CcnA, a novel non-coding RNA regulating the bacterial cell cycle

Wanassa Beroual[1], David Lalaouna[2], Nadia Ben Zaina[1], Odile Valette[1], Karine Prévost[2], Yann Denis, Meriem Djendli[1], Matteo Brilli[3], Robledo Marta[4], Jimenez-Zurdo Jose-Ignacio[4], Eric Massé[2], Emanuele G. Biondi[1*]

1. Aix Marseille Univ, CNRS, LCB, Marseille, France
2. Department of Biochemistry, RNA Group, Université de Sherbrooke, Sherbrooke, QC, Canada
3. Pediatric Clinical Research Center "Romeo ed Enrica Invernizzi", Department of Biosciences, University of Milan, Milan, Italy
4. Grupo de Ecología Genética de la Rizosfera, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Granada, Spain

* Corresponding author: ebiondi@imm.cnrs.fr

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Summary

Bacterial cells are powerful models for understanding how cells divide and accomplish global regulatory programs. In *Caulobacter crescentus* a cascade of essential master regulators regulate the correct and sequential activation of DNA replication, cell division and development of different cell types. Among them CtrA plays a crucial role coordinating all those functions. Despite decades of investigation, no control by non-coding RNAs (ncRNAs) has been linked to *Caulobacter* cell cycle. Here, for the first time we describe the role of a novel essential factor named CcnA, a ncRNA located at the origin of replication, activated by CtrA and responsible for the rapid and strong accumulation of CtrA itself. In addition CcnA is also responsible for the inhibition of GcrA translation by direct interaction with its UTR region. By a combination of probing experiments and mutagenesis, we propose a new mechanism by liberation (CtrA) or sequestration (GcrA) of the Ribosome Binding Site (RBS). CcnA role is conserved in other alphaproteobacterial species, such as *Sinorhizobium meliloti*, representing indeed a conserved and fundamental process regulating cell cycle in *Rhizobiales* and *Caulobacterales*.

Introduction

Caulobacter crescentus is a pivotal model organism to understand how basic functions of the cell physiology are organized and coordinated through the cell cycle (Collier, 2012; Skerker and Laub, 2004). *Caulobacter* combines the cultivation and genetic simplicity of a prokaryotic system with an intricacy of regulation that is paradigmatic of global regulatory programs of higher organisms. Transcriptional regulation plays a major role during cell cycle progression as several master regulators are sequentially activated in order to induce transcription of genes required at a specific phase of the cycle (Figure 1A) (Collier et al., 2006, 2007; Reisenauer and Shapiro, 2002). Each phase is under the control of a specific master regulator: (i) the initiation of the S-phase depends on DnaA, (ii) the first part of S-phase depends on GcrA and CcrM, while (iii) the second part depends on CtrA which is also the regulator of the phase G1 of swarmer cells (Panis et al., 2015). The interconnections of these four transcriptional regulators create a complex network whose behavior derives from the integration of multiple levels of regulation around master regulators. In particular, a central role is played by the essential response regulator CtrA, which controls directly or indirectly all the other master regulators of the cell cycle (Laub et al., 2002). CtrA is notably responsible for the direct transcriptional activation of key cell division genes and the biogenesis of polar structures (flagellum, stalk and pili). CtrA also activates the transcription of the orphan adenine methyl transferase CcrM encoding gene, which in turn is required for the fine-tuned regulation of the promoter P1 of *ctrA* (Reisenauer and Shapiro, 2002). Moreover CtrA indirectly blocks the activity of DnaA at the single origin of the chromosome replication (CORI) by direct binding to sites that are competing with DnaA sites required for initiation of DNA replication (Marczynski and Shapiro, 2002; Quon et al., 1998). Finally another master regulator, named GcrA, activates *ctrA* (together with the CcrM methylation as mentioned earlier), which in turns represses *gcrA* by direct binding to its promoter (Fioravanti et al., 2013; Haakonsen et al., 2015; Holtzendorff et al., 2004). CtrA encoding gene is activated by two promoters, a GcrA–dependent P1, which is activated by the conversion of a CcrM methylation site from its full to the hemi-methylation state approximately after 1/3 of DNA replication (Reisenauer and Shapiro, 2002). This P1 activation is responsible for the first weak accumulation of CtrA, which in turns starts activating a second stronger promoter P2, closer to the ATG, responsible for the robust accumulation of CtrA in the second half of DNA replication and the repression of P1 and *gcrA* transcription. Although the molecular details of this biphasic activation of *ctrA* are still partially understood, the stronger activation of P2 may underline other post-transcriptional mechanisms reinforcing CtrA accumulation. CtrA in order to be fully active requires phosphorylation by the CckA-ChpT phosphorelay (Biondi et al., 2006a). The hybrid kinase CckA has a bipolar localization (Biondi et al., 2006a; Chen et al., 2011; Jacobs et al., 2003). At the swarmer pole it acts as kinase thanks to the presence of DivL and DivK non-phosphorylated (Gora et al., 2010) by the presence of its phosphatase PleC. On the contrary CckA is a phosphatase at the stalked pole, in which the kinase DivJ keeps DivK fully phosphorylated inhibiting the phosphorelay.

As CtrA~P blocks the origin of replication, a complex degradation machinery ensures its cell cycle dependent degradation at the G1 to S-phase transition and after cell division in the stalk compartment (Joshi et al., 2015; Ryan et al., 2004). A cascade of adapter proteins (CpdR, RcdA and PopA) is responsible for the specific and highly regulated proteolysis of CtrA.

In *Caulobacter,* the regulation of gene expression by ncRNAs has revealed few examples. Initially only 27 ncRNAs were described in this organism (Landt et al., 2008). CrfA is an sRNA involved in adaptation to carbon starvation (Landt et al., 2010). GsrN is involved in the response to σ^T-dependent multiple stresses (Tien et al., 2017). Finally ChvR has been recently characterized as a ncRNA that is expressed in response to DNA damage, low pH, and growth in minimal medium (Fröhlich et al., 2018). However as new recent approaches using RNAseq and post-genomic techniques expanded the *plethora* of ncRNA candidates to more than 100 (Zhou et al., 2015), predictions of their integration into the cell cycle circuit (Beroual et al., 2018) has suggested that several new candidate ncRNAs should be deeply studied.

Here we investigated the role of a ncRNA, named CcnA, that belongs to the origin of replication of *Caulobacter* chromosome. We studied its role by the construction of deletion mutants, silencing by expression of its antisense and over expression. Results presented in this work identified the mRNAs of CtrA and GcrA, two master regulators of cell cycle, as main targets of this ncRNA. Data were

