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Replication fork passage drives asymmetric dynamics of a critical nucleoid-associated protein in *Caulobacter*

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 06 September 2016

Thank you for submitting your manuscript on the identification and characterization of a novel Caulobacter nucleoid-associated protein. Your study has now been reviewed by two expert referees, whose comments are copied below. As you will see, their recommendations are somewhat mixed - while referee 2 has only a few specific concerns, referee 1 raises a number of substantial concerns with the second part of the analysis, regarding the links between RedN and its localization dynamics to replication fork progression. Among these criticisms, key issues pertain to the need for experimental replicates and statistics, more decisive experimental data, and the clarification of discrepancies with previously reported results and models in the literature. Given these well-taken points and major reservations, I am afraid we are not in a position to consider this study a sufficiently strong candidate to warrant expedited publication in The EMBO Journal at the present stage.

However, should you be willing and able to extend your experimental analyses to address the major concerns raised in the reviews and to reconcile your present findings with previous work, we would in light of the potential interest of RedN as a replication-linked NAP certainly remain interested in considering a revised manuscript further for The EMBO Journal. In order to allow you to decisively address the referees' points, we would in this case be happy to offer an extended revision duration (beyond the standard three-months period), during which the publication of any competing work elsewhere would have no negative impact on our final assessment of the conceptual novelty of your study. Nevertheless, given that it is not clear whether all points may be addressable in a sufficiently straightforward and satisfactory manner, I would obviously also understand if you should choose to rather publish the key finding of RedN as a novel NAP rapidly and without major modifications

elsewhere.

Should you decide to revise the manuscript for The EMBO Journal, please do not hesitate to contact me ahead of resubmission to discuss revision plans or any other questions/feedback regarding the referee reports. I would also appreciate if you could keep us updated about the publication of any related work.

Thank you again for the opportunity to consider this work for The EMBO Journal. I look forward to hearing from you and/or to receiving your revision.

REFEREE REPORTS

Referee #1:

The manuscript of Arias-Cartin et al. characterizes a novel Nucleoid Associated Protein (NAP) in Caulobacter crescentus termed RedN. The manuscript makes several novel observations: (1) In contrast to previously identified C. crescentus NAPs, RedN loss causes severe pleiotropic defects in cell growth, division and chromosome dynamics; (2) RedN associates with DNA in vivo and in vitro and is shown to moderately impact gene expression, in particular stress-related genes; (3) RedN localisation pattern seems to depend on active replication activity and correlates with replisome dynamics, prompting the authors to suggest a new model driving the distribution of NAPs in bacteria.

The first part of the study on the identification of RedN as a NAP is well conducted and presented clearly. The experimental rationale, technical execution and data quality are sound. However, we have several important concerns on the second part (from Figure 5) of the study that must be revised. First, the link between replication and RedN distribution only relies on single cell examples, it is imperative that this key conclusion is backed by a statistically-relevant dataset and quantitative analysis. Second, the model only partially reproduces their own experimental results and the authors additionally suggest a chromosome configuration switch to account for replisome progression discrepancies between their experimental results and models previously proposed in the literature. This putative chromosome configuration switch doesn't seem in line with experimental results from the literature and is ignored in their model for RedN dynamic distribution in the cell.

Major points

1- In line 137 the authors say "We expected RedN to be essential for viability under standard laboratory growth conditions (30ºC, 138 PYE medium)" though at line 151 they mention cells with ΔredN deletion growing under such conditions (with some defects) while in figS1C they said that ΔredN cells grow twice slower. How are those results compatible with the protein being essential for viability?

2- In the replication model of fig 6C, at the beginning of S phase, the Caulobacter crescentus chromosome is shown to flip its configuration from longitudinal (ori-Left-right-ter) to a configuration with the ter at the center and with left and right arms located on different sides of Ter, with two independent DnaN foci (replisomes) located on either side of Ter close to the cell poles. This new chromosome configuration is interesting but is very different to what was proposed in the literature by various authors. Thus, this new configuration has to be very well validated. We have, however, several issues with the current data and interpretation:
Previous studies of chromosome choreography in C. crescentus were based on the cell cycle localization patterns of FROS tags located in different genomic positions. The authors should show that their newly proposed chromosome configuration exists under their conditions using this technology and explain why in these previous studies their configuration was not observed.
The authors explain that the new replisome localization pattern they propose was not seen previously because the second replisome has been often undetected, probably due to its much lower signal to noise ratio when another reporter of the replisome was used. The data presented in the ms relies only on cells with two replication foci, and results mostly exemplified with a few cells. It would thus be reassuring to see statistics on the percentage of cells having 0, 1, 2 or more DnaN foci in addition to the normalized distribution of their localisation along the cell axis as shown in Figure

S4.
Reaching this new configuration requires the unpairing of the two chromosomal arms (figure 6C). How is this compatible with the literature? For example, in Le et al, Science, 2013, replichore pairing was reported to persist throughout the cell cycle.
The authors should provide information on potential reasons why a second replisome focus may be much dimmer than the other. We cannot understand why this would be systematically the case. Additionally, the new chromosome configuration switch shown in fig 6C implies a redistribution of DNA within the cell and hence of its RedN stably bound to it. The terminus region, which is poorly bound by RedN (figure 4C and EV2A), is now located at the cell center, while unreplicated chromosomal arms, which should be equally covered with RedN, locate on either side of Ter. We would thus expect a symmetrical distribution of RedN along the cell longitudinal axis when this chromosomal configuration switch occurs. This view seems incompatible with RedN profiles shown on Fig 6A and kymographs of figure 6D and is totally ignored in the simulations of Figure 8.

3- ChIP circos plots on Fig EV2 show very little differences in RedN distribution between synchronized and unsynchronized populations of cells which seems in contradiction with the authors experimental and simulated results in which the replisome drives the redistribution of RedN proteins on the chromosome. Plotting ChIP profiles of RedN along the chromosome arms for distinct time-point with synchronized cells after replication arrest release would be very valuable as it would 1) confirm RedN distribution and replisome progression are linked on a population level and 2) allow a direct comparison of RedN distribution along the chromosome with the authors simulated data.

4- Demograph analysis is used to show that RedN localization is cell cycle-coordinated, however, this analysis makes the interpretation of the authors' experimental data unclear. The X axis represents the cell number, with cells classified by their length. The distribution of cell lengths being non-homogeneous, the X-axis of the figure is therefore non-linear. How are we then expected to retrieve the cell cycle stage from the X-axis? When does replication initiates/ends? How is the split in RedN distribution correlated with cell-cycle processes? And more importantly, how can we compare demographs from distinct experiments, as shown in Figure 5B, if we cannot be sure the cell length distribution is exactly the same?

5- In Figure 5B, RedN longitudinal distribution is shown for different cell lengths. For the shortest cells, in which replication hasn't or just started (G1 or early S phase), RedN seems to mainly localize near the center of the cell while the origin of replication is expected to be at the old pole. How is this distribution compatible with ChIP data showing a RedN enrichment at/near the origin?

6- All experimental results linking RedN distribution with replisome progression are represented with single cell examples (intensity profiles or single cell kymographs, Figs 6 and 7). The authors should show correlations at the population level since the approach used allows for considerable statistics. For instance, a direct comparison of population averaged RedN and DnaN kymographs of RedN and DnaN should be presented to show that RedN distribution and replisome progression are correlated at the population level, and that the correlation reported does not happen only in the example provided.

7- Figure 6A shows single cell examples of RedN redistribution with the replisome progression. Why showing this with distinct cells at different time-points since the authors' approach allow time-lapse experiments on the same cells all along the cell cycle? Also, a negative control, such as similar profiles but for HU and DnaN, should support their conclusions.

8- In Figure 6A RedN and DnaN profiles seem correlated, with a systematic shift between both distributions, supporting the authors' conclusion that RedN redistributes with the replisome progression. However, in figure 6D and 7A, RedN and the bright DnaN focus seem rather to colocalize, while it is not possible to conclude anything from the second DnaN focus which is too noisy. The authors should provide quantitative analysis of the distance separating RedN and DnaN foci along the cell-cycle.

9- FRAP experiment in Figure 7B suggests that RedN binds DNA stably, in contrast to most NAPs, and imply that its dissociation from DNA may thus occurs due to the passage of the replication fork. The experiment is performed on a timescale of 20 minutes whereas the cell cycle is 120 minutes under the manuscript's experimental conditions. The authors should provide FRAP quantification on

more relevant timescales as well as replicates.

10- The authors propose a simple model that recapitulates both the depletion of RedN behind and its accumulation in front of the replisome. However, this model seems to reproduce only part of their experimental results. For instance, displaced RedN proteins in figure 6A accumulate in front of the replisome whereas in the simulated data they very distinctly spread uniformly along the nucleoid length. Also, as mentioned before, it is unclear how RedN simulated profiles can be compared with the G1 synchronized and unsynchronized experimental ChIP profiles in Fig EV2.

11- In addition, some clarifications on the simulations should be added: line 585 : "Rebinding of RedN displaced by the replisome was modeled as a random binding with uniform probability at any genomic coordinate, including the second copy of the replicated DNA." How can displaced RedN proteins span the entire nucleoid/cytoplasm volume without rebinding to the first free DNA that they encounter? This would require a time-delay for the protein to become competent at DNA binding after dissociation which is not supported by any data in the manuscript or apparently used as an assumption of the simulations.

