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Cell cycle coordination and regulation of bacterial chromosome segregation dynamics by polarly localized proteins

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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 20 July 2010

We have now received the reports of two expert referees on your manuscript, which you will find copied below. I am pleased to inform you that both reviewers consider the paper a well-executed study with interesting and potentially important results, and therefore in principle suited for publication in The EMBO Journal. However, while referee 1 has only minor issues to be addressed, referee 2 does raise a number of more substantive points regarding the conclusiveness of the currently presented evidence. I realize that many of the major comments are likely to be addressed by clarifications, additional discussion, and qualification of various statements to avoid over-interpretation; but there are also several concerns that will likely require further experimentation to provide important controls and to allow more decisive conclusions to be drawn in some instances. Should you be able to satisfactorily address the referees' comments through a revised version of the manuscript and a detailed response letter, we should be happy to consider such a revised manuscript further for publication. I should however remind you that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be important to diligently answer to all the various points raised at this stage. When preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor

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The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This is an interesting paper that makes a significant contribution to understanding the molecular basis of chromosome segregation in bacteria. Although the ParABS systems have been implicated in the segregation of both chromosomes and plasmids in a wide range of bacteria, we still don't understand in detail how sister replicated chromosomes are moved apart. The results described in the paper reinforce the emerging general view of the ParABS systems in which the ParB component recognises and binds to the partitioning sequence, parS, and ParA acts as a cytoskeletal factor that pulls on the ParB-parS complex. ParB regulates ParA and drives its dynamics by triggering ATP hydrolysis on ParA. The results suggest that the Caulobacter parABS system operates in a similar way to that previously described for Vibrio. However, the new paper goes much further by identifying TipN as a polar marker that provides the system with directionality, and dissecting the interactions responsible for TipN function.
Overall, the paper is well written, clear and convincing. I have only a few comments for consideration.
1. p. 8, 2nd paragraph. Introduction of the concept that the misplacement of the division site and aberrant movement of MipZ are due to a chromosome segregation defect emerges quite abruptly here, and the statement that aberrant movement... was caused by a DNA segregation defect seems too strong ahead of the data. How about something along the lines... "In some bacteria, abnormal division site placement has been associated with defects in chromosome segregation. To test whether this was the case in the tipN mutant, movement of oriC was examined by...".
2. p. 10, 3rd paragraph, last 2 sentences; p. 11, first sentence, 2nd last sentence. I think the words "causing" and "resulting in" are too strong. The observed events are apparently associated, but it seems premature to assume that the connections are causal.
3. p. 14. These are interesting experiments (expressing mutant ParA proteins fused to YFP in an otherwise wild type background). However, I'm surprised that the results are so clear cut, at least for the mutants that should dimerize (D44 and R195). I would have expected heterodimers to form with the wild type protein, which might have partial function and therefore perhaps intermediate localization?
4. p. 17, lines 1-2. There is a long literature of cell size control of initiation of replication in E. coli and other bacteria.
5. p. 18, lines 6 up. The comment "as often believed" seems misplaced, since it is again well known in various bacteria, and especially well established in E. coli, that Z ring formation is temporally separated from constriction.

Trivial corrections
p 7, line 4 up. "the" new pole.
p. 9, 5 lines up. The sentence beginning "Second" seems to repeat the idea in the previous sentence?
p. 12. Fig. 7 cited out of order.
p. 18, line 3. in "a" dramatic...
p. 18, lines 5-6, and 2 up. Three sig figs seems overly precise, given the limits of standard fluorescence microscopy instrumentation.

Referee #2 (Remarks to the Author):

Comparing the dynamics of parS/ParAB and the MipZ division inhibitor in Caulobacter WT and polarity mutant (tipN) cells, Schofield et al report interesting and potentially important observations on the requirements of the movement of parS/ParAB/MipZ-complexes through the cell and the coordination of the movement with the cell cycle. Most of the experiments are well executed and the level of sophistication for the data acquisition and analysis is remarkable. Since little is known about the regulation of parS/ParAB and how this system moves chromosomal segments through cells, the topic is of broad interest, addressing an important and still mysterious question in fundamental

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bacterial cell biology.

The weaknesses are i) that the presentation and discussion of the data are for the most part phenomenological, ii) that the paper does not provide much new mechanistic depth (i.e. the mechanism of regulation of ParA-ADP by TipN and why this should cause a phenotype is not obvious) and iii) that there is a bias towards inserting unsubstantiated claims that are derived from circumstantial data and/or that are not adequately identified as speculation (see below). Also, in general I found the model of how ParA-bound DNA is stimulated by ori-bound ParB to pull or generate force (p. 12) and ultimately facilitate or guide ori movement to be confusing and not very intuitive. The fact that the tipN mutant is viable and that it has ParAB/MipZ localized to both poles, indicates that TipN plays a minor and dispensable role in the "extrinsic" regulation of ParA: chromosomes can segregate fine and ParAB/MipZ can efficiently find the poles when TipN is absent. The synthetic lethality of the popZ/tipN double mutant is interesting and definitely holds the key to understanding the basis for these phenotypes, but it is not clear from the presented data whether this synthetic lethality is due to a massive segregation defect (that is normally masked in WT and single mutant cells by the propose overlapping role of TipN and PopZ in segregation) or whether the lethality arises from an (unexplored and) synthetic division defect with TipN aiding, for example, in the late stages of cell septation/separation process and PopZ influencing the proper positioning of MipZ via ParB. Along the lines of such murky cause-vs-effect-relationships scattered in this manuscript, the authors do not make a compelling case that the division abnormalities of the tipN mutant are CAUSED by the delayed and discontinuous movement of the ParAB/MipZ complex, rather than merely being a manifestation of a secondary EFFECT of the tipN phenotype.

