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Methylation-dependent transcriptional regulation of crescentin gene (*creS*) by GcrA in *Caulobacter crescentus*

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Abstract

In *Caulobacter crescentus* the combined action of chromosome replication and the expression of DNA methyl-transferase CcrM at the end of S-phase maintains a cyclic alternation between a full- to hemi-methylated chromosome. This transition of the chromosomal methylation pattern affects the DNA-binding properties of the transcription factor GcrA that controls the several key cell cycle functions. However, the molecular mechanism by which GcrA and methylation are linked to transcription is not fully elucidated yet. Using a combination of cell biology, genetics, and in vitro analysis, we deciphered how GcrA integrates the methylation pattern of several S-phase expressed genes to their transcriptional output. We demonstrated in vitro that transcription of *ctrA* from the P1 promoter in its hemi-methylated state is activated by GcrA, while in its fully methylated state GcrA had no effect. Further, GcrA and methylation together influence a peculiar distribution of *creS* transcripts, encoding for crescentin, the protein responsible for the characteristic shape of *Caulobacter* cells. This gene is duplicated at the onset of chromosome replication and the two hemi-methylated copies are spatially segregated. Our results indicated that GcrA transcribed only the copy where coding strand is methylated. In vitro transcription assay further substantiated this finding. As several of the cell cycle-regulated genes are also under the influence of methylation and GcrA-dependent transcriptional regulation, this could be a mechanism responsible for maintaining the gene transcription dosage during the S-phase.

KEYWORDS

Caulobacter crescentus, CcrM, DNA methylation, GcrA, transcription

1 | INTRODUCTION

Bacterial cells have developed precise mechanisms to control the expression of genes temporally and localize the proteins and other macromolecules in space in the dynamic context of their cellular functions. Signals are transduced into the activation of specific transcription factors, which coordinate a response by regulating the expression of genes, creating a sequential signaling cascade. Temporal regulation of transcription is generally achieved by activation of specific transcription factors that modulate the RNA polymerase. Once proteins are translated, their localization and activity can be spatially maintained by interaction with the localization factors.

In the synchronizable alphaproteobacterium *Caulobacter crescentus* (henceforth *Caulobacter*) the chromosome positions itself in such a manner that the origin of replication (*Cori*) remains near to the old pole and the terminus (*ter*) toward the new pole (Viollier *et al.*, 2004; Umbarger *et al.*, 2011; Le *et al.*, 2013). A set of principal regulators of transcription (CtrA, DnaA, GcrA and CcrM) is responsible during the cell cycle progression for coordinating the fundamental processes such as cell division, polar morphogenesis, and

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chromosome replication (Mohapatra *et al*., 2014). As the cell cycle master regulator CtrA is degraded allowing DnaA to initiate the DNA replication, one of the two nascent chromosomes randomly segregates (Marczynski *et al*., 1990) toward the new compartment that will generate the swarmer cell by the direct interaction of the newly replicated origin of replication (*Cori*) to protein complexes at the new pole. Hence in the predivisional cells the two chromosomes are precisely located so that the origins are at the opposite poles while the terminus region is located approximately at the center of the cell (Viollier *et al*., 2004). The chromosome in *Caulobacter* is methylated on the adenosine of GAnTC sequences by the methyl-transferase CcrM (Gonzalez *et al*., 2014). The movement of the DNA replication forks during the S-phase ensures the transition of the fully methylated chromosome into two hemi-methylated copies, and they remain till the end of S-phase when the DNA methyl-transferase CcrM is produced re-methylating the daughter chromosomes. CcrM, one of the principal regulators of *Caulobacter* cell cycle plays a crucial role in coordinating the cell cycle events with that of chromosome replication (Zweiger *et al*., 1994; Stephens *et al*., 1996). Genes encoding the cell division-associated factors such as MipZ, FtsN, and FtsZ (Quardokus *et al*., 1996; Thanbichler and Shapiro, 2006; Möll and Thanbichler, 2009) are regulated by the CcrM-dependent methylation (Gonzalez and Collier, 2013; Murray *et al*., 2013). In particular, the transcription of *mipZ* and *ftsZ* was shown to be highest in the fully methylated state of their promoters (Gonzalez and Collier, 2013). This conclusion is also supported by the observation that their highest expression levels correspond to the first part of the S-phase in which the replication fork has not yet reached the terminus proximal locations of the two genes (McGrath *et al*., 2007). Moreover, one of the two promoters of cell cycle master regulator *ctrA* is under the control of CcrM-dependent methylation, as full methylation keeps the promoter in a repressed mode (Reisenauer and Shapiro, 2002). The role of full methylation is indeed either positive (*mipZ* and *ftsZ*) or, in contrast, negative for *ctrA*, suggesting that a single regulator possibly acts as an activator or repressor depending on the promoter and its methylation state or, that multiple regulators might be responsible for these opposite methylation-dependent effects. Many other genes are also affected by CcrM-dependent methylation encoding for diverse functions linked to the predivisional stage, such as the polarity factors PodJ (Viollier *et al*., 2002), TipF (Huitema *et al*., 2006), PopZ (Bowman *et al*., 2008; Ebersbach *et al*., 2008), and PleC (Wang *et al*., 1993), motility factors such as FlaY (Purucker *et al*., 1982), or the chromosome partitioning protein ParE (Ward and Newton, 1997; Wang and Shapiro, 2004), and the genes involved in DNA replication such as GyrA and GyrB (Gellert *et al*., 1976). Nevertheless, there are other genes or factors that are controlled by CcrM, such as the stalk formation regulator StaR (Kozdon *et al*., 2013; Gonzalez *et al*., 2014).

Previously, it was shown that the DNA methylation by CcrM influences the transcription of *creS* gene encoding for the intermediate filament Crescentin, as deletion of *ccrM* downregulates the *creS* expression by 1.6-fold (Gonzalez *et al*., 2014). A previous study from our group has shown by Chip-Seq analysis that the cell cycle regulator GcrA binds to the promoter region of the *creS* (Fioravanti *et al*., 2013). This study also unraveled the role of GcrA and CcrM epigenetic module in the regulation of several genes in a cell cycle-dependent manner. GcrA binds to the promoter and modulates the transcription of ca. 50 genes, such as the cell cycle master regulator *ctrA* (Holtzendorff *et al*., 2004; Fioravanti *et al*., 2013), *mipZ*, *ftsZ podJ*, *flaY* etc. (Fioravanti *et al*., 2013; Gonzalez and Collier, 2013; Gonzalez *et al*., 2014; Mohapatra *et al*., 2014) in a CcrM methylation-dependent manner. Interestingly, GcrA also showed differential binding affinities for the promoter regions (e.g., *ctrA*, *mipZ* etc.) according to their methylation status. Even though a general mechanistic model of GcrA-mediated regulation is still missing, interaction of GcrA with the cellular transcriptional machinery (RNA polymerase) has been observed in vitro (Fioravanti *et al*., 2013; Haakonsen *et al*., 2015; Wu *et al*., 2018).

Crescentin is a cytoskeletal protein belonging to the intermediate filament like proteins found in the eukaryotes, and the typical curved shape of the *Caulobacter* cells are attributed to the spatial localization of the crescentin filament toward the inner curvature of the cell (Ausmees *et al*., 2003). Previous studies have shown that the CreS subunits polymerize the forming filaments that interact with the cell membrane and localize asymmetrically toward one of the sides of the cell and this specific localization impedes the cell growth on that side leading to the typical curvature formation (Ausmees *et al*., 2003; Cabeen *et al*., 2009; Charbon *et al*., 2009). Even though the localization and polymerization process of the crescentin are understood to a great detail, the factor responsible for this process is yet to be unraveled. As previously mentioned, spatial localization of the genes in the *Caulobacter* cell might have a role in the eventual assembly of the macromolecular structure, we wanted to further explore this hypothesis using the *creS* (CC3699) as the candidate gene. In the *Caulobacter* genome *creS* is located close to the *Cori* and, therefore, the *creS* locus remains spatially confined to the poles throughout the cell cycle. Interestingly, using RNA-FISH methods it was shown previously that the *creS* transcripts are positioned spatially at the poles coinciding with its genomic location in the cell (Montero Llopis *et al*., 2010). As there is limited information about the transcriptional regulation of *creS*, it would be interesting to explore the link between spatial localization of the *creS* transcripts with that of the crescentin filaments in the cell.

