

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

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20 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*.

34 Introduction

35 *Caulobacter crescentus* is a pivotal model organism to understand how basic functions of the cell
36 physiology are organized and coordinated through the cell cycle (Collier, 2012; Skerker and Laub,
37 2004). *Caulobacter* combines the cultivation and genetic simplicity of a prokaryotic system with an
38 intricacy of regulation that is paradigmatic of global regulatory programs of higher organisms.
39 Transcriptional regulation plays a major role during cell cycle progression as several master regulators
40 are sequentially activated in order to induce transcription of genes required at a specific phase of the
41 cycle (Figure 1A) (Collier et al., 2006, 2007; Reisenauer and Shapiro, 2002). Each phase is under the
42 control of a specific master regulator: (i) the initiation of the S-phase depends on DnaA, (ii) the first
43 part of S-phase depends on GcrA and CcrM, while (iii) the second part depends on CtrA which is also
44 the regulator of the phase G1 of swarmer cells (Panis et al., 2015). The interconnections of these four
45 transcriptional regulators create a complex network whose behavior derives from the integration of
46 multiple levels of regulation around master regulators. In particular, a central role is played by the
47 essential response regulator CtrA, which controls directly or indirectly all the other master regulators
48 of the cell cycle (Laub et al., 2002). CtrA is notably responsible for the direct transcriptional activation
49 of key cell division genes and the biogenesis of polar structures (flagellum, stalk and pili). CtrA also
50 activates the transcription of the orphan adenine methyl transferase CcrM encoding gene, which in
51 turn is required for the fine-tuned regulation of the promoter P1 of *ctrA* (Reisenauer and Shapiro,
52 2002). Moreover CtrA indirectly blocks the activity of DnaA at the single origin of the chromosome
53 replication (CORI) by direct binding to sites that are competing with DnaA sites required for initiation
54 of DNA replication (Marczynski and Shapiro, 2002; Quon et al., 1998). Finally another master
55 regulator, named GcrA, activates *ctrA* (together with the CcrM methylation as mentioned earlier),
56 which in turns represses *gcrA* by direct binding to its promoter (Fioravanti et al., 2013; Haakonsen et
57 al., 2015; Holtzendorff et al., 2004). CtrA encoding gene is activated by two promoters, a GcrA-
58 dependent P1, which is activated by the conversion of a CcrM methylation site from its full to the
59 hemi-methylation state approximately after 1/3 of DNA replication (Reisenauer and Shapiro, 2002).
60 This P1 activation is responsible for the first weak accumulation of CtrA, which in turns starts
61 activating a second stronger promoter P2, closer to the ATG, responsible for the robust accumulation
62 of CtrA in the second half of DNA replication and the repression of P1 and *gcrA* transcription.
63 Although the molecular details of this biphasic activation of *ctrA* are still partially understood, the
64 stronger activation of P2 may underline other post-transcriptional mechanisms reinforcing CtrA
65 accumulation. CtrA in order to be fully active requires phosphorylation by the CckA-ChpT
66 phosphorelay (Biondi et al., 2006a). The hybrid kinase CckA has a bipolar localization (Biondi et al.,
67 2006a; Chen et al., 2011; Jacobs et al., 2003). At the swarmer pole it acts as kinase thanks to the
68 presence of DivL and DivK non-phosphorylated (Gora et al., 2010) by the presence of its phosphatase
69 PleC. On the contrary CckA is a phosphatase at the stalked pole, in which the kinase DivJ keeps DivK
70 fully phosphorylated inhibiting the phosphorelay.
71 As CtrA~P blocks the origin of replication, a complex degradation machinery ensures its cell cycle
72 dependent degradation at the G1 to S-phase transition and after cell division in the stalk compartment
73 (Joshi et al., 2015; Ryan et al., 2004). A cascade of adapter proteins (CpdR, RcdA and PopA) is
74 responsible for the specific and highly regulated proteolysis of CtrA.
75 In *Caulobacter*, the regulation of gene expression by ncRNAs has revealed few examples. Initially
76 only 27 ncRNAs were described in this organism (Landt et al., 2008). CrfA is an sRNA involved in
77 adaptation to carbon starvation (Landt et al., 2010). GsrN is involved in the response to σ^T -dependent
78 multiple stresses (Tien et al., 2017). Finally ChvR has been recently characterized as a ncRNA that is
79 expressed in response to DNA damage, low pH, and growth in minimal medium (Fröhlich et al.,
80 2018). However as new recent approaches using RNAseq and post-genomic techniques expanded the
81 *plethora* of ncRNA candidates to more than 100 (Zhou et al., 2015), predictions of their integration
82 into the cell cycle circuit (Beroual et al., 2018) has suggested that several new candidate ncRNAs
83 should be deeply studied.
84 Here we investigated the role of a ncRNA, named CcnA, that belongs to the origin of replication of
85 *Caulobacter* chromosome. We studied its role by the construction of deletion mutants, silencing by
86 expression of its antisense and over expression. Results presented in this work identified the mRNAs
87 of CtrA and GcrA, two master regulators of cell cycle, as main targets of this ncRNA. Data were

