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Integration of cell wall synthesis and chromosome segregation during cell division in Caulobacter

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January 2, 2023

Re: JCB manuscript #202211026

Dr. Erin Goley
Johns Hopkins University
725 N Wolfe St 520 WBSB
Baltimore, MD 21205

Dear Erin,

Thank you very much for submitting your manuscript entitled "Integration of cell wall synthesis activation and chromosome segregation during cell division in Caulobacter" to JCB. We have now heard back from three reviewers whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that Reviewers #1 & 2 are quite enthusiastic and feel the work provides an important advance in our understanding of the processes that regulate bacterial cell division. Reviewer #3 is supportive of the study but feels that the mechanism by which FzlA activates FtsWI remains unclear and is likely to be indirect. While we agree with this Reviewer that additional insight into the molecular mechanisms of FzlA function would be very interesting and would enhance the impact of this work, we also do not feel that it is necessary for this study. However, please revise the text to clearly acknowledge that the mechanism may be indirect and discuss limitations. There are several experimental requests which are essential for a revision - Reviewer #1 asks to experimentally confirm that Caulobacter FtsW motions are indeed bimodal rather than assuming this is the case because E. coli FtsW is known to be bimodal. Reviewer #3 asks for additional localization assays in W**I* expressing cells as well as following FtsK or FtsW depletions. Other comments from all reviewers ask for more details regarding quantifications as well as clarifications and further explanations of results and conclusions, all of these should be fully addressed.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

Thank you and we look forward to receiving your revised manuscript together with a point-by-point response to the critiques. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Best regards,
Rebecca

Rebecca Heald, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The paper Integration of cell wall synthesis activation and chromosome segregation during cell division in Caulobacter by Mahone et al. is an important work, which, among many insights, further elucidates the role of FzlA in cell division, and via its association with FtsK provides possible a link between chromosome replication and cell division. I love the "move speed" vs. time plots as measures of processivity. This work is fantastic and should be published, but a few points must be remedied (or verified) before publication.

1. The major point that needs to be addressed is their initial assumption that the motions of Caulobacter FtsW are bimodal, like E.coli, with a fast and a slow state. This has been soundly demonstrated to be the case in E. coli in the Yang 2021 paper where: 1) the wildtype FtsW data was far more clearly bimodal and subsequently backed up by B) the fast population matching the FtsZ velocity, C) chemical perturbations eliminating the slow population, and corresponding shift between these two populations with mutants.

Here, however, it is assumed at the start of the paper Caulobacter FtsW is bimodal with not a single experiment (as done in Yang) to validate the fundamental assumption throughout the paper. The only evidence to this point is the statement that E. coli (which Yang showed is bimodal) is "the phylogenetically closest relative in which PG synthase dynamics have been measured. Aside from this statement, there is no further proof of bimodality nor any experiments to test if this is the case. Their evolutionary argument is simply suggestive, not definitive: while E. coli and Caulobacter are quite evolutionarily distant, and numerous and substantial differences have been observed in many of their biological processes, many of which use different proteins. Given that other bacteria do not show bimodality in their FtsW velocities, without tests to verify bimodality, it cannot be assumed whether Caulobacter FtsW motion is bimodal.

Thus, if the authors wish to use any bimodal "fast vs. slow" population arguments in this paper, it is their duty to conclusively demonstrate to the reader that these distributions are indeed bimodal, with distinguishable fast and slow populations. As the wildtype FtsW data in no way appears bimodal in any of the graphs, bimodality should be verified by another means. The easiest and best way might be to conduct some subsets of the validating experiments done in the Yang paper on this system, such as the chemical inhibition or the shift change in populations in different media.

The underlying rationale for this request is that A) None of the WT distributions of FtsW velocity in this paper look anything like a bimodal distribution (fig 2B, 4A, 4C, 5Ai, etc.), nor is there any aspect of them that would suggest bimodality. Rather they look like a single skewed distribution, just as seen in almost every other measurement of velocity in biology, including divisome proteins in other bacteria. Thus, demonstrating the bimodality validation would make it clear if the rightward shift seen in Fig 2B is indeed a change in the proportion of fast vs. slow molecules or if there is simply a net shift in the velocity of the enzymes to a slightly higher speed.

2. In light of the curve fits used to classify the data (if that was the case), It would also help if there were some statistical reasons that the authors used two curves to fit this data. Normally with any distribution, there are tests that attempt to fit several different distributions (1, 2, 3...) to determine the best fit to the data (such as Bayesian or likelihood-based methods). Could this be done, or if it was already, can this be shown in the supplemental data?