As GcrA acts in S-phase during which the genes are duplicated and the chromosome transitions from a full- to hemi- and back to full methylation state because of a specific temporal expression and degradation of CcrM, we explored how methylation and GcrA are controlling the expression of several genes, chosen from their genomic location (timing of hemi-methylation). In particular we investigated the expression of *creS* by a combination of methods such as fluorescence microscopy, genetics, and in vitro reconstitution of the transcriptional machinery. Our study suggests that, besides a temporal methylation-dependent GcrA transcriptional control, there exists a distinct asymmetry in the localization of transcripts of the genes present in more than one copy during the S-phase, such as *creS*. And this GcrA-mediated asymmetric localization of transcripts

is dependent on the distinct methylation status of their promoter. Furthermore, we provide a model that suggests this epigenetic regulation of gene expression has a role in maintaining a gene expression dosage during the S-phase when more than one copy of several genes is present in the same compartment of the cell.

2 | RESULTS

2.1 | Expression of *creS* depends on GcrA and methylation

We have previously shown that GcrA preferentially binds to a subset of CcrM methylated sites in the *Caulobacter* genome and GcrA-binding affinity depends on the methylation state of their promoters (Fioravanti *et al.*, 2013). Subsequent studies have shown that CcrM-dependent methylation controls the specific expression of hundreds of genes (Gonzalez *et al.*, 2014). This transcriptional activation has been associated with the activation of RNA polymerase subunit σ^{70} by specifically recognizing a subset of methylation sites (Haakonsen *et al.*, 2015).

We first asked whether the GcrA-CcrM module influences the transcriptional regulation of *creS*. As the *Caulobacter* genome transitions from a fully methylated form in the beginning of the cell cycle to a hemi-methylated one, and becomes fully methylated again at the end of the cell cycle, the GcrA-CcrM module encounters changes of the methylation state of each regulated promoter. In this context, the linear distance of any genetic loci from the *Cori* would determine the amount of time it remains hemi-methylated during the S-phase (Figure S1a). *Cori*-proximal genes, such as *creS*, are duplicated at the onset of S-phase and present in two copies that are differently hemi-methylated; terminus-proximal genes are presumably fully methylated as the replication forks have not reached the terminus; finally genes located in the intervening regions on the chromosome would experience a similar time in full methylation and hemi-methylation state.

The gene *creS*, encoding crescentin, is putatively controlled by CcrM-dependent methylation (Gonzalez *et al.*, 2014). The expression of the gene peaks in S-phase and its *Cori*-proximal location suggests that the promoter should be always hemi-methylated when GcrA is expressed and presumably active in this hemi-methylated form. Two methylation sites are indeed present in the region upstream the CDS, however previous mapping of transcriptional start sites (McGrath *et al.*, 2007) revealed that only one methylation site is present in the promoter region (Figure S1b). GcrA is co-expressed with *creS* during the S-phase as previously shown by transcriptomics of a synchronized population (Figure 1a) (McGrath *et al.*, 2007). GcrA binding to the *creS* promoter was detected by Chip-Seq analysis in the previous study from our group (Fioravanti *et al.*, 2013), even though a recent analysis (Haakonsen *et al.*, 2015) did not identify *creS* as a target of GcrA. Interestingly, visual inspection of strains complemented by orthologs of GcrA from *Sinorhizobium meliloti* and *Brucella abortus* revealed an alteration of *Caulobacter* cells curvature

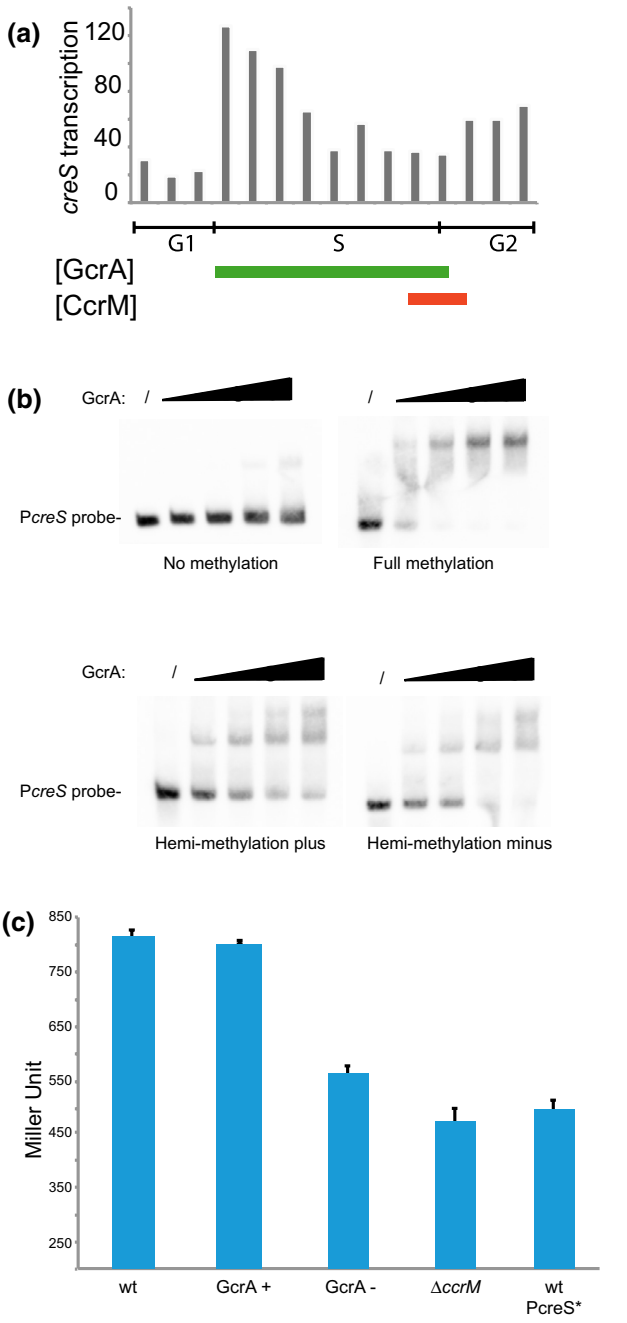

FIGURE 1 GcrA controls *creS* expression. (a) The expression level of *creS* during the S-phase (Zhou *et al.*, 2015). (b) Binding of GcrA with the *creS* promoter in different methylation states was determined by electrophoretic mobility shift assay (EMSA). The *creS* promoter region was incubated with increasing concentrations of purified GcrA. Two slow migrating GcrA-*creS* complexes in the native polyacrylamide gel indicate the interaction. (c) In vivo demonstration of GcrA-dependent transcription of *creS*. The *creS* promoter was fused with β-galactosidase reporter in the plasmid pRKlac290 and transformed into wild type, GcrA depletion, Δ*ccrM*, and P_{creS} methylation site mutant strains. In the four-hour GcrA depletion period, there was a 35% reduction in transcription from the *creS* promoter. Similarly, the *creS* transcription was reduced significantly in the Δ*ccrM* strain and the strain containing P_{creS} methylation site mutation

indicating the role of GcrA in the expression of *creS* (Fioravanti *et al*., 2013). Binding of GcrA to the P_{creS} region in all different methylation states was confirmed by EMSA assay (Figure 1b), which indicated that, as the concentration of GcrA was increased, two slow migrating complexes of GcrA and P_{creS} were formed. As GcrA was shown to dimerize previously (Fioravanti *et al*., 2013) the two complexes may correspond to the monomer and the dimer binding to the promoter region. In particular the methylation form of the promoter that showed the highest affinity was the fully methylated, while the two different hemi-methylated states, although more efficient than the non-methylated probe, showed different affinities possibly suggesting a differential transcriptional efficiency (Figure 1b). In order to demonstrate that GcrA regulates *creS* transcription, we constructed a transcription reporter fusion of P_{creS} with β-galactosidase (pRK-lac290), transformed into wild-type cells and the GcrA depletion strain (Holtzendorff *et al*., 2004). β-galactosidase assay indicated that in the absence of GcrA (4 hrs of depletion), the expression of *creS* significantly decreased by ca. 35% (Figure 1c). We further mutated the methylation site of the *creS* promoter in order to show that the methylation site is required for transcription and we compared the results with the wild-type promoter and a strain where *ccrM* was deleted. Results showed that without GcrA or CcrM methylation the expression of *creS* was reduced to 50% of the wild-type levels (Figure 1c). All these results suggested that the expression of *creS* depends on GcrA and CcrM methylation, leading to a peak of expression in S-phase. As two copies of *creS* with two chemically different hemi-methylation states are present in S-phase, we asked whether they differently responded to GcrA accumulation.