Figure 1

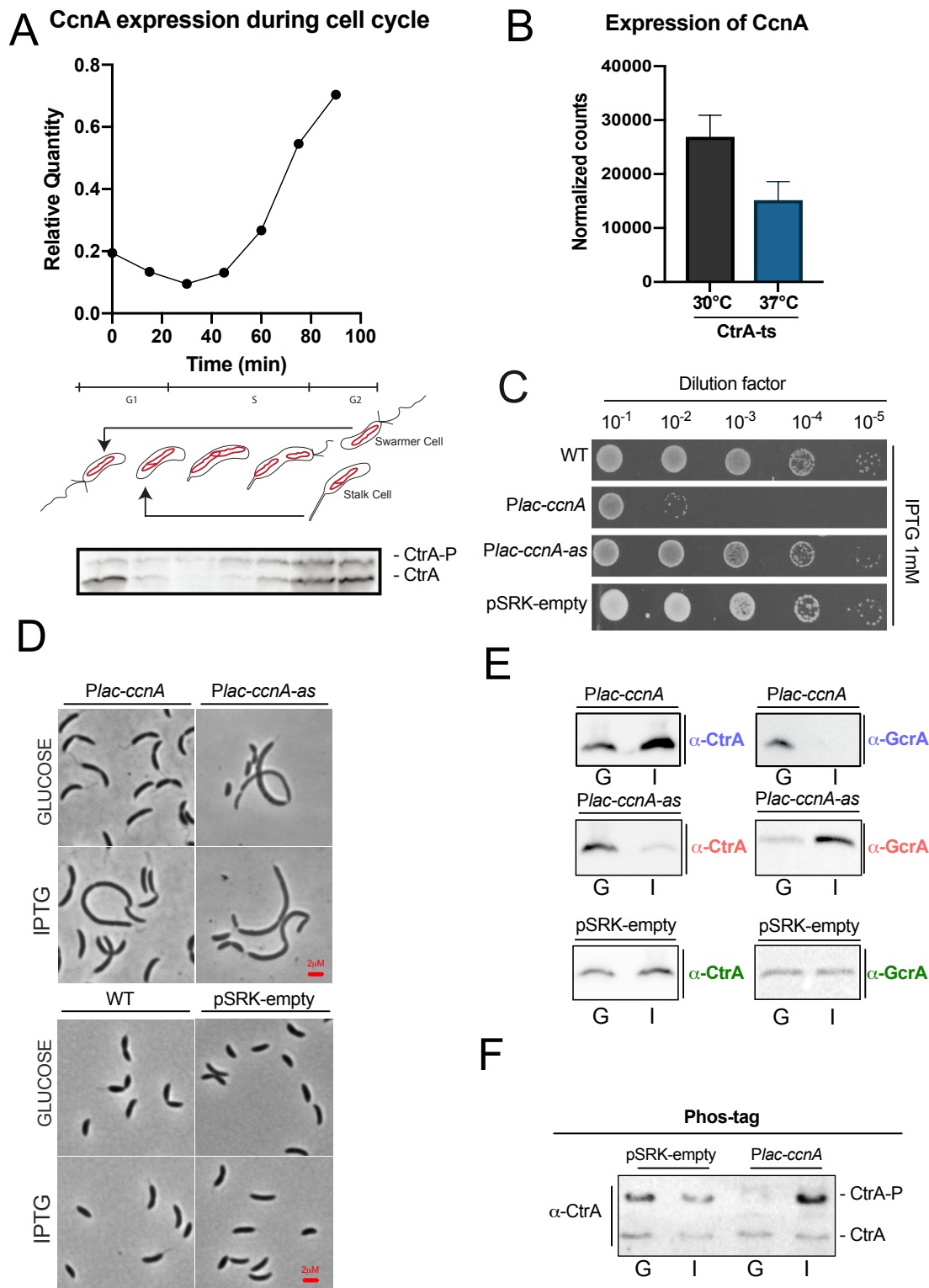


Figure 1. CcnA (Cell Cycle Non-coding RNA) regulates cell cycle. A. Levels of CcnA were compared to 16S by qRT-PCR. Samples from a synchronized population (see schematics below the plot) were isolated and RNA was extracted. On the same synchronized samples Phos-Tag anti-CtrA was performed measuring both protein and phosphorylation levels. B. A temperature sensitive allele of *ctrA* (*CtrA401*) was used to measure CcnA RNA levels at 30°C and 37°C. Expression of CcnA drops upon inactivation of CtrA. C. Phase contrast images of strains overexpressing CcnA (*Plac-ccnA*), its antisense (*Plac-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 *Plac-ccnA*, *Plac-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 *Plac-ccnA* in comparison with the strain containing the empty vector in glucose and IPTG conditions.

88 supported by a combination of MAPS, *in vitro* and *in vivo* experiments. Finally the role of CcnA in a
89 closely related organism such as *Sinorhizobium meliloti* suggested its potential conservation across
90 bacteria.
91

92 **Results**

93 **CcnA expression is regulated by CtrA**

94
95 Based on previous results (Zhou et al., 2015) we observed that CCNA_R0094, here named Cell Cycle
96 non-coding RNA A (CcnA), expression peaks after few minutes from the accumulation of *ctrA*
97 transcript and protein, in the second half of the S-phase, when P2, the second *ctrA* promoter, is
98 activated (Fig 1A).

99 We designed primers to detect and precisely quantify *ccnA* RNA in the cells by q-RT-PCR (see
100 M&M) with respect to 16S RNA levels. A synchronized population of wild type *C. crescentus* was
101 used to collect cells at 15 minutes time points of the cell cycle in rich medium (generation time is 96
102 minutes). CcnA levels start increasing at 45 minutes, coincidentally with CtrA protein levels (Figure
103 1A). More specifically we measured both protein and phosphorylation levels of CtrA by Phos-Tag
104 gels (Figure 1A). CcnA levels increase as CtrA~P levels increase (Figure 1A), suggesting that the
105 transcription of *ccnA* potentially depends on phosphorylated CtrA. This observation prompted us the
106 question whether CtrA was responsible for *ccnA* transcription. Consistently upstream of the annotated
107 Transcriptional Start Site (TSS) of *ccnA* a CtrA box was previously described (Brilli et al., 2010; Zhou
108 et al., 2015).

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

117 In conclusion CcnA is a sRNA activated by the master regulator of cell cycle CtrA peaking its
118 expression when CtrA itself strongly accumulates.
119

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

121
122 Aiming to understand the function of CcnA, we fused the sequence of *ccnA* with the +1 of a *Plac*
123 promoter in the vector pSRK (Khan et al., 2008) in order to make an IPTG-inducible version of the
124 ncRNA. This vector was introduced in *C. crescentus* cells, its induction was measured and the
125 phenotype was observed (Figure 1C, D). Even in glucose *Plac-ccnA* cells showed cell cycle defects,
126 such as slow growth, cell division abnormal morphologies, with an increased number of abnormal
127 long stalks. The level of *ccnA* in this inducible system was quantified by q-RT-PCR (see Mat and Met)
128 confirming that this strain indeed expresses a high level of *ccnA* even in glucose (Figure S1).