3. There is no clear explanation in the text as to what was used as the cutoff (or classifications) used to assign particles in Figures 2-4. into the fast and slow states - even though fractional percentages are mentioned in the text, included in table S1, and used to make arguments central to this paper. Currently, it is hard to know how these assignments were made and, importantly, how well these classifications fit the data. The only two places where a cutoff or classification is mentioned are 1) the statement in the methods "The cumulative probability density function of the directional moving speeds was further calculated and fit to a two-population model(of log-normal distribution) as described previously (Yang et al., 2021)", and 2) a cutoff of 20nm/sec on line 171. Thus, the authors should more explicitly state how these proportions were determined and what certainty underlies these classifications. Also, if CDFs were done for this filling, those graphs should be shown, along with a measure of the fit (such as residuals). If the fits from figure 5 and S4 were used for the classification of trajectories in Figs 2-4, this should also be stated.

4. In the Yang paper, the peaks of the fast and slow distributions were nearly identical across all of the experiments, allowing the reader to see the two populations and the shifts between them in a way where each experiment could be compared. However, in this paper, the peaks and widths of the fast and slow distributions that are fit in figure 5 and S4 are, in many cases, extremely different from each other. This begs the questions: 1) why are these fitted distributions so different in their maxima (peaks) and range (high and low) across these experiments? 2) More importantly, how can one conclude both within and between these multiple experiments when these peaks are very different between experiments, thereby affecting the subsequent curve fitting used to classify the peaks in each experiment? Thus, the authors should explain 1) why there is so much variation in the distributions used to fit the fast and slow enzymes in these experiments, as compared to the nearly invariant peaks of FtsW velocity they saw in E. coli, and 2) explain and why these fits and classifications from these very different distributions, can be used to classify the proportions of fast vs. slow molecules in a way so they can be compared between experiments.

5. One line 171, They state a cutoff for the analysis and interpretation of some of the data "For this analysis, we excluded molecules moving faster than 20 nm/s as they are unlikely to be actively synthesizing PG".

a) It should be made clear what data this 20nm cutoff applies to. Is it only Fig 2B? Or was this only used for the "move time vs. speed graphs"?

b) As this cutoff is the first place any classification metric is defined in the text, and there is no clear definition stated of how the "fast vs. slow" proportions were measured in fig 2-4, the reader might be confused (as I was) that the 20nm cutoff was used for not just the "move time vs. velocity" graphs, but also for all of the other data, What this cutoff applies to should be clarified to prevent any confusion.

c) Why they chose 20nm/sec as a cutoff is unclear, as it currently appears arbitrarily defined: Looking at all of the data in this paper, it is hard to see why 20nm/sec was chosen, as 1) there is no clear bimodality to hint where that cutoff should be, and 2), the 20nm/sec cutoff would cut through (nearly) through the mean (and often the peak) of all of the WT distributions (fig 2B and 5Ai), almost separating the data in half. Thus, the authors should better justify this cutoff, as it is confusing when one examines all the WT velocity data.

6. The title claims a link from division to chromosome segregation. However, it's clear from the discussion that this is a model, not a definitive conclusion. While many bacterial papers use the model as the title, they might think about rephrasing it, as this is a bit speculative.

7. lines 153-154 state they see switches in the directions of molecules. It is unclear if those "Switches" in direction are occurring only on one side of the cell (next to the coverslip, as in TIRF) or if these are arising from molecules moving around the cell. I presumed these comments related to motion only on one side of the cell (from TIRF), but this should be clarified to the reader.

8. It would be great if the fraction of FtsW tracks that switched directions were calculated, as it might be insightful data. (Not necessary to include but might be useful to the field in the future).

9. There are many points in the text where they compare the means of similarities of different distributions. However, these comparisons lack any significance metric, making it hard to understand what is significant and if the different N values of each experiment affect the significance (as the number of samples defines how well the distribution was sampled). Could the authors provide some measure of the significance of the difference between these distributions, so the reader can evaluate their certainty?

10. I laud their use of superplots. However, I found it hard to know if all the statistical comparisons shown in ALL the figures were analyzed via the superplot method or if superplots were sued for some statistics and others used analysis of pooled data points. Could this be briefly clarified in the legend, just as we would do when we note what our error bars/box plots indicate?

11. Putting the significance (*) into all panels in Fig 1 B+D and Fig 2G (and p-values in the legend) would be helpful to the reader. For example, it is very hard to understand why the difference in 1B is significant but 1C is not.

12. Lines 160-162 - they state the interesting observation that single FtsW molecules change their direction of movement or transition between states of motion and immobility. However, it is unclear if they are referring to the "fast-moving" or "slow-moving" molecules. Could this be clarified?