Figure 1

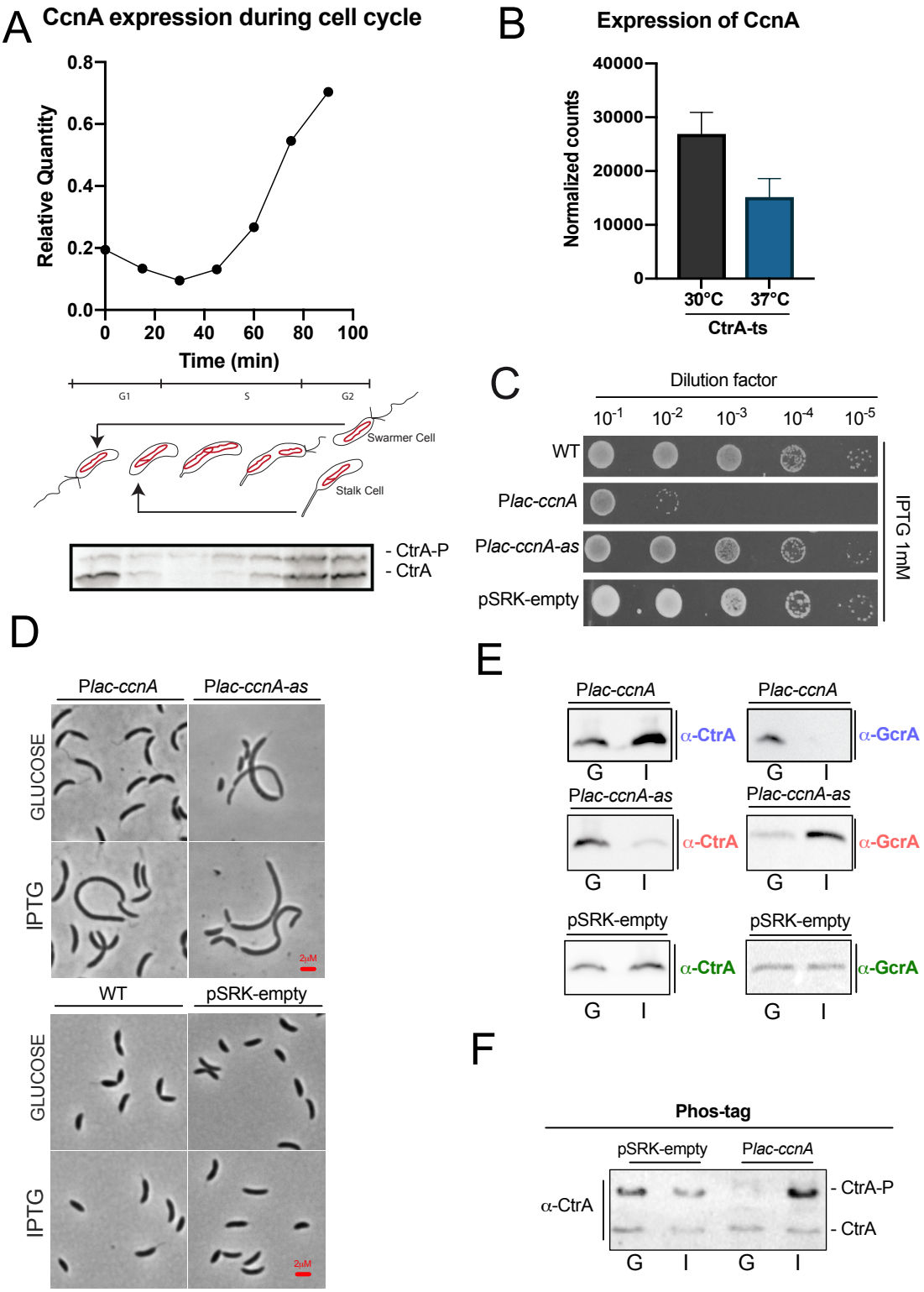


Figure 1. CcnA (Cell Cycle Non-coding RNA A) regulates cell cycle. A. Levels of CcnA were compared to 16S by qRT-PCR. Samples from a synchronized population (see schematics below the plot) were isolated and RNA was extracted. On the same synchronized samples Phos-Tag anti-CtrA was performed measuring both protein and phosphorylation levels. B. A temperature sensitive allele of ctrA (CtrA401) was used to measure CcnA RNA levels at 30ºC and 37ºC. Expression of CcnA drops upon inactivation of CtrA. C. Phase contrast images of strains overexpressing CcnA (P*lac-ccnA*), its antisense (P*lac-ccnA*-as), wild type (WT) and the empty vector (pSRK-empty). D. Cultures of the strains of panel C were grown until 0,6 and grown at different dilutions. E. Western blots using anti-CtrA and anti-GcrA antibodies in P*lac-ccnA*, P*lac-ccnA*-as and empty vector using glucose and IPTG conditions (see M&M). Upon induction of CcnA, CtrA levels increase while they decrease when expressing the antisense of CcnA. For GcrA we observed the opposite situation, while the empty vector doesn't show any variation between glucose and IPTG. F. Phos-tag gel anti-CtrA of the strain P*lac-ccnA* in comparison with the strain containing the empty vector in glucose and IPTG conditions.

supported by a combination of MAPS, *in vitro* and *in vivo* experiments. Finally the role of CcnA in a closely related organism such as *Sinorhizobium meliloti* suggested its potential conservation across bacteria.

Results

CcnA expression is regulated by CtrA

Based on previous results (Zhou et al., 2015) we observed that CCNA_R0094, here named Cell Cycle non-coding RNA A (CcnA), expression peaks after few minutes from the accumulation of *ctrA* transcript and protein, in the second half of the S-phase, when P2, the second *ctrA* promoter, is activated (Fig 1A).

We designed primers to detect and precisely quantify *ccnA* RNA in the cells by q-RT-PCR (see M&M) with respect to 16S RNA levels. A synchronized population of wild type *C. crescentus* was used to collect cells at 15 minutes time points of the cell cycle in rich medium (generation time is 96 minutes). CcnA levels start increasing at 45 minutes, coincidentally with CtrA protein levels (Figure 1A). More specifically we measured both protein and phosphorylation levels of CtrA by Phos-Tag gels (Figure 1A). CcnA levels increase as CtrA~P levels increase (Figure 1A), suggesting that the transcription of *ccnA* potentially depends on phosphorylated CtrA. This observation prompted us the question whether CtrA was responsible for *ccnA* transcription. Consistently upstream of the annotated Transcriptional Start Site (TSS) of *ccnA* a CtrA box was previously described (Brilli et al., 2010; Zhou et al., 2015).

In order to respond to this question, we performed RNAseq using a CtrA thermo-sensitive (CtrA-ts) allele specifically looking for variations of *ccnA* expression in the context of the global transcriptional changes that depended on functional CtrA (Figure 1B). A previously characterized thermo-sensitive allele of CtrA (Biondi et al., 2006a; Laub et al., 2002; Quon et al., 1996) was used at 30°C and 37°C, respectively the permissive and restrictive temperature at which CtrA completely looses its functionality. The analysis revealed that expression of CcnA significantly decreases between 37° C and 30° C, suggesting that CtrA is required for *ccnA* transcription, as suggested by the presence of the predicted binding site in *ccnA* promoter region.

In conclusion CcnA is a sRNA activated by the master regulator of cell cycle CtrA peaking its expression when CtrA itself strongly accumulates.

CcnA is responsible for the accumulation of CtrA and inhibition of GcrA

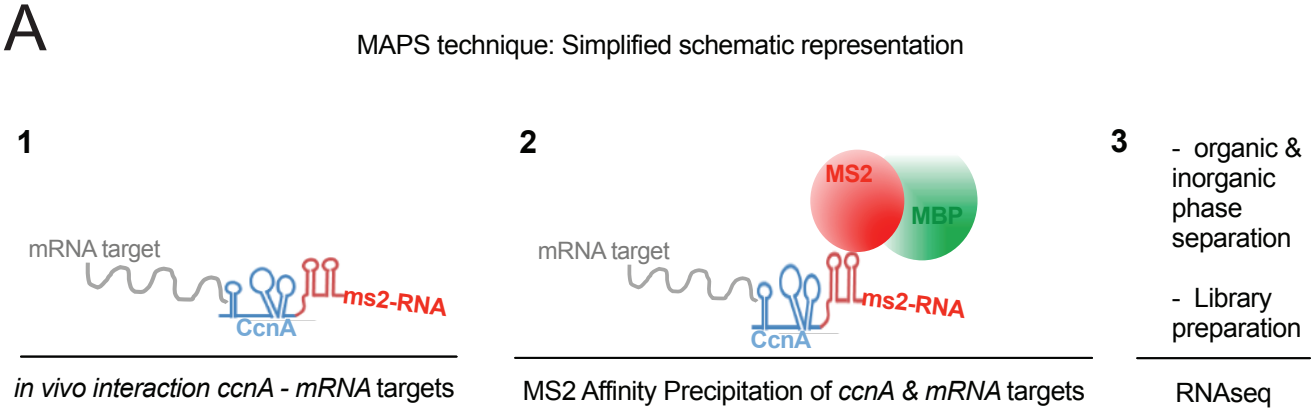
Aiming to understand the function of CcnA, we fused the sequence of *ccnA* with the +1 of a P*lac* promoter in the vector pSRK (Khan et al., 2008) in order to make an IPTG-inducible version of the ncRNA. This vector was introduced in *C. crescentus* cells, its induction was measured and the phenotype was observed (Figure 1C, D). Even in glucose P*lac-ccnA* cells showed cell cycle defects, such as slow growth, cell division abnormal morphologies, with an increased number of abnormal long stalks. The level of *ccnA* in this inducible system was quantified by q-RT-PCR (see Mat and Met) confirming that this strain indeed expresses a high level of *ccnA* even in glucose (Figure S1).

Several tests were performed in order to characterize these phenotypes. Morphologically we used MicrobeJ to quantify cell size parameters (Ducret et al., 2016). Results showed that cells were elongated with respect to wild type cells (Figure S2).

Stalk biogenesis and swarmer cells morphogenesis are both under the control of the master regulator CtrA (Biondi et al., 2006b; Quon et al., 1996) suggesting that CcnA may act on CtrA production. Therefore we quantified CtrA levels in the overexpression strain. Results showed that upon expression/overexpression of CcnA CtrA accumulates abnormally showing more CtrA than the control strain (Figure 1E). We also tested other proteins, such as DnaA, but it didn't change upon CcnA expression (Figure S3). This suggests that CcnA has an effect on the production of the master regulator and phenotypes observed may be a direct consequence of this over production of CtrA.