Minor points
_ In the literature, SMCs are typically not considered NAPs.
_ The first paragraph in the Results is largely devoted to an introduction. This should be then shifted to the correct section.
_ The authors state: "RedN-Venus had a fairly broad distribution, with some depletion near the new pole where ter is located." This is not evident from the figure quoted.
_ In the "Identification of a novel NAP" section. RedN is identified by screening proteins for features characteristics of NAPs and associated with severe fitness cost when their gene is inactivated. How many other proteins were identified with such criteria in Caulobacter crescentus?
_ In the "RedN binds to DNA" section. Fig 1D legend should specify the fact that these experiments use recombinant redN from E.coli.
_ Figure legends sometimes miss the strain genotype associated with the data presented in the figure. For instance which strain is used in figS1C with the ΔredN deletion?
_ In figS1C the authors give the doubling time of the different strains used in this study for two growth media, M2G and PYE. The two media show small growth speed differences with the wild-type. Then why showing those two media instead of minimal and rich media (such as LB)?
_ Authors verified ΔredN defects are not due to a polar effect of the downstream gene. Why checking the downstream gene only and not the upstream as well?
_ All presented demographs in the manuscript display white background. Shouldn't colorbars zero value be white?
_ I am having a hard time reconciling the kymograph data from figure 6D with the time-lapse profiles of figure 6A, since the re-distribution of RedN seems to occur on different time-scales. In fig 6A, RedN maximum reaches the center of the cell length at t=60 minutes and then moves on both sides of the cell leaving a depleted area in RedN signal profile at the cell center. In kymograph of fig 6D, RedN maximum reaches the center of the cell length solely at t~90-100 minutes and then doesn't seem to be depleted. Could the authors explain these discrepancies?
_ Experiments on FtsZ-depleted cells in which solely the chromosome at the old pole fires new round of replication are shown (the others staying unreplicated) to conclude that RedN redistribution is due to active replication. These experiments are nice and convincing though I don't understand why RedN doesn't relocalize homogeneously on the non-replicating chromosomes and appears as stripes on the kymograph. Maybe the authors could provide potential explanations on the origin of those stripes they describe? Also, it would be important to show replicates of the experiment.
_Line 155 : typo "we may expect that its absence to impact various" shold read "we may expect that its absence impacts various"
_Line 903, figure 1D legend. Typo : remove extra "See".

Referee #2:

This is a very interesting, well-executed and well-described study. It describes the identification and characterization of a new nucleoid-associated protein, widely evolutionarily conserved, that is tightly bound to replicated DNA and evicted with passage of the replication fork, with correlated effects on gene expression. These conclusions are well-documented by a variety of method.

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This reviewer has only a few comments.
1. The authors speculate that absence of this protein results in defective DNA replication. It would be interesting to understand whether/how this phenotype is related to the normal replication-promoted eviction of the protein.
2. The authors suggest that many of the genes whose expression is altered in the mutant are DNA damage-induced. The implication is that SOS induction may be required for viability in the mutant background. This could be tested.
3. The authors use DNA gyrase to block DNA replication and thereby examine spontaneous dissociation of RedN from non-replicating DNA. This approach, of course, has the disadvantage that the supercoiling status of the chromosome is altered, and this change could, in principle alter the dissociation constant, particularly if altered supercoiling in front of or just behind the replication fork causes destabilization. The data could be viewed as saying that reduced negative supercoiling such as occurs in front of the fork does not destabilize binding and thus, perhaps, it is increased negative supercoiling behind the fork that causes dissociation. If so, reduction of gyrase levels would artificially stabilize the protein. This is a significant problem for this data. Wouldn't it be possible/better to do this experiment in the ftsZ condition on the non-replicating nucleoids?

1st Revision - authors' response 05 November 2016

We thank the reviewers for their time and thorough reviews. We have addressed their comments in the revised manuscript. Please see below (in blue) for our point-by-point responses.
In addition to the revisions aimed to address the reviewers' comments, the revised manuscript includes a note after the Discussion to alert the readers about a paper from the Shapiro group that was published while we were revising our manuscript. The Shapiro paper describes the identification of the same nucleoid-associated protein in Caulobacter crescentus (Ricci et al, PNAS 2016). The only overlap with our story is the DNA-binding preference of the protein to AT-rich DNA sequences and its essential function in cell cycle progression under standard laboratory conditions (which were the only growth conditions tested in their case). In the Shapiro paper, the protein is called GapR for "Growth-associated A/T-binding protein involved in regulation. In our revised paper, we have kept the name RedN for "Replisome-dependent NAP", mostly to be consistent with the original submission and to avoid confusion for the reviewers. However, we are considering changing the protein name to GapR before publication for consistency sake. Our major concern with the name change is that the name GapR is already being used for a transcriptional regulator involved in carbohydrate metabolism in Streptomyces (Sprusansky et al, Microbiology 2001) and α-proteobacteria (Ravcheev et al, Front Microbiol 2014), the bacterial class Caulobacter belongs to. The RedN name also has the advantage of being more functionally descriptive. We would welcome the referees' advice on this issue.

Referee #1:

The manuscript of Arias-Cartin et al. characterizes a novel Nucleoid Associated Protein (NAP) in Caulobacter crescentus termed RedN. The manuscript makes several novel observations: (1) In contrast to previously identified C. crescentus NAPs, RedN loss causes severe pleiotropic defects in cell growth, division and chromosome dynamics; (2) RedN associates with DNA in vivo and in vitro and is shown to moderately impact gene expression, in particular stress-related genes; (3) RedN localisation pattern seems to depend on active replication activity and correlates with replisome dynamics, prompting the authors to suggest a new model driving the distribution of NAPs in bacteria.

The first part of the study on the identification of RedN as a NAP is well conducted and presented clearly. The experimental rationale, technical execution and data quality are sound.

Response 1: Thank you

However, we have several important concerns on the second part (from Figure 5) of the study that must be revised. First, the link between replication and RedN distribution only relies on single cell

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examples, it is imperative that this key conclusion is backed by a statistically-relevant dataset and quantitative analysis.

Response 2: In the revised manuscript, we have added several replicates as well as quantitative population analyses (Fig EV4, Appendix Fig S5, S6, S7B, and S8). This is explained in details in the responses below.

Second, the model only partially reproduces their own experimental results…

Response 3: We disagree and believe that this impression came from a misunderstanding. To clarify and avoid future confusion, we have revised the text, provided explanatory and supporting simulations (Appendix Figs S9 and S11), and modified the schematics in Fig 6B; please see also response 17 and the accompanying figure below.

…and the authors additionally suggest a chromosome configuration switch to account for replisome progression discrepancies between their experimental results and models previously proposed in the literature. This putative chromosome configuration switch doesn't seem in line with experimental results from the literature and is ignored in their model for RedN dynamic distribution in the cell.

Response 4: This is also a misunderstanding. We do not suggest a different chromosome configuration from what has been proposed in the literature. Our model explicitly considers the well-known chromosome configuration, as the location of the second replisome doesn't affect the configuration of the chromosome. We have added schematics (Fig 6B) to avoid future confusion. Please see also response 7 and the accompanying figure.

Major points

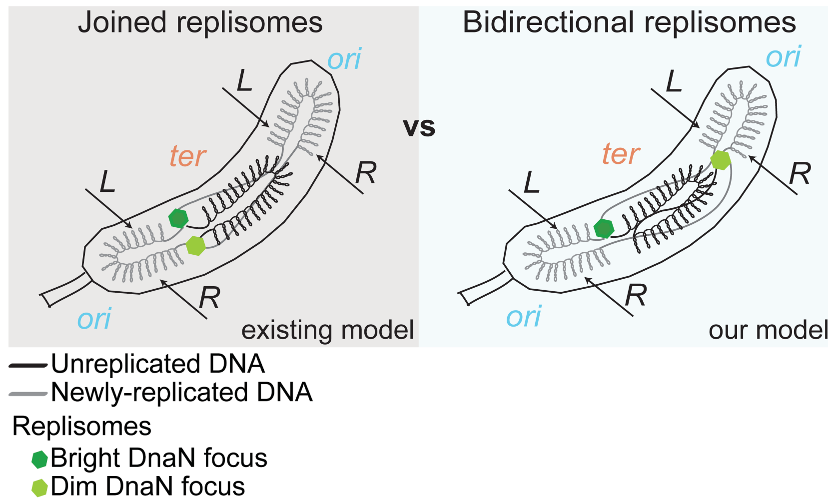
1- In line 137 the authors say "We expected RedN to be essential for viability under standard laboratory growth conditions (30ºC, 138 PYE medium)" though at line 151 they mention cells with ΔredN deletion growing under such conditions (with some defects) while in figS1C they said that ΔredN cells grow twice slower. How are those results compatible with the protein being essential for viability?

Response 5: In Fig S1C, the doubling time measurements for the Δ*redN* mutant were marked with an asterisk. The legend stated: "The asterisk denotes the caveat that the doubling time measurements do not take into consideration the difference in scattering between filamentous and normal-sized cells." In retrospect, it was probably a bad idea to provide values at all, since they are inaccurate and misleading, as indicated by the reviewer's comment. We have therefore removed these doubling time data. In addition, we have clarified the growth defect of the Δ*redN* mutants under 30ºC and PYE medium in the revised manuscript, as it was, indeed, confusing. Thank you for bringing this issue to our attention.

2- In the replication model of fig 6C, at the beginning of S phase, the Caulobacter crescentus chromosome is shown to flip its configuration from longitudinal (ori-Left-right-ter) to a configuration with the ter at the center and with left and right arms located on different sides of Ter, with two independent DnaN foci (replisomes) located on either side of Ter close to the cell poles. This new chromosome configuration is interesting but is very different to what was proposed in the literature by various authors. Thus, this new configuration has to be very well validated. We have, however, several issues with the current data and interpretation:
Previous studies of chromosome choreography in C. crescentus were based on the cell cycle localization patterns of FROS tags located in different genomic positions. The authors should show that their newly proposed chromosome configuration exists under their conditions using this technology and explain why in these previous studies their configuration was not observed.

Response 7: As mentioned above, this is a misunderstanding. We did not show or consider a flipped configuration, with left and right arms located on different sides of *ter*. In our model, the left and right arms for both unreplicated and replicated remain next to each other. Furthermore, the chromosome configuration and its cell cycle dynamics remain the same as in previous models (for example, see Figure 4 in Jensen et al, Nature Rev Mol Cell Bio 2002; Figure 7 in Hong et al, Mol Micro 2011; Figure 2A in Hong et al, PNAS 2013; and Figure 3B in Wang et al, Nat Rev Genet

2013) and are fully consistent with published FROS data (Viollier et al, PNAS 2014). The only difference between the previous model and ours is the location of the second replisome. In the previous model, the two replisomes migrate next to each other (joined replisomes). In the scenario we propose, the two replisomes migrate from opposite poles (bidirectional replisomes). The figure below shows how the difference in replisome migration does not affect chromosome configuration and dynamics.



In the revised manuscript, we have added schematics (Fig 6B) to help the readers visualize that the difference in replisome location does not affect the location of the chromosomal arms.