2.2 | Molecular basis of asymmetrical transcriptional activity by GcrA-CcrM module

As EMSA results indicated the differential binding affinity of GcrA toward different methylated forms of *creS* promoter, we explored its effect on the transcriptional activity by reconstituting the transcriptional machinery of the *Caulobacter* cells. As GcrA is present in the cell during the S-phase, we asked how it would possibly affect the transcription output from the two *creS* templates? First, we explored the GcrA-RNA polymerase interaction in vivo, using a strain where GcrA was tagged with the epitope FLAG (EB690). Using co-immunoprecipitation assay several proteins were precipitated along with GcrA (Figure 2a). Following trypsin digestion these proteins were identified by mass spectroscopy-based peptide fingerprinting. We detected the presence of most of the RNA polymerase subunits (β, α, and ω) in the immunoprecipitated samples. The interaction of RNA polymerase with GcrA was previously confirmed by a pull-down assay using a His-tagged GcrA (Fioravanti *et al*., 2013). Consistent with previous findings (Haakonsen *et al*., 2015), the housekeeping sigma factor σ^{70} (RpoD) was also precipitated in our assay suggesting that GcrA interacts with σ^{70} (Figure 2a). However, this putative partner was not particularly enriched in our analysis, pointing our interpretation of a more classic interaction of GcrA with the RNAP complex at the DNA location. Moreover, the presence of the interaction with the RNA polymerase depended on a DNase I pre-treatment (see Materials and Methods), suggesting that DNA may compete out the interaction with σ^{70}. As GcrA presumably acts on σ^{70}-dependent promoters it is reasonable to assume that these genes are constitutively expressed, although modulated by GcrA in S-phase, supporting the nonessential role of GcrA/CcrM (Murray *et al*., 2013).

In order to conclusively demonstrate the effect of GcrA on the transcript output from the differently methylated *creS* promoters, we reconstituted the transcriptional machinery of *Caulobacter* by synthesizing a template 120 bp long of *creS* containing 40 bp of its promoter and ca. 80 nucleotides of the gene (Figures 2b and S1b). Using the RNAP holo-enzyme purified from *Caulobacter* (Figure S3) we obtained transcripts (see Materials and Methods) that were of the expected size (Figure 2b). The *creS* promoter was used as a template in three different methylation states: full methylation, methylation only in the coding strand, and methylation in the noncoding strand. Increasing amounts of purified GcrA were added to the reaction and the results were always compared with the sample with no GcrA. Results showed that GcrA differently reads the templates: transcription from the fully methylated template doesn't change as GcrA increased, while the hemi-methylated templates were both activated by GcrA (Figure 2c). However, the template with the methylation site in the coding strand showed a higher increment at the same concentrations of GcrA than the one with methylation on the noncoding strand. With the increasing concentration of GcrA transcription from P_{creS} methylated in the coding strand increased almost 40-fold (2 μM GcrA), whereas transcription from the template methylated in the noncoding strand increased up to 11-fold (2 μM GcrA). This behavior, although observed in vitro, nevertheless supports the FISH results where one hemi-methylated copy of *creS* is transcribed more than the other (see the next section).

In order to exclude any unspecific effect of methylation states on the RNAP we also tested two other GcrA-dependent promoters (*ctrA* and *mipZ*). Transcription of *mipZ* is activated by full methylation (Gonzalez and Collier, 2013), while *ctrA*P1 transcription is repressed by full methylation (Reisenauer and Shapiro, 2002) but activated by GcrA in hemi-methylated state. Transcription of these two promoters was set up as *creS* with mRNA ca. 80 bp long resulting in RNAs of the expected size (Figure S4a). We then tested the dependency on the methylation (Figure S4b,c). For both genes quantification of the GcrA effect on the transcription resulted in accordance with the in vivo data, as *mipZ* showed that full methylation is the most efficient template (+3-folds) while full methylation is the least efficient one for *ctrA*P1 (−2-folds vs. +3.5-folds of hemi-methylation). The *ctrA* methylation-dependency of transcription may suggest a sharp increase of expression when the promoter goes from a fully methylated state to a hemi-methylated state in which the presence of GcrA convert the promoter from a repressed mode (GcrA completely block the −10 to −35 region) to an activated mode (GcrA binds outside the −35) allowing RNAP to access the promoter (Fioravanti *et al*., 2013).

Finally, we tested whether our in vitro results were fitting with the in vivo data of *ctrA*, *mipZ*, and *creS* expression (Viollier *et al*., 2002;

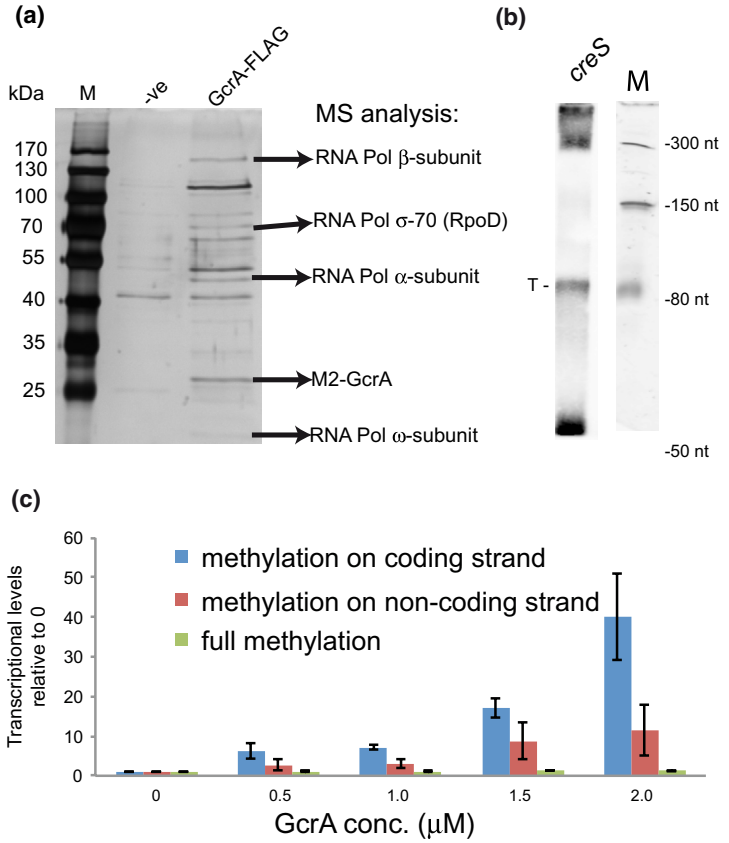

FIGURE 2 GcrA and methylation are responsible for differential *creS* transcript output. (a) GcrA interacts with RNA polymerase holoenzyme as shown in this coimmunoprecipitation assay. Strains expressing GcrA with and without FLAG-tag were used to immunoprecipitate using anti FLAG antibody. The silver stained SDS-PAGE gel indicates the proteins co-immunoprecipitated with GcrA-FLAG. The proteins were identified by mass spectroscopy based peptide fingerprinting and are indicated. (b) In vitro transcription assay to show the *creS* transcription using purified GcrA and RNA polymerase. The transcripts are run along with the RNA size markers. The expected transcript of 80 nucleotides size for the *creS* is observed in the gel. The templates were synthesized and assembled using different combinations of methylation (methylation on coding strand, noncoding strand and full methylation) and used with increasing concentrations of purified GcrA. (C) The plot shows the effect of increasing concentrations of GcrA on the differently methylated probes of *creS* promoter. Three independent experiments were conducted and the transcript intensity was calculated using the ImageJ program, the ratio of transcript intensity at a particular concentration of GcrA to the intensity of transcript without GcrA was calculated and plotted