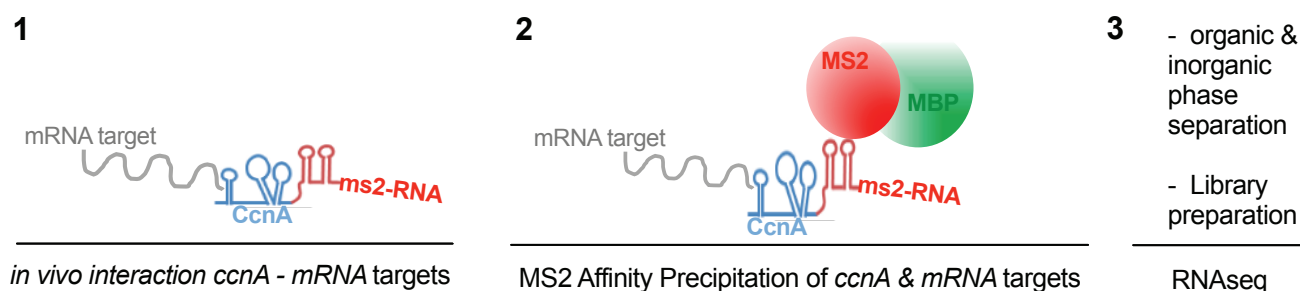
129 Several tests were performed in order to characterize these phenotypes. Morphologically we used
130 MicrobeJ to quantify cell size parameters (Ducret et al., 2016). Results showed that cells were
131 elongated with respect to wild type cells (Figure S2).

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

Figure 2

A

MAPS technique: Simplified schematic representation



B

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

139 CtrA must be phosphorylated in order to perform its functionality. Phostag technique was used, as
140 previously described (Pini et al., 2013) to quantify the levels of CtrA~P upon induction of CcnA
141 (Figure 1F). The analysis revealed that the higher level of CtrA in fact corresponds to high amount of
142 CtrA~P. As phosphorylation of CtrA is under the control of the phosphorelay CckA and ChpT we
143 tested whether levels ChpT were affected. We used YFP translational fusion of ChpT in order to
144 understand whether CcnA ectopic expression was causing a change in the protein levels.
145 Epifluorescent microscopy was used to quantify the protein level of ChpT fused with YFP and
146 expressed by its native promoter (Figure S4A). Data were further analyzed by MicrobeJ (see Mat &
147 Met) and results showed that upon CcnA induction intensity and clustering of the signal increases in
148 the ChpT-YFP strain (Figure S4B). We quantified this variation as illustrated in figure S4C.

149 Rapid accumulation of CtrA corresponds also to a decrease of GcrA levels. Therefore we also
150 quantified whether CcnA was at the same time affecting the protein level of GcrA using western blot
151 and anti-GcrA antibodies. Surprisingly the quantification of GcrA upon CcnA induction revealed a
152 significant decrease of the protein level (Figure 1E).

153 In conclusion CcnA increased expression induces a decrease of GcrA and an increase of CtrA protein,
154 specifically in its phosphorylated form. These changes in the GcrA and CtrA levels may explain the
155 cell cycle defects observed at the morphological levels, notably increase of cell length, long stalks and
156 presence of significant proportion of swarmer cells even in stationary phase (data not shown).

157

158 **Knock-out of CcnA by chromosomal deletion and antisense silencing**

159

160 The gene *ccnA* belongs to the origin of replication (Figure S5); therefore, its sequence, at least
161 partially, may play an essential role in the initiation of replication. We attempted a complete deletion
162 of the *ccnA* sequence by two-step recombination (see Mat & Met) as previously described (Skerker et
163 al., 2005) in presence of an inducible copy of *ccnA* or in the wild type background. As expected the
164 deletion of the *ccnA* sequence was not successful demonstrating that the sequence of *ccnA* is essential.
165 Same results were obtained also using the inducible copy of *ccnA*.

166 We decided to attempt different strategies allowing the inactivation of CcnA but keeping intact (most
167 of) the origin of replication. First we attempted the deletion of the promoter region including the CtrA
168 box putatively involved in *ccnA* transcription and, if successful, extending the deletion region into the
169 *ccnA* sequence. Several deletion cassettes (see Mat & Met) were prepared and used as previously
170 described for the complete deletion of *ccnA*. In order to minimize the effect of polar interference by
171 the tetR cassette we also created marker-less deletions.

172 An alternative strategy was used by overexpression of the antisense of *ccnA* (CcnAas). CcnAas was
173 fused to an IPTG-inducible promoter and its induction was tested in order to demonstrate a negative
174 effect on CcnA activity.

175 The expression of the antisense of CcnA is able to induce the opposite effect than the overexpression
176 of CcnA, that is increasing GcrA levels and decreasing CtrA levels (Figure 1C, D, E). Those results
177 strongly suggested that the expression of the antisense is able to inactivate CcnA activity and then
178 allowing the analysis of a CcnA loss of function. If the inactivation is complete, this result also
179 suggests that the inactivation of CcnA is not lethal. Supported by these results we attempted a more
180 specific deletion of *ccnA* on the chromosome by targeting its promoter region including the predicted
181 CtrA box. As shown in the previous section the *ccnA* expression should be strictly under the control of
182 CtrA, therefore the deletion of its box in the promoter region should have a mild or absent effect on
183 the origin but impair the expression of the ncRNA. Results showed that the marker less deletion of the
184 promoter of CcnA was viable but showing a severe growth defect (data not shown).

185 In conclusion both overexpression and low levels of CcnA show coherent results that suggest that
186 CcnA is indeed responsible for the activation of CtrA and the downregulation of GcrA. However we
187 asked whether this activity was due to a direct interaction of CcnA with the mRNAs of both gene and
188 moreover whether other genes were targeted by this ncRNA.