Figure 2

A



B

Name	Synonym	Product	Ratio MS/neg	CtrA controlled
bacA	CCNA_01949	bactofilin A BacA	114	
ctrA	CCNA_03130	cell cycle response regulator ctrA	99	yes
-	CCNA_01978	methyltransferase	95	
hfaD	CCNA_02713	holdfast attachment protein hfaD	71	yes
tacA	CCNA_03424	AAA-family response regulator tacA	66	yes
-	CCNA_00727	ribosomal-protein-alanine acetyltransferase	59	
flgF	CCNA_02142	flagellar basal-body rod protein flgF	49	
pleA	CCNA_02411	putative lytic transglycosylase PleA	42	
clpA	CCNA_02553	ATP-dependent clp protease ATP-binding subunit ClpA	37	yes
phoR	CCNA_00291	phosphate regulon sensor protein phoR	34	
ftsA	CCNA_02624	cell division protein FtsA	34	yes
pdeA	CCNA_03507	GGDEF/EAL phosphodiesterase PdeA	32	
mreB	CCNA_01612	rod shape-determining protein MreB	30	
ftsH	CCNA_03334	cell division protein FtsH	18	yes
-	CCNA_02080	Sec-independent protein translocase protein tatC	9	
gcrA	CCNA_02328	cell cycle regulatory protein GcrA	4	yes
dnaE	CCNA_02003	DNA polymerase III alpha subunit	4	
cheYI	CCNA_00441	chemotaxis receiver domain protein cheYI	419	yes
flgH	CCNA_02145	flagellar L-ring protein flgH	103	yes
socB	CCNA_03629	DNA replication inhibitor toxin SocB	95	
CcnA	CCNA_R0094	CcnA	253	
socA	CCNA_03630	antitoxin protein SocA	176	
-	CCNA_02425	non-essential pilus assembly protein	133	yes
flgL	CCNA_00942	flagellar hook-associated protein FlgL	121	yes
hfaB	CCNA_02712	holdfast attachment protein hfaB	85	yes
cheAI	CCNA_00442	chemotaxis histidine kinase protein cheAI	59	yes
tipF	CCNA_00747	cyclic-di-GMP phosphodiesterase, flagellum assembly factor TipF	38	
-	CCNA_02762	DNA repair protein RadC	37	
parA	CCNA_03869	chromosome partitioning protein ParA	35	
fljL	CCNA_01527	flagellin fljL	4	yes

Figure 2. CcnA binds in vivo the mRNAs of *ctrA* and *gcrA* as revealed by MAPS. A. MAPS (MS2 Affinity Purification high-throughput Sequencing) technique requires the fusion of the sRNA sequence with a RNA tag MS2. This tag has affinity for the protein MS2, which is fused to the protein maltose binding protein (MBP) that allows the binding to an amylose column. The expression of ms2-ccnA in the *Caulobacter* cells permits the formation of mRNAs-CcnA complexes that are then trapped on the amylose column. RNAs are then sequenced by RNAseq. B. Results of MAPS using MS2-CcnA in comparison with non-tagged CcnA (neg). Genes under the control of CtrA are also listed in the last column.

CtrA must be phosphorylated in order to perform its functionality. Phostag technique was used, as previously described (Pini et al., 2013) to quantify the levels of CtrA~P upon induction of CcnA (Figure 1F). The analysis revealed that the higher level of CtrA in fact corresponds to high amount of CtrA~P. As phosphorylation of CtrA is under the control of the phosphorelay CckA and ChpT we tested whether levels ChpT were affected. We used YFP translational fusion of ChpT in order to understand whether CcnA ectopic expression was causing a change in the protein levels. Epifluorescent microscopy was used to quantify the protein level of ChpT fused with YFP and expressed by its native promoter (Figure S4A). Data were further analyzed by MicrobeJ (see Mat & Met) and results showed that upon CcnA induction intensity and clustering of the signal increases in the ChpT-YFP strain (Figure S4B). We quantified this variation as illustrated in figure S4C.
Rapid accumulation of CtrA corresponds also to a decrease of GcrA levels. Therefore we also quantified whether CcnA was at the same time affecting the protein level of GcrA using western blot and anti-GcrA antibodies. Surprisingly the quantification of GcrA upon CcnA induction revealed a significant decrease of the protein level (Figure 1E).
In conclusion CcnA increased expression induces a decrease of GcrA and an increase of CtrA protein, specifically in its phosphorylated form. These changes in the GcrA and CtrA levels may explain the cell cycle defects observed at the morphological levels, notably increase of cell length, long stalks and presence of significant proportion of swarmer cells even in stationary phase (data not shown).

Knock-out of CcnA by chromosomal deletion and antisense silencing

The gene *ccnA* belongs to the origin of replication (Figure S5); therefore, its sequence, at least partially, may play an essential role in the initiation of replication. We attempted a complete deletion of the *ccnA* sequence by two-step recombination (see Mat & Met) as previously described (Skerker et al., 2005) in presence of an inducible copy of *ccnA* or in the wild type background. As expected the deletion of the *ccnA* sequence was not successful demonstrating that the sequence of *ccnA* is essential. Same results were obtained also using the inducible copy of *ccnA*.
We decided to attempt different strategies allowing the inactivation of CcnA but keeping intact (most of) the origin of replication. First we attempted the deletion of the promoter region including the CtrA box putatively involved in *ccnA* transcription and, if successful, extending the deletion region into the *ccnA* sequence. Several deletion cassettes (see Mat & Met) were prepared and used as previously described for the complete deletion of *ccnA*. In order to minimize the effect of polar interference by the tetR cassette we also created marker-less deletions.
An alternative strategy was used by overexpression of the antisense of *ccnA* (CcnAas). CcnAas was fused to an IPTG-inducible promoter and its induction was tested in order to demonstrate a negative effect on CcnA activity.
The expression of the antisense of CcnA is able to induce the opposite effect than the overexpression of CcnA, that is increasing GcrA levels and decreasing CtrA levels (Figure 1C, D, E). Those results strongly suggested that the expression of the antisense is able to inactivate CcnA activity and then allowing the analysis of a CcnA loss of function. If the inactivation is complete, this result also suggests that the inactivation of CcnA is not lethal. Supported by these results we attempted a more specific deletion of *ccnA* on the chromosome by targeting its promoter region including the predicted CtrA box. As shown in the previous section the *ccnA* expression should be strictly under the control of CtrA, therefore the deletion of its box in the promoter region should have a mild or absent effect on the origin but impair the expression of the ncRNA. Results showed that the marker less deletion of the promoter of CcnA was viable but showing a severe growth defect (data not shown).
In conclusion both overexpression and low levels of CcnA show coherent results that suggest that CcnA is indeed responsible for the activation of CtrA and the downregulation of GcrA. However we asked whether this activity was due to a direct interaction of CcnA with the mRNAs of both gene and moreover whether other genes were targeted by this ncRNA.

MS2 Affinity Purification high-throughput Sequencing (MAPS)
In order to characterize RNAs that were targeted *in vivo* by CcnA and confirm whether CtrA and GcrA mRNAs were indeed targets of CcnA, we performed the technique called MAPS (MS2 Affinity Purification high-throughput Sequencing) as previously described (Lalaouna et al., 2017) (Figure 2A).

Figure 3

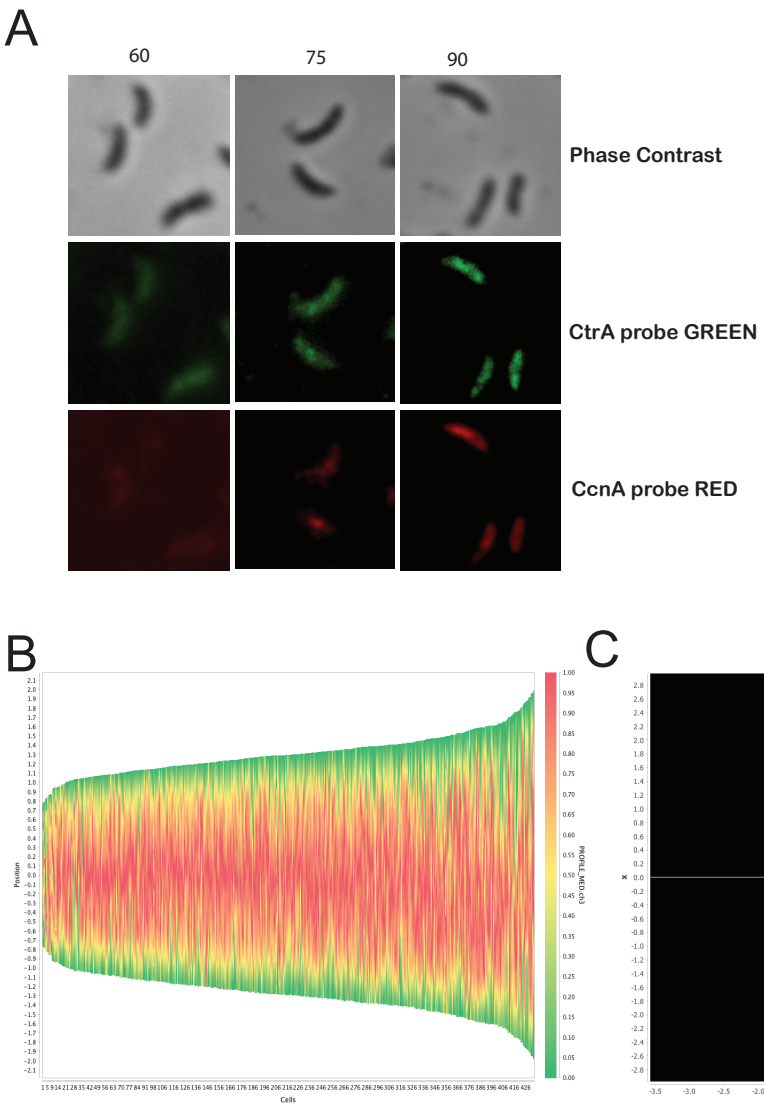


Figure 3. CcnA shows subcellular localization in late predivisional cells. A. FISH (Fluorescent in situ hybridization) was used to visualize CcnA (red) and CtrA (green) RNAs in Caulobacter cells. A population was synchronized and time points were recovered every 15 minutes. CcnA signal is undetectable until 60 minutes of the cell cycle (early S-phase) (panels not shown), starting to increase until 90 minutes as shown by q-RT-PCR (Figure 1A). B. At 90minutes of the cell cycle CcnA signal is at the highest level. In order to quantify precisely the localization of CcnA in predivisional cells, ca. 500 cells were analyzed by MicrobeJ and the signal distribution was plotted longitudinally. As clearly showed, cells present a more intense signal in the mid cell region. C. These results were plotted in a single cell showing a clear mid cell localization.

