{Age}A::\Omega \). This primer pair amplifies a 594 bp fragment when endogenous \( \text{gcrA} \) is present, while only a primer dimer band is seen for the \( \text{Age}A::\Omega \) background. As positive (\( \text{C} \)) and negative (\( \text{N} \)) controls, PCR amplifications were also carried out with NA1000 and \( \text{Age}A::\Omega \), \( \text{yl} \)/\( \text{P}_1 \)/\( \text{gcrA} \) cells. (B) Sensitivity of WT and mutant cells to the S-layer specific phage \( \varphi \text{Cr}30 \) and the pilus-specific phage \( \varphi \text{CbK} \). (C) Motility assays of WT and various mutants on PYE soft (0.3%) agar plates. Spot tests on WT and \( \text{gcrA} \) mutants carrying various plasmids (as indicated) are shown in the frame. The first column shows controls with WT, flagellin (\( \text{FlgB} \)), pilus (\( \text{PilD} \)), and rsa1 mutants (\( \text{Rsa} \)). The schematics to the right of the spot test represent test tubes showing the buoyancy of the strain. WT cells give two buoyancies (high and low). The mutants are affected in this property. The thickness of the bands reflects the number of cells obtained with a given buoyancy. (C) Motility assays of WT and various mutants on PYE soft (0.3%) agar plates. Complementation experiments of WT and \( \text{gcrA} \) mutants carrying various plasmids (as indicated) are shown in
the frame. The first column shows negative control of swarming using a flagellin (ΔgfsC) mutant.

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Figure S5 Relative cell size and chromosome number distributions analysed by FACS for NA1000 and various mutants. (A) Relative cell size distributions in NA1000 and various mutants cultivated to exponential phase in PYE or M2G medium were analysed by flow cytometry. Forward scattering values (FSC-A) were used to estimate the cell size. A total of 20,000 cells were analysed for each population, and a density heat map was used to represent the cell population distribution as a function of the FSC-A parameter. Median values are indicated (white lines) and normalized to the median value of NA1000 cultivated in PYE. (B) Same FACS samples acquired in (A) were analysed using the green fluorescence parameter (FL1-A) to estimate the relative chromosome number distribution as a function of the FL1-A parameter. Median values are indicated (white lines).

EPS

Figure S6 Depletion experiment supports the finding that GcrA is not essential. (A) Scheme summarizes the experimental approach used to prepare GcrA depleted samples in (C). NA1000 ΔgcrB ΔgcrA::Ω xylX::PxyI-gfsC grown in M2G supplemented with 0.5% xylose were harvested and washed 3 times with M2 minimal salt solution, and then resuspended in M2G (GcrA depletion) or M2GX (GcrA expression) medium for 2, 5, or 24 h at 30°C. Then, the 24 h M2G culture was supplemented with 0.3% xylose (G+X) and incubated with the 24 h M2GX culture (X) for an additional 16 h at 30°C. For the 24 h and 40 h time points, culture dilutions were done to maintain cells in exponential growth throughout the experiment. (B) Immunoblots showing steady-state levels of various proteins in WT and mutant cells in M2G (reproduced from Figure 2G). (C) Immunoblots showing steady-state levels of various proteins after 2, 5, or 24 h of GcrA depletion in M2G. Red rectangle highlights that 5 h of GcrA depletion are sufficient to reconstruct ΔgcrA::Ω protein steady-state profiles shown in (B). After 24 h of GcrA depletion in M2G, culture was supplemented with 0.3% xylose (G+X) and incubated with the 24 h M2GX culture (X) for an additional 16 h at 30°C to obtain, respectively, X and G+X 40 h samples. Blue rectangle indicates that 16 h of GcrA reinduction restores WT protein steady-state profile, confirming that prolonged depletion of GcrA phenocopies the effect of the ΔgcrB ΔgcrA::Ω strain and that WT protein levels can be restored by reinstatement of GcrA expression. This argues against the possibility that suppressive mutations accumulate in the ΔgcrB ΔgcrA::Ω strain under these conditions.

EPS

Figure S7 Tn-insertion bias at the ccrM and ftsN loci and confirmation that the ccrM::Tn insertion is a null allele. (A) Chromosomal DNA of NA1000 and of some ΔgcrA::Ω and/or ccrM::Tn variants were purified and submitted (+) or not (−) to Hinfl restriction analysis. Hinfl can only cleave unmethylated GAnTc sites. The fact that the ccrM::Tn (as with the ΔccrM::Ω extracted DNA is sensitive to restriction confirms that the ccrM::Tn mutation is a null allele. (B, C) Tn-insertion bias of ΔgexA::Ω (blue) and NA1000 (red) strains determined by Tn-seq at the ccrM (B) and the ftsN (C) loci. Abscissa shows position as function of genome position, and ordinate gives Tn-insertion value. This Tn-Seq approach confirmed the Tn-suppressor screen, Tn-integration accumulating specifically all along the ccrM coding sequence and the ftsN promoter region in ΔgexA::Ω strain compared to the WT.

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Figure S8 The ΔgcrA::Ω ccrM::Tn strain retains morphological and replicative asymmetry. (A) Fluorescence and DIC micrographs of NA1000, ΔgexA::Ω, and ΔgexA::Ω ccrM::Tn, spmX-mCherry cells after growth in PYE. In all three strains, when a stalk structure is visible on the DIC micrograph, the stalked-pole-specific marker SpmX reveals unipolar SpmX-mCherry localization at this site, confirming that elongated ΔgexA::Ω strain and ΔgexA::Ω ccrM::Tn double mutant retain morphological asymmetry. Arrowheads indicate stalks. (B) Fluorescence and DIC micrographs of NA1000, ΔgexA::Ω, and ΔgexA::Ω ccrM::Tn, egfp-parB cells after growth in PYE. Localization of the centromere binding protein GFP-ParB revealed an uneven number of foci in gcrA elongated cells, consistent with replicative asymmetry still being intact. ΔgexA::Ω ccrM::Tn double mutant is equivalent to the WT strain. (C) Fluorescence and DIC time lapse imaging of ΔgexA::Ω ccrM::Tn egfp-parB predivisional cell in PYE. First duplication of GFP-ParB foci in the new stalked compartment argues that ΔgexA::Ω ccrM::Tn double mutant retains replicative asymmetry.

EPS

Table S1 GcrA ChIP-Seq analysis on the NA1000 chromosome. (XLSX)

Table S2 ΔgcrA::Ω/NA1000 CDS Tn-insertion ratios. (XLSX)

Table S3 Global Tn-insertion location values of NA1000 and ΔgcrA::Ω datasets. (XLSX)

Text S1 Supporting experimental procedures. (PDF)

Text S2 SBML file describing the SW cell cycle model. (XML)

Text S3 SBML file describing the ST cell cycle model. (XML)

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: SM GP CF PV MH. Wrote the paper: SM GP CF PV MH.

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