13. Many of their arguments in Figures 1-5 rely on the relative proportions of different motions, and these proportions are central to many of their arguments. A few modifications would make this data, and thus their conclusions, more accessible.

a) They make many comparisons between these proportions in their text with the percentages in brackets. As these comparisons are central to their conclusions, It would be far better to display these fractional comparisons in a table in the main text. What might be better is a small figure next to each figure, which would help the reader grasp the significance of these proportions while also looking at the three other types of data in each experiment.

b) Importantly, as noted below, they should report each graph's percentage of slow, stationary, and fast molecules. Currently, it appears they are excluding one or the other of the inactive states in every measure of fractional activity they use here.

c) This work states that the 1) slow-moving molecules are active and that the 2) stationary AND the 3) fast-moving molecules are inactive. However, it does not appear the two inactive states are included in their active/inactive comparisons. Currently, it appears from the text and the table they are comparing the proportions of slow-moving molecules to the stationary ones, neglecting to include the fast-moving molecules in the inactive tally. Conversely, in figure 3B, it appears the fast-moving population is considered the inactive form, with no inclusion of the stationary molecules. To be consistent with their arguments, these relative active/inactive comparisons should include both the stationary and fast molecules as the "inactive" molecules.

d) Likewise, the fraction of fast-moving molecules is lacking in the table and most experiments. These should be included.

14. In figure 5, it is noted a computational approach is used, but no more details are given. Can the method be named or briefly described in the text or legend?

15. Lines 197-199 state- FtsW** in the ΔfzlA background appeared to have processive movement similar to FtsW** in the presence of FzlA, suggesting that FzlA does not affect the processivity of PG synthases, but rather the fraction of active PG synthases (Fig. 2 C) "while the processivity argument is clear, the reasoning behind the statement regarding the "fraction of active PG synthases" is hard to grasp as there is no plotting of the differences in the "active vs. inactive" enzymes. Even if this data can be inferred from Table S1, it would be way clearer to the reader if this was shown in the main text as a plot or a table.

Likewise, Plots (or tables) of the fractions in each class should be shown for 203-206 and 216-218.

16. It is fascinating that both FzlA and FtsN are activators and that A) FzlA moves with FtsW and B) binds to FtsZ filaments. A few recent findings about this comparison/analogy are missing from their discussion but might be insightful if they wish to include them: 1) The Loose lab showed that FtsN molecules are immobile, binding to treadmilling filaments. This appears to be a substantial difference from FzlA, where at least a subset of the FzlA moves with the filaments. Likewise, the Löwe group showed the acidic FtsN could dimerize 2 FtsA protofilaments, giving a putative mechanism for activation. These differences might be worth mentioning in the discussion if the authors wish.

But I wonder if any insights regarding activation or "stationary vs. moving" in regard to FzlA and FtsN be gained from any structural / domain information within FzlA and FtsN?

17. The effective resolution of the microscope (with instrument noise) that was used for single molecule localizations should be reported in the methods.

18. Lines 239-245 state: "We next assessed the stationary FtsW population in our depletion strain with and without FzlA. Surprisingly, depletion of FzlA in a WT background did not change the proportion of moving FtsW molecules (Table S1, ~50% in both deplete and induced), unlike in the ΔfzlA background (Table S1, 25%). These results indicate that the increase in stationary FtsW** molecules observed in the ΔfzlA background is likely due to binding the PG synthases to a stationary target rather than an inability to move dynamically about the Z-ring in the absence of FzlA".

a) The logic underlying this conclusion is unclear, most especially why this conclusion arises from differences between a depletion vs. a knockout. Can it be explained more overtly?
b) "Moving FtsW molecules" should be clarified if this is "all moving molecules," "slow-moving molecules," or "fast-moving molecules."

19. Likewise, on line 262. Likewise, they say the speed increases, but this appears to be referring to all moving molecules. Please clarify if this is the proportion of slow-moving molecules or the proportion of all moving molecules.

20. Similar to the above point, the text on lines 246-249 appears to be making a logical leap and should be better explained. To

me, it uses observations of stationary and slow-moving molecules to make a claim about how fast molecules transition into the slow-moving state. This is a confusing conclusion as there is no measure of the number of fast-moving molecules here. Can the number of fast-moving molecules be measured? Or at least this logic be spelled out more clearly?

Lines 246-249 - "The amount of time FtsW molecules remain stationary decreased when FzlA was depleted in a WT background (Table S1, Fig. 3 C, 8.8 {plus minus} 0.7 s during FzlA depletion, N = 107 vs. 10.9 {plus minus} 0.6 s at WT FzlA levels, N = 244). These tracking data are consistent with FzlA-mediated signaling acting to convert fast-moving, inactive molecules of FtsW into an activated, slow-moving state. "

21. Regarding the conclusions from the comparison of molecules between the FzlA depletion and the knockout, the first thing that comes to mind here is that there may be some other genetic difference between the strains, i.e., a possible adaptative mutation within the knockout strain. Have both strains been sequenced to see if there are any other mutations?

