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Direct single cell observation of a key *E. coli* cell cycle oscillator

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A long-standing hypothesis sees DNA replication control in *E. coli* as a central cell cycle oscillator at whose core is the DnaA protein. The consensus is that the activity of the DnaA protein, which is dependent on its nucleotide bound state, is an effector of initiation of DNA replication and a sensor of cell size. However, while several processes are known to regulate DnaA activity as a function of the cell cycle, the oscillations in DnaA expression and DnaA activity have never been observed at the single cell level, and their correlation with cell volume has yet to be established. In this study, we measured the volume-specific production rate of a reporter protein under control of the *dnaAP2* promoter in single cells. By a careful dissection of the effects of DnaA-ATP- and SeqA-dependent regulation, two distinct cell cycle oscillators emerge. The first oscillator, driven by gene dosage, DnaA activity and SeqA repression oscillates synchronously, and shows a causal relationship, with cell size and divisions, similarly to initiation events. The second one, a reporter of dosage and DnaA activity only, is strongly coupled to cell size, but loses the synchrony and causality properties, suggesting that DnaA activity peaks do not correspond directly to initiation events. These findings suggest that while transcription regulation by DnaA activity performs volume sensing, transient inhibition of gene expression by SeqA following replication fork passage keeps DnaA activity oscillations in phase with initiation events.

1 The DnaA protein is a key factor for the initiation of DNA replication and an essential protein
2 for most known bacteria ¹. When it is in its ATP-bound (“active”) form, DnaA binds to a set of
3 specific sites at the origin of DNA replication (oriC) leading to the formation of an oligomeric
4 structure and the subsequent melting of an AT-rich region required for the assembly of the DNA
5 replication forks ^{2–5}. Based on population measurements, it is believed that the amount of DnaA-
6 ATP needs to reach a threshold value once per cell cycle for this structure to form, leading to the
7 initiation of DNA replication ^{6–8}. Experiments in bulk exploring a large range of growth rates have
8 shown that cell size at initiation of DNA replication is related to the growth rate and is correlated
9 with the concentration of the DnaA protein ⁹. In *E. coli*, the DnaA-dependent regulatory circuit is
10 made of different positive and negative components ¹⁰. Several factors contribute to the decrease
11 in DnaA activity after initiation has taken place ¹¹. Firstly, the expression of the *dnaA* gene is
12 prevented for a fraction of the cell cycle by the SeqA protein binding to hemi-methylated GATC
13 sites at the promoter, and within the *dnaA* gene itself, after their replication ^{12–16}. The *dnaA* gene
14 is located close to the replication origin, and SeqA follows the forks, transiently repressing its
15 expression immediately after each initiation. Secondly, inhibition of transcription initiation by
16 the oligomerisation of DnaA-ATP itself decreases the production of DnaA ^{17–20}. This is thought to
17 occur at the time of initiation, when DnaA-ATP concentration is at its peak. Both of these processes
18 inhibit DnaA protein expression and are related to the timing of initiation of DNA replication and
19 to fork progression through the genomic position of its gene ¹³. After initiation has taken place, the
20 rate of hydrolysis of the ATP bound to DnaA is increased via an interaction with the Hda protein
21 mediated by the β -clamp during ongoing DNA replication in a process called RIDA (Regulatory
22 Inactivation of DnaA) ²¹. Finally, the binding of DnaA-ATP to the *datA* site also contributes to the
23 conversion of DnaA-ATP to DnaA-ADP after initiation, via *datA*-dependent DnaA-ATP hydrolysis
24 (DDAH) ²². The increase in DnaA-ATP required for the initiation of a new DNA replication cycle
25 depends on the timely accumulation of newly expressed protein ^{7,8,13} and on the binding of DnaA
26 to the DARS1 and DARS2 sites, contributing to a further increase in the DnaA-ATP to DnaA-ADP
27 ratio by favoring the exchange of the DnaA-bound nucleotide ^{10,23–25}.

28 Together, these processes lead to the belief that DnaA activity is a cell cycle oscillator.
29 Specifically, oscillations in DnaA activity are believed to play a key role at faster growth rates,
30 when the frequency of initiation increases relative to the time of genome replication ^{12,26–28}. How-
31 ever, given the complexity of this regulatory circuit, one of the major challenges in the field has
32 been to find a way to measure the oscillations in DnaA activity in real time, particularly because

33 they occur at the level of the single cell and they are not synchronized across cells in a normally
34 dividing population. Moreover, while the different mechanisms regulating DnaA activity via ATP
35 hydrolysis have been carefully characterized, less is known on the real time dynamics of DnaA's
36 gene expression as a function of the cell cycle and its coupling to cell size. In this study, we rely
37 on a chromosomal promoter-reporter system based on the *dnaA* promoter itself as a reporter of
38 the relative contributions of DnaA-ATP and SeqA on the expression of DnaA and to measure their
39 relationship with cell size and cell division.

40 Monitoring these oscillations in single cells is particularly important because it makes it
41 possible to compare the oscillator with known single cell observations of replication-initiation
42 reporters²⁹⁻³⁶. These studies reported a constant (independent from cell size at initiation) added
43 size between initiation events and also between the initiation of DNA replication and cell
44 division³³. However, a direct link is still missing between possible single cell oscillations in DnaA
45 activity and cell size correlation patterns of initiation events.

46 **The *dnaA* promoter as a reporter of the changes in DnaA-ATP activity and SeqA *in vivo***

47 Since DnaA in the cell exists under two forms, ATP and ADP bound, only the former being the
48 one that can initiate DNA replication, we looked for a reporter of DnaA-ATP activity to study
49 the regulation of DNA replication in *E. coli* in real time *in vivo*. We have chosen to use the
50 role of DnaA as a transcription factor^{39,40} to report on the changes in DnaA activity. One of
51 the best characterized targets of transcription regulation by DnaA is its own promoter^{18,19}. We
52 have constructed a reporter cassette where the fast-folding *mut2-gfp* gene is under control of the
53 *dnaAP2* promoter sequence (from -136 to +48 relative to the transcription start site). This GFP
54 protein is highly soluble and stable⁴¹. This construct includes a Kanamycin resistance cassette
55 expressed divergently upstream from the *dnaA* promoter. In order to obtain an effect of SeqA and
56 gene dosage on our reporter as similar as possible to the endogenous promoter, we have inserted
57 the *dnaAP2* promoter reporter cassette in the genome within the Ori macrodomain, at the "Ori3"
58 locus downstream of the *aidB* gene (4413507 bp)³⁷, which was used in a previous study⁴². The
59 coordinate of this site is at 21% of the right replicore, the replication fork should pass through it
60 on average about 8 minutes after initiation of DNA replication.

61 Regulation of expression of the *dnaA* gene depends on a promoter region that includes two

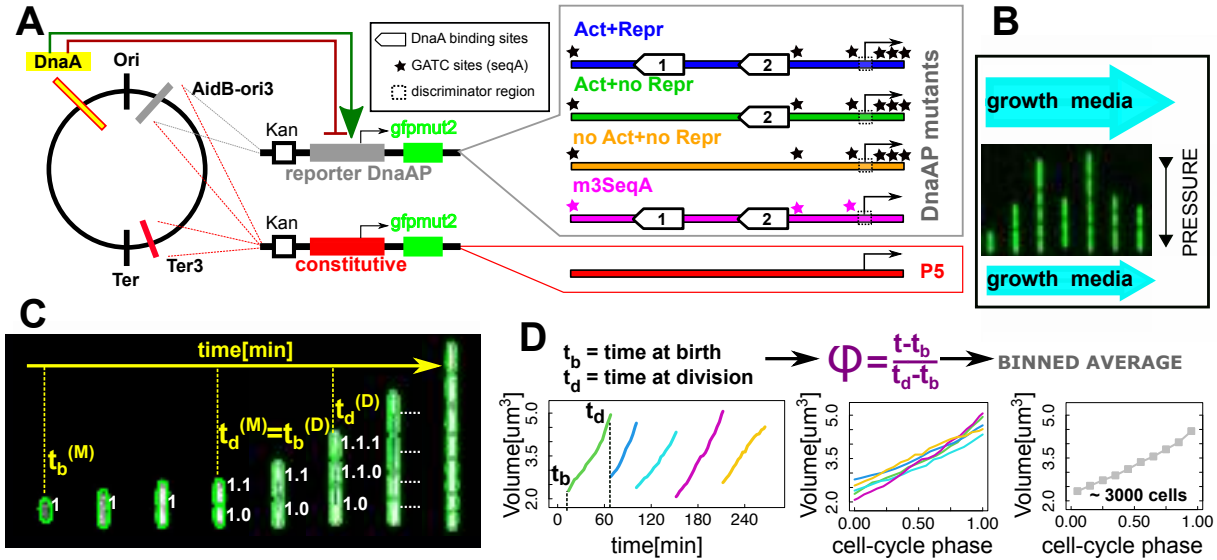


Figure 1: Robust and long-term single-cell tracking in fast growth conditions. (A) We inserted a reporter construct of the *mut2gfp* gene at a specific site downstream of the *aidB* gene in the Ori macrodomain³⁷. Expression of the reporter protein is under control of the native *dnaAP2* promoter region. A Kanamycin resistance cassette is expressed divergently upstream of the promoter. Different promoter mutants with different levels of regulation by DnaA and SeqA were considered. As a reference for baseline gene expression we used a constitutive promoter (see Methods). (B) The experimental device is a two-ended "mother machine" microfluidic channel where growth media flows constantly at the top and bottom of tapered-end microchannels, ensuring a constant environment³⁸. The picture shows a field of view with 7 micro-channels. Differences in flow rates between the two large channels generate a pressure that keeps the bacteria (which can only escape from the larger top end) inside the channels. (C) Segmentation/tracking algorithms follow the changes in cell size and fluorescence over time and across generations. The image shows snapshots of the same channel at subsequent times, and $t_{b,d}$ represent the times of birth/division of mother/daughter (M,D) in a lineage. (D) To examine the effects of cell cycle progression, we aligned growth and gene expression data with respect to cell cycle phase (fraction of the cell cycle), defined as cell cycle time normalized by the cell's division time.