The authors explain that the new replisome localization pattern they propose was not seen previously because the second replisome has been often undetected, probably due to its much lower signal to noise ratio when another reporter of the replisome was used. The data presented in the ms relies only on cells with two replication foci, and results mostly exemplified with a few cells. It would thus be reassuring to see statistics on the percentage of cells having 0, 1, 2 or more DnaN foci in addition to the normalized distribution of their localisation along the cell axis as shown in Figure S4.

Response 8: As we noted in the text, the replisome localization pattern we described had been seen before (Jensen et al, EMBO J 2001; Wang et al, PNAS 2004; Collier et al, J Bacteriol 2009; Hong et al, Mol Microbiol 2011; and Fernandez-Fernandez et al, Microbiology 2013), but had been left out of models because the observation of cells with two separated replisome foci was infrequent relative to the observation of cells with only one replisome focus.
Figure S4 did not show the distribution of DnaN localization along the cell axis. Instead, it shows the frequency of cells with 0, 1 and 2 DnaN foci, which is, unless we are mistaken, what the reviewer is requesting. This frequency is shown as a function of cell length because cells with zero DnaN focus should be expected for the shortest and longest cells (i.e., before replisome assembly and after replisome disassembly), whereas cells with intermediate lengths should have one or two DnaN foci, as the plot shows. Importantly, going back to the reviewer's request, the plot shows that there is about the same percentage of cells with either one or two DnaN foci in still images of a cell population. However, we expect that the percentage of cells with two DnaN foci in a population is an underrepresentation, since we found by time-lapse microscopy that the formation of a second DnaN focus is transient (Fig 6D and Appendix Figs S5, S6 and S7B). In any case, as discussed in response 7 and as shown in the new schematics (Fig 6B), the mode of replisome migration (joined or bidirectional) does not affect our conclusions about RedN dynamics or the current of view of chromosome configuration.
This said, we acknowledge that the two mode of replisome dynamics shown in the new Fig 6B could co-exist and probably do. We have revised the text to present this possibility.

Reaching this new configuration requires the unpairing of the two chromosomal arms (figure 6C). How is this compatible with the literature? For example, in Le et al, Science, 2013, replichore pairing was reported to persist throughout the cell cycle.

Response 9: As mentioned above, we do not suggest a new chromosome configuration or the unpairing of the two chromosomal arms. Please see responses 7 and 8.

The authors should provide information on potential reasons why a second replisome focus may be much dimmer than the other. We cannot understand why this would be systematically the case.

Response 10: We do not know why there are less DnaN subunits associated with the replisome migrating from the new-pole side relative to the replisome migrating from the old-pole side. It is just an observation that we thought is worth mentioning. We note that it is not unusual for a fluorescently-labeled replisome to be dimmer than the other, as reported in other bacteria (Reyes-Lamothe et al, Cell 2008; Reyes-Lamothe et al, Science 2010; Su'etsugu et al, Mol Cell 2011; Trojanowski et al, mBio 2015). In those cases, it was not known whether it was always the same one that was dimmer, as the identity of the poles wasn't assessed, unlike in our study.

Additionally, the new chromosome configuration switch shown in fig 6C implies a redistribution of DNA within the cell and hence of its RedN stably bound to it. The terminus region, which is poorly bound by RedN (figure 4C and EV2A), is now located at the cell center, while unreplicated chromosomal arms, which should be equally covered with RedN, locate on either side of Ter. We would thus expect a symmetrical distribution of RedN along the cell longitudinal axis when this chromosomal configuration switch occurs. This view seems incompatible with RedN profiles shown on Fig 6A and kymographs of figure 6D and is totally ignored in the simulations of Figure 8.

Response 11: This comment was based on the confusion that we were proposing a different chromosome configuration, which we weren't. The well-accepted view of chromosome organization during the cell cycle, which we fully support, is in close agreement with the RedN profiles and kymographs shown in Fig 6 and Appendix Fig S5, and is absolutely considered in the simulations of Fig 8.

3- ChIP circos plots on Fig EV2 show very little differences in RedN distribution between synchronized and unsynchronized populations of cells which seems in contradiction with the authors experimental and simulated results in which the replisome drives the redistribution of RedN proteins on the chromosome. Plotting ChIP profiles of RedN along the chromosome arms for distinct time-point with synchronized cells after replication arrest release would be very valuable as it would 1) confirm RedN distribution and replisome progression are linked on a population level and 2) allow a direct comparison of RedN distribution along the chromosome with the authors simulated data.

Response 12: We agree that, in the original manuscript, the simulated RedN profiles directly related to synchronized cells and that it may not be easy for readers to use this information to infer how the RedN profile should look like for an asynchronous cell population. To address this issue, we used our simulations to calculate the average RedN profile along the chromosome in an asynchronous population (for explanations and details, please see Appendix Supplementary Methods). This calculation shows that i) the resulting RedN profile remains asymmetric between *ori* and *ter*, and ii) this asymmetry agrees well with the ChIP-seq profiles of asynchronous populations (Appendix Fig S12).
In addition, we have addressed the reviewer's points using time-lapse microscopy data. We believe that this method is better (and cheaper) than ChIP to examine the relationship between replisome progression and the RedN profile given the significant variability in the timing of DNA replication initiation and in the speed of replisome motion, even when starting with a pure population of swarmer cells (for single-cell examples, see Fig 6D and Appendix Figs S5, S6, and S7B). First, we have added multiple single-cell time-lapse data to show that the dynamics between Replisome and RedN are very well correlated (Appendix Figs S5 and S7B). Second, we developed an algorithm to shift signal profiles in time until their DnaN profiles are maximally correlated, which computationally reduces the cell-to-cell variability in replisome dynamics (something that cannot be done with ChIP experiments). Thereby, we were able to provide a population analysis of RedN dynamics relative to replisome assembly and disassembly (Fig EV4).
Please note that it is important to distinguish the binding profile of RedN at the chromosome (large-scale) level, which our study primarily addresses, from the profile of RedN binding at the nucleotide (small-scale) level. Our data suggest that the progression of the replication forks contributes to the asymmetric binding of RedN along the chromosome. It does not, however, explain why some specific chromosomal coordinates (e.g., *parS* and *dif* regions) display large ChIP peaks. We

acknowledge that other layers of regulation exist to explain all aspects of the RedN binding profile. This is now more clearly stated in the Discussion section of the revised manuscript.

4- Demograph analysis is used to show that RedN localization is cell cycle-coordinated, however, this analysis makes the interpretation of the authors' experimental data unclear. The X axis represents the cell number, with cells classified by their length. The distribution of cell lengths being non-homogeneous, the X-axis of the figure is therefore non-linear. How are we then expected to retrieve the cell cycle stage from the X-axis? When does replication initiates/ends? How is the split in RedN distribution correlated with cell-cycle processes? And more importantly, how can we compare demographs from distinct experiments, as shown in Figure 5B, if we cannot be sure the cell length distribution is exactly the same?

Response 13: At this stage of the manuscript, we are simply showing that the localization profile of RedN during the cell cycle is clearly different from that of a protein (like HU2) that binds the DNA uniformly. Demographs are commonly used in the literature to show a cell cycle localization profile (e.g., Hocking et al, J Bacteriol 2012, van der Ploeg et al, Mol Microbiol 2013; Cameron et al, mBio 2014; Möll et al, J Bacteriol 2014; Beaufay F et al EMBO J, 2015; Vichner et al, Front Microbiol 2015). More detailed cell cycle analysis is performed in Fig 6, Fig EV4 and Appendix Figs S5 and S6. We have revised the text to avoid future confusion.

5- In Figure 5B, RedN longitudinal distribution is shown for different cell lengths. For the shortest cells, in which replication hasn't or just started (G1 or early S phase), RedN seems to mainly localize near the center of the cell while the origin of replication is expected to be at the old pole. How is this distribution compatible with ChIP data showing a RedN enrichment at/near the origin?

Response 14: The demograph does not show a midcell localization in young cells, and the localization profile is consistent with the ChIP data showing depletion at *ter* relative to *ori*. But we think that the confusion was probably caused by the presence of low (blue) RedN signal close to the old cell pole. This low signal is simply due to the fact that the cell outline (determined from the phase contrast image) is bigger than the DNA signal. This is why the visual comparison should be with the HU2-mCherry demograph provided below the RedN-Venus demograph. HU2 binds to the DNA uniformly, thereby showing the expected localization profile of the DNA. The reviewer will note that in short/young cells, there is about as much low (blue) signal of HU2 near the old pole as there is low signal of RedN at the same location; this is simply because there is little to no DNA there. In contrast, there is a larger space with low (blue) signal on the opposite side (new pole) for RedN compared to HU2. In other words, there is more RedN near the origin of replication (close to old pole) relative to the terminus (close to new pole), consistent with the average RedN profile in short (synchronized swarmer) cells shown in Fig 4C. We have revised the text to help prevent confusion.

6- All experimental results linking RedN distribution with replisome progression are represented with single cell examples (intensity profiles or single cell kymographs, Figs 6 and 7). The authors should show correlations at the population level since the approach used allows for considerable statistics. For instance, a direct comparison of population averaged RedN and DnaN kymographs of RedN and DnaN should be presented to show that RedN distribution and replisome progression are correlated at the population level, and that the correlation reported does not happen only in the example provided.

Response 15: In the original submission, we showed a single-cell kymograph rather than an average kymograph because there is too much cell-to-cell variability in the timing of replisome assembly (DnaN spot formation) and in the speed of replisome migration. To address the reviewer's concerns, we have provided additional examples of single-cell kymographs (Appendix Figs S5 and 7B). In addition, we now provide a population analysis of RedN localization relative to replisome dynamics (Fig EV4). To do this population analysis, we had to minimize the aforementioned cell-to-cell variability in replisome dynamics by developing an algorithm that shifts single-cell signals according to the DnaN signal.