189

190 **MS2 Affinity Purification high-throughput Sequencing (MAPS)**

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

Figure 3

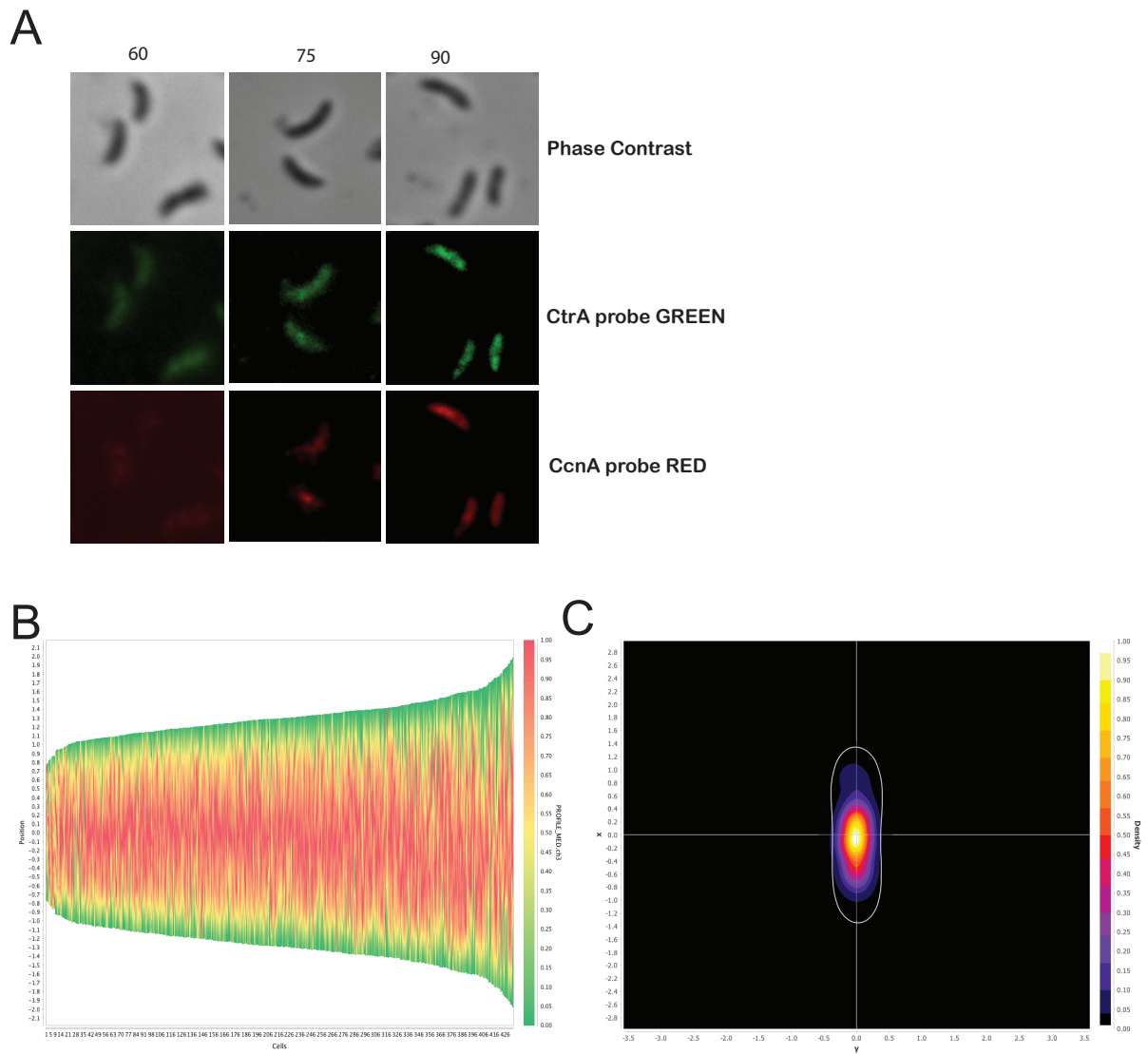


Figure 3. CcnA shows subcellular localization in late predivisinal cells. A. FISH (Fluorescent in situ hybridization) was used to visualize CcnA (red) and CtrA (green) RNAs in *Caulobacter* cells. A population was synchronized and time points were recovered every 15 minutes. CcnA signal is undetectable 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 90 minutes of the cell cycle CcnA signal is at the highest level. In order to quantify precisely the localization of CcnA in predivisinal 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.

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

215

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

217

218 Aiming to understand the expression dynamics of CcnA during cell cycle and visualize the subcellular
219 localization of CcnA and its target CtrA mRNA, we used Fluorescent *in Situ* hybridization (FISH) to
220 image RNAs in the cells. Probes for CcnA and CtrA mRNA were designed and labeled with different
221 fluorochromes (see Mat & Met). First, we synchronized a population of *Caulobacter* and visualized
222 CcnA and CtrA mRNA every 15 minutes of the cell cycle in rich medium until 90 min (Figure 3).
223 Results for CcnA (Figure 3) are perfectly matching with qPCR data (Figure 1A) increasing after 60
224 minutes and reaching a high level around 90 minutes of the cell cycle that corresponds to a late
225 predivisional stage (Figure 3A). The increased level of CcnA RNA and its accordance to qPCR levels
226 strongly support the specificity of the probe. CtrA mRNA levels are also changing over the cell cycle
227 with a similar dynamics accordingly to previous results (Laub et al., 2000; Reisenauer and Shapiro,
228 2002; Reisenauer et al., 1999).

229 Surprisingly CcnA RNA showed a clear diffused midcell localization (Figure 3B) at 90 min that may
230 suggest a condensation in this area as its levels increase and presumably the target RNA also
231 increases. It's fascinating to speculate that the localization factor for CcnA is the presence of multiple
232 copies of target mRNAs in that region of the predivisional cell.

233 We also tested FISH on the strains expressing high levels of CcnA (as showed in previous figures) as
234 showed by qPCR. Accordingly, to high RNA levels the signal of the CcnA probe is considerably
235 higher than in wild type cells and remarkably showing a patchy diffused level of CcnA RNA (data not
236 shown).

237

238 **Modulations of CcnA are able to complement mutants of cell cycle**

239

240 As methylation sites are connected to *ctrA* transcription by its own P1 promoter, we asked whether the
241 expression of CcnA was also interacting genetically with the delta-*ccrM* mutant (Murray et al., 2013).
242 We introduced *Plac-ccnA* and *Plac-ccnAas* in the CcrM mutant and analyzed the phenotypes. First we
243 were not able to stably introduce CcnA antisense into delta-*ccrM* suggesting an incompatibility
244 between the two genetic constructions. The expression of CcnA was indeed able to completely
245 suppress the sick phenotype of the delta-*ccrM* mutant (Figure 4B). Notably the severe morphological
246 defects of delta-*ccrM* were rescued (Figure 4B, C) as well as the motility defect (Figure 4D). We
247 asked whether in the CcrM deletion background CcnA was indeed able to increase CtrA levels (Figure
248 4F). Results clearly showed that CcnA is able to increase CtrA levels in the delta-*ccrM* mutant and