62 promoters, P1 and P2⁴³. P2 is found downstream of P1 and includes a GC-rich discriminator
 63 region overlapping with the transcription start site, making transcription initiation negatively reg-
 64 ulated by ppGpp⁴⁴. P2 is the stronger promoter in exponential phase and is thought to provide
 65 the main growth-rate-dependent regulation of DnaA expression, while P1 provides a basal level of
 66 constitutive expression, similarly to what is found at ribosomal promoter regions^{19,44,45}.

67 Expression from P2 is negatively regulated by a high concentration of DnaA-ATP, and posi-
 68 tively regulated by DnaA when its concentration decreases^{18,19,46}, making it an effective sensor of
 69 DnaA-ATP levels. More specifically, two high-affinity sites for DnaA, Box1 and Box2, are found

70 upstream of the *dnaAP2* promoter. The binding of DnaA-ATP to these two high-affinity sites ac-
71 tivates transcription when DnaA-ATP activity is low. As DnaA-ATP concentration increases, the
72 DnaA bound to Box1 and 2 becomes the scaffold for the formation of an oligomeric structure that
73 represses transcription by occluding the RNA polymerase binding site^{18,19,46}. Specific mutations
74 in Box1 and Box2 disrupt the DnaA-binding consensus sequence. These mutants decrease the
75 binding affinity for DnaA and thus result in promoters that are only positively regulated (Box1
76 mutation) or neither positively nor negatively regulated by DnaA-ATP (Box1-Box2 mutation)¹⁹.
77 Finally, transcription initiation is inhibited by SeqA binding to five GATC sites, two overlapping
78 with the -10 and -35 sequences of P2 and three closely spaced sites found downstream of the
79 transcription start site.

80 An additional set of mutations (“m3SeqA”, Fig. 1A) change the sequence of these three
81 GATC sites. A previous study has shown that the set of these three mutations does not affect the
82 synchrony of initiation of DNA replication, but causes a decrease in growth rate and DNA content
83 in rich media²⁷. The two GATC sites overlapping with the -10 and -35 sequence were left intact in
84 order not to affect the binding of RNA polymerase, but a previous study has shown that they have
85 little effect on repression by SeqA and DNA replication parameters⁴⁷. As a further reference, we
86 considered the expression of the same reporter fluorescent protein under control of a constitutive
87 promoter used in a previous study⁴². This phage-derived constitutive promoter, “P5” in Fig. 1A,
88 has consensus -10 and -35 sequences and lacks regulation by specific transcription factors, there-
89 fore its GFP production rate can be considered to be largely representative of the change in gene
90 copy number with the passage of the DNA replication fork. To verify that its expression depends
91 on the change in gene copy number as a function of the cell cycle we have inserted the P5-*gfp*
92 construct at the same origin-proximal locus as the *dnaAP2*-GFP construct (Ori3) as well as at the
93 terminus-proximal locus (Ter3) downstream of the *uspE* gene (see Methods).

94 The effect of the *dnaAP2* promoter mutations on GFP expression from the chromosomal
95 insertion site have been measured by a plate reader assay and are consistent with the previously
96 published results obtained on a plasmid¹⁹ (Supplementary Fig. S1). Mutation of Box1 increases
97 expression relative to the wild type sequence, while mutation of both Box1 and 2 decreases ex-
98 pression back to wild type levels. What is important for this study is that (i) the original pro-
99 moter (“Act+Repr” in Fig. 1A) senses both DnaA-ATP levels and the transit of the replication
100 forks via the negative effect of SeqA binding (ii) the promoter stripped of both DnaA binding

101 sites (“noAct+noRepr” in Fig. 1A) only senses the binding of SeqA, and (iii) the m3SeqA variant
102 without the three GATC sites is only regulated by DnaA-ATP levels.

103 To achieve single cell resolution and capture the dynamic changes in cell growth, cell di-
104 vision and gene expression as a function of the cell cycle as well as across several generations,
105 we used an integrated microfluidics and time-lapse microscopy approach³⁸. In this device, an air
106 pressure-controlled flow system provides a constant environment where cells can grow steadily
107 for several days as the growth medium flows continuously at a constant rate (Fig. 1B and Supple-
108 mentary Fig. S2). We studied cells in a fast growth condition (M9 minimal medium with glucose
109 and casamino acids at 30°C), where the cells have a mean doubling time of 45 minutes and ini-
110 tiate DNA replication at 2 origins. Thousands of single cells were segmented and tracked from
111 movies with frames obtained every 3 minutes to examine cell cycle dependent changes in fluores-
112 cent protein expression and cell size in lineages comprising up to 15 generations, as described in⁴²
113 (Fig. 1C). Supplementary Table S1 provides a complete list of measured parameters and computed
114 variables. Each experiment yielded 2-8000 full cell cycles with a good reproducibility (Supple-
115 mentary Fig. S3).

116 **In absence of transcription regulation, GFP production rate increases with gene copy num-** 117 **ber and cell volume**

118 To establish a solid reference for monitoring the cell cycle dependence of gene expression from
119 the *dnaAP2* promoter, our first goal was to characterize the “null” relationships between cell cycle
120 progression and gene expression, i.e., the cell cycle variability of an unregulated promoter. To
121 estimate the promoter activity in single cells, we defined a GFP production rate as the discrete
122 time-derivative of fluorescence dF/dt from the time series of total fluorescence $F(t)$. Protein pro-
123 duction rate can vary along the cell cycle because the replication of a gene at a specific moment
124 in time doubles the probability that it will be expressed. Since gene replication occurs at a time
125 in the cell cycle that depends on the gene’s distance from the origin of DNA replication, the cell
126 cycle dependence of its expression rate will depend on the gene’s location along the genome. The
127 copy number of a gene, g , therefore doubles during the cell cycle, and its timing can be estimated
128 quantitatively by a standard model⁴⁸, which also takes into account the case of overlapping DNA
129 replication rounds, where replication forks from different initiation events are active in the same
130 cell (see Methods). Additionally, a protein’s production rate has been observed to be proportional

131 to cell size ⁴⁹, probably in connection with the fact that cell size tends to be proportional to ri-
132 bosome amounts. In our data, cell volume was computed considering a cell as a cylinder with
133 two hemispherical caps where the radius was estimated from the segmented projected area (see
134 Methods).

135 Supplementary Fig. S4A reports scatter plots of GFP production rate versus volume for
136 origin- and terminus-proximal P5 constructs. These single-cell data show an average linear pro-
137 portionality between the GFP production rate and cell volume, leading to an increase along the
138 cell cycle. As expected from the estimation of average gene copy number, the same unregulated
139 promoter shows an increased production rate when it is placed close to the replication origin com-
140 pared to when it is found near the terminus. Normalizing the data by the estimated mean gene copy
141 number (Eq. 3) removes most of this offset (Supplementary Fig. S4B), but the volume dependency
142 remains.

143 In order to quantify the change in gene expression due to the increase in gene copy number as
144 a function of cell cycle progression, we averaged the same data conditioning by cell cycle phase ⁵⁰,
145 defined as cell cycle time rescaled by the time between two consecutive divisions, i.e., t/τ (Supple-
146 mentary Fig. S4AB). This procedure makes it possible to average together cells with all doubling
147 times (3000-8000 cells in our case), hence increasing the statistical power. We found that the GFP
148 production rate from the constitutive promoter is biphasic, which appears more clearly when the
149 promoter is inserted close to the replication origin (Supplementary Fig. S4B). The beginning of
150 the second phase correlates with the expected value of the cell cycle phase at which the gene is
151 copied by DNA replication, supporting an effect of gene dosage. Rescaling the GFP production
152 rate by cell volume and mean gene copy number highlights measurable oscillations that are pre-
153 sumably due to cell cycle dependent gene dosage (Supplementary Fig. S4C). In particular, both
154 the ori-proximal and the ter-proximal reporters increase their production after they are replicated.
155 These small changes can be observed thanks to the large number of cell cycles analysed in our
156 data.

157 In summary, GFP production from an unregulated promoter involves “null” cell cycle depen-
158 dent trends due to volume and gene dosage, which in a regulated promoter have to be disentangled
159 from any regulatory signal.