We constructed a version of CcnA tagged with an MS2 RNA tag able to bind the protein MS2-MBP trapped in an amylose column. As a negative control the same version was cloned without tagging in order to compare results presumably specific to the MS2 technique. Strains expressing *ms2-ccnA* or *ccnA* were grown in exponential phase and *ms2-ccnA*/*ccnA* induced by IPTG for half an hour. Cells were lysed and soluble cell content was loaded onto an amylose column containing MS2-MBP fusion. RNA molecules and proteins were separated and purified as previously described (see Mat & Met).
RNAs trapped in the amylose column in presence of *ms2-ccnA* or non-tagged CcnA were characterized by RNAseq and results were analyzed (see Mat & Met). Among other targets, the region mRNA of CtrA and GcrA were detected (Figure 2B). This result consistently supports our previous findings that CcnA mutations were indeed affecting CtrA and GcrA expression. The extent of CcnA regulated targets is wider than just *ctrA* and *gcrA* mRNAs. As illustrated in figure 2, other mRNAs are targeted by CcnA. A general observation of genes controlled by CcnA is that most of them generally belong to the CtrA regulon, such as motility genes.
We also tagged UTR regions of *ctrA* (mRNAs generated by the promoter P1 or promoter P2), and *dnaA* (as a putative negative control) in order to confirm (or exclude) interactions with CcnA. If CcnA really interacts with *ctrA* P1 and P2 UTR regions but it doesn't bind the UTR of *dnaA*, this reverse-MAPS should be able to confirm the specific interaction. We expressed the MS2 tagged UTR regions in *C. crescentus* cells, comparing results with MAPS using a wild type strains and strains expressing non-tagged UTRs (see Mat & Met). We looked for the presence of CcnA (Data not shown) demonstrating that only the mRNA of the UTR region of CtrA corresponding to the P2 promoter is able to recover CcnA.

Fluorescent in situ hybridization (FISH) of CcnA and CtrA mRNA

Aiming to understand the expression dynamics of CcnA during cell cycle and visualize the subcellular localization of CcnA and its target CtrA mRNA, we used Fluorescent *in Situ* hybridization (FISH) to image RNAs in the cells. Probes for CcnA and CtrA mRNA were designed and labeled with different fluorochromes (see Mat & Met). First, we synchronized a population of *Caulobacter* and visualized CcnA and CtrA mRNA every 15 minutes of the cell cycle in rich medium until 90 min (Figure 3). Results for CcnA (Figure 3) are perfectly matching with qPCR data (Figure 1A) increasing after 60 minutes and reaching a high level around 90 minutes of the cell cycle that corresponds to a late predivisional stage (Figure 3A). The increased level of CcnA RNA and its accordance to qPCR levels strongly support the specificity of the probe. CtrA mRNA levels are also changing over the cell cycle with a similar dynamics accordingly to previous results (Laub et al., 2000; Reisenauer and Shapiro, 2002; Reisenauer et al., 1999).
Surprisingly CcnA RNA showed a clear diffused midcell localization (Figure 3B) at 90 min that may suggest a condensation in this area as its levels increase and presumably the target RNA also increases. It's fascinating to speculate that the localization factor for CcnA is the presence of multiple copies of target mRNAs in that region of the predivisional cell.
We also tested FISH on the strains expressing high levels of CcnA (as showed in previous figures) as showed by qPCR. Accordingly, to high RNA levels the signal of the CcnA probe is considerably higher than in wild type cells and remarkably showing a patchy diffused level of CcnA RNA (data not shown).

Modulations of CcnA are able to complement mutants of cell cycle

As methylation sites are connected to *ctrA* transcription by its own P1 promoter, we asked whether the expression of CcnA was also interacting genetically with the delta-*ccrM* mutant (Murray et al., 2013). We introduced P*lac-ccnA* and P*lac-ccnAas* in the CcrM mutant and analyzed the phenotypes. First we were not able to stably introduce CcnA antisense into delta-*ccrM* suggesting an incompatibility between the two genetic constructions. The expression of CcnA was indeed able to completely suppress the sick phenotype of the delta-*ccrM* mutant (Figure 4B). Notably the severe morphological defects of delta-*ccrM* were rescued (Figure 4B, C) as well as the motility defect (Figure 4D). We asked whether in the CcrM deletion background CcnA was indeed able to increase CtrA levels (Figure 4F). Results clearly showed that CcnA is able to increase CtrA levels in the delta-*ccrM* mutant and

Figure 4

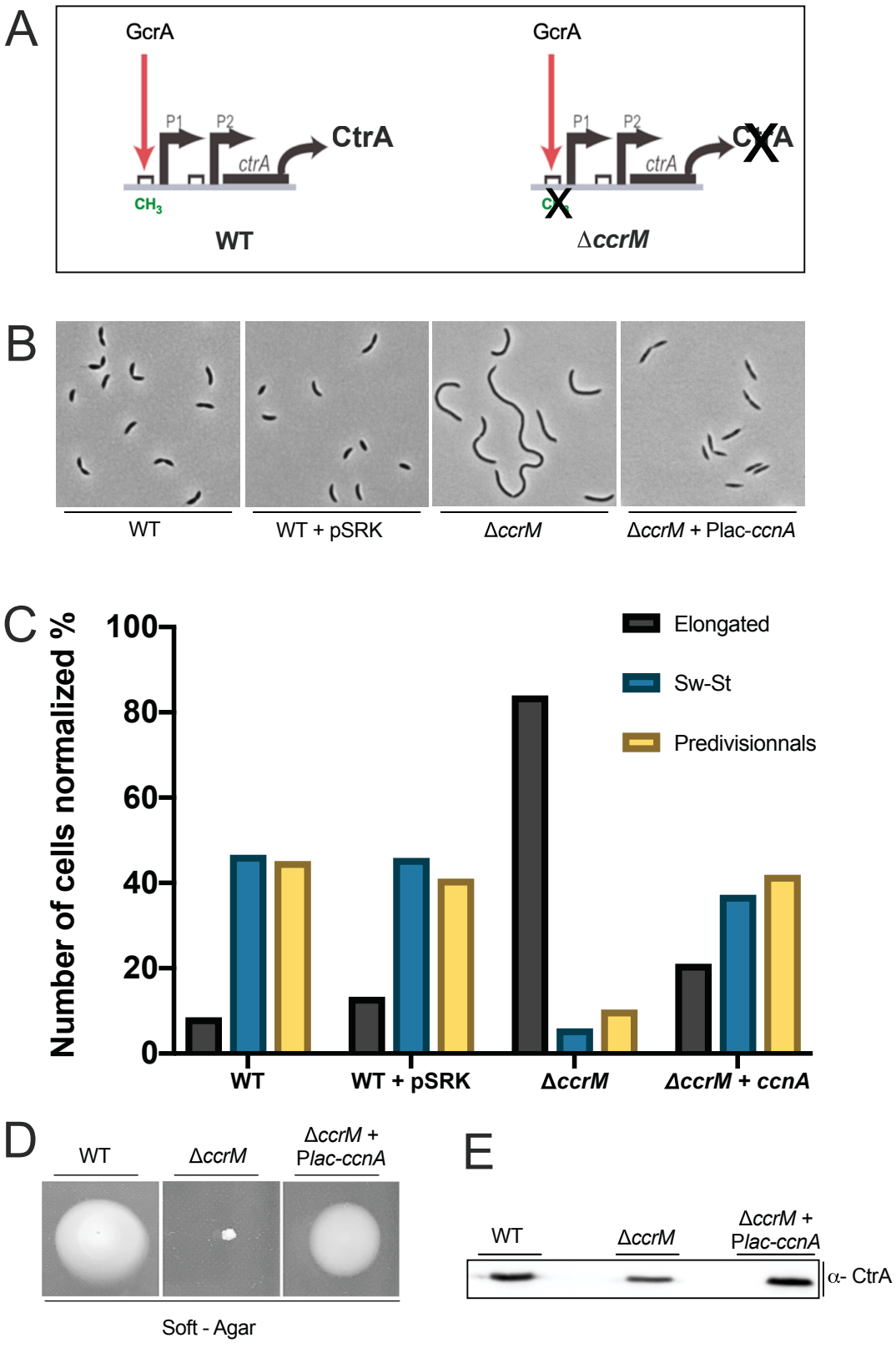


Figure 4. CcnA rescues the CcrM loss of function phenotype. A. Regulatory mechanism of P1 promoter activation by GcrA and CcrM methylation in wild type (WT) and Δ*ccrM*. The absence of CcrM causes a loss of the methylation at the P1 promoter, decreasing the affinity of GcrA for the promoter region. B. Phase contrast microscopy of wild type (WT), a strain with the empty vector (WT + pSRK), Δ*ccrM* and Δ*ccrM* with the plasmid expressing CcnA (Δ*ccrM* + ccnA). C. Quantification of morphological types in different genetic backgrounds. D. Complete suppression of the motility defect of Δ*ccrM*. F. Western blotting using CtrA antibodies in WT, Δ*ccrM* and Δ*ccrM* expressing CcnA.