22. Many of the graphs have "speed" on one axis. Do they mean to use speed as the measure (the average frame-to-frame displacement) or the velocity (the sustained movement in one direction, as determined by MSD vs. t?) This should be clarified or the axes corrected.

Reviewer #2 (Comments to the Authors (Required)):

This manuscript describes a novel pathway that allows the coordination of chromosome segregation and septum synthesis in Caulobacter crescentus, a model alpha proteobacterium. The authors elegantly show that in Caulobacter, the FtsZ-interacting protein FzlA, which is specific to alpha proteobacteria, fulfils a key role in mediating the activation of the essential peptidoglycan synthesising proteins FtsW and FtsI. Using a combination of genetics and single molecule tracking they show that FzlA influences the speed of FtsW molecules and promotes the slow movement of FtsW which has been shown to be associated with active PG synthesis. Using in vivo pull-down and two-hybrid interaction studies they further identify a novel interaction partner of FzlA, the essential DNA translocase FtsK. I find this part of the work particular exciting. The authors elegantly show show that FzlA plays a crucial role in coordinating the licencing of division septum formation with the completion of DNA segregation and that misregulation of this step leads to DNA damage with lethal consequences.

This work is exciting and uncovers a new layer of regulation of cell division, demonstrating the diverse strategies and molecular players bacteria employ to ensure faithful propagation. The presented experimental work is thorough and the experiments are well executed experiments, with appropriated controls and clear data presentation. The manuscript is well written, however in some places a bit dense for none-specialist reader. Perhaps the few minor comments I have is that although this work aims to unravel the FzlA-FtsW/I activation pathway, the molecular basis of this activation is still unclear. For example, for most part of the manuscript, the authors seem to imply that FzlA directly influences FtsW activity/movement speed but there is no evidence that the proteins physically interact and thus, this activation mechanism is probably more complex and may involve additional components. This aspect could probably be made a bit clearer throughout the manuscript.

I have a couple of minor more specific components and comments that should be addressed.

Introduction
It would be beneficial to the reader to briefly provide some more background about the nature/activity associated with the FtsW**/I* variants.

Main text
L169/170: Do FtsW** molecules move at the same rate in the WT and the FtsW**/I* background? Can this be simply assumed or has this been demonstrated elsewhere?

Line 175: active FtsW** are more processive than active WT FtsW molecules
Line 182/183: Perhaps tone down this statement as this data does not provide direct evidence that more PG is made.

How does FzlA affect FtsW mobility? Directly or via FtsZ?
L195/196: FtsW**/I* can still receive input from FzlA even though it is not required for activation indirect or direct? Unfortunately, this is not further addressed in the manuscript

Line 205-206: Not quite clear to my why authors consider the stationary FtsW complexes as "poised for FzlA activation" and not the fast-moving FtsW complexes.

Line 209 and 219: FzlA and FtsWI activation - has it been established that FzlA directly interact with FtsW and FtsI? These proteins do not come up in the co-IP analysis.

Line 262-263 and Line 273-274: The authors state that overexpression of FzlA does not alter the proportion of moving/stationary

FtsW and FtsW** molecules. If stationary FtsW/FtsW** molecules are poised for turning into active complexes, wouldn't access level of FzlA be expected to reduce the number of stationary molecules?

Line 280: The use of the computational approach needs a bit more explanation. Are the graphs shown in Figure based on a simulation (text) or a re-analysis of data (as indicated in the figure legend)?

Line 294: "solidifying" seems a rather strong expression considering that this data has only been modelled.

Do the authors have additional SMT data of FtsW molecules in an FtsK depletion background to support their hypothesis that FtsK is involved in activating FtsW/I.

Do the authors envision that FtsK moves with FzlA/active FtsW/I complexes and how would this kind of dynamics affect chromosome segregation dynamics?

Line 530-531: This statement is slightly confusing because in other places of the manuscript, the authors suggest that stationary FtsW complexes are poised for activation (e.g. Figure 10 legend, results and text below).

Line 560: But Caulobacter has a FtsN homolog (see sentence above and DOI: 10.1111/j.1365-2958.2009.06706.x).

Line 563: Do the authors have any experimental evidence that the highlighted residues in in the FzlA C-terminus are indeed crucial for the interaction with FtsK?

Figure S1, A and B:
What does D227K, NB2 mean?
Panel A: FzlA protein levels in induced sample of the WT and the hyp variant - why are FtzLA leves in the WT(+)/diluted sample higher than in the undiluted sample and the hyp(+)/diluted sample?