Figure 4

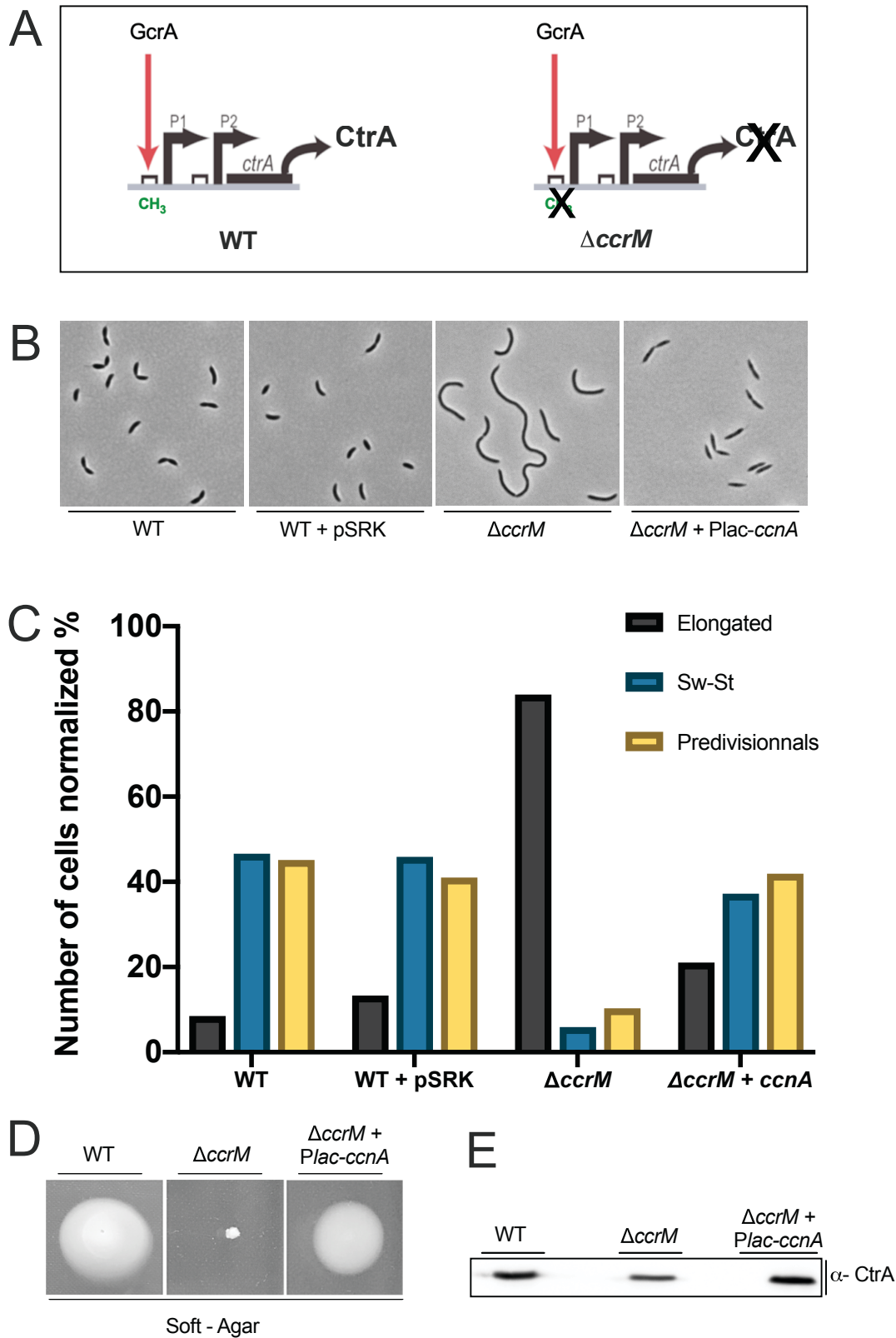


Figure 4. CcnA rescues the CcrM loss of function phenotype. A. Regulatory mechanism of P1 promoter activation by GcrA and CcrM methylation in wild type (WT) and $\Delta ccrM$. The absence of CcrM causes a loss of the methylation at the P1 promoter, decreasing the affinity of GcrA for the promoter region.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

249 that indeed the mechanism by which CcnA increases CtrA protein levels is independent from CcrM,
250 so presumably acting on the P2 promoter.

251 Supported by the results, we also decided to combine mutants of CcnA with $\Delta pleC$, a mutant involved
252 in the negative control of DivK phosphorylation level. As DivK~P inhibits CtrA stability and activity,
253 in the deletion mutant of DivK phosphatase, PleC, CtrA levels are extremely low (Figure 5A).
254 Therefore high CcnA activity should compensate the PleC deletion mutant's defects, restoring the
255 wild type phenotypes.

256 We introduced *Plac-ccnA* or *Plac-ccnAas* in $\Delta pleC$ mutant and observe the morphology, motility in
257 soft agar plates, sensitivity to the phage CbK and stalk length (Figure 5).

258 The expression of CcnA was able to rescue $\Delta pleC$ defects, restoring stalks and motility while the
259 expression of the CcnA antisense caused a very severe phenotype (Figure 5B, C). We asked whether
260 this suppression was just increasing the level of CtrA or it was indeed able also to affect the
261 phosphorylation of CtrA. We measured CtrA~P by Phos-Tag technique (Figure 5D). This analysis
262 revealed that CcnA was indeed able to increase the CtrA~P levels in $\Delta pleC$.

263 Finally we measured the sensitivity to the phage CbK, which is entering the cells by attachment to the
264 pili structures (Figure 5E). As the main subunit PilA of the pilus is completely under the control of
265 CtrA, a mutant of PleC completely lacks of pili and therefore it is resistant to CbK infection (Panis et
266 al., 2012; Sommer and Newton, 1988). Results showed that the expression of CcnA was completely
267 able to restore the sensitivity to CbK at the levels of wild type while the expression of CcnA antisense
268 did not change the resistance to the phage infection of the *pleC* mutant (Figure 6). Finally we
269 measured the stalk length of the $\Delta pleC$ in which we expressed CcnA (Figure 5F). The expression of
270 CcnA indeed restores the stalk production (absent in $\Delta pleC$), while the expression of the antisense
271 does not.

272

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

274

275 In order to understand the molecular bases of CcnA-CtrA interactions we performed Probing
276 experiments using *ctrA* (promoters P1 and P2), *gcrA* and *dnaA* UTR regions. MAPS and molecular
277 analysis revealed a putative interaction between CcnA and CtrA/GcrA mRNAs, while the interaction
278 with the UTR region of *dnaA* was only predicted (Beroual et al., 2018), but no *in vivo* link was found
279 and therefore we included as a negative control.