McGrath *et al.*, 2007). Transcriptional mathematical models of the three different methylation states (Full, hemi-methylation-coding and hemi-methylation-noncoding strands) were constructed based on the gene gate modeling approach as previously described (Blossey *et al.*, 2006; 2008). For all three genes the activation curves fit the model of transcription (Figure S5a). For *creS* and *mipZ*, as GcrA accumulates, no variation in methylation state is observed (*creS* has two hemi-methylated copies, while *mipZ* has only one copy in fully methylated state). However, as also previously reported (Reisenauer and Shapiro, 2002), *ctrA* transcription from the promoter P1 is methylation dependent going from a repressed mode (full methylation) to an activated double copy (hemi-methylated) in the first half of the S-phase. Therefore, we asked if we could fit the delay in activation and reinitiation of transcription of *ctrA* observed in vivo using our in vitro data as GcrA accumulates. At the onset of S-phase CtrA levels are clearly dropping (in order to free the origin of replication) while GcrA levels are rapidly accumulating (due to the DnaA activation of GcrA transcription) (Holtzendorff *et al.*, 2004; Collier *et al.*, 2007). Data of real GcrA and CtrA concentrations were used and compared with the theoretical behavior of the *ctr*AP1 fully methylated template and the two hemi-methylated copies combined together (Figure S5b). Results showed that our in vitro model fully supports the GcrA accumulation in vivo at the onset of S-phase.

2.3 | GcrA and CcrM methylation are responsible for asymmetric localization of *creS* transcripts

Previously, it was shown using RNA-FISH that *creS* mRNAs are localized to the cell poles corresponding to the gene location (Montero Llopis *et al.*, 2010). Interestingly, the study found two spots of *creS* transcripts (corresponding to the two copies of the genes in S-phase) with different intensities localized at the two poles of the cells, however no further study was performed to address how this localization pattern was generated and its significance. Here we explored this issue in order to understand the mechanism responsible for the

spatially organized transcripts of the *creS*. First, we designed RNA-FISH probes for *creS* using the previously described method (Montero Llopis *et al*., 2010). Hybridizing the wild-type *Caulobacter* cells with a *creS-cy3* FISH probes reproduced the results obtained by the above cited study, whereas no signal was detected in a strain where *creS* was deleted (*ΔcreS*) confirming the specificity of our hybridization procedure (Figure 3a). We further explored if the *creS* expression was cell cycle-dependent by collecting samples at different time points in a synchronized population, and observing the transcripts by RNA-FISH. Our results indicated that the *creS* transcripts start to accumulate at the poles at around 20–30 min after synchronization coinciding with the beginning of the S-phase where GcrA becomes available in the cell. As the cell cycle progressed, more and more cells showed localized transcripts of *creS* at the pole (Figure 3b). Toward the second half of S-phase, transcript levels were difficult to detect although GcrA was still present suggesting other repressing mechanisms. For example, CtrA, which accumulates at the same time when *creS* transcription drops, may be responsible for this regulation as CtrA binding sites were also identified in the *creS* promoter region (Murray *et al*., 2013). Fluorescence intensity between 30 and 60 min of the cell cycle was measured along the length of individual cells ($n > 200$ cells), showing different transcripts localization. This analysis indicated that cells possessed a single focus (localized) especially at 45 min, while at 30 and 60 min the fluorescence was more diffused along the length of the cell (diffused) (Figure S2). This result also suggests that localization of FISH signal is not artificially induced by the technique.

2.4 | Localization of *creS* transcript confirms the random segregation of nascent chromosomes

As *creS* transcripts are localized toward the polar regions of the *Caulobacter* cells, we asked whether the localization was possibly stalked- or swarmer-pole specific. In order to map *creS* mRNAs with respect to the poles, we used a *Caulobacter* strain with a polar marker (SpmX) fused with mCherry (Radhakrishnan *et al*., 2010). SpmX is the localization factor for the stalk and therefore it is always present at the stalked (old) pole. Using a synchronized population of the strain expressing SpmX-mCherry we followed the *creS* transcripts using RNA-FISH during the cell cycle. By measuring the FISH signal we found that the localization of the two *creS* transcripts foci were not associated with a specific pole, with the most intense *creS* foci were either toward the old pole (41%) (near the SpmX-mCherry spot) or toward the new pole (40%) (away from the SpmX-mCherry spot). The rest 19% cells showed a more diffused or bipolar localization of the *creS* transcripts (Figure 4a). It's been shown before that the *Caulobacter* DNA segregation is random as the newly formed DNA molecule, having different hemi-methylation patterns, can segregate to any of the two cellular compartments in the pre-divisional cell (Marczynski *et al*., 1990). Our results with the localization of the *creS* transcripts followed a pattern that is consistent with the random segregation of the nascent chromosomes, as the transcripts were distributed almost equally at each of the two poles. This result indeed demonstrates that the intensity difference is not due to the local context of each pole but it could depend on the state of each *creS* promoter. As *creS* expression depends on methylation, this result may suggest that the two hemi-methylation states are responsible for this different transcript pattern.

To further investigate the random localization of the two hemi-methylated copies of the *creS* gene, we constructed strains that were having *creS* in the inverted orientation of the wild type under the control of the native promoter. We hypothesized that since genes are segregating randomly, changing the orientation of the *creS* gene would not affect the localization of the *creS* transcripts, unless the localization of the *creS* mRNAs depends on some genetic determinants close to the *creS* locus. The inverted strains showed

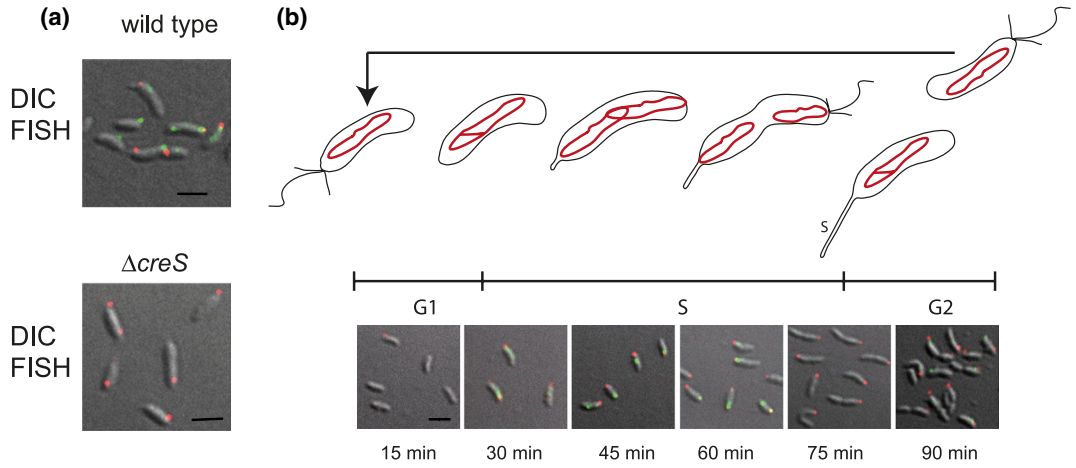

FIGURE 3 The expression of *creS* is cell cycle regulated. Lines in microscopy pictures corresponds to 2 μm. (a) Localization of *creS* transcripts (Green) in the cell. Cy3 tagged *creS* RNA-FISH probes were hybridized with the *Caulobacter* wild type and *ΔcreS* strains to observe the localization pattern. The strains also express a mCherry tagged SpmX protein (Red) that serves as the old pole marker in this experiment. (b) Localization of *creS* transcripts during the cell cycle progression. RNA-FISH experiment was done using the synchronized population of *Caulobacter* cells expressing the SpmX-mCherry. Samples were collected from different times of synchronization as indicated, and hybridized with *creS* RNA-FISH probes. Lines in microscopy pictures corresponds to 2 μm