Figure 10: Please label all the components shown in the figure panels
Panel B: do black arrows indicate direct interaction?
How does the model explain the influence FzlA has on the different populations of FtsW (static, fast, slow)?
Does the interaction between FtsK and FtsWI change depending on the movement/activity?

Video 1 did not play for me.

Reviewer #3 (Comments to the Authors (Required)):

This work focuses on FzlA, which is a division protein conserved in alphaproteobacteria. Previous work has shown that FzlA is essential in Caulobacter, but in its absence the divisome is apparently assembled but inactive. The need for FzlA can be suppressed if the strain contains a mutation that is hyperactive for division (W**I*). Knowing that the divisome appears fully assembled in the absence of FzlA, along with knowing it can be bypassed by a hyperactive mutant (a W**I* mutant has a wild type length in the absence of FzlA), suggests that FzlA is required for activation of the divisome. In a sense it mimics the activation mutations). What is examined in this paper is how FzlA activates the divisome, i.e. how does it contribute to the activation of FtsWI. The mechanism is not clear and it is likely that FzlA acts very indirectly.

The authors show that increasing FzlA enhances constriction speed and FzlA is observed to be mostly present in slow moving complexes in a W**I* strain (indicative of it moving with an active complex). Also, FzlA overexpression in the W**I* strain leads to the death of an FtsK deletion mutant. This is likely due to DNA getting in the way of the accelerated constriction leading to cell death by a combination of DNA damage and cell lysis. Eliminating the ability of FzlA to interact with FtsZ or FtsK does away with the overexpression killing indicating FzlA has to interact with both of these division proteins to have an effect. Also, FzlA appears to interact with the C-terminus of FtsK. Overall there is good correlation between the speed of WI and active vs inactive complexes that indicates that FzlA is an activator. However, the mechanism is far from clear and is likely to be very indirect with layers of regulation. For example, initiation of constriction is not earlier its just that septation is faster as the first section of the results show. Also, according to Goley et al, 2011 FzlA arrives very eary in assembly of the divisome (20 min before start of constriction) whereas in E. coli FtsN (thought to be the trigger) arrives last (expected of a trigger). In Caulobacter FtsK appears to arrive more closely to the time of initiation of constriction and could be the trigger. Perhaps FslA enhances the recruitment of FtsK (since it interacts with FtsK and FtsZ) and that is what's going on. Also, Caulobacter has FtsN which is essential.

FzlA is interesting since it binds to both FtsZ and FtsK. Thus, one would think FzlA should be either stationary or being propelled by FtsZ (like fast moving WI complexes). In cells with W**I* most FzlA is slow moving indicating it is likely moving with W**I* meaning it is no longer with FtsZ. Is it with FtsK? Is FtsK with W**I*? Where is FzlA when FtsK or FtsW are depleted.

Overall some aspects of this work are clear, e.g. FzlA activates FtsWI by somehow converting fast moving WI (inactive) to slow

moving WI (active). Consistent with this W**I* bypasses FzlA. However, when it comes to a mechanistic understanding of what FzlA does and how it converts fast moving complexes to slow ones, it is not so clear. Since it binds FtsZ and FtsK, perhaps it stabilizes the Z ring in some way that it is easier for WI to be activated. Zap mutants e.g., which have a less coherent Z ring, have a hard time activating WI for example. As I said perhaps it helps recruit FtsK.

Line 265 to 274 and again around 290. I have a question about terminology and the effect of FzlA, especially in the W**I* background. The terms are activation and also hyperactivaiton. W**I* suppresses the loss of FzlA and cell length is said to be normal. Thus, activation is not needed in this case- W**I* are active. When FzlA is added the average speed is slower but there is no change in processivity or the proportion of moving molecules - lines 269-271. The authors then state W**I* are primed for activation, but as stated they can be active without FzlA - some other term needs to be used - even hyperactivation is not clear. Also, around 292 it is stated that a greater proportion of W**I* is moving slower. In the discussion it is stated FtsW** is primed for activation.

Fig. 7 and S5. Does FzlA interact with E. coli FtsZ? Does FtsK interact with. E. coli FtsZ or FtsK. If they do they might localize in E. coli and affect the BACTH results.

In the results the author use SEM to analyze the results of the speed of the various constructs. Is it known why there is such wide variation - the scale in most figures go from ~3 to 90 nm/s?

How can one accurately measure the time of constriction initiation? I assume this is from phase microscopy?

I do not understand why stationary molecules represent. The authors suggest they are poised for activation (lines 180-1)? I thought the fast moving molecules are poised for activation, since one is arguing that activation involves molecules going from fast moving to slow.

Line 67. This should be Table S1.

Line 144. What do you mean by more frequently? Do you mean more FtsWs are active?

Line 182. What is the greater proportion of active W**I* vs WT? Give numbers in text.