Major comments:
A) Generally, the impressive quantitative analysis of MipZ/ParAB/FtsZ should not only be done with WT and tipN cells, but also with the complemented mutant to show that these phenotypes are solely due to the absence of the TipN protein. These control experiments are missing.

B) The authors show that tipN cells divide aberrantly and that the mutant cells are altered in the proper placement of FtsZ. They also find that the movement of parS/ParB is delayed and erratic and they observe that there is a correlation with impaired localization of ParA. These results are solid, but they are only correlative in nature and they do not distinguish between cause versus effect (see above): do these parS/ParAB deficiencies give rise to the tipN division phenotype or could they be the result of a secondary effect? In support of the former possibility, the authors show that ParA can be pulled down along with TipN from crosslinked Caulobacter extracts, but many controls are missing from this experiment and the supporting FRET experiments are everything but convincing. Is the pull-down specific for ParA? Is MipZ or any random integral inner membrane protein pulled down as well? How about the PopZ polar organizer? PopZ is an ideal control since it has previously been shown to interact with ParB? With respect to the FRET experiments, subtraction of the SEM from the relative signal values essentially nullifies the FRET signals relative to those from the control strain. Only the spread is affected. I don`t know enough about FRET experiments to easily appreciate ho such values could indicate a positive and significant interaction between TipN and ParA relative to the control FRET pair. What are the p-values for these FRET data sets?

C) An example of the aforementioned overstatement is on the bottom of page 12: "We show that segregation of chromosomal parS/ParB depends on a regulatory mechanism that prevents the released ParA molecules from reforming a functional ParA-ATP nucleoprotein structure behind the translocating parS/ParB complex to avoid backward motions."
The authors have not shown i) that ParA is released, ii) that the TipN-dependent segregation mechanism prevents this release, and iii) that ParA forms and reforms a ParA-ATP nucleoprotein complex in Caulobacter cells. Also, what exactly is meant by "functional" here? Do the authors imply that force is being generated by ParA bound to DNA, and if so, how?

Also, on page 16: "However, our findings imply that these intrinsic, self perpetuating dynamics are inconsistent with chromosome segregation".
I am not aware that "self-perpetuating dynamics" has been shown for Caulobacter ParAB. Fig. 2F certainly does not make the point that these dynamics are self-perpetuating and that only 3 components (parS/ParAB) are sufficient for such dynamics.

A final example is on Page 2: "Our data provide a mechanistic framework for adapting a self-

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organizing oscillator to create motion suitable for chromosome segregation."
What kind of movement is "suitable" for segregation? Is it known whether Caulobacter parS/ParAB is in fact a self-organizing oscillator? As the results don`t provide much mechanistic insight, it is unclear how it allows for the formulation of a mechanistic framework.

D) ParAB/MipZ eventually do localize to the new pole in tipN cells. If ParA interacts with TipN in WT cells and polar TipN guides ParA to the new pole, what does ParA interact with at the pole in the tipN mutant and how does ParA get there when TipN is not around? It puzzles me that this subtle effect on ParA localization seen in the tipN mutant can result on such an overt effect on division since ParAB/MipZ is at the correct place in the tipN mutant. Can the authors comment on both of these issues?

E) Another faux-pas is that the authors claim these observations to be causal without providing hard evidence to support it.
For example, (last para of Intro) "We show here that TipN exerts its long-distance effect on cell division positioning by regulating ParA and parS/ParB segregation dynamics". This is an over-interpretation and not supported by the data. I have no objections to the statement that TipN somehow regulates parS/ParAB, but I disagree with the claim that the authors have shown that the division abnormality of the tipN strain is the result of this misregulation of parS/ParAB movement. In fact, the authors later show that although the timing of FtsZ-ring formation is delayed in the tipN strain versus WT, the timing of constriction is not altered. So then what is the basis for the division defect?

F) Also, it has not been shown that the erratic movement of the parS/ParAB complex does indeed result in the misplacement of FtsZ rings. How does this occur? Can it be shown that the altered position of the MipZ minimum is responsible of the aberrant assembly of FtsZ? Though MipZ is a prime candidate responsible for this effect, can the authors exclude that misregulation of another (known or unknown) regulator of FtsZ instead causes the division phenotype?

G) "First, the ParA-YFP cloud did not condense rapidly to the new pole (Figure 2B) and the protein accumulated at the old pole (Figure 2B, pink arrowhead). Second, unlike in WT cells, the ParA-YFP signal failed to accumulate at the new pole in tipN cells (Figure 2B)." This is important, but since TipN encodes a polarity factor could it simply be that the polarity is reversed in the TipN, i.e. that the stalk is at the new pole instead of the old one. If this were the case, then the localization of FtsZ would still occur at the correct location, relative to the old pole.

H) page 11, last para
The sequestration of ParA to the new poles that are generated by TipN overproduction is nice. Is PopZ at these sites as well and could the recruitment of ParA into these clusters be a consequence of PopZ being at these sites and interacting with ParB and perhaps ParA?

I) page 16:" tipN swarmer cells display a shorter G1 phase, thereby considerably reducing the extra period of growth during G1 and partially compensating for the cell length increase at birth."

Could this not simply be the result of a problem of tipN cells finishing septation/separation of the outer envelope layer(s) rather than the presence of a novel type of cell sensing mechanism? As a consequence the daughter cell program would be initiated earlier (before cell separation) and, as a result, replication would start earlier in the mutant daughter vs WT.

J) bottom of page 17. "Mutant analysis suggests that PopZ has some affinity for non-DNA-binding forms of ParA (Figure S6; Supplemental Information). This property, together with the polar attachment of parS/ParB, minimizes the tipN defects. Such synergistic function between PopZ and TipN is supported by the synthetic lethal phenotype of a double popZ tipN mutation (Ebersbach et al.,)."