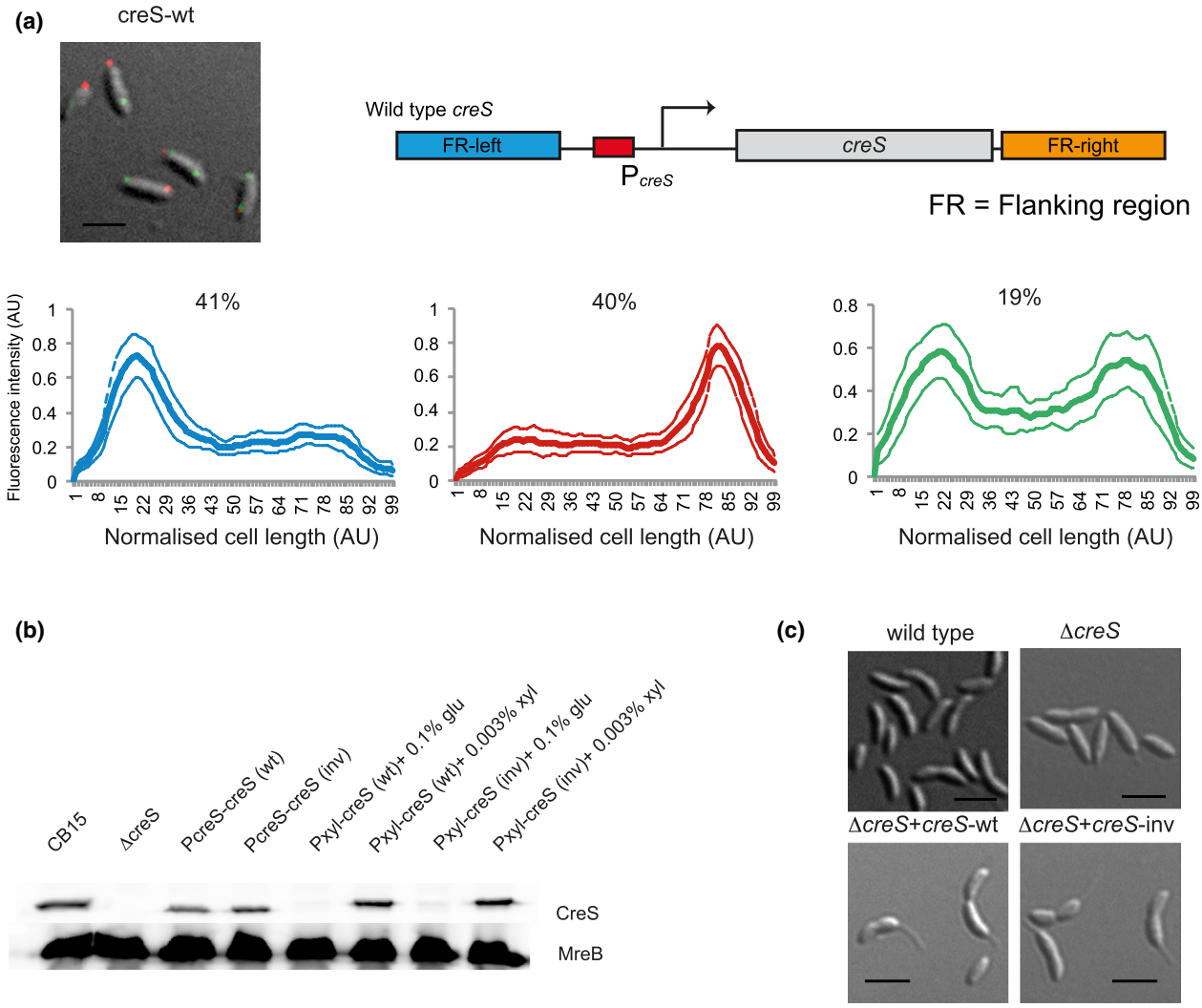

FIGURE 4 Polar transcription and localization of *creS* transcripts. (a) Fluorescence intensity profiling of synchronized *Caulobacter* cells (45 min) to understand *creS* transcript localization. More than 200 cells were measured for the fluorescence intensity across the axial length using ImageJ program and plotted. Cell lengths were normalized to an arbitrary unit 100 and plotted along the X-axis. Similarly, the fluorescence intensity was normalized to an arbitrary unit 1 and plotted on the Y-axis. (b) Immunoblotting to demonstrate the expression of CreS in different genetic backgrounds. (c) The phenotypes of *Caulobacter* cells having different *creS* genotypes. Lines in microscopy pictures corresponds to 2 μm

similar expression levels of CreS as measured by immunoblots in comparison with MreB antibodies as loading control (Figure 4b). The transcription of *creS* in the strains having either *creS-wt* or *creS-inv* was similar to the wild type explaining the full restoration of the wild-type cell curvature (Figure 4c). Therefore, results show that the asymmetric localization of *creS* transcripts depends solely on the *creS* gene.

In order to further demonstrate that this localization pattern of *creS* transcripts depends on the promoter of *creS*, we replaced the promoter with a methylation/GcrA independent promoter (P_{xyl}) (Figure 5a). In the absence of the inducer, the cells looked like the *ΔcreS* strain having rod shaped morphology demonstrating that in these strains the transcription of *creS* depends on xylose. We measured the *creS* mRNA by FISH and found that the signal was still localized toward the cell pole but the two copies of the gene were expressed at a similar level (Figure 5b). Synchronized population of cells (n > 200 cells) expressing *creS* from the P_{xyl} promoter showed 85% cells having bipolar expression, whereas, the rest 15% were monopolar (Figure 5c). The results indicate that asymmetric localization pattern of *creS* transcripts depends on the promoter of *creS*, which is controlled by GcrA and CcrM.

2.5 | Is asymmetric localization of *creS* transcripts required for proper curvature?

As the cytoskeleton protein crescentin is localized toward the inner curvature of the cell, we wanted to understand if the asymmetric localization pattern of *creS* transcripts has a role in the proper assembly and polymerization of crescentin filaments. We ectopically expressed CreS under different conditions. First, we checked the effect of different levels of expression of CreS in the *creS* chromosomal