Line 197 and entire paragraph. It is argued that FzlA does not activate FtsW**I* but causes more to be moving.(i.e. line 205-6; it is stated that W (referring to W**I*) is waing to be activated by FzlA.

Line 218-21. Conclude that FzlA mains associated with active, slow moving. However, the fast moving population is there. Is fast moving FzlA associated with FtsZ but maybe not with FtsW?
How does FzlA act as a switch? FtsN (Ecoli) is only associated with slow moving.

Line 248 or so. Do you see stationary FzlA? If you don't what does this mean?

Line 259-60. Should you say the 'average' speed?

Line 263-274. What keeps FzlA from having an effect?

What happens to FzlA movement when a FtsI inhibitor is added or FtsW is depleted?

Line 270.5 How can overexpresed FzlA impact the W**I* background if there is no change in processivity or proportion of stationary W.

Line 277. Cluster movement. Why not say treadmilling?

Line 304. This is a nice section

Line 387-91. I am not sure that a failure to interact in one orientation is a sign of reduced affinity. The mutant still interacts in one orientation.

Line 394-7. Perhaps mention that both conditions are lethal just that the terminal phenotypes are different.

Response to reviewers

Reviewer #1 (Comments to the Authors (Required)):

The paper Integration of cell wall synthesis activation and chromosome segregation during cell division in Caulobacter by Mahone et al. is an important work, which, among many insights, further elucidates the role of FzlA in cell division, and via its association with FtsK provides possible a link between chromosome replication and cell division. I love the "move speed" vs. time plots as measures of processivity. This work is fantastic and should be published, but a few points must be remedied (or verified) before publication.

We thank this reviewer for the positive words and for their thorough and thoughtful suggestions for improvement.

1. The major point that needs to be addressed is their initial assumption that the motions of Caulobacter FtsW are bimodal**,** like E.coli, with a fast and a slow state. This has been soundly demonstrated to be the case in E. coli in the Yang 2021 paper where: 1) the wildtype FtsW data was far more clearly bimodal and subsequently backed up by B) the fast population matching the FtsZ velocity, C) chemical perturbations eliminating the slow population, and corresponding shift between these two populations with mutants.

Here, however, it is assumed at the start of the paper Caulobacter FtsW is bimodal with not a single experiment (as done in Yang) to validate the fundamental assumption throughout the paper. The only evidence to this point is the statement that E. coli (which Yang showed is bimodal) is "the phylogenetically closest relative in which PG synthase dynamics have been measured. Aside from this statement, there is no further proof of bimodality nor any experiments to test if this is the case. Their evolutionary argument is simply suggestive, not definitive: while E. coli and Caulobacter are quite evolutionarily distant, and numerous and substantial differences have been observed in many of their biological processes, many of which use different proteins. Given that other bacteria do not show bimodality in their FtsW velocities, without tests to verify bimodality, it cannot be assumed whether Caulobacter FtsW motion is bimodal.

Thus, if the authors wish to use any bimodal "fast vs. slow" population arguments in this paper, it is their duty to conclusively demonstrate to the reader that these distributions are indeed bimodal, with distinguishable fast and slow populations. As the wildtype FtsW data in no way appears bimodal in any of the graphs, bimodality should be verified by another means. The easiest and best way might be to conduct some subsets of the validating experiments done in the Yang paper on this system, such as the chemical inhibition or the shift change in populations in different media.

The underlying rationale for this request is that A) None of the WT distributions of FtsW velocity in this paper look anything like a bimodal distribution (fig 2B, 4A, 4C, 5Ai, etc.), nor is there any aspect of them that would suggest bimodality. Rather they look like a single skewed distribution, just as seen in almost every other measurement of velocity in biology, including divisome proteins in other bacteria. Thus, demonstrating the bimodality validation would make it clear if the rightward shift seen in Fig 2B is indeed a change in the proportion of fast vs. slow molecules or if there is simply a net shift in the velocity of the enzymes to a slightly higher speed.

We thank the reviewer for bringing up this important concern. In retrospect, we agree it is vital that we provide more direct evidence for a two-track model in *Caulobacter*. As suggested, we undertook additional single molecule tracking experiments of FtsW under perturbed conditions. Specifically, we monitored FtsW dynamics in conditions where we (1) inhibited FtsW activity by depleting its lipid II substrate with fosfomycin or (2) slowed FtsZ dynamics using a GTPase-deficient mutant of FtsZ. As documented in updated Figure 2, inhibiting cell wall synthesis led to an increase in FtsW speeds on average and production of GTPase-deficient FtsZ led to a decrease in FtsW speeds on average. We performed one- and two-population fitting and found that, while two populations are difficult to resolve unequivocally in the WT unperturbed condition (i.e. one- and two-population models fit equally well), two-populations fit most of the perturbed conditions the best (see below). Using two-population fitting, fosfomycin depleted the slow-moving population while GTPase-deficient FtsZ eliminated fast-moving molecules (in this case, a single, slow population fit the data best). This is exactly in line with the *E. coli* two-track model, where slow-moving FtsW is active for PG synthesis and fast-moving FtsW is driven by FtsZ treadmilling. We appreciate the reviewer making this suggestion as we believe these new data significantly strengthen our argument that FtsW follows two tracks in *Caulobacter* similar to *E. coli*. This is especially important in light of recent additional reports on PG synthase dynamics in Gram-positive species that suggest FtsZ and PG synthases are largely uncoupled in many of those organisms.