that indeed the mechanism by which CcnA increases CtrA protein levels is independent from CcrM, so presumably acting on the P2 promoter.
Supported by the results, we also decided to combine mutants of CcnA with Δ*pleC*, a mutant involved in the negative control of DivK phosphorylation level. As DivK~P inhibits CtrA stability and activity, in the deletion mutant of DivK phosphatase, PleC, CtrA levels are extremely low (Figure 5A). Therefore high CcnA activity should compensate the PleC deletion mutant's defects, restoring the wild type phenotypes.
We introduced P*lac-ccnA* or P*lac-ccnAas* in Δ*pleC* mutant and observe the morphology, motility in soft agar plates, sensitivity to the phage CbK and stalk length (Figure 5).
The expression of CcnA was able to rescue Δ*pleC* defects, restoring stalks and motility while the expression of the CcnA antisense caused a very severe phenotype (Figure 5B, C). We asked whether this suppression was just increasing the level of CtrA or it was indeed able also to affect the phosphorylation of CtrA. We measured CtrA~P by Phos-Tag technique (Figure 5D). This analysis revealed that CcnA was indeed able to increase the CtrA~P levels in Δ*pleC.*
Finally we measured the sensitivity to the phage CbK, which is entering the cells by attachment to the pili structures (Figure 5E). As the main subunit PilA of the pilus is completely under the control of CtrA, a mutant of PleC completely lacks of pili and therefore it is resistant to CbK infection (Panis et al., 2012; Sommer and Newton, 1988). Results showed that the expression of CcnA was completely able to restore the sensitivity to CbK at the levels of wild type while the expression of CcnA antisense did not change the resistance to the phage infection of the *pleC* mutant (Figure 6). Finally we measured the stalk length of the Δ*pleC* in which we expressed CcnA (Figure 5F). The expression of CcnA indeed restores the stalk production (absent in Δ*pleC*), while the expression of the antisense does not.

CcnA interacts *in vitro* with the mRNAs of CtrA, GcrA and DnaA

In order to understand the molecular bases of CcnA-CtrA interactions we performed Probing experiments using *ctrA* (promoters P1 and P2), *gcrA* and *dnaA* UTR regions. MAPS and molecular analysis revealed a putative interaction between CcnA and CtrA/GcrA mRNAs, while the interaction with the UTR region of *dnaA* was only predicted (Beroual et al., 2018), but no *in vivo* link was found and therefore we included as a negative control.
CtrA UTR region was analyzed considering the two Transcription start sites (TSS), P1 and P2, controlled by GcrA/CcrM methylation and CtrA, respectively. For GcrA we used data derived from 5' race experiments at the genome scale (Zhou et al., 2015). Results showed regions of protection for both CtrA UTR from the promoter P1 and the promoter P2 (Figure 6). They both share most of the annealing regions except for an extra distal region in P1 that is in fact outside the P2 UTR. A common element of annealing was found looking into the GcrA UTR region annealing with CcnA. A stretch of CCCC, present in a loop region of CcnA, is also protecting both CtrA and GcrA UTR regions. Regarding DnaA that was previously predicted to be the real target of CcnA (Beroual et al., 2018), Probing experiments indeed showed a clear protection that corresponded to the predicted region (Figure 6). Whether this interaction is real or not, we can speculate that (i) CcnA and *dnaA* mRNA are not usually expressed at the same time; (ii) MAPS did not revealed any interaction between CcnA and the UTR region of DnaA; (iii) None of phenotypes of the loss or overexpression of CcnA are physiologically connected to DnaA. Therefore we excluded that CcnA binds DnaA during the cell cycle. However it's possible that this interaction becomes relevant during a specific response in which the expression of CcnA is indeed directed to DnaA regulation. Moreover we cannot exclude that the UTR region of *dnaA* is indeed inversely regulating CcnA.
In order to consolidate these observations we decided to check whether the CtrA UTR region was indeed able to bind CcnA using a reverse MAPS in which the mRNA of *ctrA* was tagged with MS2. Results clearly showed that CcnA appears among targets of *ctrA* UTR reinforcing the discovery of the CcnA-CtrA mRNA interaction. As a putative negative control (based on westerns on DnaA that showed no variation at the protein level) we also tested the UTR region of DnaA tagged with MS2. Although this region is able to bind CcnA *in vitro* (Figure 6), by using an *in vivo* MAPS technique, we were not able to detect any interaction with DnaA mRNA suggesting that in normal physiological

Figure 5

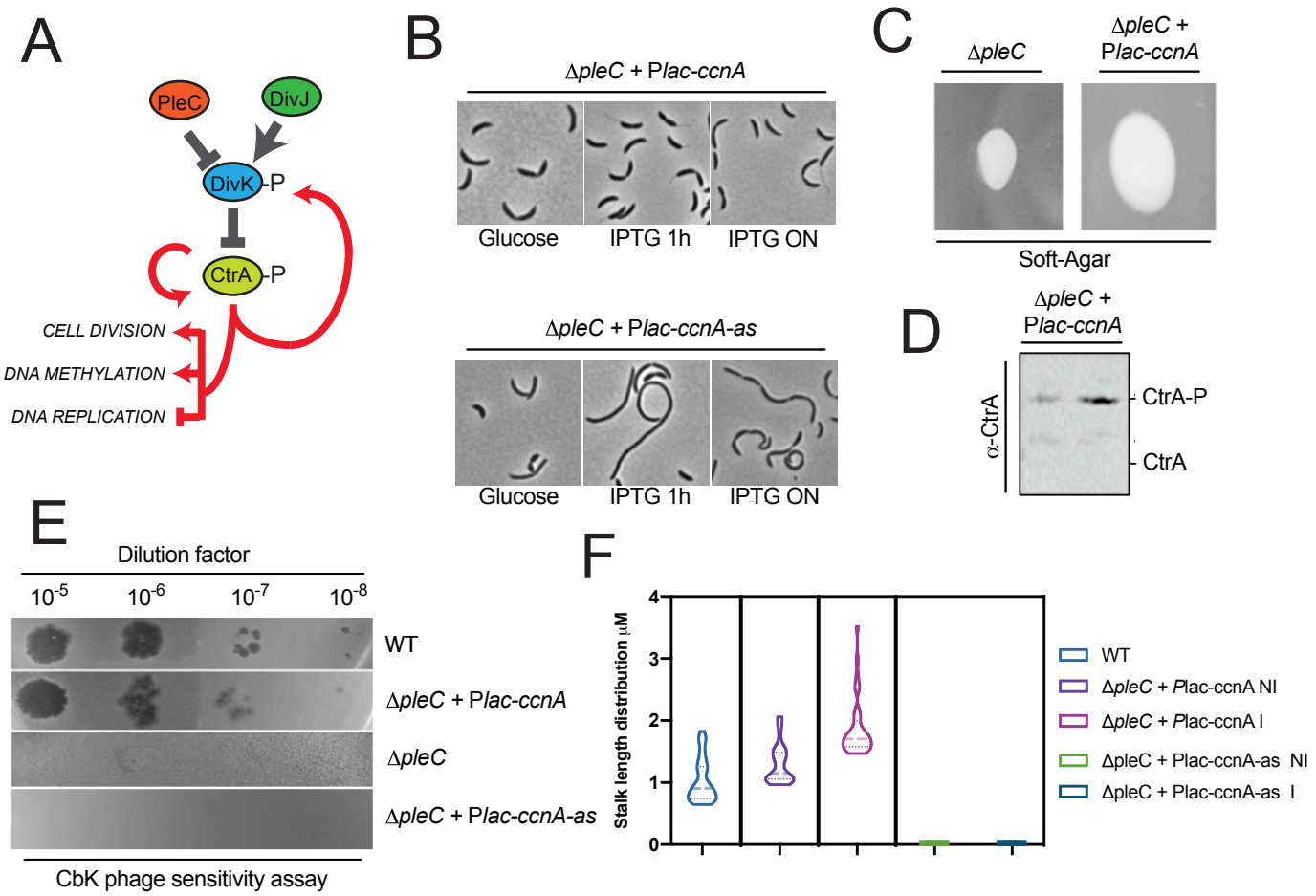


Figure 5. CcnA rescues the pleiotropic phenotypes of Δ*pleC*. A. Schematics of CtrA-DivK negative feedback loop. DivK phosphorylation levels are controlled by the kinase DivJ and the phosphatase PleC. Absence of PleC causes a decrease of CtrA, both at the protein and phosphorylation levels. B. Phase contrast microscopy of the Δ*pleC* mutant expressing CcnA (Δ*pleC* + P*lac-ccnA*) and its antisense (Δ*pleC* + P*lac-ccnA*-as) in glucose (G) and IPTG 1h and IPTG overnight (IPTG ON). C. Soft agar assay using Δ*pleC* and Δ*pleC* expressing CcnA. D. Phos-tag gel measuring the levels of CtrA~P in the Δ*pleC* background expressing CcnA in glucose and IPTG (1h). E Infection by the phage CbK in different backgrounds, wild type (WT), Δ*pleC*, Δ*pleC* mutant expressing CcnA (Δ*pleC* + P*lac-ccnA*) and its antisense (Δ*pleC* + P*lac-ccnA*-as). F. Measures of stalk length using BacStalk.

conditions CcnA is not interacting with DnaA mRNA. This result is also compatible with the observation that CcnA exists in the cell in the second half of the S-phase in which no role of DnaA has never been described.