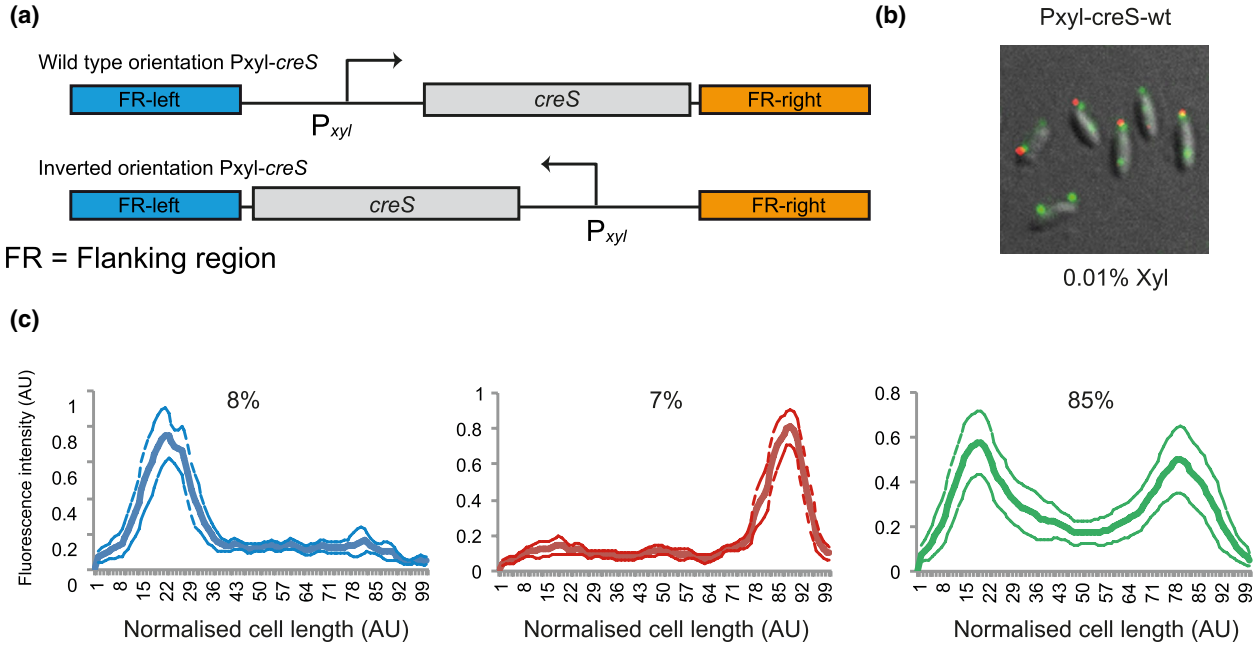

FIGURE 5 Monopolar expression of *creS* depends on its promoter and methylation. (a) Native promoter of *creS* replaced with a xylose inducible (P_{xyl}) one. (b) RNA-FISH to show the expression and localization of *creS* transcripts (green) from the P_{xyl} promoter in xylose induced condition (0.01%), the SpmX-mCherry fusion protein is indicated as red. (c) More than 200 cells are measured for their fluorescence intensity across the length of the cell and plotted as shown. Lines in microscopy pictures corresponds to 2 μm

deletion background using a xylose inducible system integrated in the same locus as wild-type *creS*. Several concentrations of xylose were initially tested, first checking the CreS protein level by western blotting in comparison with wild-type conditions, selecting the growth condition expressing the same amount of CreS as the wild type. As expected, the curvature of cells dramatically changed when comparing wild type, in strain carrying the deletion of *creS* (rod cells) or overexpression of *creS* (Figure 6a,b). Strains with wild-type levels of CreS but having a different genetic arrangement visually look very similar (Figure 6a). In particular, we compared a strain with P_{xyl}-*creS* integrated in the *creS* deleted locus with a strain having the same integration with the native *creS* promoter. The difference between these two strains is, respectively, the bipolar versus monopolar expression of *creS* gene as revealed by FISH in (Figures 4a and 5b). We analyzed more than 1,000 cells all taken after synchronization at 45 min, which corresponds to the highest level of expression of CreS in the wild type. Cell curvature was then calculated and results were plotted (Figure 6c). Analysis showed that in all strains most of the cells have a curvature equivalent to wild type.

3 | DISCUSSION

A model of GcrA transcriptional control has been proposed suggesting that CcrM methylation and the RNA polymerase subunit σ^{70} direct GcrA specific activity to a set of genes, including for example *mipZ* (Haakonsen *et al.*, 2015). However, considering that σ^{70} is constitutively expressed, this model does not explain the relationship between GcrA and the changing methylation pattern, such as for the gene *ctrA* or for the asymmetrical distribution of *creS* transcripts, that most of the genes of the *Caulobacter* chromosome encounters during the S-phase. Here we first focused our investigation on the gene *creS* that is proximal to the origin of replication and present as two different hemi-methylated templates located at the two different poles of the predivisional cell. In fact, during the S-phase, GcrA would hardly encounter the P_{creS} in a full methylated form; by the time GcrA accumulates in the cell P_{creS} would already be in a hemi-methylated state. Due to the GAnTC CcrM methylation sequence, the two copies will not be structurally identical (bases surrounding the methylated nucleotide are different) opening the possibility to a differential transcriptional behavior. This phenomenon has been previously described in bacteria, for example, in *Salmonella enterica* in which the expression of *traJ*, coding a transcriptional activator of the transfer operon, depends on the methylation of a GATC site by the leucine-responsive regulatory protein (Lrp). This regulator binds the hemi-methylated form with the methyl group in the noncoding strand while methylation in the coding strand does not lead to transcriptional activation (Camacho and Casadesús, 2005).

The gene *creS* would remain hemi-methylated for almost the entire length of S-phase till CcrM is again produced and rapidly re-methylates the genome. In case of intermediate genes (equally distant between origin and terminus), such as *ctrA* (located at 3 Mb of the genome that corresponds to ¾ and ¼ of the predivisional cell), promoters with methylation sites will experience a rapid transition between full to hemi-methylation when GcrA is already at the highest level of expression.