7- Figure 6A shows single cell examples of RedN redistribution with the replisome progression. Why showing this with distinct cells at different time-points since the authors' approach allow time-lapse

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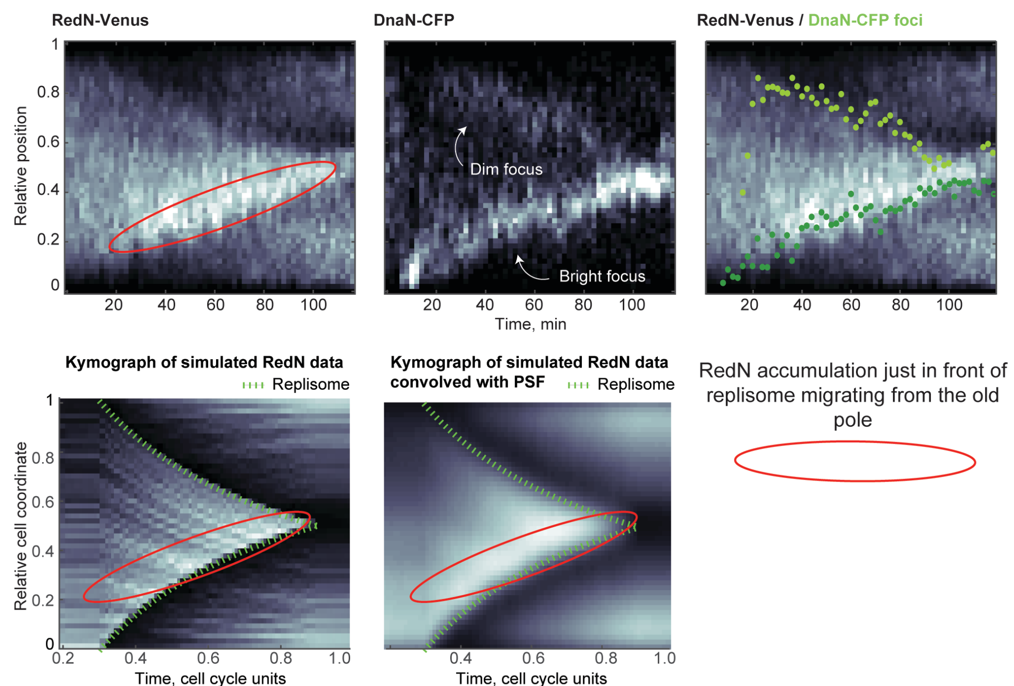
experiments on the same cells all along the cell cycle? Also, a negative control, such as similar profiles but for HU and DnaN, should support their conclusions.
Response 16: Fig 6D showed the time-lapse experiment requested. The reason for Fig 6A was to show the same results in two different ways: 1) still images of a population at different time points of the cell cycle (Fig 6A), and 2) time-lapse of single cells during the course of their cell cycle (Fig 6D and Appendix Fig S5). As mentioned in response 15, we have added a population analysis of single-cell time-lapse data (Fig EV4) to support our claim. We have also added multiple time-lapse experiments of DnaN and HU2 as requested (Fig 5C and Appendix Fig S6).

8- In Figure 6A RedN and DnaN profiles seem correlated, with a systematic shift between both distributions, supporting the authors' conclusion that RedN redistributes with the replisome progression. However, in figure 6D and 7A, RedN and the bright DnaN focus seem rather to colocalize, while it is not possible to conclude anything from the second DnaN focus which is too noisy. The authors should provide quantitative analysis of the distance separating RedN and DnaN foci along the cell-cycle.

Response 17: The 'colocalization' that the reviewer refers to is predicted by our model. In addition to the cone-shaped condensation of the RedN signal, the replisome-dependent model predicts an accumulation of RedN right next to the replisome migrating from the old pole (see figure below, which compares experiment and model). In the experiment, this signal accumulation right in front of the replisome will appear as colocalization because of the point spread function of the DnaN and RedN fluorescent signals. The accumulation of RedN in front the bright DnaN focus is due to the slanted distribution of RedN in the G1 phase, as predicted by the model (Fig 8B) and as shown by the experiments (Fig 4).



In addition to providing several replicates of single-cell kymographs (Appendix Fig S5 and Fig S7), we have added a quantitative analysis of RedN and DnaN dynamics (Fig EV4), as mentioned in response 16.

9- FRAP experiment in Figure 7B suggests that RedN binds DNA stably, in contrast to most NAPs, and imply that its dissociation from DNA may thus occurs due to the passage of the replication fork. The experiment is performed on a timescale of 20 minutes whereas the cell cycle is 120 minutes under the manuscript's experimental conditions. The authors should provide FRAP quantification on more relevant timescales as well as replicates.

Response 18: The 20-min time scale we provided would be considered as already extremely long as far as FRAP experiments go. Quantitative interpretation of FRAP experiments assumes that cell growth, *de novo* RedN synthesis and DNA rearrangement/motion can be ignored. The longer the

FRAP experiment, the more we violate these assumptions. Experiments that are 120-min long would not be interpretable. FRAP experiments are generally done at small timescales to minimize this problem. Once the time of recovery is determined at a small timescale, it is easy to extrapolate how much protein redistribution there would be at longer time scales.
To address the reviewer's concern about timescale, we have performed a quantitative analysis of FRAP data and found that the recovery time is $\tau = 105 \pm 35$ min (mean ± SD, n = 9). This value likely is an underestimation of the spontaneous dissociation time of RedN from the DNA (τ_{off}) as *de novo* synthesis of RedN-Venus during the 20-min FRAP experiment contributes to the fluorescence recovery we measured. In any case, even if we consider that $\tau_{off} = 100$ min, it does not affect the results, as we now show in new simulations (Appendix Fig S13).
It is also important to note that the kymographs of FtsZ-depleted cells in Fig 8A and Appendix Fig S8 show very long streaks of RedN signal on the non-replicating DNA for period over 150 min, further supporting our claim that there is very little DNA dissociation during the cell cycle timescale. If there were significant DNA dissociation, long signal streaks would not be apparent in kymographs. We have added 'didactic' simulations to explain this concept (Appendix Fig S9).

10- The authors propose a simple model that recapitulates both the depletion of RedN behind and its accumulation in front of the replisome. However, this model seems to reproduce only part of their experimental results. For instance, displaced RedN proteins in figure 6A accumulate in front of the replisome whereas in the simulated data they very distinctly spread uniformly along the nucleoid length.

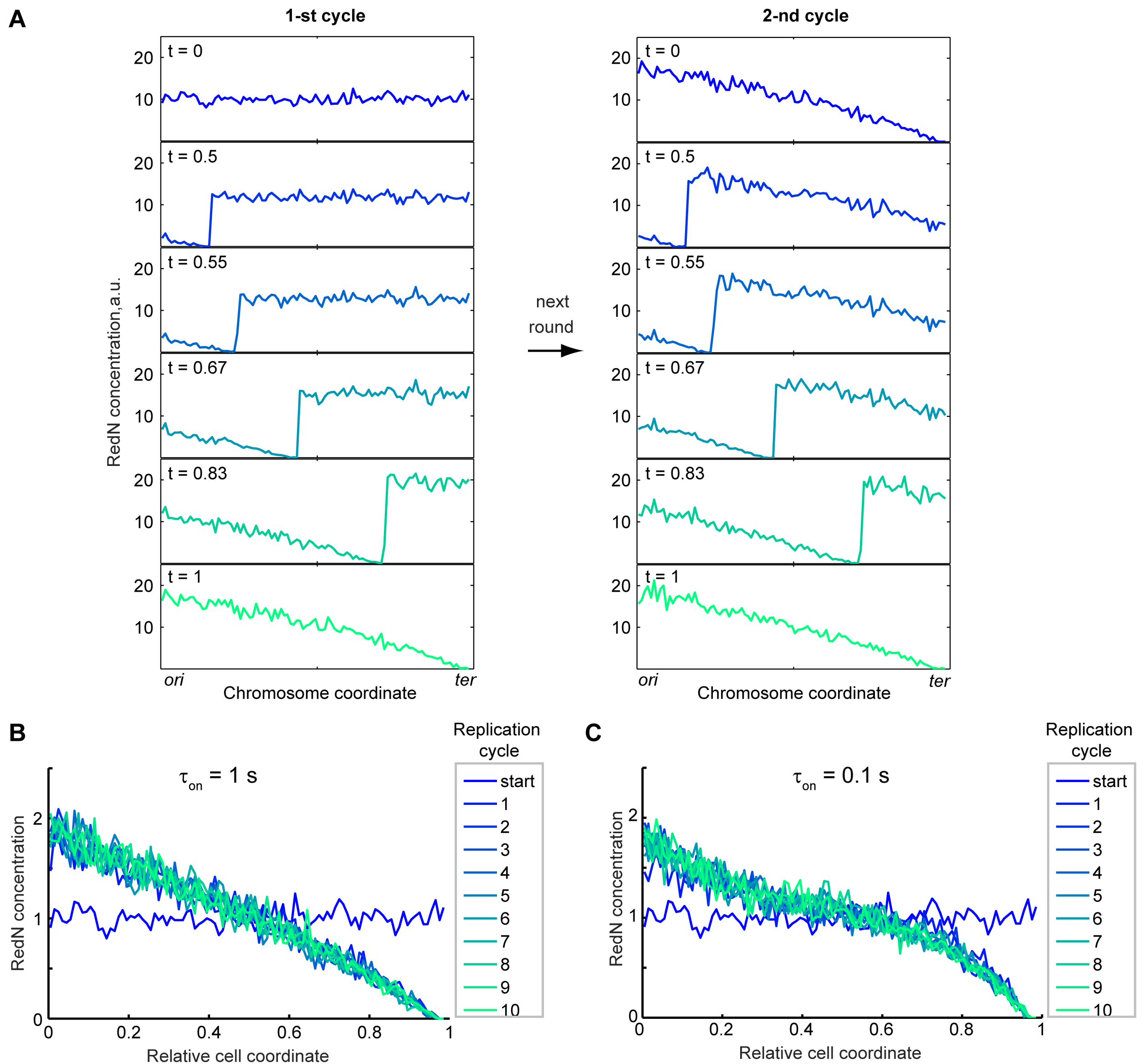
Response 19: We disagree. RedN proteins very distinctly accumulate in front of the replisome in the simulated data. Please see response 17 and its accompanying figure above.
In the original manuscript, we wrote: "In addition, the replisome-displacement model produced the accumulation of RedN in front of the replisome migrating from the old pole (Fig 8B-D), similar to what we observed experimentally (Fig 6D). This accumulation was due to the slanted distribution of RedN in G1 phase caused by the previous replication round, as shown by our model (Fig 8A-B)." In our opinion, the similarity between the experiment and the simulated data is remarkable (please compare Fig 6D to Fig 8C-D).

Also, as mentioned before, it is unclear how RedN simulated profiles can be compared with the G1 synchronized and unsynchronized experimental ChIP profiles in Fig EV2.

Response 20: Please see response 12.