Based on these results we constructed mutant versions of CcnA in which the two active loops "CCCC" (Loop A) and "ATCAA" (Loop B) were individually mutated or introduced together in the CcnA sequence (Figure 7A). These mutated versions of CcnA were then tested *in vivo* using the same expression system that was used before as shown in figure 1. These mutant versions were expressed in *C. crescentus* and results are reported in figure 7B as a series of dilutions. The mutation of the site "CCCC" is already able to abolish completely the sick phenotype of CcnA overexpression, while the mutation of the Loop B in which growth of *C. crescentus* cells is similar to the over CcnA strain. These results suggest that the sick phenotype observed when inducing the WT version of CcnA is mainly due to the binding of the Loop A of CcnA to the mRNA.

We did test whether expressing mutant versions of CcnA was causing the same CtrA protein variations when expressing CcnA wild type (Figure 7C). Results showed that mutation of Loop A and both A and B do not show the same CcnA induction-dependent variation of CtrA. When we looked at the Western blotting results, the mutation of both loops is needed to restore a normal amount of CtrA in comparison with the WT strain. However the over expression of the version mutated of the Loop A still induces an increase of CtrA. This suggests also that the presence of the non-mutated Loop B alone is still able to increase CtrA.

Conservation of CcnA among alphas

Considering the key role of CcnA in *Caulobacter* coordinating two of the principal master regulators of cell cycle, we asked whether its function was conserved in bacteria that share the regulatory mechanisms. We considered a well known bacterial model, *Sinorhizobium*, a symbiotic nitrogen fixing organism. *S. meliloti* shares with Caulobacter most of the regulatory circuit driving cell cycle including CtrA (Pini et al., 2013, 2015). Therefore we took advantage of the inducible system we used for Caulobacter, which is also working in the *Sinorhizobium* (Khan et al., 2008). We electroporated *S. meliloti* with a plasmid containing *Caulobacter* CcnA. Expressing CcnA from *Caulobacter* slowed the growth of *S. meliloti* and caused an abnormal morphology (Figure 8B). We therefore asked whether this alteration in cell morphology was due to a change in CtrA levels in comparison with a negative control (Figure 8C). Indeed results showed that CcnA of *C. crescentus* is able to induce a cell cycle defect, that is branched cells and a clear cell division retard, indeed similar to that observed in a delta-*divJ* mutant (Pini et al., 2013).

The activity of Caulobacter CcnA in these two alphaproteobacterial species suggested that a putative homologous gene should be present in *Sinorhizobium*. We therefore scanned the genomes of the alphaproteobacterial species using GlassGo (Lott et al., 2018) aiming to find CcnA homologs. We found a conservation of CcnA in several closely related species (Figure 8A).

Figure 6

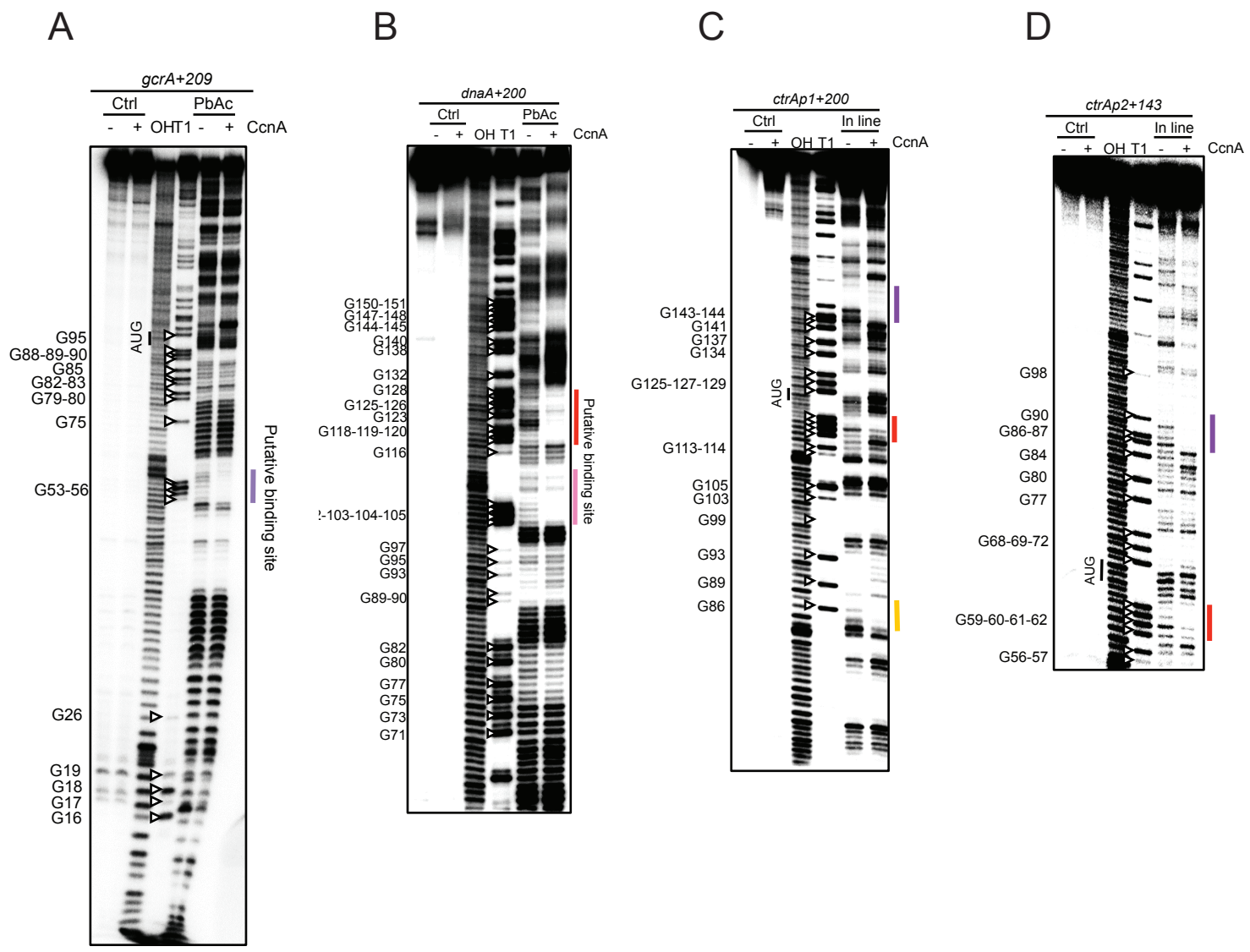


Figure 6. Probing of CcnA against *ctrA* P1 and P2, *dnaA* and *gcrA* UTR regions. A. Probing experiments (see Materials and Methods) were performed using a probe corresponding to the GcrA UTR. B. Probing experiments using a probe corresponding to the DnaA UTR. C. Probing experiments using a probe corresponding to the CtrA P1 UTR. D. Probing experiments using a probe corresponding to the CtrA P2 UTR.

Discussion

The origin of replication of *Caulobacter* chromosome represents one of the most important regions of the genome as it's absolutely necessary for replication of the genetic material and hence life. The origin itself may be empty of genes as its function was completely dedicated to DNA replication. However transcriptomic data revealed that some parts were transcribed; for example a short gene was present (CCNA_R0094) and its sequence was essential as no transposition event was ever selected in a TnSeq approach (Christen et al., 2011; Schrader et al., 2014; Zhou et al., 2015). This gene is surrounded by CtrA boxes at -23 bp from the TSS and at the very end of the gene (Brilli et al., 2010). CtrA binding sites at the origin of replication play an inhibitory role on the replication of DNA as they allow CtrA~P to compete out the binding of DnaA (Frandi and Collier, 2019). Here we investigated the role of CcnA in controlling cell cycle. Its role has emerged as central in the regulation of two master regulators of cell cycle, CtrA and GcrA.