160 **Transcription regulation by DnaA-ATP and SeqA causes strong oscillations in volume-specific**
 161 **GFP production rate**

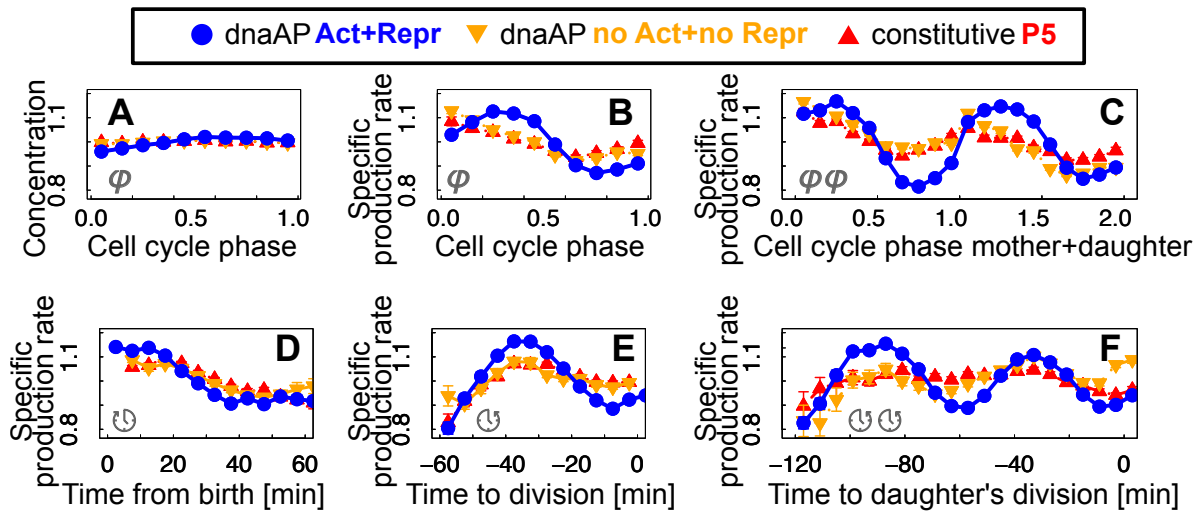


Figure 2: **Regulation of the *dnaAP2* promoter by DnaA-ATP causes an oscillation in GFP production rate beyond the effect of gene dosage.** (A) Oscillations in GFP concentration as a function of cell cycle phase are weak for the constitutive promoter (red triangles), the *dnaAP2* promoter (blue circles) and *dnaAP2*-Box1-Box2, the promoter not regulated by DnaA (orange triangles). (B) The fold-change in volume-specific GFP production rate from *dnaAP2* shows a clear peak that is not present for a constitutive promoter or the *dnaAP2*-Box1-Box2 promoter, which follow similar weak trends. (C) A sinusoidal oscillation for GFP expression from *dnaAP2* is observed when taking into account the cell cycle phase of lineages with two consecutive generations. (D,E) Volume-specific GFP production rate averaged conditionally to time from birth is very similar for the three promoters, while oscillations are enhanced for the *dnaAP2* promoter when the same data are averaged conditionally as a function of the time to division. (F) Oscillations in GFP expression from *dnaAP2* are enhanced when averaged conditionally to time to daughter division. If these oscillations correspond to changes in DnaA activity, they are consistent with the model where DNA replication initiating in the mother cell and terminating in the daughter cell will influence the timing of cell division of the daughter⁴⁸. Error bars (often smaller than symbol size) are standard errors of the mean from a re-sampled distribution obtained by bootstrapping from the experimental data for each bin.

162 We next set out to ask how the expression of GFP under control of the *dnaAP2* promoter
 163 differs from that of a constitutively expressed gene as a function of the cell cycle. There is
 164 only a small reproducible difference in the concentration of GFP as a function of the cell cycle
 165 phase when the constitutive and *dnaAP2* promoters are compared (Fig. 2A). However, the volume-
 166 specific GFP production rate from *dnaAP2* clearly shows an oscillation that is not present in the
 167 data for the constitutive P5 promoter or the promoter mutant without DnaA-ATP regulation (“no
 168 Act+no Repr”, with mutated DnaA Box1 and 2 binding sites in Fig. 1A), which behave similarly
 169 in these conditional averages, despite of the repression from SeqA and the different promoter se-

170 quences (Fig. 2B). Using lineages of two generations we also tested whether these cell cycle phase
171 oscillations are detectable in mother-daughter lineages, which is indeed the case (Fig. 2C).

172 The near-equivalence of the promoter stripped of DnaA regulation and the constitutive one
173 in Fig. 2BC suggests that the cell cycle dependent SeqA repression alone does not suffice in estab-
174 lishing the reported oscillations, while DnaA-ATP oscillations are essential. The *dnaAP2* promoter
175 includes a GC-rich discriminator region at the transcription start site which makes it a target for
176 both ppGpp and negative supercoiling dependent regulation⁴⁵. ppGpp levels and negative super-
177 coiling have a strong effect on the regulation of DNA replication as a function of growth rate⁵¹. As
178 a control, we measured the expression of GFP from a minimal ribosomal promoter (*rrnBP1*) that
179 also contains a GC-rich discriminator region. Expression of GFP from this promoter does not show
180 a cell cycle dependent oscillation beyond dosage effects (Supplementary Fig. S5), confirming that
181 ppGpp levels and DNA supercoiling are not sufficient to create the observed volume-dependent
182 oscillation.

183 We can gain more insight on the relationship between these oscillators and the cell cycle by
184 performing averages of the volume-specific production rate that are conditioned on specific cell cy-
185 cle variables. Specifically, by averaging the data as a function of the time from cell birth the specific
186 GFP production rate from *dnaAP2* becomes almost indistinguishable from that of the constitutive
187 promoter (Fig. 2D). However, the amplitude of the oscillation is much greater than that of the con-
188 stitutive promoter once we average the data as a function of the time to division (Fig. 2E). The
189 two findings suggest that the wild type *dnaAP2* oscillations are somewhat agnostic of cell birth (or
190 not synchronized with it), and prognostic of (or synchronized with) the next cell division. Fig. 2F
191 shows that the difference between *dnaAP2* and the constitutive control promoter P5 becomes more
192 evident when the time to daughter's division is used to bin the data. The reason for this is simple
193 if we assume that minima of this oscillator can correspond to the effect of the increase in gene
194 copy number, and thus initiations of DNA replication, amplified by DnaA-dependent regulation.
195 The replication cycle spans two cell cycles under these growth conditions, therefore the time of
196 initiation within the mother cell can have an effect on the time of division of the daughter cell. The
197 results over two-generations lineages also show more clearly how oscillations in volume-specific
198 GFP production rate are symmetric and sinusoidal when under control of the *dnaAP2* promoter.

199 Once again, the oscillations are also lost for the construct where both the DnaA binding

200 sites have been mutated (“no Act + no Repr”, orange triangles in Fig. 2DEF). However, in the
201 m3SeqA promoter, when the three downstream SeqA sites are mutated in the context of the wild
202 type *dnaAP2* sequence, the oscillations do not disappear, but they change in timing and amplitude
203 (Supplementary Fig. S6). This result is in accordance with the idea that SeqA repression of tran-
204 scription creates a delay in GFP production after the gene has been copied by DNA replication and
205 before its expression is increased by the doubling of gene copy number. The residual oscillation
206 measured by GFP production rate from the m3SeqA promoter should be prominently driven by
207 changes in DnaA activity, on top of dosage changes due to replication and cell division.

208 These results show that volume-specific GFP production rate from the *dnaAP2* promoter is a
209 *bona fide* cell cycle oscillator, coupled to the cell division event, and driven by DnaA-ATP levels
210 and SeqA repression. SeqA-dependent repression plays a role in the oscillations but, alone, is
211 insufficient to drive it.

212 **Oscillations in *dnaAP2* promoter activity are a cell size sensor**

213 The results in Figure 2 show that oscillations emerge when lineages are aligned by cell cycle phase
214 or time to divisions, but not when they are aligned with respect to time from birth. Cell cycle
215 phase aligns the data from individual cells as a function of the the fractional cell cycle progression
216 between the times of cell birth and division and it has been shown to align the time of gene doubling
217 by DNA replication⁵⁰. Given the consensus on the links between cell size and replication initiation,
218 the *dnaAP2* oscillator can be also expected to follow cell size. If that were the case, binning the
219 gene expression data as a function of cell volume should result in an improved synchronization of
220 individual cells’ oscillations.

221 We thus proceeded to test the hypothesis that DnaA activity and/or production rate may be a
222 cell size sensor by binning the data as a function of cell volume instead of cell cycle phase. Fig. 3A
223 shows that volume-binned averages in GFP volume-specific production rate from *dnaAP2* follow
224 strong oscillations reaching maxima and minima at multiples of a characteristic volume. The same
225 analysis with the data from the constitutive promoter and the mutant *dnaAP2* promoters also shows
226 oscillations, however of smaller amplitude (Supplementary Fig. S7).