So, is ParA bound by both TipN and PopZ? Which contributes more? Could the synthetic lethality underlie misregulation of another protein(s) instead, that plays a role in septation/separation? The popZ mutant that cannot fasten MipZ at the poles is not dead. Why should a tipN/popZ double mutant be lethal if the ultimate effect is on MipZ? MipZ is not essential either and minicells are formed?

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"Depletion of TipN in popZ cells caused a dramatic increase in cell division events producing cells lacking a partition complex (Figure 5D-E), suggesting that the synthetic lethal phenotype is due to a severe chromosome segregation defect."
Or it could be due to a role of TipN in promoting the late events of septation/separation.

What is the evidence for a severe segregation defect and why should the PopZ/TipN - cells die because of that? Are the authors suggesting that PopZ also plays an active role in origin segregation, not just capturing? Again the capturing function is apparently dispensable.

"Suppression of cell filamentation was correlated with a rescue of the defects in ParA localization and parS/ParB/MipZ-mCFP polar attachment (Figure 5G). This is not surprising as the cells are smaller. Is the number of ParAB complexes or their relative distances different in short vs filamentous TipN/PopZ- cells? If so, this would be a clear indication for a segregation defect, all other effects could also be accounted for by a septation/separation defect.

K) A control kymograph is missing- there is no kymograph with MipZ-CFP and ParA(WT)-YFP in a wild type cell to compare the mutants in Figure 4 to. Is the intention that the same figures shown in 2F,G showing the overexpression serve as controls for the kymograph of ParA mutants shown in Fig. 4? If this is the case, why do the mutants not cause oscillations of MipZ between the poles? All these mutants (Figure 4) were induced from the chromosomal Pxyl promoter by addition of 0.3% xylose for 1.5 hr, but Figure 2G shows that overexpression of WT ParA-YFP from Pxyl (on the chromosome) with this amount of xylose for 2 hours is sufficient to disrupt partitioning and make MipZ oscillate between the old and new poles. So the abnormal localization is not necessarily due to the mutations.

L) ParA is abundant in the cell (Fig S3) and binds over the whole nucleoid (Fig S5), but it is apparently not degraded (Fig S3D), so for it to condense at the new pole it must be very efficiently sequestered there by TipN. Does this suggest that each molecule of TipN is binding multiple ParA monomers? With other words what is the stochiometry of ParA-binding to TipN and are there enough TipN molecules to sequester the free ParA?

M) The level of filamentation of popZ mutants (tipN+) shown in Panel 5 D and 5 F is very different. Why?

Minor comments
p. 3
"ParA-ATP dimers bind to non-specific DNA cooperatively " I don't` know what non-specific DNA is. Do the authors want to say that ParA binds to DNA non-specifically and in a cooperative manner?

"Recently, some in vivo mechanistic insight".
How much is "some"?

p. 4 "What generates this difference is unclear."
For one, the size (molecular mass) difference of plasmid DNA versus the chromosome might cause the difference.

"Another key aspect of chromosome segregation that cannot be addressed with plasmid models is its necessary temporal and spatial coordination with other cell cycle events". Why can this not be addressed with plasmids?

Delete "yet it underpins the remarkable ability of bacteria to proliferate." There is no value in this sentence.

P5. "and before division delocalizes",
replace division with cell separation

Results:
P8. "We found that the aberrant movement of MipZ in tipN cells was caused by a DNA

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segregation defect".
Segregation is not defective. It still occurs, but it is altered or perturbed

P9: "was cleanly substituted".
What does "clean" mean in scientific terms?

Figure 2G is not referred to in the main text (pg 10)

Pg. 13 "by sequestering the released ParA at the new pole."
i.e. ParA-ADP?

Page 14: "The only expected difference is that D44A should be able to bind the DNA, unlike the other two mutants. This argues"
What argues?

Page 17:
"Mutant analysis suggests that PopZ has some affinity for non-DNA-binding forms of ParA (Figure S6; Supplemental Information).
What does "some" mean in scientific terms?

Materials and Methods:

Correct "French pressPress" pg 22 line 8

(YFP and YFP), pg 22 penultimate line and pg 23 line 8, should this be (CFP and YFP)?

Figure legends:

remove "from" from last line of pg 24

1st Revision - authors' response 30 July 2010

We thank the reviewers for their prompt and constructive reviews. We have addressed their comments with textual revision and additional experimentation, as described in our point-by-point response below.

Referee #1 (Remarks to the Author):

This is an interesting paper that makes a significant contribution to understanding the molecular basis of chromosome segregation in bacteria. Although the ParABS systems have been implicated in the segregation of both chromosomes and plasmids in a wide range of bacteria, we still don't understand in detail how sister replicated chromosomes are moved apart. The results described in the paper reinforce the emerging general view of the ParABS systems in which the ParB component recognises and binds to the partitioning sequence, parS, and ParA acts as a cytoskeletal factor that pulls on the ParB-parS complex. ParB regulates ParA and drives its dynamics by triggering ATP hydrolysis on ParA. The results suggest that the Caulobacter parABS system operates in a similar way to that previously described for Vibrio. However, the new paper goes much further by identifying TipN as a polar marker that provides the system with directionality, and dissecting the interactions responsible for TipN function.
Overall, the paper is well written, clear and convincing. I have only a few comments for consideration.
1. p. 8, 2nd paragraph. Introduction of the concept that the misplacement of the division site and aberrant movement of MipZ are due to a chromosome segregation defect emerges quite abruptly here, and the statement that aberrant movement... was caused by a DNA segregation defect seems too strong ahead of the data. How about something along the lines... "In some bacteria, abnormal

division site placement has been associated with defects in chromosome segregation. To test whether this was the case in the tipN mutant, movement of oriC was examined by...".

Response 1: Good suggestion. The text has been revised.