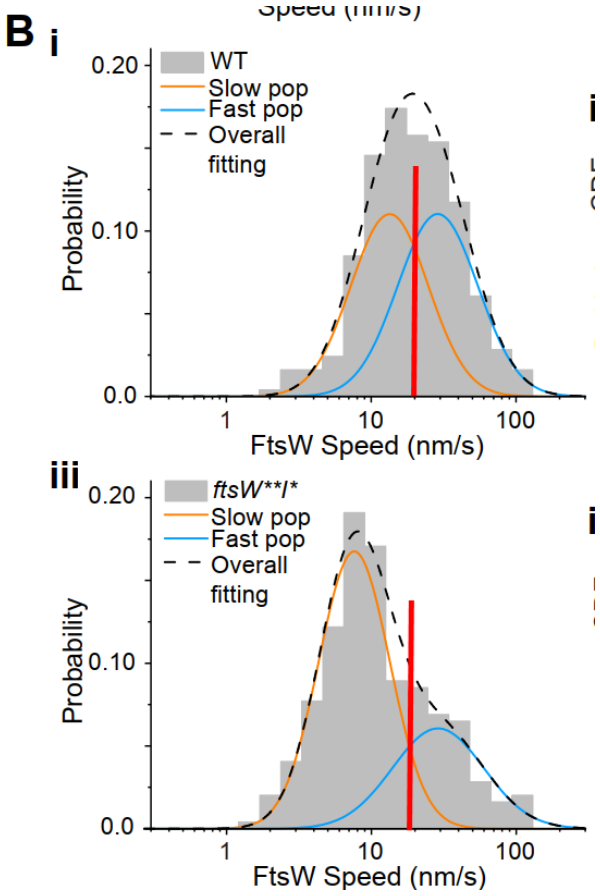
2. In light of the curve fits used to classify the data (if that was the case), It would also help if there were some statistical reasons that the authors used two curves to fit this data. Normally with any distribution, there are tests that attempt to fit several different distributions (1, 2, 3...) to determine the best fit to the data (such as Bayesian or likelihood-based methods). Could this be done, or if it was already, can this be shown in the supplemental data?

We have incorporated two key elements into our analysis: fitting residuals for visualization and Akaike Information Criterion (AIC) test to facilitate quantitative comparisons between single- and two-population fitting approaches. AIC estimates the quality of the fitting model and penalize for overfitting. As depicted in Figures 2, 3, 5, and Supplementary Figure S4, we observed that four distinct datasets exhibited a robust fit when employing the single-population model. These are: FtsW in wildtype cells (EG3052), FtsW in cells with backgrounds of $FtsZ^{WT}$ and $FtsZ^{D216A}$ (EG3929), as well as FtsW in cells with FzlA repletion (EG3523 + xylose). Our rationale for selecting the single-population model for these cases is rooted in the close proximity between slow- and fast-moving speeds, rendering the two-population fitting less suitable. To fortify this assertion, we computed the AIC values for both fitting approaches across each condition. It is statistically favored to opt for the model with a smaller AIC. As indicated by our visualizations, the four aforementioned conditions exhibit a superior fit with the single-population model, whereas **all other conditions favor the two-population fitting approach** (refer to Table S1, Tab 1, last two columns for details).

3. There is no clear explanation in the text as to what was used as the cutoff (or classifications) used to assign particles in Figures 2-4. into the fast and slow states - even though fractional percentages are mentioned in the text, included in table S1, and used to make arguments central to this paper. Currently, it is hard to know how these assignments were made and, importantly, how well these classifications fit the data. The only two places where a cutoff or classification is mentioned are 1) the statement in the methods "The cumulative probability density function of the directional moving speeds was further calculated and fit to a two-population model(of log-normal distribution) as described previously (Yang et al., 2021)", and 2) a cutoff of 20nm/sec on line 171. Thus, the authors should more explicitly state how these proportions were determined and what certainty underlies these classifications. Also, if CDFs were done for this filling, those graphs should be shown, along with a measure of the fit (such as residuals). If the fits from figure 5 and S4 were used for the classification of trajectories in Figs 2-4, this should also be stated.