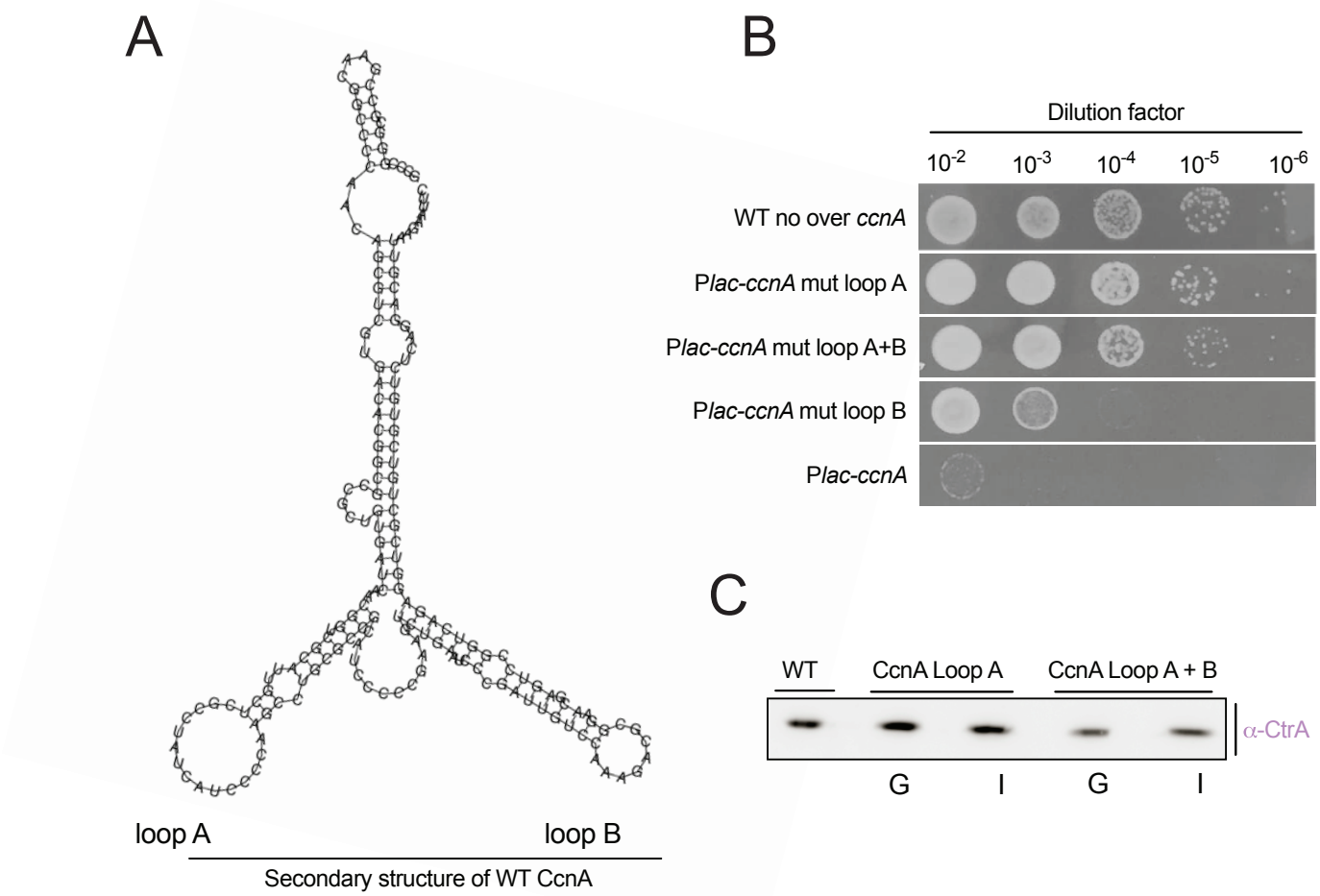
Using qPCR and FISH we clearly showed that CcnA starts accumulating in the second half of the S-phase coincidentally with CtrA itself being accumulated, presumably by the transcription driven by the CtrA promoter P1. We showed that the expression of *ccnA* depends on CtrA, as *ccnA* is one of the genes that are affected in the temperature sensitive allele of CtrA (Thr-170 to Ile). Once CcnA starts being accumulated, it binds *in vivo* (MAPS results) the mRNA of CtrA by at least two regions belonging to loops (Figure 7A), in particular Probing experiments showed that a stretch of CCCC is particularly active on target sequences, possibly stabilizing the interaction. We hypothesize that this binding of CcnA on CtrA UTR region causes a liberation of the RBS followed by an increase of protein levels. We predicted the structure of the UTR regions starting from the TSS of promoter P1 and P2 of the gene *ctrA* with and without CcnA (Data not shown). At the pure prediction level it appears evident that the mRNA of CtrA has the RBS blocked in a stem region while binding of CcnA frees the RBS.

Examples of ncRNAs controlling and enhancing key functions can be found elsewhere (Fröhlich and Vogel, 2009), such as in *Pseudomonas stutzeri* A1501 with NfiS, a positive regulator of the Nitrogenase (Zhan et al., 2016). In this system, a compact ncRNA structure acts on the mRNA of *nifK*, encoding the β-subunit of the MoFe protein of the nitrogenase enzymatic complex, enhancing the translation.

Indeed CcnA plays a role in the regulation of the expression of CtrA as an activator of translation. The regulatory circuit created by CtrA-CcnA and back to CtrA represents a positive feedback loop in which the regulatory layer controlled by CcnA acts on top of a second layer of transcriptional auto-activation of CtrA on its second strong promoter P2. In parallel CtrA has an inhibitory activity on GcrA, creating a negative feedback loop in which GcrA activates CtrA that in turn blocks GcrA. CcnA acts as well on this feedback reinforcing a reduction of translation by direct binding onto the UTR region of GcrA. Therefore CcnA in principles doesn't create new connections between master regulators of cell cycle but in fact acts on a preexisting circuit increasing presumably the robustness of the system. This behavior by sRNAs has been described before (Dutta and Srivastava, 2018; Mandin and Guillier, 2013; Nitzan et al., 2017). The role of sRNAs therefore is to consolidate the robustness of transcriptional circuits by introducing a fast translational control on the mRNAs produced by transcription factors. From this point of view CcnA may indeed act as key trigger for protein production linking transcription to translation. The importance of CcnA emerges when redundant mechanisms of CtrA control are not present, such as the absence of GcrA (primary activator of CtrA expression in the second half of S-phase).

Is this CcnA dependent mechanism, controlled by CtrA itself, also conserved in organisms in which CtrA regulates cell cycle? We investigated *S. meliloti* where the role of CtrA has been investigated (Pini et al., 2013, 2015). In these two organisms CtrA is essential and controls key cell cycle functions such as cell division and DNA replication. Accordingly to our hypothesis the expression of *Caulobacter* CcnA causes the same molecular alterations as described here. Although more molecular

Figure 7



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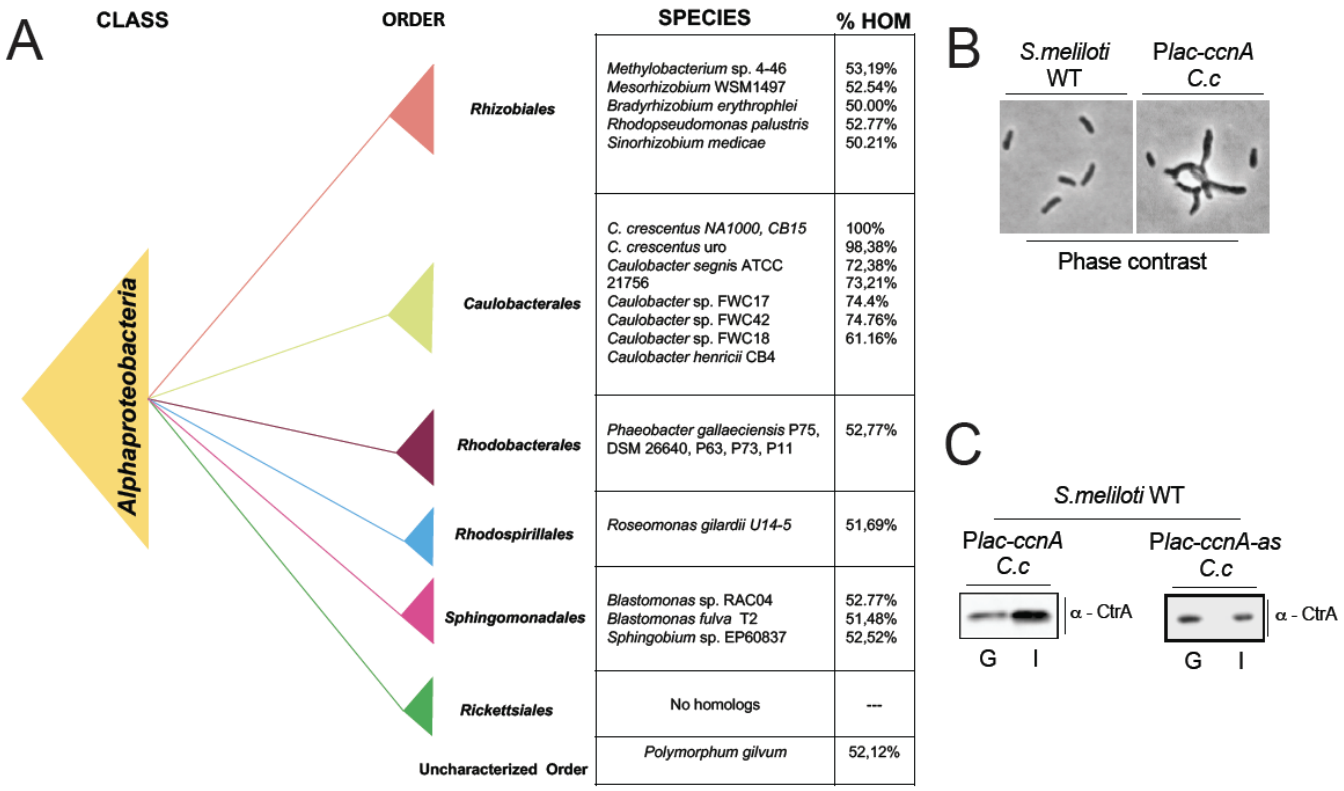
investigation of homologous ncRNAs in these organisms must be explored, we can easily conclude that CcnA activity may be indeed a conserved mechanism of the regulation of cell cycle. This new system of complex regulatory circuits carried out by CcnA indeed expand the key role of ncRNAs in bacteria opening a new crucial activity that will need a thorough molecular investigation of mechanistic activity of this Y-shaped RNA. The CcnA structure and consequent activity may be a new class of ncRNAs whose role is still at its beginning of study, presenting interesting structural similarities with tRNAs. Interestingly a prediction of target genes among several homologs have shown that targets usually falls into the chemotaxis and motility classes of genes suggesting a common function. This is not surprising considering that CtrA itself is considered in *Caulobacter* and most of alphaproteobacteria as a regulator of motility (Brilli et al., 2010; Greene et al., 2012).