227 The underlying oscillation of the constitutive and unregulated *dnaAP2* promoters reflects

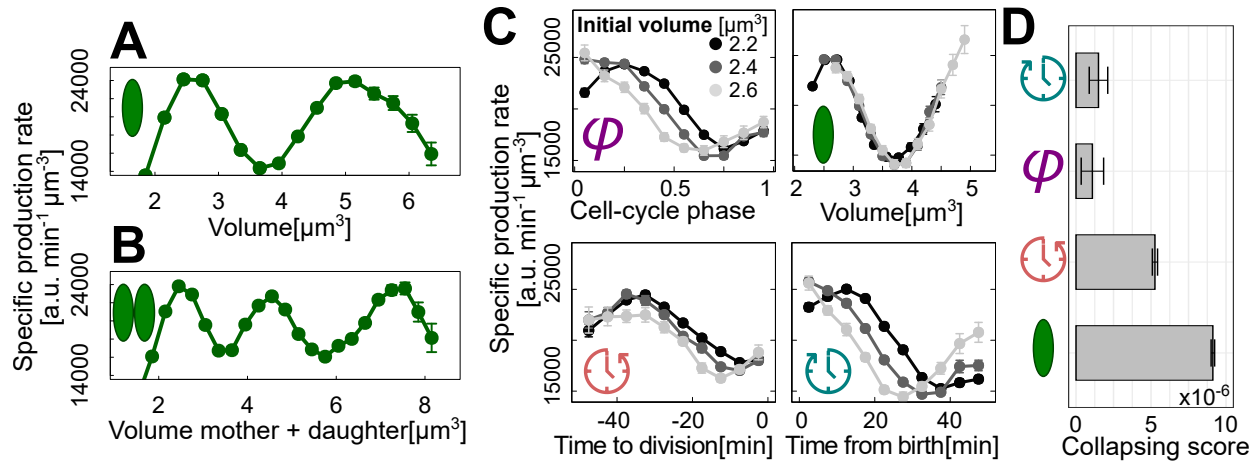


Figure 3: *dnaAP2* oscillations sense cell volume (A) Plot of the conditional average of GFP volume-specific production rate from the *dnaAP2* promoter as a function of cell volume. (B) The same average across mother+daughter lineages shows two minima at multiples of a characteristic volume, as expected from replication initiations. (C) Volume-specific *dnaAP2* promoter activity oscillations for cells of different initial size show different degrees of overlap when conditionally averaged as a function of cell cycle phase, time to division, time from birth and cell volume. The differently shaded curves result from data binned according to cell size (volume) at birth ($2.2 \pm 1 \mu\text{m}^2$, black, $2.4 \pm 1 \mu\text{m}^2$, dark-grey and $2.6 \pm 1 \mu\text{m}^2$, light-grey). If the variable in the x axis is the sensed variable, the averages should change independently of the cell size at birth. Data for cell volume shows the best collapse, indicating that the *dnaAP2* oscillator is a volume sensor. (D) Quantification of the collapse of the curves of panel C relative to 11 bins of cell sizes at birth (see Methods). Binning the data by cell size shows the best collapse and binning the data as a function of the time to division shows a good collapse. Error bars are standard errors of the mean obtained by bootstrapping from the experimental data for each bin.

228 increased expression upon the doubling of gene dosage by the passage of the replication fork
 229 followed by a decrease as cell size further increases⁵⁰. Hence, part of these oscillations must be
 230 due to dosage effects and dosage-volume correlations, independently from specific regulation by
 231 DnaA or SeqA. The dosage-volume correlation is indicative of the strong cell-size dependence of
 232 initiation of DNA replication.

233 The effect of the mutation of the DnaA binding sites determines a visible change in amplitude
 234 and phase of the average oscillation (when averaged conditionally with respect to cell volume),
 235 consistent with a volume dependent regulation by the concentration of free DnaA-ATP, increasing
 236 gene expression rate after the increase in gene copy number and decreasing it before initiation
 237 (Supplementary Fig. S7). Thus, our data give an unprecedented view of the regulatory effect of the
 238 oscillations of DnaA activity in single cells. In the absence of DnaA-dependent regulation (noAct
 239 + noRepr) the promoter is only repressed by SeqA. In this case volume-specific production rate

240 shows a visible change in phase compared to the constitutive promoter (Supplementary Fig. S7).
241 On the other hand, mutation of the SeqA sites in the presence of DnaA-dependent regulation
242 shows a visible shift in the volume at which oscillation minima occur compared to the wt *dnaAP2*
243 promoter (Supplementary Fig. S6). These results are consistent with repression by SeqA delaying
244 gene expression from the newly replicated promoter.

245 In order to shed more light into the size-sensing properties of the *dnaAP2* promoter, we
246 performed joint conditional averages of volume-specific GFP production rate considering different
247 cell cycle variables (Fig.3B). We performed these averages by further grouping cells based on their
248 size at birth, as we figured that the variables that are more directly coupled to the oscillations should
249 be insensitive to variations of any extrinsic variable. In particular, if the oscillator is a true volume
250 sensor, it should have no memory of size at birth. We divided cells into 11 different birth size
251 classes, and considered binned averages of specific GFP production rate oscillations as a function
252 of cell cycle phase, time to division, time from birth or cell volume. Fig. 3B shows three of the
253 birth size bins relative to an average birth size of $2.2 \pm 0.1 \mu\text{m}^3$, $2.4 \pm 0.1 \mu\text{m}^3$ and $2.6 \pm 0.1 \mu\text{m}^3$.
254 The plots show that volume-binned oscillations are strongly insensitive to birth size, since the data
255 for different birth size bins overlap, while the other variables are sensitive (i.e., the plots relative
256 to different birth size classes do not collapse). Binning by time from birth shows that cells born
257 larger reach the minimum of the oscillation in a shorter time than cells born smaller. Binning by
258 time to division shows the same trend, but results in an intermediate level of collapse. To quantify
259 this behavior, we defined a score of the collapse of different birth-size classes as the inverse of
260 the sum of SE-normalized distances between the oscillations in specific production rate for all 11
261 birth-size bins (Fig. 3C). The higher the score, the greater the collapse of the oscillations for cells
262 with a different birth size. Fig. 3C shows that cell volume gives the highest score. Time to division
263 gives an intermediate score (still a factor of two higher than those for cell cycle phase and time
264 from birth). Finally, we compared the collapse score using different proxies of cell size (length,
265 surface, volume) as candidates to be sensed by the *dnaAP2* oscillator, finding that volume is the
266 best candidate⁵² (Supplementary Fig. S8).

267 **Volume-sensitive *dnaAP2* oscillations require activation and repression by DnaA-ATP and**
268 **are linked to cell division via repression by SeqA.**

269 As noted above, the average specific GFP production rate binned as a function of cell volume
270 for the constitutive promoter, the promoters stripped of DnaA-ATP binding sites and the m3SeqA
271 promoter show oscillations that are lower than the wild-type *dnaAP2* promoter, but still have an
272 amplitude of 30-40% (Supplementary Fig. S7). Crucially, however, the analysis on the joint con-
273 ditional averages as a function of birth volume reveals direct volume sensing more effectively
274 (Supplementary Fig. S9).

275 The comparison of the results of this volume-sensing analysis obtained with the different pro-
276 moter constructs shows that the collapse score for volume is the highest for the wild-type *dnaAP2*
277 sequence, remains high for the mutants that are still activated by DnaA and is lost when both DnaA
278 binding sites are mutated (Supplementary Fig. S9). Removing cell-cycle dependent repression by
279 mutating the SeqA regulatory sites (m3SeqA reporter mutant) has a small effect on the collapse
280 with volume but loses the collapse with the time to division (Supplementary Fig. S10). In other
281 words, regulation by DnaA-ATP alone is not sufficient to link the oscillation in GFP production
282 rate to the time of cell division, which seems to require repression by SeqA.

283 Finally, regulation by SeqA alone (in the mutant promoter not regulated by DnaA) is insuffi-
284 cient to couple the oscillations to either volume or cell size at division independently of birth size
285 (Supplementary Fig. S9). Note that the population average gene expression level for this mutant is
286 very similar to that of the wild type (Supplementary Fig. S1). In summary, both positive and neg-
287 ative regulation by DnaA are required to maintain a good coordination between the oscillator and
288 cell volume. An improved level of collapse of the curves is obtained by transcription repression
289 from SeqA.

290 Together, these data lead us to conclude that the *dnaAP2* oscillator sets its phase through the
291 timing of SeqA regulation and the changes in gene copy number. These oscillations are amplified
292 by volume-coupled DnaA-dependent transcription regulation due to the cycle of DnaA activity.