2. p. 10, 3rd paragraph, last 2 sentences; p. 11, first sentence, 2nd last sentence. I think the words "causing" and "resulting in" are too strong. The observed events are apparently associated, but it seems premature to assume that the connections are causal.

Response 2: We have revised the text to indicate correlation or association instead of causation.

3. p. 14. These are interesting experiments (expressing mutant ParA proteins fused to YFP in an otherwise wild type background). However, I'm surprised that the results are so clear cut, at least for the mutants that should dimerize (D44 and R195). I would have expected heterodimers to form with the wild type protein, which might have partial function and therefore perhaps intermediate localization?

Response 3: We agree but we donít think that we can exclude the possibility of heterodimerization with the WT protein for either mutant. The D44A mutant confers a dominant-negative effect on translocation of the parS/ParB partition complex (Fig. 4C), possibly through heterodimerization. Perhaps it does not form a cloud structure at an "intermediate" level because both subunits of the dimer have to be proficient for ATP hydrolysis to do so. Along the same lines, a R195E/WT heterodimer may not be able to bind DNA because DNA binding may require both subunits of the dimer to bind and the R195E cannot bind (Fig. S5B). Without DNA binding, we would not expect a cloud structure to form.

4. p. 17, lines 1-2. There is a long literature of cell size control of initiation of replication in E. coli and other bacteria.

Response 4: We meant to say that this mechanism what previously unknown in C. crescentus. We also note that it seems different in E coli since E. coli initiates DNA replication at variable cell length (Donachie Nature 1968). Nevertheless, we have deleted the words "previously unknown".

5. p. 18, lines 6 up. The comment "as often believed" seems misplaced, since it is again well known in various bacteria, and especially well established in E. coli, that Z ring formation is temporally separated from constriction.

Response 5: We understand how our comment can be misleading. We have deleted it.

Trivial corrections
p 7, line 4 up. "the" new pole.

Response 6: Thanks.

p. 9, 5 lines up. The sentence beginning "Second" seems to repeat the idea in the previous sentence?

Response 7: We have fixed this issue.

p. 12. Fig. 7 cited out of order.
Response 8: This is true and we hope that this one-time infringement to save figure space is OK.

p. 18, line 3. in "a" dramatic...
Response 9: Thank you

p. 18, lines 5-6, and 2 up. Three sig figs seems overly precise, given the limits of standard fluorescence microscopy instrumentation.
Response 10: True but our program allows us to make measurements at the sub-pixel resolution. In the original version, the error corresponded to the standard deviations, which now have been

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replaced by the standard error of the mean (SEM), in response to the second review.. We have adjusted the number of significant figures based on the obtained SEM values.

Referee #2 (Remarks to the Author):

Comparing the dynamics of parS/ParAB and the MipZ division inhibitor in Caulobacter WT and polarity mutant (ΔtipN) cells, Schofield et al report interesting and potentially important observations on the requirements of the movement of parS/ParAB/MipZ-complexes through the cell and the coordination of the movement with the cell cycle. Most of the experiments are well executed and the level of sophistication for the data acquisition and analysis is remarkable. Since little is known about the regulation of parS/ParAB and how this system moves chromosomal segments through cells, the topic is of broad interest, addressing an important and still mysterious question in fundamental bacterial cell biology.

The weaknesses are i) that the presentation and discussion of the data are for the most part phenomenological, ii) that the paper does not provide much new mechanistic depth (i.e. the mechanism of regulation of ParA-ADP by TipN and why this should cause a phenotype is not obvious) and iii) that there is a bias towards inserting unsubstantiated claims that are derived from circumstantial data and/or that are not adequately identified as speculation (see below).
Response 11: We respectfully disagree. Our work provides mechanistic insight into i) the regulation of chromosome segregation through the action of polarly localized factors on the parS/ParB/ParA system, and ii) cell cycle coordination by providing evidence that specific and distinct properties of the segregation process regulate not only cell division, but also cell growth. We have added experimentation to address the reviewer's concerns and have revised the manuscript to avoid undue speculation and to clarify when we do speculate.

Also, in general I found the model of how ParA-bound DNA is stimulated by ori-bound ParB to pull or generate force (p. 12) and ultimately facilitate or guide ori movement to be confusing and not very intuitive. The fact that the ΔtipN mutant is viable and that it has ParAB/MipZ localized to both poles, indicates that TipN plays a minor and dispensable role in the "extrinsic" regulation of ParA: chromosomes can segregate fine and ParAB/MipZ can efficiently find the poles when TipN is absent.

Response 12: We provide evidence (Figure 5) that at least two processes partially compensate for the loss of TipN function, masking the severity of the tipN mutation. The first one is due to a cell size-dependent sensing mechanism that regulates the timing of DNA replication and consequently the length of the growth cycle, and the other is mediated by PopZ. While viable alone, tipN and popZ deletions become synthetic lethal when combined. These two layers of compensatory mechanisms provide support to the notion that extrinsic regulation of the parS/ParB/ParA system is important to the cell.

The synthetic lethality of the popZ/tipN double mutant is interesting and definitely holds the key to understanding the basis for these phenotypes, but it is not clear from the presented data whether this synthetic lethality is due to a massive segregation defect (that is normally masked in WT and single mutant cells by the propose overlapping role of TipN and PopZ in segregation) or whether the lethality arises from an (unexplored and) synthetic division defect with TipN aiding, for example, in the late stages of cell septation/separation process and PopZ influencing the proper positioning of MipZ via ParB.

Response 13: We would not expect a synthetic division defect to increase the number of cells without chromosomal origins (Fig. 5D and E) while this increase is consistent with a segregation defect. We have already shown that TipN does not influence the timing of initiation of cell constriction (Figure 6A). To rule out the alternative proposed by the reviewer that TipN affects late stages of cell division, we have added two panels in Figure 6 (D and E) showing that TipN has no effect on the speed of cell constriction or on the timing of cell separation.