Figure 8



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Materials and Methods

Strains and growth conditions

Caulobacter strains were routinely cultured in peptone-yeast extract (PYE) medium with appropriate amount of antibiotics (Kanamycin 25 µg/ml, Tetracycline 2 µg/ml) and 0.3% xylose or 0.2% glucose whenever necessary. The cultures were grown at 30°C or 37°C as required for different experiments. Synchronization of the *Caulobacter* cells was done using Percoll or Ludox 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, Tetracycline 10 µg/ml) as necessary. *Caulobacter* cells were transformed with different plasmids by electroporation.

MAPS

MAPS has been performed as previously described (Lalaouna et al., 2017).

RNAseq

Cultures were harvested at 0.6 DO600 and frozen in liquid nitrogen as previously described (Pini et al., 2015). Total RNA was prepared using RNeasy Mini Kit (Qiagen). Ribosomal RNAs were removed using the Bacterial RiboZero (Illumina) and libraries for MiSeq (V3 cassette) were prepared using the Stranded True Seq RNAseq Kit (Illumina).

Probing experiments

Templates for in vitro probing, containing a T7 promoter, were obtained by PCR amplification. Lead acetate degradation and In-line probing assays were performed as previously described (Lalaouna et al., 2015). In brief, 0.2 μ M of *in vitro*-generated *gcrA+209, dnaA+200, ctrAp1+200 and ctrAp2+143, 5′* -end-labeled were incubated with or without 1µM CcnA sRNA. Radiolabeled RNA was incubated 5 min at 90°C with alkaline buffer or 5 min at 37°C with ribonuclease T1 (0.1 U; Ambion) to generate the Alkaline (OH) ladder and the T1 ladder, respectively. RNA was analyzed on an 8% acrylamide/7M urea gel.

Microscopy analysis

Cells were observed on a 24x50 mm coverslip under a 0.15% agarose-PYE "pad" to immobilize the cells. Samples were observed thanks to an epifluorescent-inverted microscope Nikon Eclipse TiE E PFS (100 x oil objective NA 1.45 Phase Contrast). Cells morphologies and fluorescent images were analysed using ImageJ and MicrobeJ (Ducret et al., 2016; Schneider et al., 2012).

Fluorescent in situ Hybridization (FISH)

RNA- experiments were conducted following the protocols described previously (Montero Llopis et al., 2010; Russell and Keiler, 2009) , 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 1X 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 1X PBS + 0.05% Tween 20, followed by once with 1X PBS. Cells resuspended in 1x PBS. To a clean and sterile cover slip (round ones) 10 □l poly-L-lysine (Sigma) applied and kept at room temperature for 10 min. Excess poly-L-lysine was removed with kim-wipes. For super resolution microscopy cells were spread on Super resolution coverslips. 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 of cold acetone (-20°C) was

added and kept for 30 seconds. 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 2X SSC) to each coverslip and incubating at 37°C for 1 hr. RNA-FISH probes (Table S5) for CcnA (CAL Fluor Red 610) or CtrA (Fluorescein) by Stellaris were mixed at 200 uM concentration with 25 µl of hybridization buffer I (2X 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 [2X 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 + 2X SSC solution. Then the coverslips were washed 5 times, each with 100 µl of 1X PBS for 1 min. The coverslips were mounted on 10 µl of mounting medium (REF) 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.

Acknowledgments

We thank Yann Denis of the Transcriptomic platform of IMM for qPCR analysis. We thank also Patrick Viollier for the phage CbK.

Figure Legends

Figure 1. CcnA (Cell Cycle Non-coding RNA A) regulates cell cycle. A. Levels of CcnA were compared to 16S by qRT-PCR. Samples from a synchronized population (see schematics below the plot) were isolated and RNA was extracted. On the same synchronized samples Phos-Tag anti-CtrA was performed measuring both protein and phosphorylation levels. B. A temperature sensitive allele of *ctrA* (CtrA401) was used to measure CcnA RNA levels at 30ºC and 37ºC. Expression of CcnA drops upon inactivation of CtrA. C. Phase contrast images of strains overexpressing CcnA (P*lac-ccnA*), its antisense (P*lac-ccnA-as*), wild type (WT) and the empty vector (pSRK-empty). D. Cultures of the strains of panel C were grown until 0,6 and grown at different dilutions. E. Western blots using anti-CtrA and anti-GcrA antibodies in P*lac-ccnA*, P*lac-ccnA-as* and empty vector using glucose and IPTG conditions (see M&M). Upon induction of CcnA, CtrA levels increase while they decrease when expressing the antisense of CcnA. For GcrA we observed the opposite situation, while the empty vector doesn't show any variation between glucose and IPTG. F. Phos-tag gel anti-CtrA of the strain P*lac-ccnA* in comparison with the strain containing the empty vector in glucose and IPTG conditions.

Figure 2. CcnA binds *in vivo* the mRNAs of *ctrA* and *gcrA* as revealed by MAPS. A. MAPS (MS2 Affinity Purification high-throughput Sequencing) technique requires the fusion of the sRNA sequence with a RNA tag MS2. This tag has affinity for the protein MS2, which is fused to the protein maltose binding protein (MBP) that allows the binding to an amylose column. The expression of *ms2-ccnA* in the Caulobacter cells permits the formation of mRNAs-CcnA complexes that are then trapped on the amylose column. RNAs are then sequenced by RNAseq. B. Results of MAPS using MS2-CcnA in comparison with non-tagged CcnA (neg). Genes under the control of CtrA are also listed in the last column.

Figure 3. CcnA shows subcellular localization in late predivisional cells. A. FISH (Fluorescent in situ hybridization) was used to visualize CcnA (red) and CtrA (green) RNAs in *Caulobacter* cells. A population was synchronized and time points were recovered every 15 minutes. CcnA signal is undetectable until 60 minutes of the cell cycle (early S-phase), starting to increase until 90 minutes as shown by q-RT-PCR (Figure 1A). B. At 90minutes of the cell cycle CcnA signal is at the highest level. In order to quantify precisely the localization of CcnA in predivisional cells, ca. 500 cells were analyzed by MicrobeJ and the signal distribution was plotted longitudinally. As clearly showed, cells present a more intense signal in the mid cell region. C. These results were plotted in a single cell showing a clear mid cell localization.

Figure 4. CcnA rescues the CcrM loss of function phenotype. A. Regulatory mechanism of P1 promoter activation by GcrA and CcrM methylation in wild type (WT) and Δ*ccrM*. The absence of CcrM causes a loss of the methylation at the P1 promoter, decreasing the affinity of GcrA for the promoter region. B. Phase contrast microscopy of wild type (WT), a strain with the empty vector (WT + pSRK), Δ*ccrM* and Δ*ccrM* with the plasmid expressing CcnA (Δ*ccrM* + *ccnA*). C. Quantification of morphological types in different genetic backgrounds. D. Complete suppression of the motility defect of Δ*ccrM*. F. Western blotting using CtrA antibodies in WT, Δ*ccrM* and Δ*ccrM* expressing CcnA.

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Author Contribution

WB and EGB conceived the experiments and wrote the manuscript. KP performed Probing. DL and WB performed MAPS. WB performed RNAseq. WB and MD performed microscopy analysis. NB, OV participated in the cloning and western blotting, respectively.

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