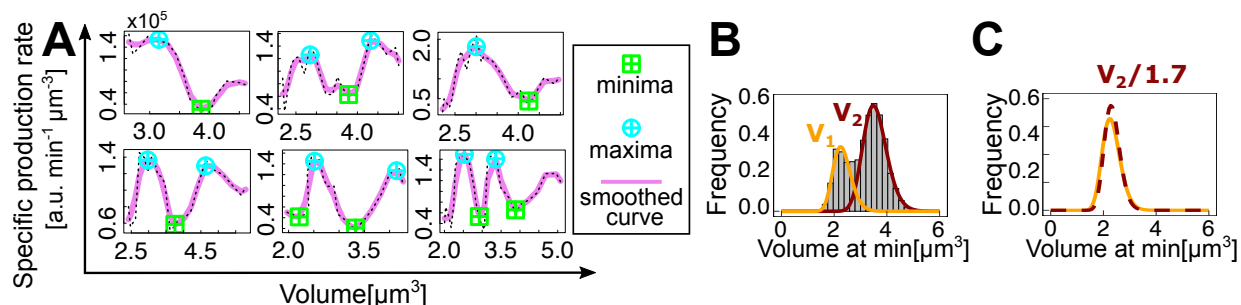


Figure 4: **In single cells, minima and maxima of *dnaAP2* oscillations appear at multiples of a characteristic volume similarly to DNA replication initiations.** (A) Oscillations are detectable at the level of single cells. The plots show some single cells *dnaAP2* tracks vs volume (dashed lines), as well as the smoothed tracks (purple solid lines) and the local maxima and minima (symbols) found by our automated algorithm (see Methods). (B) The distribution of cell sizes at minima is bimodal with peaks at discrete cell volumes. The bar plots show histograms of cell size at *dnaAP2* minima, and the solid lines are a fit with a log-normal mixture model, which results in two log-normal distributions. (C) If the minima are consistent with initiations, the two distributions should collapse if we divide the mean of the second peak by 2. The best collapse is obtained for a factor of 1.7.

293 **At the single cell level, *dnaAP2* oscillation minima are compatible with initiations**

294 While all the above analyses firmly establish the coupling of the *dnaAP2* oscillator with cell size
 295 and cell division using conditional means, more can be learned considering behavior of single
 296 cells⁵³. It is important to realize that when using conditional (binned) averages, if some oscillations
 297 exist but are not synchronized with respect to the conditioning variable, they cancel out. In other
 298 words, a flat conditional average results both if the oscillations are not there and when they are
 299 present but not in phase with each other. Instead, direct analysis of single cell lineages resolves
 300 this ambiguity. In particular, the correlation signatures linking the key events of DNA replication
 301 and cell division are well known^{30,32,33}, and our data allows us in principle to ask how single cell
 302 *dnaAP2* and m3SeqA oscillations relate to cell cycle progression.

303 Fig. 4A and Supplementary Fig. S11 show that in our data *dnaAP2* oscillations are detectable
 304 at the level of a single cell track. We defined an automated algorithm that extracts for every cell
 305 and lineage the local minima of the *dnaAP2* oscillations. The procedure to detect the minima
 306 is delicate, as it requires taking derivatives of noisy data and smoothing, hence subject to false
 307 positives (see Methods). Despite this limitation, our data gave us the cell cycle events related
 308 to *dnaAP2*, plus cell division, for each cell and lineage, where we know instantaneous cell size,
 309 growth rate, and interdivision time of each cell. Our data lack a direct proxy for the initiation of

310 DNA replication, but we could ask whether the *dnaAP2* oscillations minima in single cells follow
311 similar patterns to the ones recently observed for initiation of DNA replication^{29,32,33}. We focus
312 in particular on the minima, which could occur downstream of initiation of DNA replication, the
313 moment where SeqA represses transcription and DnaA-ATP is near its highest activity.

314 In the fast growth condition of our experiments, we found that the distribution of cell sizes
315 at minima is bimodal (Fig. 4B). This is because in some cases, at fast growth, initiation of DNA
316 replication takes place before cell division has occurred, and thus at twice the number of origins
317 and at a cell volume that should be proportional to the number of origins. This is in agreement
318 with the fact that the distribution of cell sizes at initiation of DNA replication in the presence of
319 overlapping rounds of DNA replication should be approximately the sum of two log-normal dis-
320 tributions with means that are one the double of the other⁵⁴. Using a log-normal mixture model to
321 separate the two distributions, we tested whether they would collapse by dividing the mean of the
322 second distribution by two. In our data, the best collapse is achieved by dividing by a factor of 1.7.
323 The small discrepancy could be due to false-positives in our detected minima and to correlations
324 between the volumes at initiation and the probability of extra rounds of replication, previously
325 observed computationally⁵⁴. Additionally, the knowledge of *dnaAP2* oscillations minima along
326 single cell lineages allowed us to repeat the analysis reported in Fig. 3 and test for direct sensing
327 of added volume using doubly-conditioned averaged with added volume and volume at birth. Sup-
328plementary Fig. S12 shows that the collapse of added volume is as good as for volume itself, hence
329 the analysis does not select which of the two variables is more tightly connected with the oscillator.

330 Next, we decided to investigate the correlation patterns that link consecutive *dnaAP2* oscil-
331 lation minima across one cell division. As a first control, we verified that our cells show adder
332 correlation patterns between cell birth and division independently of cell size at birth (Supplemen-
333 tary Fig. S13)^{55,56}. Supplementary Fig. S14 shows that the added volume between two minima
334 weakly correlated with cell volume at the first minimum. Considering the main mode of the distri-
335 bution, the slope of the conditional average was -0.266 ± 0.004 and consistent across one replicate
336 (-0.19 ± 0.09). Considering the uncertainty due to measurement noise, derivatives, and minima
337 detection, these results can be considered in line with the adder-like inter-initiation correlation
338 pattern found by labeling origins or replication fork proteins in single cells^{29,31-33,54}.

339 To summarize, the minima extracted from *dnaAP2* oscillations in single cells follow single-

340 cell correlation patterns that are roughly in line with replication initiations, supporting our inter-
341 pretation that the *dnaAP2* cell cycle oscillator is intimately linked to the single cell replication
342 cycle.

343 Performing the same analysis on the m3SeqA oscillator minima, (Supplementary Fig. S14)
344 we found a slightly stronger slope, suggesting that the two minima follow different rules. We note
345 however that this difference was found only in one of the two experimental replicates for m3seqA
346 at fast growth, probably due to uncertainties in minima detection in the lower-quality dataset. This
347 observation is mirrored by the shift of the minima at smaller volumes and cell cycle phase observed
348 in conditionally averaged m3SeqA oscillations (Supplementary Fig. S6).

349 In summary, the results of the analysis at the single-cell level support those obtained from
350 conditional averages, they indicate that the wild type *dnaAP2* oscillator acts in synchrony with
351 volume and with cell division, while the m3seqA oscillator, deprived of SeqA repression at fork
352 passage, may loose some synchronization properties with the cell division cycle.

353 **Causal links with cell volume and division differ between *dnaAP2* and m3seqA oscillations.**

354 Since the detection of the minima requires smoothing the data and taking derivatives, then smooth-
355 ing again to detect minima reliably, this procedure is particularly sensitive to false positives due
356 to propagated measurement noise. Additionally, the analyses so far are insufficient to hypothesize
357 any causal links between the oscillators and the cell division cycle.

358 To address these problems, we used synchronization analysis, cross-covariance, and causal
359 inference of *dnaAP2* time series along lineages of single cells. The two latter techniques in par-
360 ticular have the advantage of considering the whole time series (hence leveraging all the data) and
361 not just relying on minima detection. For each lineage, we considered the *dnaAP2* and m3SeqA
362 cell cycle oscillators, the cell volume time series, and the cell division time series, as three *a priori*
363 independent signals, to investigate their synchronization and to test whether a causality between
364 these signals exists (Fig. 5A and Supplementary Fig. S11). Each of these parameters displays some
365 periodicity throughout several generations.

366 To test the presence of a time hierarchy connecting specific *dnaAP2* activity and volume os-

367 cillations, we first considered the cross-covariance between these two time series, computed along
368 lineages⁵⁷. Fig. 5B shows that this function is markedly periodic, suggesting a strong coupling,
369 with higher-amplitude peaks for positive time delays. This asymmetry of the cross-covariance
370 function could suggest a potential time hierarchy between volume and *dnaAP2* whereby changes
371 in volume may be used to predict future changes in *dnaAP2* oscillations in volume-specific pro-
372 duction. Importantly, in promoter mutants with mutated DnaA binding sites or in the constitutive
373 promoter the cross-covariance are strongly reduced (Supplementary Fig. S15). Mutation of SeqA
374 binding sites (m3seqA) mildly reduces the amplitude of the delayed cross-covariances, but alters
375 the pattern of the time asymmetry (Fig. 5B and Supplementary Fig. S15).

376 Since the results on the m3SeqA promoter lead us to hypothesize that the wild-type *dnaAP2*
377 oscillator is in synchrony with cell cycle progression through SeqA, hence through sensing of the
378 initiation event by fork transit, we tested the phase locking of the two oscillators as follows⁵⁸⁻⁶⁰.
379 We characterized each oscillator with a phase, a linearly increasing variable reset at each cycle,
380 which advances by 1 between successive cycles. Specifically, we defined a *dnaAP2* phase variable
381 Θ , where $\Theta = 0$ corresponds to the minima of *dnaAP2* promoter activity and a cell cycle phase Φ
382 as described above in this text ($\Phi = 0$ represents birth). These two variables define a periodic square
383 (a torus) $[0, 2\pi) \times [0, 2\pi)$. Subsequently, as time t increases, the combined phase (Θ, Φ) from each
384 single cell lineage traces a trajectory in this phase space. If their orbits are phase-locked, single
385 lineages follow a diagonal trajectory in this space. Hence, we took a heatmap of the trajectory
386 density from all available data (Supplementary Fig. S16). For the wt *dnaAP2* oscillator, the phase
387 difference, $\Delta = \Theta - \Phi$ fluctuates around a constant value, hence the heatmap shows two juxtaposed
388 diagonal stripes whose slope is one. Hence the oscillations only depend on the phase difference, as
389 holds for a wide class of synchronized oscillators⁶¹⁻⁶³. Conversely the m3SeqA mutant breaks this
390 dependency, likely as a result of the loss of complete synchrony with the cell cycle phase. Note
391 however that contrary to the cross-covariance analyses, definition of a phase relies once again on
392 delicate minima detection.