Along the lines of such murky cause-vs-effect-relationships scattered in this manuscript, the authors do not make a compelling case that the division abnormalities of the ΔtipN mutant are

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CAUSED by the delayed and discontinuous movement of the ParAB/MipZ complex, rather than merely being a manifestation of a secondary EFFECT of the ΔtipN phenotype.

Response 14: It has been shown that MipZ is an inhibitor of FtsZ assembly and that its translocation of MipZ (through association with ParB) to the new pole displaces FtsZ from that location, favoring formation of the FtsZ ring structure where the MipZ concentration is the lowest (Thanbichler et al, Cell 2006). Our observations in wild-type cells are consistent with this notion. Fig. 1B shows that MipZ arrival at the new pole is accompanied with the displacement of FtsZ from the new pole and with its migration to cell interior at a location that is in good agreement with the measured MipZ minimum (Fig. 1D, green arrow). Fig. 1C shows that in tipN cells, the erratic motion of MipZ/ParB complex is associated with correlated motions of FtsZ. As shown in the kymograph (purple arrow) and in the selected time-lapse images (purple arrow, time-points 42 and 45 min), the backwards movement of MipZ toward the cell interior (due to the erratic motion of the parS/ParB partition complex) shifts his minimum closest to the old pole (Fig. 1D, blue arrow); this shift is correlated with the FtsZ ring moving closer to the old pole. In the revised text, we have added the measured values for the position of MipZ minima (0.530±0.004 in WT (n=94) and 0.460±0.008 in tipN (n=64)) to demonstrate how well they agree with the measured values of FtsZ ring position (0.54±0.003 in WT (n=1433) and 0.45±0.004 in tipN (n=1766)). With the support of this quite remarkable agreement between MipZ minima and FtsZ ring location, we think that the simplest explanation is that the erratic motion of the MipZ-associated partition complex is responsible for the misplacement of the FtsZ cytokinetic ring.

To make it clear that this is an interpretation of the data, we have revised our original text as follows. "In wild-type cells (n=94), the minimum of the MipZ-YFP signal was slightly biased toward the new pole with a mean value of 0.530±0.004 (Figure 1D, green arrow), whereas in tipN cells (n = 63), this minimum was shifted towards the old pole with a mean value of 0.460±0.008 (Figure 1D, blue arrow) most likely because of the common backwards motions of MipZ following FtsZ displacement from the new pole (Figure 1C, see images at time points 42 and 45 min for examples). This likely contributes to the difference in relative FtsZ-YFP ring positioning along the cell length between wild-type and tipN cells."

Major comments:
A) Generally, the impressive quantitative analysis of MipZ/ParAB/FtsZ should not only be done with WT and ΔtipN cells, but also with the complemented mutant to show that these phenotypes are solely due to the absence of the TipN protein. These control experiments are missing.

Response 15: The complementation experiments are now provided as Figures S2D and S3G.

B) The authors show that ΔtipN cells divide aberrantly and that the mutant cells are altered in the proper placement of FtsZ. They also find that the movement of parS/ParB is delayed and erratic and they observe that there is a correlation with impaired localization of ParA. These results are solid, but they are only correlative in nature and they do not distinguish between cause versus effect (see above): do these parS/ParAB deficiencies give rise to the ΔtipN division phenotype or could they be the result of a secondary effect? In support of the former possibility, the authors show that ParA can be pulled down along with TipN from crosslinked Caulobacter extracts, but many controls are missing from this experiment and the supporting FRET experiments are everything but convincing. Is the pull-down specific for ParA? Is MipZ or any random integral inner membrane protein pulled down as well? How about the PopZ polar organizer? PopZ is an ideal control since it has previously been shown to interact with ParB? With respect to the FRET experiments, subtraction of the SEM from the relative signal values essentially nullifies the FRET signals relative to those from the control strain. Only the spread is affected. I don`t know enough about FRET experiments to easily appreciate ho such values could indicate a positive and significant interaction between TipN and ParA relative to the control FRET pair. What are the p-values for these FRET data sets?

Response 16: This is our mistake. For the FRET results, we were showing the mean ± the standard deviation (SD) of our distributions instead of the mean ± the standard error of the mean (SEM), as rightfully assumed by the reviewer. We have corrected this mistake. The nFRET/YFP value for TipN-CFP and ParA-YFP (Fig. 3B) is 0.21±0.03 versus 0.01±0.005 for our negative control. The nFRET/YFP value for CFP-ParB and ParAD44A-YFP is 0.31± 0.03 versus -0.008±0.002 for the

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negative control. As the reviewer will now appreciate, our FRET data are statistically significant with nFRET/YFP values 10 times over the SEM and $p < 0.0001$ (K-S test). We have replaced the SD values by the SEM values throughout the text, and have added the p value for the FRET experiments.

Regarding the specificity of the ParA pull-down with TipN-FLAG (Fig. 3A), we think that this is best shown in Fig. 4G with the G16V and D44ER195E ParA mutants. Both localize at the poles, but our analysis in the tipN mutant (Fig. 4E-F) suggests that TipN interacts preferentially with G16V. In accord, TipN-FLAG is better able to pull down the G16V mutant over the D44ER195E mutant (Figure 4G). If the pull-down was not specific, we would expect the two mutant proteins to be pulled down equally well. We feel that this is more stringent evidence of specificity than showing that a random protein does not get pulled-down, which would be a negative result. We also show that the ParA pull-down is dependent on the presence of TipN-FLAG (Figure 3A), indicating that ParA is not simply sticking to the beads.

In summary, we show that 1) ParA does not accumulate at the new pole when new-pole localized TipN is absent (Fig. 2A-C); 2) TipN pulls-down ParA; 3) TipN is sufficient to recruit ParA at (Fig. 3C); and 4) TipN and ParA interact at new pole based on in vivo FRET experiments.