280 CtrA UTR region was analyzed considering the two Transcription start sites (TSS), P1 and P2,
281 controlled by GcrA/CcrM methylation and CtrA, respectively. For GcrA we used data derived from 5'
282 race experiments at the genome scale (Zhou et al., 2015). Results showed regions of protection for
283 both CtrA UTR from the promoter P1 and the promoter P2 (Figure 6). They both share most of the
284 annealing regions except for an extra distal region in P1 that is in fact outside the P2 UTR. A common
285 element of annealing was found looking into the GcrA UTR region annealing with CcnA. A stretch of
286 CCCC, present in a loop region of CcnA, is also protecting both CtrA and GcrA UTR regions.
287 Regarding DnaA that was previously predicted to be the real target of CcnA (Beroual et al., 2018),
288 Probing experiments indeed showed a clear protection that corresponded to the predicted region
289 (Figure 6). Whether this interaction is real or not, we can speculate that (i) CcnA and *dnaA* mRNA are
290 not usually expressed at the same time; (ii) MAPS did not revealed any interaction between CcnA and
291 the UTR region of DnaA; (iii) None of phenotypes of the loss or overexpression of CcnA are
292 physiologically connected to DnaA. Therefore we excluded that CcnA binds DnaA during the cell
293 cycle. However it's possible that this interaction becomes relevant during a specific response in which
294 the expression of CcnA is indeed directed to DnaA regulation. Moreover we cannot exclude that the
295 UTR region of *dnaA* is indeed inversely regulating CcnA.

296 In order to consolidate these observations we decided to check whether the CtrA UTR region was
297 indeed able to bind CcnA using a reverse MAPS in which the mRNA of *ctrA* was tagged with MS2.
298 Results clearly showed that CcnA appears among targets of *ctrA* UTR reinforcing the discovery of the
299 CcnA-CtrA mRNA interaction. As a putative negative control (based on westerns on DnaA that
300 showed no variation at the protein level) we also tested the UTR region of DnaA tagged with MS2.
301 Although this region is able to bind CcnA *in vitro* (Figure 6), by using an *in vivo* MAPS technique, we
302 were not able to detect any interaction with DnaA mRNA suggesting that in normal physiological

Figure 5

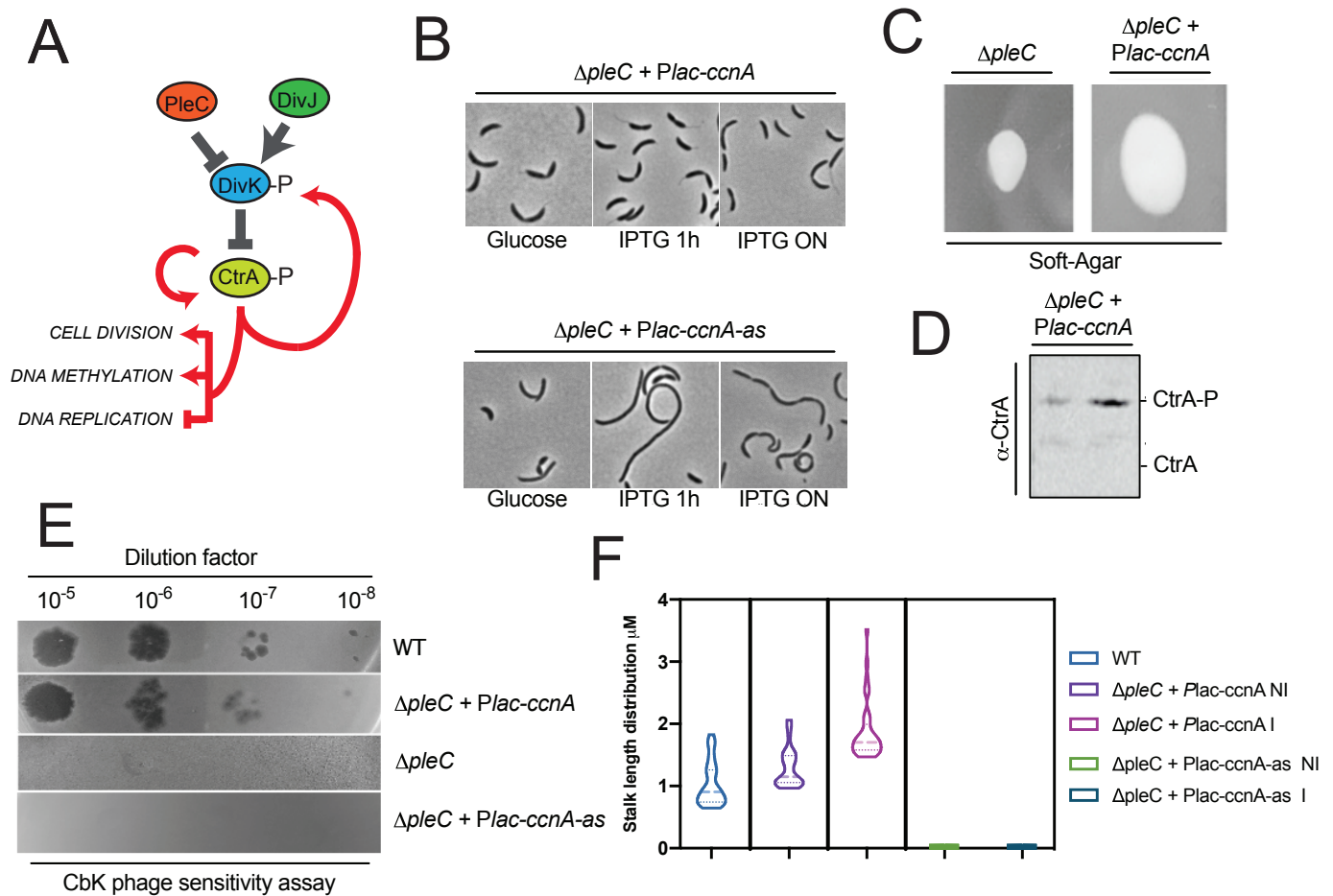


Figure 5. CcnA rescues the pleiotropic phenotypes of $\Delta pleC$. A. Schematics of CtrA-DivK negative feedback loop. DivK phosphorylation levels are controlled by the kinase DivJ and the phosphatase PleC. Absence of PleC causes a decrease of CtrA, both at the protein and phosphorylation levels. B.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