11- In addition, some clarifications on the simulations should be added: line 585: "Rebinding of RedN displaced by the replisome was modeled as a random binding with uniform probability at any genomic coordinate, including the second copy of the replicated DNA." How can displaced RedN proteins span the entire nucleoid/cytoplasm volume without rebinding to the first free DNA that they encounter? This would require a time-delay for the protein to become competent at DNA binding after dissociation which is not supported by any data in the manuscript or apparently used as an assumption of the simulations.

Response 21: Even without considering a time delay, our assumption is valid as long as the re-association time of RedN to the DNA is slow relative to its diffusion time through the cytoplasm. The fastest DNA reassociation constant (k_{on}) that we could find in the literature for a NAP is *E. coli* H-NS with $k_{on} = 10^5$ M^{-1} s^{-1} (Pelletier et al, PNAS 2012 and Dame et al, Nature 2006). Considering this k_{on} value, 10,000 H-NS binding sites per chromosome (Kahramanoglou et al, Nucleic Acids Res 2011) and one chromosome copy per 1 μm^3, we estimate that the characteristic time for H-NS rebinding time (τ_{on}) is 0.7 s. Since the timescale of a protein ($D = 10$ μm^2 s^{-1}) to travel the length of the cell ($L = 2$ µm) is about 100 ms, H-NS would typically cross the cell multiple times before re-binding, indicating that our assumption is reasonable.
To examine how the characteristic time of RedN rebinding affects its distribution along the chromosome, we have performed new simulations of the replisome-dependent model in which diffusion of RedN and kinetics of RedN association and dissociation were explicitly considered. The figure below shows that, for a rebinding time τ_{on}= 1s, a uniform distribution of RedN at t = 0 quickly converges to an asymmetric shape following DNA replication (see figure below, panels A and B). Even if the rebinding time was 10 times faster (τ_{on} = 100 ms), the RedN profile remained highly asymmetric (figure below, panel C).



One-dimensional simulation of the replisome-displacement model that explicitly considers RedN diffusion prior to DNA binding.
A. Shown is the evolution of RedN profile through the first two replication cycles, starting with uniform distribution of RedN for the case of diffusion coefficient D = 7 $\mu m^2 s^{-1}$ and characteristic DNA-binding time τ_{on} = 1 s.
B. Time evolution of RedN profiles at the start of the simulation and at the end of each replication cycle for 10 consecutive cycles for the simulation shown in A.
C. Same as B except the characteristic DNA-binding time is 10-fold shorter τ_{on} = 0.1 s.

Minor points
_ In the literature, SMCs are typically not considered NAPs.

Response 22: We agree that SMCs are different from many other NAPs, which we indicate in the Introduction. But SMCs have been reported as NAPs in several reviews (e.g., Dame RT, Mol Microbiol 2005, Dillon and Dorman, Nat Rev Micro 2010, and Wang et al, Nat Rev Genet 2013). So we wanted to be inclusive to be on the safe side.

_ The first paragraph in the Results is largely devoted to an introduction. This should be then shifted to the correct section.

Response 23: We have revised the text accordingly.

_ The authors state: "RedN-Venus had a fairly broad distribution, with some depletion near the new pole where ter is located." This is not evident from the figure quoted.

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Response 24: We think that our description of Fig 4C is correct. The RedN signal 'hugs' the DAPI (DNA) signal (meaning broad distribution), but deviates with a lower signal (meaning depletion) when approaching the new pole where the terminus is located. We would be happy to revise, but we would need clarification on the problem.

_ In the "Identification of a novel NAP" section. RedN is identified by screening proteins for features characteristics of NAPs and associated with severe fitness cost when their gene is inactivated. How many other proteins were identified with such criteria in Caulobacter crescentus?

Response 25: Zero.

_ In the "RedN binds to DNA" section. Fig 1D legend should specify the fact that these experiments use recombinant redN from E.coli.

Response 26: Done. Thank you.

_ Figure legends sometimes miss the strain genotype associated with the data presented in the figure. For instance which strain is used in figS1C with the ΔredN deletion?

Response 27: We have verified that strain names are provided in legends when appropriate.

_ In figS1C the authors give the doubling time of the different strains used in this study for two growth media, M2G and PYE. The two media show small growth speed differences with the wild-type. Then why showing those two media instead of minimal and rich media (such as LB)?

Response 28: M2G and PYE are the minimal and rich media for *Caulobacter*. This bacterium does not grow on LB (too high osmolality).

_ Authors verified ΔredN defects are not due to a polar effect of the downstream gene. Why checking the downstream gene only and not the upstream as well?

Response 29: Polar effects on multicistronic transcripts (operon) generally only concern downstream genes.

_ All presented demographs in the manuscript display white background. Shouldn't colorbars zero value be white?

Response 30: The white background is not data; it is just background. It can be in any color we choose and should not be included in the color scale of the protein signal.

_ I am having a hard time reconciling the kymograph data from figure 6D with the time-lapse profiles of figure 6A, since the re-distribution of RedN seems to occur on different time-scales. In fig 6A, RedN maximum reaches the center of the cell length at t=60 minutes and then moves on both sides of the cell leaving a depleted area in RedN signal profile at the cell center. In kymograph of fig 6D, RedN maximum reaches the center of the cell length solely at t~90-100 minutes and then doesn't seem to be depleted. Could the authors explain these discrepancies?

Response 31: The overall temporal sequence can be compared, but not the exact timing, because the cells are not growing under the same conditions (growth medium and temperature) and therefore have different cell cycle lengths. We are now clearly stating the growth conditions in the legend to avoid future confusion.

_ Experiments on FtsZ-depleted cells in which solely the chromosome at the old pole fires new round of replication are shown (the others staying unreplicated) to conclude that RedN redistribution is due to active replication. These experiments are nice and convincing though I don't understand why RedN doesn't relocalize homogeneously on the non-replicating chromosomes and appears as stripes on the kymograph. Maybe the authors could provide potential explanations on the origin of those stripes they describe? Also, it would be important to show replicates of the experiment.

Response 32: We expect that RedN relocalize with equal probability anywhere on the DNA. Even with an equal probability of relocalization, there will be accumulations at certain DNA locations because of stochasticity. They will then persist for extended amount of time (forming long streaks of fluorescent signal in kymographs) due to RedN displaying very little spontaneous dissociation from the DNA. The fact that these long streaks (over 150 min) are seen is further evidence of a very slow spontaneous dissociation. We have generated 'didactic' simulations to explain this concept (Appendix Fig S9).
In addition, we have added several replicates of this experiment, including cases when replication occasionally occurs at the new pole (Appendix Fig S8).

_Line 155 : typo "we may expect that its absence to impact various" shold read "we may expect that its absence impacts various"

Response 33: Corrected. Thank you.

_Line 903, figure 1D legend. Typo : remove extra "See".

Response 34: Corrected. Thank you.

Referee #2:

This is a very interesting, well-executed and well-described study. It describes the identification and characterization of a new nucleoid-associated protein, widely evolutionarily conserved, that is tightly bound to replicated DNA and evicted with passage of the replication fork, with correlated effects on gene expression. These conclusions are well-documented by a variety of method.

This reviewer has only a few comments.
1. The authors speculate that absence of this protein results in defective DNA replication. It would be interesting to understand whether/how this phenotype is related to the normal replication-promoted eviction of the protein.

Response 1: We agree; this will be very interesting to examine in future studies. We hope that the reviewer will agree that this is beyond the scope of this study.

2. The authors suggest that many of the genes whose expression is altered in the mutant are DNA damage-induced. The implication is that SOS induction may be required for viability in the mutant background. This could be tested.

Response 2: Indeed, even under slow-growth conditions, we were able to consistently get mutant colonies when transducing the Δ*redN* mutation in the wild-type background, while we reproducibly could not get any Δ*redN* transductants in a Δ*recA* or $lexA_{K203A}$ background. RecA is essential for SOS induction in *C. crescentus* (Galhardo et al, Nucleic Acids Res 2005; da Rocha et al, J Bac 2008; and Modell et al, Plos Biol 2014) and the $LexA_{K203A}$ mutant is a constitutive inhibitor of SOS induction in *C. crescentus* (Modell et al, Plos Biol 2014). As a control, we confirmed that the Δ*recA* and $lexA_{K203A}$ mutants have no viability defect in a wild-type background under slow- or fast-growth conditions, consistent with previous reports (Modell et al, Plos Biol 2014).

3. The authors use DNA gyrase to block DNA replication and thereby examine spontaneous dissociation of RedN from non-replicating DNA. This approach, of course, has the disadvantage that the supercoiling status of the chromosome is altered, and this change could, in principle alter the dissociation constant, particularly if altered supercoiling in front of or just behind the replication fork causes destabilization. The data could be viewed as saying that reduced negative supercoiling such as occurs in front of the fork does not destabilize binding and thus, perhaps, it is increased negative supercoiling behind the fork that causes dissociation. If so, reduction of gyrase levels would artificially stabilize the protein. This is a significant problem for this data. Wouldn't it be possible/better to do this experiment in the ftsZ condition on the non-replicating nucleoids?

Response 3: We have performed the requested FRAP experiment with untreated (no novobiocin) FtsZ-depleted cells (Fig 7B-D) and found a characteristic time of fluorescence recovery $\tau = 110 \pm$

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28 min (mean ± SD, n = 10), which is comparable to the experiment with novobiocin (τ = 105 ± 35 min, n = 9). Note that these τ values are likely underestimations of the true spontaneous dissociation time τ_{off} since RedN-Venus is synthesized during the 20-min experiment and this *de novo* protein synthesis likely contributes to fluorescence recovery. In any case, we now show, in new simulations, that even when we considered a spontaneous dissociation of τ_{off} =100 min, we still obtained an asymmetric distribution of RedN (Appendix Fig S12), as before (Fig 8).

2nd Editorial Decision 25 November 2016

Thank you for submitting your revised manuscript for our consideration. We have now received the below comments from the two original referees, who have once more assessed the study and your responses. I am pleased to say that both consider the study significantly improved and would now in principle support publication. Nevertheless, there are still a number of -mostly presentational- issues that would appear important to be addressed in order to substantiate the findings and strengthen the impact of this work. In addition, there are also several editorial points to be addressed in order to make the manuscript suitable for publication:

1) Please satisfactorily address referee 1's three remaining concerns with the presentation and discussion of the data, which also affect interpretation of the results.