C) An example of the aforementioned overstatement is on the bottom of page 12: "We show that segregation of chromosomal parS/ParB depends on a regulatory mechanism that prevents the released ParA molecules from reforming a functional ParA-ATP nucleoprotein structure behind the translocating parS/ParB complex to avoid backward motions."
The authors have not shown i) that ParA is released, ii) that the TipN-dependent segregation mechanism prevents this release, and iii) that ParA forms and reforms a ParA-ATP nucleoprotein complex in Caulobacter cells. Also, what exactly is meant by "functional" here? Do the authors imply that force is being generated by ParA bound to DNA, and if so, how?

Response 17: To clarify, we do not claim that TipN-dependent segregation mechanism prevents ParA release. We propose that TipN binds the ParA molecules that have been released from the nucleoprotein structure, thereby preventing reformation of such structure behind the translocating partition complex. But otherwise we agree with the reviewer that we should have used the term "suggest" instead of "show" since it is an interpretation based on cumulative knowledge derived from our data and previous studies. We have revised the text accordingly.

We deleted the term "functional" as we did not mean to imply that there were non-functional ParA-ATP nucleoprotein structures.

Also, on page 16: "However, our findings imply that these intrinsic, self perpetuating dynamics are inconsistent with chromosome segregation".
I am not aware that "self-perpetuating dynamics" has been shown for Caulobacter ParAB. Fig. 2F certainly does not make the point that these dynamics are self-perpetuating and that only 3 components (parS/ParAB) are sufficient for such dynamics.

Response 18: Our sentence was in the following context. "Plasmid-encoded parS/ParB/ParA systems are thought to be self-organizing and to act without host factors (Gerdes et al., 2010). Without extrinsic interference, the dynamics of these systems lead to back-and-forth motion of plasmids over short times and hence equidistribution when time-averaged. These dynamics are analogous to the oscillatory behavior of the related, self-organizing Min system in E. coli. However, our findings imply that these intrinsic, self-perpetuating dynamics are inconsistent with chromosome segregation, where duplicated origins are partitioned to opposite poles in one single, rapid event during the cell cycle. We propose that…".

This goes back to what we wrote in the introduction when we compare plasmid pB171 and chromosome I of Vibrio cholera and ask how perpetual back-and-forth plasmid movement and the single DNA translocation event of chromosome can be achieved using the same Par system.

Showing that the C. crescentus parS/ParB/ParA system only needs these three components to achieve self-perpetuating dynamics would be a huge undertaking. This has not even been done for plasmid-encoded parS/ParB/ParA systems even though they are widely believed to be self-

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organizing (see Gerdes et al, Cell 2010 for a review). We propose that TipN and PopZ functions prevent the back-and-forth behavior that is observed for plasmids that lack extrinsic regulation. This model predicts that saturation of TipN binding sites by ParA overproduction would result in oscillation of parS/ParB complexes that are unable to attach to PopZ. This is what we observed for the unattached parS/ParB complex in ParA-overproducing cells (Fig. 2G). We have revised the text (p.18) as follows: "Since large overproduction of ParA causes cell filamentation (Mohl and Gober, 1997), the elongated ParA-YFP-overproducing cells often displayed more than two partition complexes, with the unattached complexes óbut not the polarly localized, PopZ-attached complexesó moving back and forth synchronously with ParA oscillations (Figure 2G). Overproduction of ParA may presumably saturate TipN binding sites, resulting in oscillatory behavior of unattached parS/ParB, as observed for plasmids."

A final example is on Page 2: "Our data provide a mechanistic framework for adapting a self-organizing oscillator to create motion suitable for chromosome segregation."
What kind of movement is "suitable" for segregation? Is it known whether Caulobacter parS/ParAB is in fact a self-organizing oscillator? As the results don`t provide much mechanistic insight, it is unclear how it allows for the formulation of a mechanistic framework.

Response 19: See response 18 above.

D) ParAB/MipZ eventually do localize to the new pole in ΔtipN cells. If ParA interacts with TipN in WT cells and polar TipN guides ParA to the new pole, what does ParA interact with at the pole in the ΔtipN mutant and how does ParA get there when TipN is not around? It puzzles me that this subtle effect on ParA localization seen in the ΔtipN mutant can result on such an overt effect on division since ParAB/MipZ is at the correct place in the tipN mutant. Can the authors comment on both of these issues?

Response 20: We present evidence that PopZ works together with TipN (Fig. S6 and Fig. 5D-G). The effect on ParA localization in the tipN mutant is not subtle but significant (please compare Fig. 2A with 2B). The data suggest that the misplacement in division along the cell body is caused by the common backwards motions of MipZ-associated parS/ParB complex (see Fig. 1C, kymograph and selected images); please see response 14 above.

E*) Another faux-pas is that the authors claim these observations to be causal without providing hard evidence to support it.*
For example, (last para of Intro) "We show here that TipN exerts its long-distance effect on cell division positioning by regulating ParA and parS/ParB segregation dynamics". This is an over-interpretation and not supported by the data. I have no objections to the statement that TipN somehow regulates parS/ParAB, but I disagree with the claim that the authors have shown that the division abnormality of the ΔtipN strain is the result of this misregulation of parS/ParAB movement. In fact, the authors later show that although the timing of FtsZ-ring formation is delayed in the ΔtipN strain versus WT, the timing of constriction is not altered. So then what is the basis for the division defect?