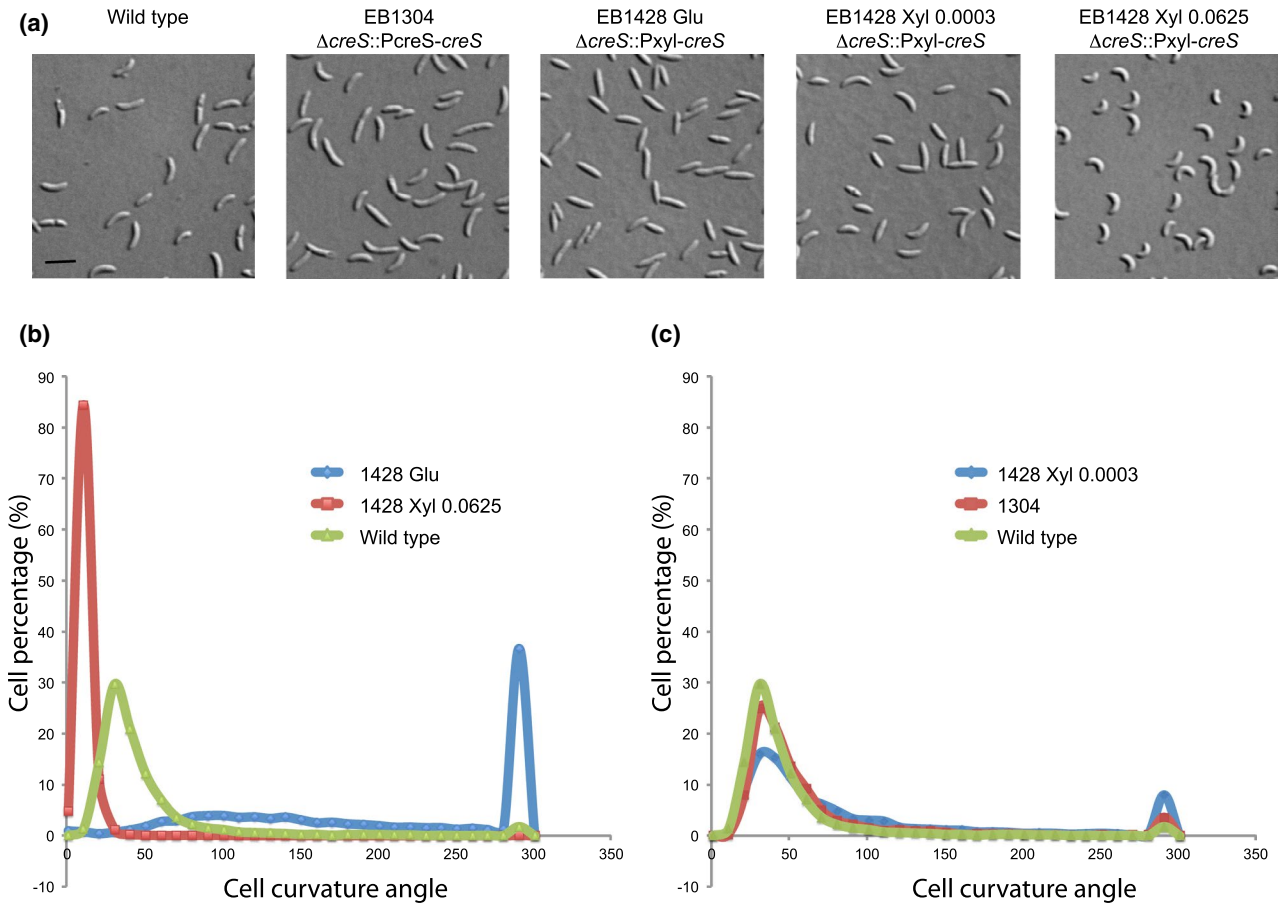

FIGURE 6 Effect of mono-/bi- polar expression of *creS* on the curvature of the cells. (a) Phenotypes (curvature) of cells expressing different levels of CreS in different genetic backgrounds. The first panel shows the wild-type cells. The third, fourth, and fifth panel shows effect of CreS expression from a chromosomally integrated xylose inducible promoter. In presence of glucose the cells show no curvature at all (second panel), whereas mild induction with xylose (0.0003%) produced a curvature pattern very similar to the wild type. With very high induction (0.0625% xylose) the cells became extremely curved (fifth panel). Chromosomal integration of wild-type *creS* in the *ΔcreS* fully reproduced the wild-type phenotype (second panel). (b) Cell curvature pattern of strains showing bipolar expression of CreS. The cell curvature angles were calculated using the method implemented in microbetracker (Sliusarenko *et al.*, 2011) and plotted along with the wild-type strains. (c) Cell curvature pattern of strains showing monopolar expression of CreS. Small angles correspond to highly curved cells while angles around 300° correspond to straight rod shape cells. Lines in microscopy pictures corresponds to 2 μm

Here the in vitro analysis of three distinct GcrA and CcrM methylation-dependent genes, *creS*, *ctrA*, and *mipZ* propose a more general model about the significance of having two layers of control of gene expression. Methylation transition, which depends on gene location and accumulation of GcrA, can be compared with a similar transcriptional control in which only the accumulation of the transcription factor affects gene expression. For *mipZ* the two conditions are in principle identical as the methylation state of the gene/copy number doesn't change during the accumulation of GcrA. However, for *ctr*AP1 and *creS* the consequences are more pronounced. For the *ctr*AP1, we showed that, in presence of GcrA, full methylation doesn't activate its transcription, while at the passage of the replication fork two hemi-methylated copies of *ctrA* are produced resulting in 5× more transcription, leading to a sharp increase of expression. Obviously, this on/off activation suggests that expression of the master regulator CtrA must be fired with a sharp dynamic. For *creS* the existence of a methylation driven transcriptional control has consequences at two levels: (a) dosage control and (b) spatial organization of transcription. The first mechanism derives from the observation in vitro and in vivo that only one hemi-methylated state is transcriptionally active, buffering completely the duplication of the gene *creS*. The second mechanism apparently has no consequences on cell curvature (Figure 6), but it introduces a clear subcellular localization of crescentin transcripts that may have consequences in specific subcellular conditions that have not been deciphered yet. For example, the function of crescentin has been recently associated with the colonization of surfaces (Persat *et al.*, 2014) in which small variations of cell curvature may have a relevant role in the ecology of *Caulobacter* cells. It is interesting to speculate that although polar expression of a single gene may have no specific role, however more genes under the control of methylation and GcrA may create a choreography of expression that may globally modulate developmental functions that are hitherto unknown.

4 | MATERIALS AND METHODS

4.1 | Plasmids and strains construction and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1. *Caulobacter* strains were routinely cultured in peptone-yeast extract (PYE) medium with appropriate amount of antibiotics (Kanamycin 25 μg/ml, Streptomycin 5 μg/ml, Spectinomycin 100 μg/ml, Tetracycline 2 μg/ml) and 0.1% xylose or 0.1% glucose whenever necessary. The cultures were grown at 30 or 37°C as required for different experiments. Synchronization of the *Caulobacter* cells was done using percoll as described before (Marks *et al*., 2010). *E. coli* strains were grown at 37°C in LB broth or solid medium with required amount of antibiotic supplements (Ampicillin 100 μg/ml, Kanamycin 50 μg/ml) as necessary. *Caulobacter* cells were transformed with different plasmids by electroporation. The primers used for cloning and constructions of strains are listed in Table S2.

For P_{xyl}-controlled and inverted *creS* strains, the native *creS* gene was first deleted. Then *creS* gene was integrated both in wild type and inverted orientations. All the different backgrounds (*ΔcreS*, *creS-wt*, and *creS-inv*) were moved into *Caulobacter* CB15N and the strain expressing SpmX-mCherry fusion by transduction. *ΔcreS* strain lost its typical curvature and became rod shaped (Figure 4c), as has been observed earlier (Ausmees *et al*., 2003).

The data that supports the findings of this study are available in the supplementary material of this article.

4.2 | β-galactosidase reporter assay

β-galactosidase reporter assays were done at 30°C to check the role of GcrA on the transcription of *creS* following the protocol described previously (Huitema *et al*., 2006; Fioravanti *et al*., 2013).

4.3 | Fluorescent in situ hybridization (FISH) & microscopy

RNA-FISH experiments were conducted following the protocols described previously (Russell and Keiler, 2009; Montero-Llopis *et al*., 2010) with few modifications. The method is described as follows. *Caulobacter* cells grown up to mid-exponential phase or isolated from different stages of a synchronized population were fixed with 4% formaldehyde (in 1× PBS, pH 7.4) for 15 min at room temperature followed by 30 min on ice. Then cells were briefly centrifuged and supernatant removed. The pellet washed thrice with 1× PBS + 0.05% Tween 20, followed by once with 1× PBS. Cells resuspended in 1× PBS. To a clean and sterile cover slip (round ones) 10 μl poly-L-lysine (Sigma) applied and kept at room temperature for 10 min. Excess poly-L-lysine was removed with kim-wipes. Then 10 μl of cell suspension was added and kept at room temperature for 10 min. Excess liquid removed with kim-wipes. To the coverslip with attached cells, 100 μl cold methanol (−20°C) was added and incubated for 1 min. Methanol removed slowly with micropipette and then, 100 μl cold acetone (−20°C) was added and kept for 30 s. Acetone was removed with micropipette. Coverslips were kept in open to become dry. Pre-hybridization and hybridization were set up in small petri dishes, each containing a single coverslip, and the petridishes were kept in a humidified chamber incubated at the required temperature. Pre-hybridization was done by adding 100 μl of pre-hybridization buffer (40% formamide in 2× SSC) to each coverslip and incubating at 37°C for 1 hr. RNA-FISH probes for *creS* were mixed with 25 μl of hybridization buffer I (2× SSC, 80% formamide, 70 μg/ml Salmon Sperm DNA, 1 mg/ml *E. coli* tRNA) to a concentration 250 nM and heated at 65°C for 5 min, to which equal volume (25 μl) of hybridization buffer II [2× SSC, 20% Dextran Sulfate, 10 mM Vanadium Ribonucleoside Complex (VRC) (NEB), 0.2% BSA, 40 U RNase Inhibitor] was added. 50 μl of the hybridization buffer added to each coverslip and the whole humidified chamber was incubated at 37°C for overnight. Following the hybridization, the coverslips were washed twice, each for 15 min, with 100 μl of 50% formamide + 2× SSC solution. Then the coverslips were washed 5×, each with 100 μl of 1× PBS for 1 min. The coverslips were mounted on 8 μl of mounting medium (mowiol with anti-fade reagent) on glass slides. The slide was kept at room temperature for at least 1 hr followed by 3–4 hrs at 4°C to stabilize the medium.

4.4 | Electrophoretic mobility shift assay

Electrophoretic mobility shift assays were performed using the protocol as described previously (Fioravanti *et al*., 2013) using LightShift Chemiluminescent EMSA Kit (Thermo Scientific).