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

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

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

322

323 **Conservation of CcnA among alphas**

324

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

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

341

342 Discussion

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

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

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

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

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

Figure 7

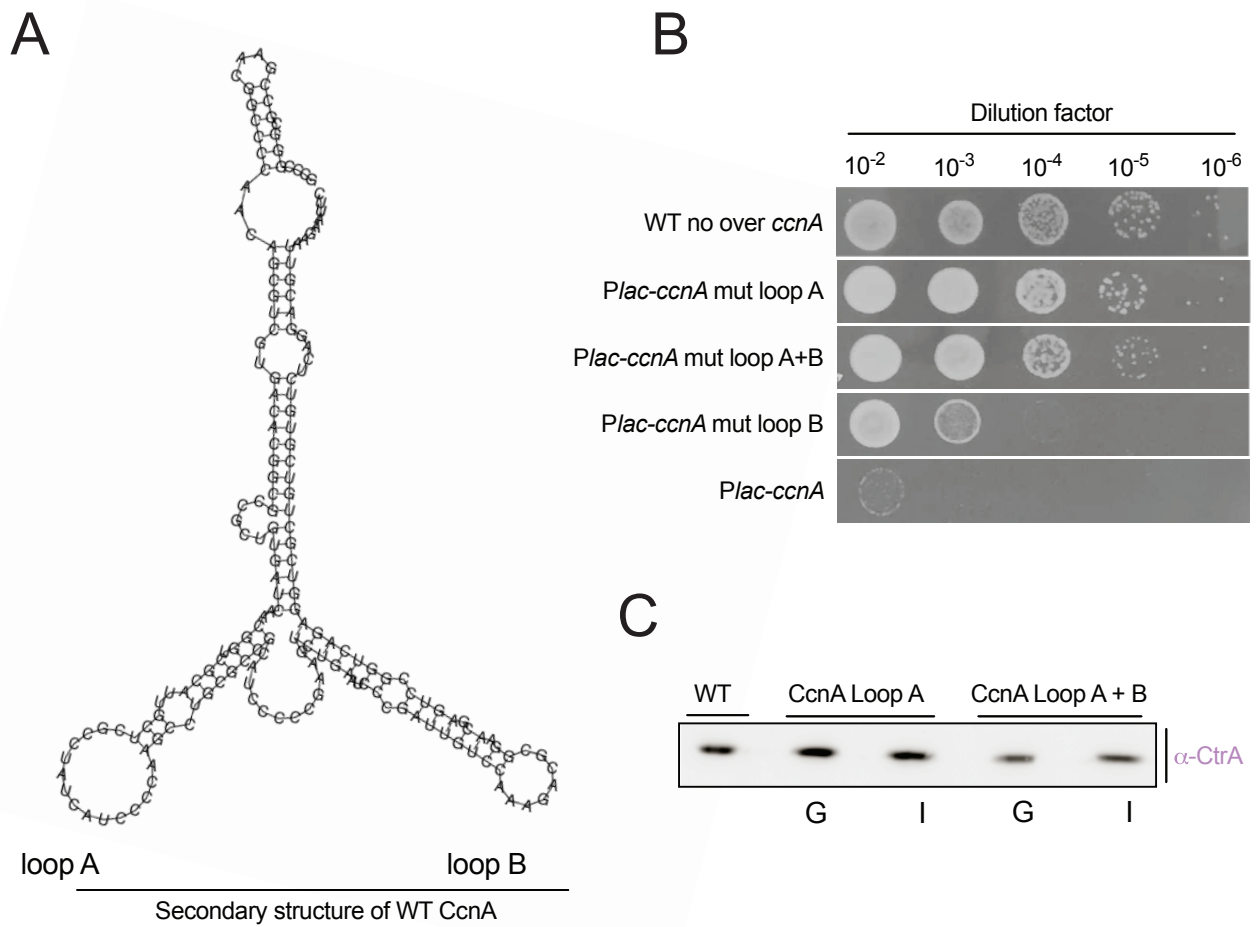


Figure 7. Mutation of loop regions of CcnA. A. Structure of CcnA, loops are marked as "Loop A" and "Loop B". B. Expression of mutated CcnA sequences in comparison with wild type in wild type, as measured by dilution series. C. Western blotting anti-CtrA on strain expressing mutated versions of CcnA (with loopsA and A+B).