393 Due to the loss of synchronization with the cell cycle of the m3SeqA oscillator, and due to
394 its loss of delay-time asymmetry in the cross-covariance with volume, we figured that the causal
395 links between the oscillator and the other proxies of the cell cycle may be stronger for the wild-
396 type *dnaAP2* oscillator. While asymmetric cross-covariances reveal a time hierarchy, one has to
397 be careful when inferring causal relations between two observables because of the existence of a

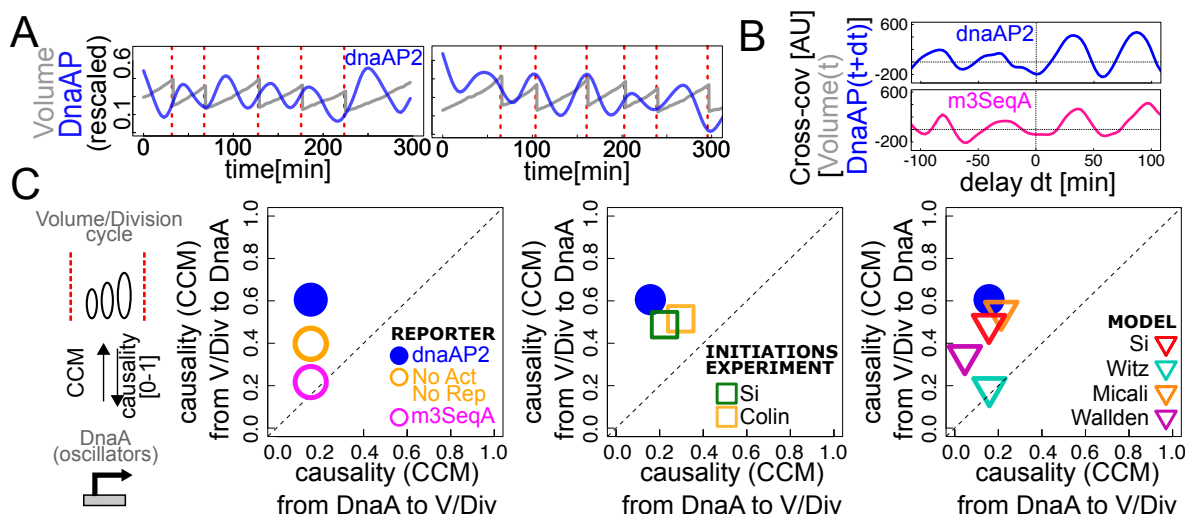


Figure 5: *dnaAP2* promoter activity and growth-division are coupled oscillators. (A) Lineages of single cells show a clear oscillation of *dnaAP2* promoter activity (blue), compared here to volume growth (grey) and division events (red), which we considered as three *a priori* independent time series. Volume and *dnaAP2* promoter activity are normalized here by their average value in order to show them in the same plot. (B) Cross-covariance between *dnaAP2* promoter activity and volume growth is periodic, supporting synchronization between the two oscillators, and asymmetric, supporting a stronger effect of volume changes on future *dnaAP2* changes than *viceversa*. Asymmetry and cross-covariance are weaker for the *m3SeqA* oscillator. (C) Convergent Cross-Mapping (CCM) was used to detect causal relationships between oscillators. Left panel: For the wild-type promoter, volume/division are strong causes of *dnaAP2* changes, but the strength of this causal link decreases in the mutants of the DnaA binding sites, and the causality becomes completely symmetric in the mutant of the SeqA binding site. Middle panel: CCM derived from experimental replication-initiation datasets is consistent with our data for the wild-type promoter. Right panel: CCM from simulated initiation datasets is most consistent with our data for the wt *dnaAP2* promoter for the models from refs. ^{29,30,32,33,35}.

398 non-zero correlation between two signals does not necessarily imply a causal link ⁶¹. To investigate
 399 the directionality of the coupling, we used the Convergent Cross Mapping (CCM) technique, con-
 400 sidering *conditional* correlations of one variable with a second one, under the constraint that the
 401 former is constrained to its attractor manifold, as reconstructed from the time series using Takens'
 402 theorem ^{64,65} (see Methods and Supplementary Fig. S17). Importantly, this analysis is based on
 403 the whole time series and does not rely on minima detection. The output of this analysis, given
 404 two time series *A* and *B* is a pair of a directional parameters ρ_{AB} and ρ_{BA} , between 0 and 1, that
 405 represent the strength of the causality link from *A* to *B* and from *B* to *A*. A causal link is witnessed
 406 by unequal causality parameters in the two directions $\rho_{AB} \neq \rho_{BA}$.

407 Fig. 5C summarizes the results of this analysis on our data. In order to try to disentangle the

408 effects of cell volume from cell division events, we first tested the causality between cell volume
409 and an oscillatory signal constructed to have a maximum at division. This analysis returns that
410 volume and cell division are always in a strong symmetric relationship (Supplementary Fig. S18),
411 hence, they are causally indistinguishable. Crucially, Fig. 5C shows instead a strongly asymmetric
412 causality from volume (or division) to *dnaAP2*. This asymmetric causal link is weakened in the
413 promoter without negative and positive DnaA regulation, and disappears in the m3SeqA promoter,
414 for which the correlation becomes causally symmetric. It is important to point out that these
415 results do not refer to the mutant of the endogenous *dnaA* promoter, but only report how the same
416 endogenous DnaA-ATP oscillations and SeqA transit are read by our reporters when there are
417 mutations of the binding sites. Hence, the observation that causality is lost when SeqA binding
418 sites (and thus sensing of the passage of the replication fork) are mutated indicates once again that
419 the endogenous *dnaA* promoter as well as our reporter takes relevant input from the fork transit.

420 Since we have found that *dnaAP2* oscillatory minima in single cells follow similar patterns
421 to those found for initiation events, we devised a way to compare the oscillator's causality patterns
422 with those observed for initiations, both in data from refs ^{29,32} and in cell-cycle mathematical mod-
423 els proposed in the literature ^{29-33,35} (Fig. 5C). To do this, we constructed time series connecting
424 measured or simulated initiation events by sinusoids, in such a way that the minima coincide with
425 initiations in time series taken from data or mathematical models (see Methods). This procedure
426 produces a differentiable oscillatory curve, which can be compared to volume and division time
427 series using convergent cross-mapping. Fig. 5C shows that the predicted causality pattern from
428 experimental data on initiations is completely consistent with the one shown by *dnaAP2*. Fig. 5C
429 shows that only the models from refs ^{30,32} are consistent with the causal asymmetry. Both mod-
430 els assume that the pattern between initiations is an adder per origin. The model proposed by Si
431 and coworkers assumes that division is agnostic of the chromosome, and not linked to replication-
432 segregation. The concurrent processes model proposed by Micali and coworkers assumes concu-
433 rency of time scales between a cellular process and chromosome replication-segregation setting
434 division through an AND gate.

435 Going back to our experimental data from wild type *dnaAP2*, our causality analysis using
436 convergent cross mapping shows that cell division or cell size cause *dnaAP2* oscillations in a
437 much stronger fashion than *dnaAP2* causes division. This perhaps unintuitive result may have two
438 explanations, (i) there is a strong symmetric coupling between cell division and cell size, and the

439 *dnaAP2* promoter is a strong size sensor, or (ii) there is a strong symmetric coupling between cell
440 division and cell size, and the *dnaAP2* promoter is a strong cell division sensor. However, the causal
441 equivalence of cell division and volume in our data do not allow us to distinguish between the two
442 hypotheses. Note that given the essentiality of the SeqA binding sites for the causal asymmetry, it
443 seems reasonable to assume that the crucial sensed event is fork transit, hence (ii) can be restated
444 by saying that replication initiation itself may be a strong sensor of the previous cell division, and
445 (i) that it may be a strong sensor of cell size.

446 In order to attempt to resolve this question, we performed additional experiments inhibiting
447 division by adding cephalixin (Supplementary Fig. S19). Cephalixin-treated cells do not divide,
448 but keep growing and initiating DNA replication, producing an array of nucleoids. Hence, their
449 cell cycle is still somewhat operative. Convergent cross mapping analysis is technically impossi-
450 ble under this perturbation, because the volume time series increases monotonically and lacks an
451 attractor. Interestingly this perturbation does not ablate time-periodic *dnaAP2* oscillations (Sup-
452 plementary Fig S19), but conditional averages become flat as soon as the cell sizes exceed the
453 physiological range, suggesting that the synchrony with cell volume is lost ⁶⁶.