2) Following referee 2's request, please include the observations related to the SOS response in the manuscript text.

3) For production purposes, please upload individual files for each main figure and each Expanded View figure.

4) In the manuscript text, please make sure to reference Appendix Figure S12 at least once (currently it is only referred to in the point-by-point response)

5) The text refers to "Appendix Code 1" and "Appendix Code 2" but the provided file instead contains "ComputerCodeEV1A/B/C" - please clarify and make sure all three codes are references at least once in the manuscript. Also, it would be preferable to supply the code in text rather than PDF format. Therefore, please upload the three codes as separate Expanded View files "Computer Model/Code EV1/2/3" in .txt or .xml format, providing the title/legend in the main manuscript (as for EV figures/datasets/movies), and making sure to reference each of the three in the text as such.

6) In light of the referees' equivocal feedback on RedN/GapR naming and in the interest of avoiding unnecessary confusion, we would like to suggest the following compromise: in the abstract, refer to the protein as "RedN/GapR". At the first mention in the text, where you introduce the RedN naming, please add a sentence that during the revision process of this work, Ricci et al (citation) independently identified this protein and called it GapR. For the remainder of the text, we would suggest to refer to it as GapR to avoid confusion, but given that this may also mean relabelling all figures, you may alternatively refer to it as "RedN/GapR" throughout. In the discussion, it appears warranted to briefly but properly compare and contrast your findings to those of Ricci et al, instead of just including a brief note.

7) Finally, the most important remaining conceptual concerns are raised by referee 2, and urge for a some major changes in the writing and organization of the paper. On one hand, the referee (pt. 4) is concerned that the some very important novel findings and implications of the paper are inadequately emphasized, discussed and fleshed out in the final models. On the other hand (pt. 3), the referee raises doubts about the decisiveness of RedN-replisome link models and the possibility of equally likely alternative interpretations, urging for a more factual presentation of results followed by discussion and evaluation of possible models and interpretations.
Please note that we normally avoid bringing up new points during a revision that had not been raised already during the initial round; and our referee in fact appreciates this and apologized also to us for originally having overlooked these deeper conceptual issues. Nevertheless, I realize that their points are actually very well-taken, and agree that the study may really become much more compelling if reorganized and reinterpreted along the lines suggested in this report. Given that the publication of partly overlapping work after your initial submission does not impact on our decision on your

manuscript, and that further changes are anyway needed to address referee 1's remaining concerns, I think it would be highly beneficial to also invest this additional time and effort now, in order to further strengthen the impact of this work and to differentiate it even better from a mere identification and characterization of RedN. Such rethinking should obviously also be reflected in altered title, abstract and synopsis text/model figure.

I am therefore returning the manuscript to you once again for a final round of revision. As always, I'd be happy to discuss these points further, so please do not hesitate to get back to me in case this should consider this helpful.

REFEREE REPORTS

Referee #1:

Most of the comments in the original review have been addressed appropriately in the revision.

Regarding the name of the protein, we would stick to GapR. We agree that the choice of name was unfortunate, but it will be much better to stick to a single name as otherwise this will complicate the literature in future.

We have still a few points that were not addressed appropriately in the revision and that we would strongly recommend the authors to address.

We have asked to see the statistics on the percentage of cells having 0, 1, 2 or more DnaN foci. The authors (response 8) argue that this is shown in Figure S4. However, in this figure what is shown is the distribution of cell lengths for cells with 0, 1 or 2 foci. It is not clear what the normalisation is in this figure: Frequency axis is obscure; curves are not normalised so that their amplitude or area is unity/ and it is not clear whether the distributions are normalised to respect to each other. The information we request is important. As in one model (joined replisomes) one would expect to see that in all cell cycle stages (cell lengths) cells have either 1 or 0 replication foci. However, if the bidirectional replisome model was predominant one would expect, after replication initiation, that cells shift from 0 to 2 foci (with a low proportion of cells having 1 foci). We do not think this information can be retrieved from S4, unless there is something we don't understand: for a specific cell length, what is the number of cells with 0, 1 and 2 foci? This can provide a clear indication of whether there is a predominant mechanism.

Authors argue that their way of plotting demographs has been used before. We could quote a longer list of publications where the x-axis is normalized by nucleoid/cell length to represent cell cycle stage/ time. The current x-axis in their figures is non-linear so it is impossible to compare demographs in different conditions, as shown in Figs. 5B, S7, or EV3. The authors argue in the text that " demograph analysis-in which fluorescence profiles of individual cells are sorted by cell length as a proxy for cell cycle progression ". As the x-axis is non-linear, then this is a poor representation of cell cycle progression... We would strongly encourage the authors to implement this change.

We have previously argued that "RedN seems to mainly localize near the center of the cell while the origin of replication is expected to be at the old pole. How is this distribution compatible with ChIP data showing a RedN enrichment at/near the origin?". The authors response is that " The demograph does not show a midcell localization in young cells, and the localization profile is consistent with the ChIP data showing depletion at ter relative to ori.". We understand that the patterns appearing in the demograph are complex, but they should be properly explained (e.g. the influence of cell edge detection) so that a reader does not misinterpret their data. They could have, for instance, added dashed lines from the profile of the cell, from the signal of a pole marker such as TipN-GFP used in figure EV3A or from the HU2-GFP signal to make this plot more understandable.

Referee #2:

1. I think the authors should keep their name, for all of the reasons they mention and others that they do not.

2. It would be useful to put observations regarding SOS-dependence in the text.

With apologies for having been delinquent: I now have some further comments on the latter part of the paper, stimulated in part by comments of Reviewer 1. There are two main points:

3. About RedN, I think that the authors are likely being too specific in their model and that they should confine themselves to: (a) stating the phenomenon in the Results and then speculating in the Discussion; and (b) considering the possibility that polymerase does NOT actively evict RedN in quite the way they describe (or at least as I understood their description). For example, it seems equally likely that they are detecting a global change in nucleoid state (eg in supercoiling). They can't tell about this or any other mechanism. Moreover, there is a fundamental issue that they don't discuss which is the overall asymmetry of the situation. Another red flag is the ftsZ experiment. There is alot more RedN on the non-replicating genome than on the replicating genome. They use this to analyze RedN binding, but it actually is mysterious per se and does not, at face value, support their replication-eviction model and was not discussed, that I could see. My guess is that RedN binding is favored by negative supercoiling. It will be evicted from replicating DNA by positive supercoiling in front of the fork and it will be favored in the quiescent state which is likely to be hyper-supercoiled. This does not explain why the effect is "permanent" and thus has significant implications for the global effect of replication. Personally, I don't think that the modeling adds much. It is always possible to make a model to explain observations, but there is no guarantee that the model is right. I appreciate that this is not the point of view of the authors, but nonetheless, this could go in the SI - it does not provide support for any particular model beyond what is apparent in the data.

4. More generally: the authors are doing themselves a dis-service. As far as I understand, this is the first time anyone in the Caulobacter field has actually looked at global nucleoid dynamics (e.g. HU)and the DnaN studies are of higher quality than previously. Irrespective of RedN, these are new data. In particular, there are three phenomena that are obvious: (i) In the HU data, there is a "duality transition" about half way through the cell cycle and this transition puts the remaining material in the middle of the cell (actually as marked by RedN). In fact, this is exactly what happens during the E.coli cell cycle and there are other close analogies between the two systems re origin splitting and dynamics (as described by papers and a review from the Bates and Kleckner laboratories). Seeing a convergence is nice and could be mentioned (ii) The DnaN data are lovely. I have no concerns. The authors should not "bow" to less good data from others. The authors should distinguish three phases: (a) an early phase where there may be one/two close foci and their dynamics can't tell; (b) as is clear in panel 6D, there is a rapid splitting and movement of the replisome early in the cell cycle. There is no chance that, after this transition, there is only "one replisome" marked - it is only flickering of the faint focus. (c) finally, there is one focus - can't rule out two close foci, as at the early stage. But at both (a) and (c) it is expected that there is a one-focus stage, at the start of initiation and as forks converge and/or one complex is lost before the other. This is a detail, but data are fully consistent. Then there is real global transition, the nature of which remains to be determined but it should be mentioned. In any case, the dogma of the replication factory idea has been suspect for awhile (see review article by Bates some years ago). It would be useful to have a more sensible interpretation of the data. (iii) The asymmetry of DnaN brightness is mirrored by the asymmetry of RedN brightness. This is likely not a coincidence. But the authors never mention this asymmetry at all, in any context. This should be mentioned and discussed, even though highly mysterious.
In summary: the paper would be greatly enhanced if the authors could comment clearly on novel features revealed by the particular combination of analyses done for this project and simply say that they raise interesting questions for the future. Along these lines, the Results should include an unbiased description of interesting findings rather than being so targeted to the issues re RedN. It would be clearer to start by describing HU and DnaN results, irrespective of RedN; then go on to RedN. (And the model can be moved to SI and Discussion).

2nd Revision - authors' response 29 November 2016

Please see my response below each comment (in blue).

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Thank you for submitting your revised manuscript for our consideration. We have now received the below comments from the two original referees, who have once more assessed the study and your responses. I am pleased to say that both consider the study significantly improved and would now in principle support publication. Nevertheless, there are still a number of -mostly presentational- issues that would appear important to be addressed in order to substantiate the findings and strengthen the impact of this work. In addition, there are also several editorial points to be addressed in order to make the manuscript suitable for publication:

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We have addressed them. They do not affect the interpretations of the results.

2) Following referee 2's request, please include the observations related to the SOS response in the manuscript text.
Done

3) For production purposes, please upload individual files for each main figure and each Expanded View figure.
Certainly

4) In the manuscript text, please make sure to reference Appendix Figure S12 at least once (currently it is only referred to in the point-by-point response)
Sorry for this oversight.
This is now done

5) The text refers to "Appendix Code 1" and "Appendix Code 2" but the provided file instead contains "ComputerCodeEV1A/B/C" - please clarify and make sure all three codes are references at least once in the manuscript. Also, it would be preferable to supply the code in text rather than PDF format. Therefore, please upload the three codes as separate Expanded View files "Computer Model/Code EV1/2/3" in .txt or .xml format, providing the title/legend in the main manuscript (as for EV figures/datasets/movies), and making sure to reference each of the three in the text as such.
Each code is now saved as a text file and is referred to in the text.