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

401

Figure 8

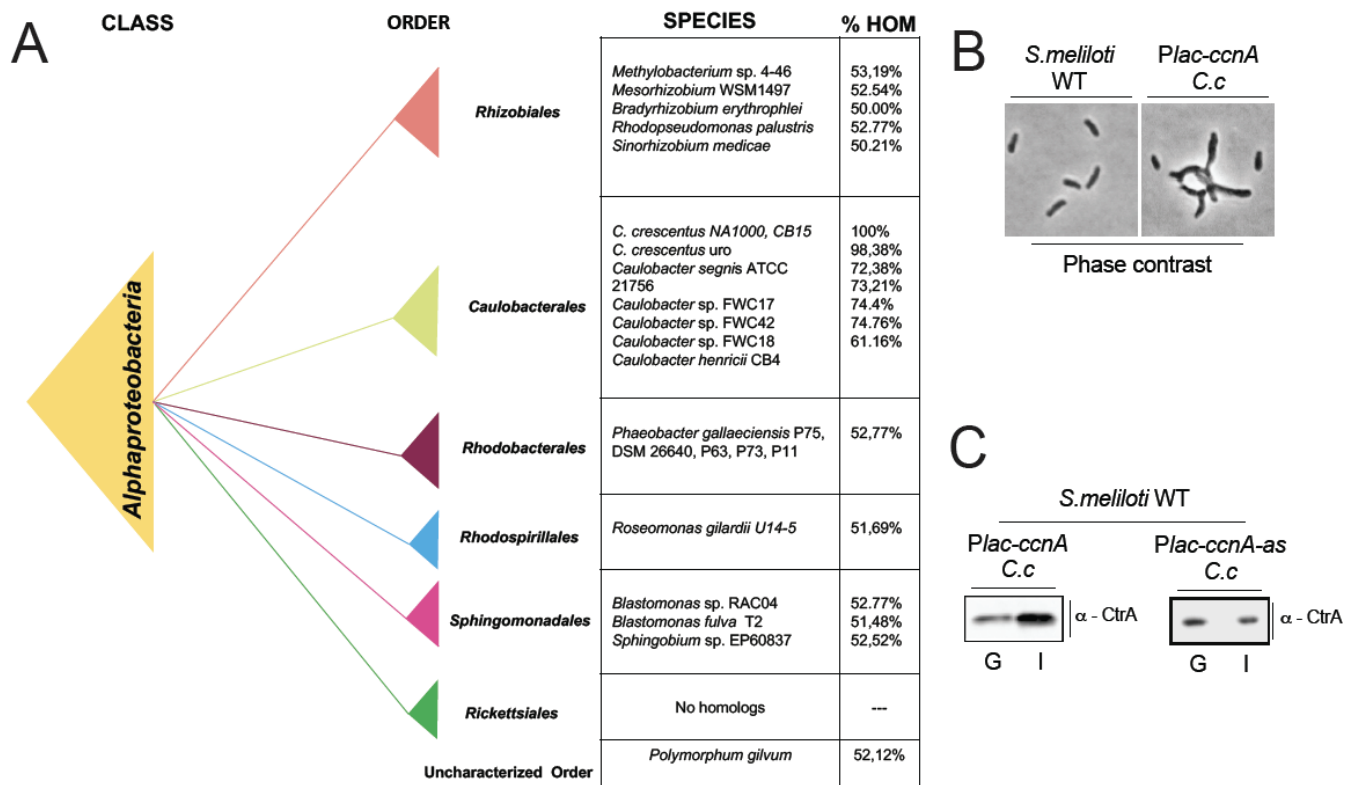


Figure 8. Conservation of CcnA among alphaproteobacterial species and expression of CcnA in *S. meliloti*. A. Homologs of CcnA in several species of the class *Alphaproteobacteria*. B. Expression of CcnA from *C. crescentus* (*C. c.*) causes a severe cell cycle defect upon induction. C. Western blotting against CtrA shows that expression of *C. crescentus* CcnA induces an increase of CtrA levels in *S. meliloti* while its antisense has no effect.

402 **Materials and Methods**

403 **Strains and growth conditions**

404 *Caulobacter* strains were routinely cultured in peptone-yeast extract (PYE) medium with appropriate
405 amount of antibiotics (Kanamycin 25 µg/ml, Tetracycline 2 µg/ml) and 0.3% xylose or 0.2% glucose
406 whenever necessary. The cultures were grown at 30°C or 37°C as required for different experiments.
407 Synchronization of the *Caulobacter* cells was done using Percoll or Ludox as described before (Marks
408 et al., 2010). *E. coli* strains were grown at 37°C in LB broth or solid medium with required amount of
409 antibiotic supplements (Ampicillin 100 µg/ml, Kanamycin 50 µg/ml, Tetracycline 10 µg/ml) as
410 necessary. *Caulobacter* cells were transformed with different plasmids by electroporation.

411 **MAPS**

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

413 **RNAseq**

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

418 **Probing experiments**

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

426 **Microscopy analysis**

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

431 **Fluorescent in situ Hybridization (FISH)**

432 RNA- experiments were conducted following the protocols described previously (Montero Llopis et
433 al., 2010; Russell and Keiler, 2009) , with few modifications. The method is described as follows.
434 *Caulobacter* cells grown up to mid-exponential phase or isolated from different stages of a
435 synchronized population were fixed with 4% formaldehyde (in 1X PBS, pH 7.4) for 15 min at room
436 temperature followed by 30 min on ice. Then cells were briefly centrifuged and supernatant removed.
437 The pellet washed thrice with 1X PBS + 0.05% Tween 20, followed by once with 1X PBS. Cells
438 resuspended in 1x PBS. To a clean and sterile cover slip (round ones) 10 µl poly-L-lysine (Sigma)
439 applied and kept at room temperature for 10 min. Excess poly-L-lysine was removed with kim-wipes.
440 For super resolution microscopy cells were spread on Super resolution coverslips. Then 10 µl of cell
441 suspension was added and kept at room temperature for 10 min. Excess liquid removed with kim-
442 wipes. To the coverslip with attached cells, 100 µl cold methanol (-20°C) was added and incubated for
443 1 min. Methanol removed slowly with micropipette and then 100 µl of cold acetone (-20°C) was

444 added and kept for 30 seconds. Acetone was removed with micropipette. Coverslips were kept in open
445 to become dry. Pre-hybridization and hybridization were set up in small petri dishes, each containing a
446 single coverslip, and the petridishes were kept in a humidified chamber incubated at the required
447 temperature. Pre-hybridization was done by adding 100 μ l of pre-hybridization buffer (40%
448 formamide in 2X SSC) to each coverslip and incubating at 37°C for 1 hr. RNA-FISH probes (Table
449 S5) for CcnA (CAL Fluor Red 610) or CtrA (Fluorescein) by Stellaris were mixed at 200 μ M
450 concentration with 25 μ l of hybridization buffer I (2X SSC, 80% formamide, 70 μ g/ml Salmon Sperm
451 DNA, 1 mg/ml E. coli tRNA) to a concentration 250 nM and heated at 65°C for 5 min, to which equal
452 volume (25 μ l) of hybridization buffer II [2X SSC, 20% Dextran Sulfate, 10 mM Vanadium
453 Ribonucleoside Complex (VRC) (NEB), 0.2% BSA, 40 U RNase Inhibitor] was added. 50 μ l of the
454 hybridization buffer added to each coverslip and the whole humidified chamber was incubated at 37°C
455 for overnight. Following the hybridization, the coverslips were washed twice, each for 15 min, with
456 100 μ l of 50% formamide + 2X SSC solution. Then the coverslips were washed 5 times, each with
457 100 μ l of 1X PBS for 1 min. The coverslips were mounted on 10 μ l of mounting medium (REF) on
458 glass slides. The slide was kept at room temperature for at least 1 hr followed by 3-4 hrs at 4°C to
459 stabilize the medium.

460

461 **Acknowledgments**

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

464 **Figure Legends**

465

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

479

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

488

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

498

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

506

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

516 **Figure 6. Probing of CcnA against *ctrA* P1 and P2, *dnaA* and *gcrA* UTR regions.** A. Probing
517 experiments (see Materials and Methods) were performed using a probe corresponding to the GcrA
518 UTR. B. Probing experiments using a probe corresponding to the DnaA UTR. C. Probing experiments
519 using a probe corresponding to the CtrA P1 UTR. D. Probing experiments using a probe
520 corresponding to the CtrA P2 UTR.
521

522 **Figure 7. Mutation of loop regions of CcnA.** A. Structure of CcnA, loops are marked as “Loop A”
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526

527 **Figure 8. Conservation of CcnA among alphaproteobacterial species and expression of CcnA in**
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531 in *S. meliloti* while its antisense has no effect.
532
533
534

535 Author Contribution

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

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