454 Discussion and Conclusions

455 An oscillation in DnaA-ATP activity coupled to the cell cycle in *E. coli* is assumed by most, but so
456 far supported only by indirect population-level data ^{67,68}. Our data provide a first-time observation
457 of a cell cycle oscillator of gene expression in single *E. coli* cells through the volume-specific
458 production rate of a reporter protein under control of the *dnaAP2* promoter. This promoter was
459 chosen because it integrates, similarly to the endogenous DnaA production rate, both changes in
460 DnaA activity, via activation and repression by DnaA-ATP, and the timing of initiation of DNA
461 replication via SeqA repression following gene duplication.

462 The changes in gene dosage across the cell cycle affect the transcription rate and set up an
463 underlying cell cycle oscillation of gene expression rate ^{50,69}. We see this in our data as a change in
464 the volume-specific production rate from a constitutive promoter depending on its genome position
465 (Fig. 2).

466 When we look at the volume-specific production rate from the *dnaAP2* promoter and its

467 mutants through conditional averages, we see a clear oscillation as a function of cell cycle phase
468 as well as cell volume (Fig. 2 and 3). The oscillations have a considerably larger amplitude than
469 those of the constitutive promoter, showing that the additional regulation of gene expression by
470 DnaA amplifies the effect of the gene copy number. The idea that these oscillators may be strong
471 volume (or added volume) sensors is confirmed by our analyses with doubly-conditional averages.
472 Furthermore, changes in the volume-specific production rate measured by binning the data as a
473 function of cell volume have a larger amplitude than conditional average taken as a function of
474 cell-cycle phase, suggesting a stronger synchronization of the change in gene copy number with
475 respect to cell volume than the fractional duration of the cell cycle.

476 The main insights in our study stem from comparing the wild type *dnaAP2* reporter to pro-
477 moter mutants of the DnaA and SeqA binding sites, allowing us to measure their effect gene
478 expression separately. SeqA represses DnaA's gene expression for a window of time (about 10
479 minutes) after the passage of the replication forks¹². Accordingly, the comparison of the m3SeqA
480 mutant promoter with the wt *dnaAP2* shows that the oscillations are shifted to smaller volumes
481 or an earlier cell cycle phase (Supplementary Fig. S6). This also shows that direct regulation by
482 SeqA is not required for relevant cell cycle oscillations, but regulation by changes in DnaA-ATP
483 concentration are sufficient. The comparison of the oscillations of the constitutive promoter and
484 the *dnaA* promoters with mutations of the DnaA binding sites is consistent with a scenario where
485 repression by DnaA-ATP takes place mainly before the increase in gene copy number and activa-
486 tion by DnaA-ATP takes place after SeqA has dissociated (Supplementary Fig. S7). Connecting to
487 standard views on this oscillator^{68,70} we can speculate that a rapid decrease in the free DnaA-ATP
488 concentration after initiation is likely due to the combined effects of RIDA, DDAH, titration to
489 newly replicated genomic sites and transient SeqA repression of DnaA expression coupled with
490 dilution due to cell growth¹¹. Future experiments monitoring the behavior of our reporters in
491 mutants could help elucidating these details.

492 Based on our analyses of conditional averages for the sensing of different variables, both
493 the wild type *dnaAP2* oscillator and the m3SeqA oscillator sense cell volume (Fig. 3 and Supple-
494 mentary Fig. S10). Interestingly, the m3SeqA promoter, while showing a strong collapse with cell
495 volume, shows poor collapse with time to division. Hence, while the relationship of the oscillator
496 with volume depends on regulation by DnaA, the relationship of the oscillator with time to division
497 appears to rely on the negative regulation by SeqA. Hence, we conclude that DnaA activity oscil-

498 lations likely sense cell volume. Interestingly, expression from the promoter that is activated but
499 not repressed by DnaA-ATP and repressed by SeqA retains a significant level of volume sensing
500 and coupling with cell division time (Supplementary Fig. S9) despite an increased average expres-
501 sion rate (Supplementary Fig. S1). It seems that in these growth conditions timely repression by
502 SeqA is sufficient to maintain some response to volume change in the absence of repression by
503 DnaA-ATP. Negative regulation of gene expression by DnaA-ATP can integrate on the promoter
504 the effects of the different regulatory factors of DnaA activity, setting an upper limit of DnaA-ATP
505 concentration via the expression rate.

506 Most importantly, the high amplitude of the oscillations in the strains where GFP expression
507 is under control of the promoters regulated by DnaA-ATP allows for an analysis of the minima
508 at the level of single-cell lineages. Minima can be thus reliably detected in lineages of the wild
509 type and m3SeqA *dnaAP2* promoter constructs. In single cells, the minima of the oscillators
510 pinpoint key cell cycle times: for the m3SeqA reporter these are related to the joint contributions
511 of dosage oscillations and DnaA activity-based regulation, and for the wt reporter they are due
512 to SeqA repression as well, and are expected to co-occur with fork passage a few minutes after
513 replication initiation (the delay should be about eight minutes and the time difference between
514 consecutive frames is three minutes in our experiments). Minima for the wt *dnaAP2* oscillator have
515 a resemblance with initiations^{29,32,33}, in that they show near-adder correlations between subsequent
516 minima (Supplementary Fig. S14). Finally, when analyzed as a phase oscillator, only the wt
517 *dnaAP2* oscillator shows strong synchronization properties with the cell cycle phase, while the
518 m3SeqA oscillator shows a more irregular synchronization behavior (Fig. S16).

519 However, since these analyses relied on possibly fragile minima-detection techniques, we
520 made use of techniques that rely on the full time series. Cross-covariance analysis confirms a
521 difference in the relationship of wt *dnaAP2* and m3seqA oscillations with cell cycle progression.
522 Equally, when we detect causality by convergent cross mapping between size oscillations and the
523 wt *dnaAP2* oscillator, we see the same asymmetric causal pattern as for a sinusoidal oscillator
524 based on experimental initiation events, robustly across data sets (Fig. 4), and once again this is
525 not the case for the m3SeqA oscillator.

526 Hence, it appears from our data that the combination of only DnaA activity and dosage, prox-
527 ied by the m3SeqA oscillator, does not encode initiations, but the *dnaAP2* oscillator may do, and

528 the additional SeqA repression is crucial for this by coupling the induction of gene expression to
529 the initiation event, thus coupling DnaA-ATP production with the initiation mass of the following
530 initiation. A further implication of these results is that peaks in DnaA-ATP concentration, defin-
531 ing the minima in the absence of SeqA, do not appear to correspond to initiation events. This is
532 consistent with previous data showing that over-expression of DnaA does not have a strong effect
533 on the initiation of DNA replication^{71,72} unlike under-expression, where DnaA becomes limiting
534 6, 8, 32, 67, 72–74.

535 In the absence of direct measurements of the activity of the regulators of initiation of DNA
536 replication, its coupling to cell size has been the object of several mathematical models^{26,54,68,70,75}.
537 Most models predict a peak either in DnaA-ATP amount or concentration at the time of initiation.
538 Our results provide the first evidence at the single cell level that there is an oscillation in DnaA-
539 ATP concentration that is coupled with cell size and the cell cycle. The results obtained by the
540 comparison of the different variants of the *dnaAP2* promoter are consistent with a rapid decrease
541 in DnaA activity following initiation. This takes place at the same time as repression of gene ex-
542 pression by SeqA, independently of the specific cell size or DnaA activity. This delay in *dnaA* gene
543 expression further decreases DnaA-ATP levels. The tight coupling of the minima in gene expres-
544 sion with initiation events shows that the subsequent induction of DnaA promoter activity occurs
545 rapidly after the dissociation of SeqA. Transient transcription repression by SeqA and titration of
546 DnaA have been proposed to lead to steeper oscillations in DnaA activity that decrease the noise
547 in initiation⁷⁰. Activation of gene expression by DnaA can further contribute to a step function in
548 the induction of transcription prior to initiation.

549 Finally, while here we measured the expression of a reporter protein under control of the
550 *dnaA* promoter, we can speculate based on our data that the expression of the actual *dnaA* gene
551 depends on both volume sensing, via autoregulation by DnaA-ATP concentration, and sensing of
552 a successful initiation, via SeqA repression. In a recent study, the mRNA of the *dnaA* gene was
553 shown to oscillate with the cell size of fixed cells in a similar pattern to the one observed here for
554 our reporter construct, with an effect of transcription shutoff by SeqA and a different phase from
555 the one expected just from the increase in gene dosage due to DNA replication⁶⁹. However,
556 their technique does not have dynamic resolution along single-cell lineages and makes it possible
557 to access only the relatively weak concentration oscillations shown in Fig. 2A.

558 In this study we have focused on using specific promoter variants that report on DnaA activity
559 and the replication cycle in the wild type background and in the absence of perturbations. In the
560 future, this approach can be used to more directly measure the effect of perturbations of regulators
561 of DnaA-ATP of DnaA-ADP ratio on both the oscillations of DnaA activity and initiation of DNA
562 replication.