Response 21: Please see response 14 above. As mentioned by the reviewer below, MipZ, the FtsZ ring assembly inhibitor, is the prime candidate and as demonstrated before (Thanbichler et al 2006), MipZ affects the positioning of FtsZ (Fig. 1B and C); their correlated movement is quite remarkable for being coincidental. Furthermore, as mentioned in response 14, there is a very good agreement between the position of MipZ minima and that of FtsZ ring location, supporting a causal effect rather than a coincidence. The difference between the distributions for MipZ minimum position in wild-type and tipN backgrounds is significant (K-S test, $p<0.00001$). Regarding the basis of the cell division defect, we indicate that TipN affects the positioning of cell constriction along the cell body and not its timing. This is actually an important point. We have revised the text to ensure that this is clear.

F) Also, it has not been shown that the erratic movement of the parS/ParAB complex does indeed result in the misplacement of FtsZ rings. How does this occur? Can it be shown that the altered position of the MipZ minimum is responsible of the aberrant assembly of FtsZ? Though MipZ is a prime candidate responsible for this effect, can the authors exclude that misregulation of another

(known or unknown) regulator of FtsZ instead causes the division phenotype?

Response 22: Please see responses 14 and 21 above.

G) "First, the ParA-YFP cloud did not condense rapidly to the new pole (Figure 2B) and the protein accumulated at the old pole (Figure 2B, pink arrowhead). Second, unlike in WT cells, the ParA-YFP signal failed to accumulate at the new pole in ΔtipN cells (Figure 2B)." This is important, but since TipN encodes a polarity factor could it simply be that the polarity is reversed in the TipN, i.e. that the stalk is at the new pole instead of the old one. If this were the case, then the localization of FtsZ would still occur at the correct location, relative to the old pole.

Response 23: It has been shown that the position of stalk and other old-pole markers is unaltered in tipN cells (Lam et al, Cell 2006; Huitema et al, Cell 2006). It has also shown that the differential localization of the cell fate determinant CtrA is similarly unaltered (Lam et al, Cell 2006), indicating that in that respect, the "identity" of the daughter cells is unchanged in tipN cells. We have revised the introduction to include this valuable information.

H) page 11, last para
The sequestration of ParA to the new poles that are generated by TipN overproduction is nice. Is PopZ at these sites as well and could the recruitment of ParA into these clusters be a consequence of PopZ being at these sites and interacting with ParB and perhaps ParA?

Response 24: We show in Fig. 5G that overproduction of TipN also recruits ParA in a popZ background. The label of Fig. 5G had a mistake (" " was missing in front of "popZ"), which may have confused the reviewer. This has been fixed.

I) page 16:" ΔtipN swarmer cells display a shorter G1 phase, thereby considerably reducing the extra period of growth during G1 and partially compensating for the cell length increase at birth."

Could this not simply be the result of a problem of ΔtipN cells finishing septation/separation of the outer envelope layer(s) rather than the presence of a novel type of cell sensing mechanism? As a consequence the daughter cell program would be initiated earlier (before cell separation) and, as a result, replication would start earlier in the mutant daughter vs WT.

Response 25: As mentioned in response 13 above, we have included new data (Figure 6D and E) showing that the timing and rate of cell constriction and cell separation are normal in tipN cells.

J) bottom of page 17. "Mutant analysis suggests that PopZ has some affinity for non-DNA-binding forms of ParA (Figure S6; Supplemental Information). This property, together with the polar attachment of parS/ParB, minimizes the ΔtipN defects. Such synergistic function between PopZ and TipN is supported by the synthetic lethal phenotype of a double ΔpopZ ΔtipN mutation (Ebersbach et al.,)."

So, is ParA bound by both TipN and PopZ? Which contributes more? Could the synthetic lethality underlie misregulation of another protein(s) instead, that plays a role in septation/separation? The ΔpopZ mutant that cannot fasten MipZ at the poles is not dead. Why should a tipN/popZ double mutant be lethal if the ultimate effect is on MipZ? MipZ is not essential either and minicells are formed?

Response 26: Please see response 13 and Figure 6D and E for additional data.
MipZ is essential for viability (Thanbichler and Shapiro Cell 2006).

"Depletion of TipN in ΔpopZ cells caused a dramatic increase in cell division events producing cells lacking a partition complex (Figure 5D-E), suggesting that the synthetic lethal phenotype is due to a severe chromosome segregation defect."
Or it could be due to a role of TipN in promoting the late events of septation/separation.
Response 27: Please see response 13 and Figure 6D and E for additional data.

What is the evidence for a severe segregation defect and why should the PopZ/TipN - cells die because of that? Are the authors suggesting that PopZ also plays an active role in origin segregation, not just capturing? Again the capturing function is apparently dispensable.

Response 28: DNA segregation is essential for viability (e.g., parA and parB are essential genes). We think that these questions are addressed in the text p.16-18 under "Existence of partially compensatory mechanisms".

"Suppression of cell filamentation was correlated with a rescue of the defects in ParA localization and parS/ParB/MipZ-mCFP polar attachment (Figure 5G). This is not surprising as the cells are smaller. Is the number of ParAB complexes or their relative distances different in short vs filamentous TipN/PopZ- cells? If so, this would be a clear indication for a segregation defect, all other effects could also be accounted for by a septation/separation defect.

Response 29: We are not sure that we understood what the reviewer meant here but the new data (Figure 6D and E in response 13 above) rules out a septation/separation defect; please see response 13.

K) A control kymograph is missing- there is no kymograph with MipZ-CFP and ParA(WT)-YFP in a wild type cell to compare the mutants in Figure 4 to. Is the intention that the same figures shown in 2F,G showing the overexpression serve as controls for the kymograph of ParA mutants shown in Fig. 4? If this is the case, why do the mutants not cause oscillations of MipZ between the poles?

Response 30: In Fig. 2F, the wild-type protein is indeed under similar level of expression, but the mutants are all defective and thus we should not expect any oscillations, which require ParA activity. The mutants should be compared among themselves. We have revised the text as follows: "All three ParA-YFP mutants, which were produced as stable, full-length proteins (Figure S3E-F), exhibited distinct localization pattern during the cell cycle (Figure 4A-D).". Please see also response 31 below.