6) In light of the referees' equivocal feedback on RedN/GapR naming and in the interest of avoiding unnecessary confusion, we would like to suggest the following compromise: in the abstract, refer to the protein as "RedN/GapR". At the first mention in the text, where you introduce the RedN naming, please add a sentence that during the revision process of this work, Ricci et al (citation) independently identified this protein and called it GapR. For the remainder of the text, we would suggest to refer to it as GapR to avoid confusion, but given that this may also mean relabelling all figures, you may alternatively refer to it as "RedN/GapR" throughout. In the discussion, it appears warranted to briefly but properly compare and contrast your findings to those of Ricci et al, instead of just including a brief note.
Thank you for the suggestion, but we think that using two names will be confusing. We have renamed the protein GapR and change the text and figures accordingly.

We have removed the note, and are now mentioning the Shapiro paper in the beginning of the Discussion. We explain that the only overlap with our story is the DNA-binding preference of the protein to AT-rich DNA sequences and its essential function in cell cycle progression under standard laboratory conditions. The readers will understand from this that everything else in our study is novel.

7) Finally, the most important remaining conceptual concerns are raised by referee 2, and urge for a some major changes in the writing and organization of the paper. On one hand, the referee (pt. 4) is concerned that the some very important novel findings and implications of the paper are inadequately emphasized, discussed and fleshed out in the final models.
We agree with the reviewer that the DnaN data revises the current model of replisome dynamics in *Caulobacter*, and we dedicate 26 lines of text and 5 figure panels (Figure 6 and Appendix Fig S4), including a schematic (Fig 6B) to explain it. Adding to it will detract from the GapR story whose is conceptually far more novel and generalizable than the revision of the model of replisome dynamics in *Caulobacter*.

On the other hand (pt. 3), the referee raises doubts about the decisiveness of RedN-replisome link models and the possibility of equally likely alternative interpretations, urging for a more factual presentation of results followed by discussion and evaluation of possible models and interpretations.
We disagree. What the reviewer proposes relies on the replication fork passage evicting GapR from the DNA, which is our model. He just goes one step further than us by proposing a mechanism by which GapR is evicted by the replisome (through generation of positive supercoils in front of the replication fork). We, on the other hand, leave it at the phenomenological level because different mechanisms are possible and it does not matter which one it is as long as protein eviction happens.

This said, the comment from the reviewer made us realize that the manuscript should be rearranged to present the FRAP data before the replisome eviction idea is considered. Indeed, if the spontaneous dissociation of GapR was relatively fast (time scale of minute or under) as it is generally assumed for DNA-binding proteins, the correlated dynamics between the replisome and GapR could be due to a difference in binding affinity between replicated vs. unreplicated DNA (because of a difference in "nucleoid state" like supercoiling or whatever), without evoking eviction by the replisome. But the negligible spontaneous dissociation of GapR excludes this possibility. We have reorganized the paper accordingly, and have added discussion to explain the implications of the FRAP results

Please note that we normally avoid bringing up new points during a revision that had not been raised already during the initial round; and our referee in fact appreciates this and apologized also to us for originally having overlooked these deeper conceptual issues. Nevertheless, I realize that their points are actually very well-taken, and agree that the study may really become much more compelling if reorganized and reinterpreted along the lines suggested in this report. Given that the publication of partly overlapping work after your initial submission does not impact on our decision on your manuscript, and that further changes are anyway needed to address referee 1's remaining concerns, I think it would be highly beneficial to also invest this additional time and effort now, in order to further strengthen the impact of this work and to differentiate it even better from a mere identification and characterization of RedN. Such rethinking should obviously also be reflected in altered title, abstract and synopsis text/model figure.
We do not think that the new requests by reviewer 2 are justified, and we disagree that reviewer 1"s remaining concerns have any effect on data interpretation. Nevertheless, we have revised the text, and added data to avoid future confusion. See below for more explanations.

I am therefore returning the manuscript to you once again for a final round of revision. As always, I'd be happy to discuss these points further, so please do not hesitate to get back to me in case this should consider this helpful.

Referee #1:

Most of the comments in the original review have been addressed appropriately in the revision. Regarding the name of the protein, we would stick to GapR. We agree that the choice of name was unfortunate, but it will be much better to stick to a single name as otherwise this will complicate the literature in future.
Response 1: Okay

We have still a few points that were not addressed appropriately in the revision and that we would strongly recommend the authors to address. We have asked to see the statistics on the percentage of cells having 0, 1, 2 or more DnaN foci. The authors (response 8) argue that this is shown in Figure S4. However, in this figure what is shown is the distribution of cell lengths for cells with 0, 1 or 2 foci. It is not clear what the normalisation is in this figure: Frequency axis is obscure; curves are not normalised so that their amplitude or area is unity/ and it is not clear whether the distributions are normalised to respect to each other. The information we request is important. As in one model (joined replisomes) one would expect to see that in all cell cycle stages (cell lengths) cells have either 1 or 0 replication foci. However, if the bidirectional replisome model was predominant one would expect, after replication initiation, that cells shift from 0 to 2 foci (with a low proportion of cells having 1 foci). We do not think this information can be retrieved from S4, unless there is something we don't understand: for a specific cell length, what is the number of cells with 0, 1 and 2 foci? This can provide a clear indication of whether there is a predominant mechanism.

Response 2: The frequency means probability, which was calculated so that the integral of each curve is 1, which is very standard. We have replaced "frequency" by "probability", and added to the legend how it was calculated.

Regarding the added request, the results cannot affect the main conclusions of the paper, as the dynamics of GapR are the same whether the replisomes migrate bidirectionally or in pair. We have, nevertheless, performed the analysis, which is now provided as Appendix Fig S4B.

Authors argue that their way of plotting demographs has been used before. We could quote a longer list of publications where the x-axis is normalized by nucleoid/cell length to represent cell cycle stage/ time. The current x-axis in their figures is non-linear so it is impossible to compare demographs in different conditions, as shown in Figs. 5B, S7, or EV3. The authors argue in the text that " demograph analysis-in which fluorescence profiles of individual cells are sorted by cell length as a proxy for cell cycle progression ". As the x-axis is non-linear, then this is a poor representation of cell cycle progression... We would strongly encourage the authors to implement this change.
Response 3: It would have been helpful to get the reference for at least one paper where "the x-axis is normalized by cell length", as we could not find it, except for one paper that simply converted the number of cells by cell cycle units, assuming that the two are linearly correlated, which, as the reviewer pointed out, are not.

We agree that the x-axis is non-linear, but don't understand why this makes it problematic for the comparison we wish to make. First, comparisons and measurements can be done with non-linear scales. Second, and more importantly, our only goal at that stage was to demonstrate, at the population level, that the localization of GapR was different from that of a protein (like HU2) that binds DNA uniformly. This difference was then used as a rationale for detailed analysis of their localization during the cell cycle by time-lapse microscopy (Fig 5C, Fig 6, Appendix Figure S5-S6). So, even if the reviewer wasn't convinced with the demographs, he or she has the time-lapse experiments for comparison.

In any case, even though we strongly believe that the demographs provide valuable cell cycle information, we have revised the text to remove any interpretation about the cell cycle localization of GapR from the demograph data. They are now only used to show that the localization of GapR is different from that of HU, which is irrefutable. 5

In addition, we have converted the x-axis to "percentile of the population", which may make the demographs easier to understand. We have also converted the y-axis to relative cell length to address the concern below.

We have previously argued that "RedN seems to mainly localize near the center of the cell while the origin of replication is expected to be at the old pole. How is this distribution compatible with ChIP data showing a RedN enrichment at/near the origin?". The authors response is that " The demograph does not show a midcell localization in young cells, and the localization profile is consistent with the ChIP data showing depletion at ter relative to ori.". We understand that the patterns appearing in the demograph are complex, but they should be properly explained (e.g. the influence of cell edge detection) so that a reader does not misinterpret their data. They could have, for instance, added dashed lines from the profile of the cell, from the signal of a pole marker such as TipN-GFP used in figure EV3A or from the HU2-GFP signal to make this plot more understandable.
Response 4: The cell edge should be very easy to see; it is the border between the signal (color scale) and the white background. As mentioned above, we now provide demographs in which the y-axis is the relative cell length, which may help. In any case, the concern does not affect the interpretation of our results. Even if the demographs are difficult for the reviewer to interpret, he or she has the time-lapse data showing the cell cycle localization of GapR and HU2 (Fig 5C, Fig 6, Appendix Figure S5-S6).

Referee #2:

1. I think the authors should keep their name, for all of the reasons they mention and others that they do not.

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Response 5: Reviewer 1 suggested that we go with GapR and the editor proposed that we use both names. The lack of unified view is consistent with the fact that there is no great solution…At the end, we think that it is probably best to go with the GapR name.

2. It would be useful to put observations regarding SOS-dependence in the text.
Response 6: Done.

With apologies for having been delinquent: I now have some further comments on the latter part of the paper, stimulated in part by comments of Reviewer 1. There are two main points:

3. About RedN, I think that the authors are likely being too specific in their model and that they should confine themselves to: (a) stating the phenomenon in the Results and then speculating in the Discussion; and (b) considering the possibility that polymerase does NOT actively evict RedN in quite the way they describe (or at least as I understood their description). For example, it seems equally likely that they are detecting a global change in nucleoid state (eg in supercoiling). They can't tell about this or any other mechanism.
Response 7: No, it is not equally likely that GapR is changing its binding activity because of a global change in nucleoid state such as supercoiling because of the negligible spontaneous dissociation of GapR from the DNA. If GapR had a fast rate of spontaneous dissociation (minute time scale or under), as it is generally assumed for DNA-binding proteins, then, a change in location of binding sites (because of a difference in supercoiling, higher-order structure, methylation, acetylation, or whatever) would be accompanied with a change in GapR binding location, without evoking eviction of GapR by the replication forks. This is because the rapid spontaneous dissociation of GapR from the DNA would permit for such a rearrangement to occur. But, as the FRAP data show, the spontaneous dissociation of GapR is very slow, negligible in the timescale of the cell cycle. The data indicate that GapR proteins will remain on the DNA for as long as they are not evicted from the DNA by the replication fork passage. Note that we never implied a mechanism by which eviction occurs. It could be through collision between replisome and GapR, or through a local change in DNA structure (e.g., supercoiling) right in front of the replisome. The result is the same phenomenologically: the replisome passage evicts GapR from the DNA.