4.5 | Co-immunoprecipitation assay

Co-immunoprecipitation assay was done to confirm the interaction of GcrA with the RNA polymerase subunits in vivo using the following procedure. *Caulobacter* strains EB689 (−ve control) and EB690 (expressing GcrA-FLAG) were grown in PYE broth up to an OD_{600} of 0.6 in a 50 ml volume. Centrifuged at 8,000 ×*g* for 10 min at 4°C. Supernatant was discarded and re-suspended in 1 ml of 1× TBS (50 mM Tris-Cl, pH 7.5, 150 mM NaCl). Samples were pelleted again, and kept at −20°C for 30 min before proceeding for cell lysis. All the following steps were done on ice. Pellet was re-suspended in 1 ml of cell lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1% Triton X-100) by thorough vortexing. Lysozyme was added (0.1 mg/ml final conc.) to the above and the samples passed through 18 gz and 27 gz needles to facilitate cell lysis and then, kept on ice for 30 min. DNase I (10 μg/ml) and $MgCl_2$ (5 mM final conc.) was added. Samples were centrifuged at 12,000 ×*g* for 15 min to remove the cell debris. Supernatant was transferred to a new eppendorf tube. For pre-clearing of the samples, 50 μl of protein A sepharose (CL-4B, GE Healthcare) was added to the supernatant and incubated at 4°C in a rotary shaker for 30 min.

Centrifuged for 1 min at 500 ×*g* and supernatant was removed into a new eppendorf tube leaving the sepharose beads. To the pre-cleared cell lysate 15 μl of anti-FLAG resin (Sigma) was added and incubated for 2 hr at 4°C in a rotary shaker. Anti-FLAG resin was prepared following the manufacturer's instructions. The cell lysate and the resin mix were centrifuged at 5,000 ×*g* for 1 min at 4°C, supernatant was removed leaving the resin. The resin was washed 3 × with 500 μl of cell suspension buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0) by centrifuging at 5,000 ×*g* for 30 s each. Immunoprecipitated proteins were eluted from the resin by incubating it with 30 μl of FLAG peptide (100 μg/ml in 1× TBS) at 4°C for 1 hr. Centrifuged at 6,000 ×*g* for 1 min and supernatant collected in a new eppendorf tube. Samples were run in SDS-PAGE gel, and protein bands were selected and sent for analysis by mass spectroscopy. The immunoprecipitated samples were also probed against anti- *E. coli* RNA polymerase β subunit to detect the presence of RNA polymerase.

4.6 | Purification of crescentin and immunoblotting

To raise antibody against crescentin, a 6×His tagged form of crescentin was purified following the method described before (Esue *et al*., 2010). Purified crescentin was sent for raising antibody (Davids biotech, Germany) and it was used at a concentration of 1:5,000 in immunoblotting experiments.

4.7 | Purification of *Caulobacter* RNA polymerase

RNA polymerase was prepared from 2-L volume of an exponentially growing *Caulobacter* culture (ML 1799) by tandem affinity purification method (Rigaut *et al*., 1999). The preparation was checked with SDS-PAGE for the presence of the major subunits of RNA polymerase and stored at −80°C in a buffer consisting of 10 mM Tris-HCl, pH 7.9, 500 mM NaCl, 50% glycerol, 0.1 mM EDTA and 0.1 mM DTT. The preparation could be used for in vitro transcription assay till one month without losing its activity.

4.8 | In vitro transcription assay

The in vitro transcription assay was performed to detect the effects of GcrA and CcrM methylation on the transcription of *creS*, *ctrAP1*, and *mipZ* promoters. The promoter regions (around 120 nucleotides consisting of at least 80 nt from the coding regions) were synthesized as single stranded forms containing m^6A sites, which were later constituted into double stranded ones to result in desired methylated forms such as hemi-methylation either in the coding or noncoding strands, and fully methylated forms. Approximately 250 ng of different methylated templates were pre-incubated with increasing concentrations of purified GcrA (0.125–0.5 μM) at room temperature for 10 min in a reaction buffer containing 66 mM Tris-Acetate (pH 7.9), 40 mM potassium acetate, 20 mM magnesium acetate, 5 mM dithiothreitol (DTT), and 100 μg/ml bovine serum albumin (BSA) (Biswas and Mohapatra, 2012). After incubation, 1 μl of *Caulobacter* RNA polymerase (from a preparation of 0.75 mg/ml) was added and incubation was continued for 5 min at room temperature to form the open complex. Transcription was initiated by adding to a final concentration 1 mM each of ATP, CTP, and GTP, and 0.25 mM UTP along with 0.75 mM biotin labeled UTP (Biotin-16-UTP, Epicentre) to the reaction mixture. Heparin (10 μg) was added to inhibit the reinitiation of transcription. The reaction volume was maintained at 10 μl. The reaction was continued for 30 min at 37°C, following which the templates were degraded using 2 U DNaseI (Epicentre) for 10 min at 37°C. Equal volume of 2× RNA loading dye (95% formamide, 0.5 mM EDTA, 0.025% SDS, 0.025% bromophenol blue and 0.025% xylene cyanoll) was added to the reaction followed by denaturing the samples by heating at 65°C for 3 min. Samples were resolved in a 8% denaturing polyacrylamide gel (8M Urea) in 0.5× Tris-Borate-EDTA (TBE) buffer running at 200 V for 75 min. The gel was washed twice each for 5 min, in 0.5× TBE buffer with shaking to remove excess urea. The transcripts from the gel were transferred to a 0.45 μm Biodyne B nylon membrane (Thermo Scientific) at a constant voltage of 20 V for 45 min at 4°C. The membrane was crosslinked in a UV crosslinker using a setting of 0.120 mJ. Membranes were processed as recommended in the Chemiluminescent Nucleic Acid Detection Module Kit (Thermo Scientific). The images were processed using ImageJ (Schneider *et al*., 2012) to calculate the fold changes in transcription with the increasing GcrA concentration. Three independent experiments were conducted for each promoter template to calculate the effect of GcrA on the transcription of different methylated promoters.

4.9 | Mathematical modeling

The starting point for the mathematical modeling of the transcriptional activity of the genes is the gene gate model as developed previously (Blossey *et al*., 2006; 2008). In this model, the concentration of an mRNA transcript under control of a dimeric transcription factor t of concentration $[t]$ is given by the formula $[\text{mRNA}] = (1/\delta)(\varepsilon + r[t]^2)/(1 + \nu[t]^2)$, in which δ is the degradation rate of the transcript, ε the basal transcription rate, r the activation rate of the gene, and ν the repression rate of the gene. This formula covers three types of behaviors of mRNA-concentration [mRNA] as a function of transcription factor concentration $[t]$: (a) quadratic growth with $[t]$ for small ν; (b) saturation of transcript concentration for $\nu \neq 0$; (c) for $r = 0$, decay as $1/[t]^2$. These behaviours cover all observed properties of the three genes/promoters *creS*, *ctr*AP1 and *mipZ* by varying only two parameters, r and ν, as is shown in Figure S5a. The fitting parameters can be used to relate the translational activity based on methylation during S phase. This is shown for the in vivo data on CtrA and GcrA in Figure S5b. The transcriptional model above is valid in the vicinity of the switch from the repressed promoter *ctr*AP1. In the course of this window, GcrA levels rise while CtrA levels fall and start to

rise. In the figure, CtrA data over this interval is plotted in blue, while the brown and green curves, respectively, are derived from the model for the case of full methylation and the sum of the hemi-methylated promoters on the positive and negative strands. This calculation was performed by taking the GcrA levels at each time point, and computing the corresponding CtrA level according to the above formula. The data have been rescaled by common factors to account both for the differences between the in vitro and the in vivo situations and the conversion from mRNA transcript to protein concentrations. This procedure has been applied to the two model cases, full methylation and the sum of hemi-methylated promoters with one corresponding set of two parameters in order to fit to experiment. Given the non-monotonous behavior this fit has to cover, the agreement of the model for full methylation for the time interval (20, 40) min and the model for hemi-methylation for (40, 60) min the result is consistent with expectations. For the data later than 60 min the model cannot be applied anymore, as the falling GcrA levels clearly indicate that cell volume effects, not taken into account in the model, become relevant. Figure S5c describes the qualitative evolution of the protein concentrations during the cell cycle.

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AUTHOR CONTRIBUTIONS

SSM and EGB designed and led the project. AF, PV, CS, FP, OV, and CB performed some experiments. RB performed the computational analysis. SSM and EGB wrote the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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