All these mutants (Figure 4) were induced from the chromosomal Pxyl promoter by addition of 0.3% xylose for 1.5 hr, but Figure 2G shows that overexpression of WT ParA-YFP from Pxyl (on the chromosome) with this amount of xylose for 2 hours is sufficient to disrupt partitioning and make MipZ oscillate between the old and new poles. So the abnormal localization is not necessarily due to the mutations.

Response 31: All the mutants were expressed under the same conditions; yet they exhibited a different localization pattern. We therefore think that it is fair to assume that the difference is caused by the mutation. Comparison among the mutants indicate that D44A binds to ParB strongly and while the others do not, and that only G16V has a new-pole localization that TipN-dependent.

L) ParA is abundant in the cell (Fig S3) and binds over the whole nucleoid (Fig S5), but it is apparently not degraded (Fig S3D), so for it to condense at the new pole it must be very efficiently sequestered there by TipN. Does this suggest that each molecule of TipN is binding multiple ParA monomers? With other words what is the stochiometry of ParA-binding to TipN and are there enough TipN molecules to sequester the free ParA?

Response 32: Fig. S3 shows Western blots of ParA-YFP from which the abundance cannot be deduced. Fig. S5 shows nucleoid-localization of C. crescentus ParA artificially expressed from a high-copy-number plasmid and a strong heterologous promoter in E. coli. This shows that ParA binds to DNA non-specifically, but it does not inform us about the normal abundance of ParA inside C. crescentus. With the available data, we do not think that there is reason to believe that there is more ParA than TipN (or vice versa) inside cells. We hope that these clarifications, together with the multiple lines of evidence of a ParA/TipN interaction (see response 16), are satisfying.

M) The level of filamentation of popZ mutants (tipN+) shown in Panel 5 D and 5 F is very different. Why?

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Response 33: Good point. This is due to a difference in media, which we should have pointed out. For Fig. 5D-E, cells were grown in minimal M2G medium where filamentation is less severe whereas cells were grown in PYE for Fig. 5F where cell filamentation is more severe. We use M2G for fluorescence microscopy experiments (as in Fig. 5D-E) to alleviate the background fluorescence observed with rich PYE medium. Fig. 5F shows that TipN overproduction suppresses the cell filamentation phenotype of popZ cells even in PYE medium in which this phenotype is particularly dramatic.
We now provide a supplementary figure (Figure S6E) showing the cell filamentation phenotype of the popZ strain in these two media. The text has been revised accordingly.

Minor comments
p. 3
"ParA-ATP dimers bind to non-specific DNA cooperatively " I don't` know what non-specific DNA is. Do the authors want to say that ParA binds to DNA non-specifically and in a cooperative manner?
Response 34: Yes. Thanks.

"Recently, some in vivo mechanistic insight".
How much is "some"?
Response 35: We have deleted "some".

p. 4 "What generates this difference is unclear."
For one, the size (molecular mass) difference of plasmid DNA versus the chromosome might cause the difference.
Response 36: Oscillations of chromosomal regions can be observed under some conditions as shown in Fig. 2G; so the size difference does not seem to be the main contributing difference. Regardless, it is safe to say that the origin of the difference is not clear; we have replaced "unclear" by "not clear".

"Another key aspect of chromosome segregation that cannot be addressed with plasmid models is its necessary temporal and spatial coordination with other cell cycle events". Why can this not be addressed with plasmids?
Response 37: To our knowledge, replication and segregation of plasmids do not influence cell cycle events of the host. We have changed the sentence into: "Another key aspect of chromosome segregation that is difficult to address with plasmid models is its necessary temporal and spatial coordination with other cell cycle events."

Delete "yet it underpins the remarkable ability of bacteria to proliferate." There is no value in this sentence.
Response 38: Done

P5. "and before division delocalizes", replace division with cell separation
Response 39: Good point. Fixed.

Results:
P8. "We found that the aberrant movement of MipZ in ΔtipN cells was caused by a DNA segregation defect".
Segregation is not defective. It still occurs, but it is altered or perturbed
Response: OK

P9: "was cleanly substituted".
What does "clean" mean in scientific terms?
Response: We meant to say that we did not leave any plasmid sequence or truncated parA sequence behind. We have deleted the term "cleanly".

Figure 2G is not referred to in the main text (pg 10).
Response: It is referred to in p. 17.

Pg. 13 "by sequestering the released ParA at the new pole."
i.e. ParA-ADP?

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Response: It could be ParA-ADP, ParA without nucleotide or ParA-ATP monomer before dimerization. It is an open question, but importantly it is non-DNA binding form.

Page 14: "The only expected difference is that D44A should be able to bind the DNA, unlike the other two mutants. This argues"
What argues?
Response: "This argues" has been replaced by "This result suggests".

Page 17:
"Mutant analysis suggests that PopZ has some affinity for non-DNA-binding forms of ParA (Figure S6; Supplemental Information).
What does "some" mean in scientific terms?
Response: These types of experiment do not inform us whether the affinity is strong or weak. So we used the term "some" to suggest this uncertainty. We have replaced "some" by "an".
Materials and Methods:

Correct "French pressPress" pg 22 line 8
Response: Thanks.

(YFP and YFP), pg 22 penultimate line and pg 23 line 8, should this be (CFP and YFP)?
Response: Thanks.

Figure legends:
remove "from" from last line of pg 24
Response: Fixed.

Acceptance letter 04 August 2010

Thank you for submitting your revised manuscript. Referee 2 has now looked at it once again, and I am happy to inform you that there are no further objections towards its publication in The EMBO Journal. You shall receive a formal letter of acceptance shortly.

With best regards,
Editor

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