563 METHODS

564 **Strains and growth media.** The experiments were carried out with the wild-type *E. coli* strain
565 BW25113, the parent strain of the Keio collection ⁷⁶, which has been fully sequenced ⁷⁷. Promoter-
566 reporter constructs were inserted in the chromosome close to the origin of replication at Ori3
567 (4413507 bp, in the region downstream of the converging *aidB* and *yjfN* genes). The *gfpmut2*
568 gene, coding for a fast folding green fluorescent protein ⁴¹ is placed downstream of the chosen
569 promoter sequence. A kanamycin resistance cassette (KanR) is divergently expressed upstream
570 from the promoter region. The constitutive promoter cassette was also inserted in the chromosome
571 close to the replication terminus, at the Ter3 site (1395706 bp, downstream of the converging
572 *uspE* and *ynaJ* genes). Bacteria were grown overnight in M9+0.4% glucose at 30°C. Overnight
573 cultures were diluted 500:1 in new growth medium and returned to the incubator for 3-4 h. This
574 is important to guarantee bacteria to be in exponential phase when injected into the microfluidic
575 device. Experiments were carried out at 30°C in M9+0.4% glucose + 0.2% casamino acids, with
576 an average doubling time of 45 ± 5 min. We verified that the different levels of expression of the
577 GFP in the different strains do not have an effect on cell growth. Doubling time and cell size are
578 consistent between all the mutants (Supplementary Fig. S20).

579 **Mother machine experiments.** We used a microfluidic "mother machine" device where the 1
580 micron channels are found between two large feeding channels ⁷⁸. The bacteria are trapped in the
581 microfluidic channel thanks to a narrower opening on one side. PDMS (Polydimethylsiloxane)
582 devices from the mold were obtained by standard procedure and attached to a microscope slide
583 by treatment with a plasma cleaner. Before loading bacteria into the device, each chip was treated
584 with a solution of bovine serum albumin (BSA) to minimize bacterial interactions and binding
585 to the glass or PDMS components. Devices were injected with around 150 μ L of 2% BSA and
586 incubated at 30°C for 1 h. Passivated chips were rinsed with freshly filtered medium and 1ml of
587 bacterial culture was injected manually. Each feeding channel is coupled with a flow sensor. Using
588 its feedback loop, we can monitor and control the flow rate in our microfluidic setup while keeping
589 stability and responsiveness of pressure driven flows (Elveflow). This technology enables us to
590 set up robust and long term microfluidic experiments. A home-built temperature control system
591 is used to maintain the entire setup at 30°C. We use Nikon Inverted Microscope ECLIPSE Ti-E
592 with 100X oil objective high, 1.4, NA (Numerical Aperture) lens, coupled with a Nikon Perfect
593 Focus System (PFS) to rectify drift in focus. A xy motion plate is used to memorize and loop over

594 different Regions Of Interest (ROIs) at a specified interval of time.

595 The camera captured 16 bit images at 512 x 512 pixel resolution with the length of one pixel
596 equal to $0.1067\mu\text{m}$. The motorized stage and camera were programmed to cycle between at most
597 40 fields of view, each spanning roughly 8 microchannels, every 3 min.

598 **Data analysis pipeline.** The data obtained are in the .nd2 format and are imported and analysed
599 with the Fiji software. Background subtraction is performed using a 50 pixel rolling ball tech-
600 nique, and different positions are stored separately as a set of .tiff image files. Channels with a
601 good number of bacteria are selected manually and stored in different folders. For segmentation
602 and tracking, we started from codes developed by Mia Panlilio ⁴² and we added necessary modi-
603 fications to optimize them for our experimental setup. Before starting, we select the experimental
604 time window where bacteria were growing in a steady growth rate in a given growth medium. This
605 window was defined by observing sliding averages of population interdivision time, growth rate
606 and cell size at birth ⁴². To correct for segmentation and tracking errors we applied a set of filters.
607 First, we considered only cells where both mother and daughter(s) were at least partially tracked.
608 Second, we excluded division events where daughters had a volume outside of the interval 40-60%
609 of the volume of the mother. This step aims to eliminate filamentous cells and segmentation arte-
610 facts. Third, we excluded cell cycles with interdivision times lower than 15 mins (the physiological
611 lower limit is 15-20 min). Lastly, we considered cell cycles where initial/final volume, initial/final
612 width and initial/final growth rate were inside the 95% tails of the distribution. Before computing
613 the discrete derivative of fluorescence and volume (as central derivatives defined across three sub-
614 sequent frames) we removed outliers (defined by subtracting a trend line by binned averages and
615 identifying points lying more than 1.5 standard deviations from the baseline) from fluorescence
616 and volume traces and we substituted them by linear interpolation. Minima were detected after
617 removing outliers from the differentiated data, and smoothing with a 3-point average.

618 All datasets were grouped based on strain in different R objects. We performed an ex-
619 ploratory data analysis to check if datasets were consistent and if filters worked as desired. Custom
620 functions were written to handle and analyse these large, non-uniform datasets. In particular, func-
621 tions were written to compute the discrete derivatives and the different normalizations.

622 **Volume and gene dosage estimation.** We calculate the volume describing a cell as a cylinder
 623 with two hemispherical caps,

$$V(t) = \pi(l(t) - w) \left(\frac{w}{2}\right)^2 + \frac{4}{3}\pi \left(\frac{w}{2}\right)^3, \quad (1)$$

624 where w was taken as a cell cycle average of the average measured cell width. The expected gene
 625 copy number was computed from the Cooper-Helmstetter model⁷⁹. Replication of the *E. coli*
 626 chromosome begins from a single origin and oppositely oriented replication forks proceed sym-
 627 metrically along the genome to complete replication. Since, on average, a cell divides at a time
 628 $C + D$ (≈ 60 min) after replication initiation, an average time lag B before initiation is necessary
 629 to make the total replication time $B + C + D$ an integer multiple of the doubling time τ . Thus,
 630 defining $n = \text{Int}(C + D/\tau)$ as the integer number of times that τ divides $C + D$, one has that
 631 $B + C + D = (n + 1)\tau$. More generally, we can consider a gene at a chromosomal position defined
 632 by its normalized distance from Ori, i.e. $l = 0$ represents a gene in Ori and $l = 1$ a gene in Ter.
 633 The copy number of this gene, g , changes during the cell cycle following

$$g(t) := \begin{cases} 2^{n'} & \text{if } 0 < t < (n' + 1)\tau - (c(1 - l) + D) \\ 2^{n'+1} & \text{if } (n' + 1)\tau - (c(1 - l) + D) < t < \tau, \end{cases} \quad (2)$$

634 where $n' = \text{Int}\left[\frac{C(1-l)+D}{\tau}\right]$. By averaging over the cell cycle one gets the expected gene

$$g = \langle g(t) \rangle_{\text{cell cycle}} = \frac{1}{\tau} \int_0^\tau g(t) dt = 2^n \{1 - n + \mu[C(1 - l) + D]\}. \quad (3)$$

635 **Convergent cross mapping.** Convergent Cross Mapping (CCM) is a method for causality in-
 636 ference based on Takens' theorem⁶⁴ and developed in ref.⁶⁵. Takens' theorem shows that time-
 637 delay embedding of a one-dimensional time series provides a 1–1 mapping of system dynamics
 638 from the original phase space (constructed with all system variables) to the reconstructed shadow
 639 phase space, as long as the latter has sufficient dimensions to contain the original attractor. Sup-
 640 plementary Figure S17 summarizes the main steps of the CCM method. Shortly, it reconstructs
 641 a shadow attractor from one time series at a time and uses these coordinates to compute condi-
 642 tional correlations at fixed values of the shadow attractor of the variable. Since they are based
 643 on different constraints, the conditional correlations are not symmetric, and reveal causal links.
 644 We used the R package `multispatialCCM` to implement CCM (<https://CRAN.R-project>).

645 `org/package=multispatialCCM`)⁸⁰. CCM was used on our experimental data on volume,
646 divisions, and *dnaAP2* oscillation time series, using DNA replication initiation data from the lit-
647 erature and on simulated data. In our data, we used volume and *dnaAP2* oscillator time series,
648 and a cell division time series was defined as a continuous process with narrow peaks around each
649 experimental division event. For initiation of DNA replication data, we used two datasets from
650 the literature where initiation of DNA replication and cell division were tracked within the cell cy-
651 cle ^{29,32}, and we defined a putative *dnaAP2* oscillator time series by assuming a sinusoid between
652 0 and 1 with the minima at consecutive initiations (Supplementary Fig. S17B). We also simulated
653 four different models described in the literature: a model where replication initiation sets cell di-
654 vision through a timer, from ref. ³⁵; a model where DNA replication initiation set cell division
655 through an adder ³³; a model where replication and an inter-division concurrently limit cell divi-
656 sion ^{30,31}; and a model where DNA replication has no direct influence on the timing of division ³².
657 The codes and parameter values are those used in ref. ²⁹.

658 **Data and code availability.** Data sets of segmented and tracked cells, together with exam-
659 ple code for data analysis, were made available through the Mendeley Data Repository DOI:
660 [10.17632/hhp6g5zt8j.1](https://doi.org/10.17632/hhp6g5zt8j.1)

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853 **Competing Interests** The authors declare that they have no competing financial interests.

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