Moreover, there is a fundamental issue that they don't discuss which is the overall asymmetry of the situation.
Response 8: We absolutely discuss it; it is a major point of our story (e.g., lines 425-428, 445-449, 491-493). The model (Figure 8) recapitulates the asymmetry observed experimentally. Please see also response 17 in our previous rebuttal.

Another red flag is the ftsZ experiment. There is alot more RedN on the non-replicating genome than on the replicating genome. They use this to analyze RedN binding, but it actually is mysterious per se and does not, at face value, support their replication-eviction model and was not discussed, that I could see.
Response 9: The asymmetry in RedN/GapR amount between replicating and non-replicating genome is absolutely consistent with our model. Let's consider the simple analogy of two pots of money, each containing $50. If you take all the money from one pot and redistribute it equally between the two pots, one pot will have $25 and the other $75. Repeat the process, and one pot will now have 12.5 and the other $87.5. In other words, you will observe a progressive depletion of money (RedN) in one pot (the replicating DNA) and an accumulation of money (RedN) in the other pot (the unreplicating DNA). RedN only gets depleted from the replicating genome where replication occurs, but gets re-distributed on both replicating and non-replicating genomes, leading to "a lot more RedN on the non-replicating genome than on the replicating genome", as observed.

My guess is that RedN binding is favored by negative supercoiling. It will be evicted from replicating DNA by positive supercoiling in front of the fork and it will be favored in the quiescent state which is likely to be hyper-supercoiled. This does not explain why the effect is "permanent" and thus has significant implications for the global effect of replication. Personally, I don't think that the modeling adds much. It is always possible to make a model to explain observations, but there is no guarantee that the model is right. I appreciate that this is not the point of view of the authors, but nonetheless, this could go in the SI - it does not provide support for any particular model beyond what is apparent in the data.

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Response 10: The reviewer's guess that „[GapR] will be evicted from replicating DNA by positive supercoiling in front of the fork" is consistent with our replisome-eviction model.

We understand that the reviewer is not a fan of mathematical modeling. But we use the pots-of-money analogy above to explain that our model works, not because it includes a hidden weird assumption that makes it "magically" work, but because it makes perfect sense in retrospect. The model is very simple, and is mostly used for didactic reason.

For example, let's consider the reviewer's best guess of what might be happening: 1) "[GapR] will be evicted from replicating DNA by positive supercoiling in front of the fork" and 2) "it will be favored in the quiescent state which is likely to be hyper-supercoiled." His first point is 100% consistent with what we propose: eviction of GapR by the replication fork passage. Our model shows that his second point, which is speculation, is not needed. Even if the dissociated GapR protein re-bind replicated and unreplicated DNA with equal probability (the simpler case), we already get the asymmetry in GapR binding between ori and ter. This is simply because DNA replication is asymmetric, always going from ori to ter. GapR gets removed only from the replicated DNA, and even if it gets re-distributed equally between replicated and unreplicated DNA, it will lead to a depletion from the replicated DNA and an accumulation on the unreplicated DNA (think about the pots-of-money analogy). By the end of replication, the ter region has been freshly depleted of GapR, and thus has a lower level of GapR. Conversely, the regions near ori have the largest amount of GapR bound, simply because they have had more time to replete in GapR than regions close to ter. Again, the model is not proposing anything strange than logic cannot explain. The modeling remains useful, because it helps realize/visualize that the directionality of DNA replication (from ori to ter) causes an asymmetry in GapR binding even if the binding sites are uniformly distributed. In our opinion, this is conceptually new and exciting, as it is generalizable to any DNA-binding protein with a slow spontaneous dissociation rate.

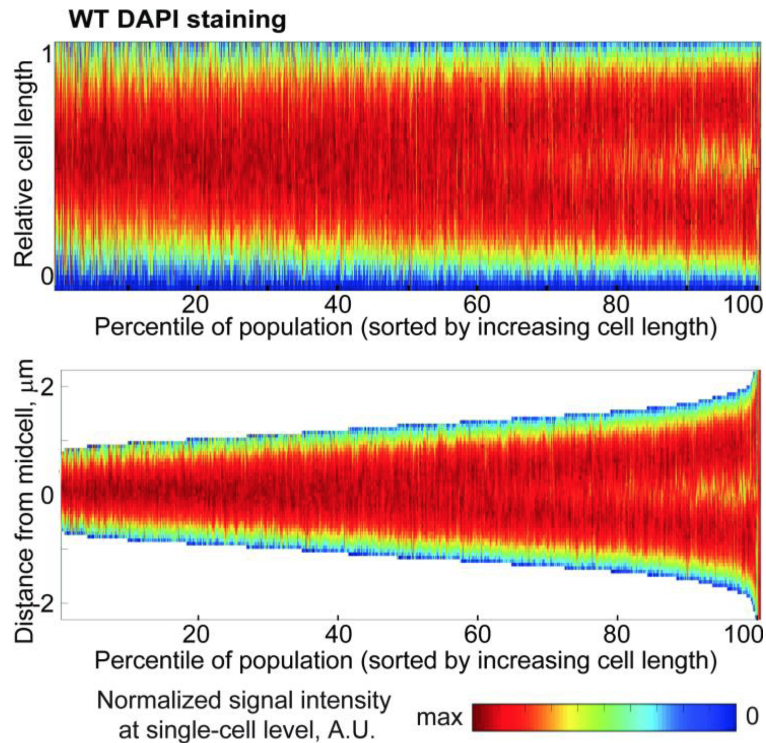
The reviewer might be right that unreplicated DNA is more supercoiled and that GapR preferentially binds to hyper-supercoiled DNA. If true, it would enhance the asymmetry in binding. But again, there is no need to evoke something that we don't know when what we know (the most parsimonious model) is already sufficient to produce an asymmetry in binding. We do mention in the discussion that other layers of regulation (such as supercoiling) may exist to affect (positively or negatively) the asymmetry generated by the simple replisome-eviction mechanism.

4. More generally: the authors are doing themselves a dis-service. As far as I understand, this is the first time anyone in the Caulobacter field has actually looked at global nucleoid dynamics (e.g. HU)and the DnaN studies are of higher quality than previously. Irrespective of RedN, these are new data. In particular, there are three phenomena that are obvious: (i) In the HU data, there is a "duality transition" about half way through the cell cycle and this transition puts the remaining material in the middle of the cell (actually as marked by RedN). In fact, this is exactly what happens during the E.coli cell cycle and there are other close analogies between the two systems re origin splitting and dynamics (as described by papers and a review from the Bates and Kleckner laboratories). Seeing a convergence is nice and could be mentioned (ii) The DnaN data are lovely. I have no concerns. The authors should not "bow" to less good data from others. The authors should distinguish three phases: (a) an early phase where there may be one/two close foci and their dynamics can't tell; (b) as is clear in panel 6D, there is a rapid splitting and movement of the replisome early in the cell cycle. There is no chance that, after this transition, there is only "one replisome" marked - it is only flickering of the faint focus. (c) finally, there is one focus - can't rule out two close foci, as at the early stage. But at both (a) and (c) it is expected that there is a one-focus stage, at the start of initiation and as forks converge and/or one complex is lost before the other. This is a detail, but data are fully consistent. Then there is real global transition, the nature of which remains to be determined but it should be mentioned. In any case, the dogma of the replication factory idea has been suspect for a while (see review article by Bates some years ago). It would be useful to have a more sensible interpretation of the data.

Response 11: We agree that our DnaN data challenge the current models of replisome dynamics in *Caulobacter*. We dedicate a big section in the results to explain it, and the different steps in DnaN dynamics described by the reviewer are represented in our schematic of the bidirectional replisome mode (Fig 6B). So, the information is there.

We are not sure what the reviewer means by "In the HU data, there is a "duality transition" about half way through the cell cycle and this transition puts the remaining material in the middle of the

cell (actually as marked by RedN)". But HU2 demographs look very similar to demographs of cells stained with the DNA stain DAPI (see below), suggesting that HU2 localization mirrors that of the DNA.



Regarding the "joined replisomes" vs. "bidirectional replisomes" models, we think that they may co-exist in the populations. In still images, we still see too many cells with a single DnaN-CFP focus to exclude the possibility that there is no instance of replisomes traveling in pair (joined replisome mode, Fig 6B). It could be detection problem, but we cannot exclude the possibility that it reflects joined replisomes. Furthermore, in time-lapse experiments, we occasionally see a transient disappearance of the dim DnaN focus accompanied with an increase in signal at the bright DnaN focus, suggesting a temporary re-joining of the replisomes. Therefore, we are not comfortable with the reviewer's suggestion to exclude the joined-replisomes model. Importantly, the joined-replisomes model does not argue for the "replication machinery" idea as the joined replisomes are still traveling from the old pole to midcell.

(iii) The asymmetry of DnaN brightness is mirrored by the asymmetry of RedN brightness. This is likely not a coincidence. But the authors never mention this asymmetry at all, in any context. This should be mentioned and discussed, even though highly mysterious.
Response 12: We do not know why the DnaN focus coming the old pole is brighter that the DnaN focus coming from the new pole. But we do explain the asymmetry in RedN brightness several times (lines 425-428, 445-449, 491-493). It is not mysterious at all. Our model predicts it (Fig 8) and we discussed it, with explanation and figures in response 17 in our first response letter.

In summary: the paper would be greatly enhanced if the authors could comment clearly on novel features revealed by the particular combination of analyses done for this project and simply say that they raise interesting questions for the future. Along these lines, the Results should include an unbiased description of interesting findings rather than being so targeted to the issues re RedN. It would be clearer to start by describing HU and DnaN results, irrespective of RedN; then go on to RedN. (And the model can be moved to SI and Discussion).
Response: We thank the reviewer for this suggestion, but we disagree. In our view, the paper should focus on the conceptual advance of our work, which is that 1) DNA-binding proteins with negligible spontaneous dissociation from the DNA will display an *ori*-to-*ter* asymmetry in binding along the chromosome even if their binding sites are uniformly distributed, and 2) their activity will be exquisitely coordinated with the passage of the replication fork.

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Is there an estimate of variation within each group of data?	Yes, probability density and or and stardart deviation was used was calculated for populations when needed. Also significance of the mesurements were assesed with p-values and e-values when appropriate
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