All percentages of fast- and slow-moving populations were estimated from the non-linear two-population fitting. As the histograms show, a molecule moving at a certain speed (30 nm/s for example), could be either in the “fast” or “slow” population but with different probabilities, as the distributions of both populations are wide. The percentage of each population, however, can be obtained by fitting the histogram. As suggested, we now include plots of the CDF and residuals figures displaying one- and two-population fitting.

For speed vs lifetime time comparisons (Figure 3C, 4B and D, and 5C), however, we wanted to plot only the molecules likely to be actively synthesizing PG. To exclude molecules that are more likely to be inactive and fast-moving by association with FtsZ, we set a threshold of 20 nm/s based on the fitting results (Figure 2B and below). Molecules moving slower than 20 nm/s are statistically more likely to be slow-moving/active than fast-moving/inactive We have added this reasoning in the Method section (Lines 747-750).



The cross point of the orange and blue curves (slow- and fast- population fitting) is a reasonable speed to threshold the two different types of moving states.

4. In the Yang paper, the peaks of the fast and slow distributions were nearly identical across all of the experiments, allowing the reader to see the two populations and the shifts between them in a way where each experiment could be compared. However, in this paper, the peaks and widths of the fast and slow distributions that are fit in figure 5 and S4 are, in many cases, extremely different from each other. This begs the questions: 1) why are these fitted distributions so different in their maxima (peaks) and range (high and low) across these experiments? 2) More importantly, how can one conclude both within and between these multiple experiments when these peaks are very different between experiments, thereby affecting the subsequent curve fitting used to classify the peaks in each experiment? Thus, the authors should explain 1) why there is so much variation in the distributions used to fit the fast and slow enzymes in these experiments, as compared to the nearly invariant peaks of FtsW velocity they saw in E. coli, and 2) explain and why these fits and classifications from these very different distributions, can be used to classify the proportions of fast vs. slow molecules in a way so they can be compared between experiments.

In our previous work in *E. coli*, we fixed the fast- or slow-moving peak speed in the two-population fitting according to values determined in control experiments. In the original version of the current manuscript, we simply fit the CDF curves without fixing the parameters, leading to more variability in peak values. After carrying out additional suggested control experiments, we are better able to fix the fast-moving population speed as described below.

Due to the small diameter of *Caulobacter crescentus* cells, we have been unable to directly measure FtsZ's treadmilling speed. Instead, we opted to track the motion of FtsW molecules following treatment with fosfomycin, which inhibits peptidoglycan synthesis and consequently leads to an accumulation of the fast-moving population. As described earlier in point #2 (the new Figure 2C), the two-population fitting approach outperforms the one-population

model for fos-treated cells, providing further support for this distinction. We posit that this fast-moving population corresponds to molecules that move with FtsZ, akin to the behavior observed in *E. coli*.

Our previous study showed that FtsZ's speed does not change unless the GTPase activity varies (Yang et al., 2017). Therefore, we now fix the parameter μ_2 (indicating the median speed) of the fast-moving population in all subsequent two-population fittings throughout the manuscript. However, we have refrained from fixing σ_2 (indicating the width of the distribution), as the width of this distribution may fluctuate under different experimental conditions.

$$CDF = 0.5 \cdot P_1\left(1 + erf\left(\frac{\ln v - \mu_1}{\sqrt{2}\sigma_1}\right)\right) + 0.5 \cdot (1 - P_1)\left(1 + erf\left(\frac{\ln v - \mu_2}{\sqrt{2}\sigma_2}\right)\right)$$

With this refined fitting methodology (described in Lines 730-746), we confirmed a disparity in the slow-moving speeds, often associated with active synthesis, between FtsW and the FtsW** variant. Notably, the FtsW** variant exhibits a slower pace compared to wild-type FtsW, as detailed in Table S1. This is similar to the effect of some hyperactive mutations in FtsW or FtsI observed in *E. coli*, as reported by Yang et al. in 2021. The variation in our previous fitting results, where all parameters were allowed to vary freely, can be attributed to the impact of different slow-moving speeds.

5. One line 171, They state a cutoff for the analysis and interpretation of some of the data "For this analysis, we excluded molecules moving faster than 20 nm/s as they are unlikely to be actively synthesizing PG".

a) It should be made clear what data this 20nm cutoff applies to. Is it only Fig 2B? Or was this only used for the "move time vs. speed graphs"?

Yes, this cut-off was used for (and only for) the move time vs speed plots (now Fig 3C, 4B and D, 5C). See response to point 3 for complete explanation. This is clearly stated in the methods now.

b) As this cutoff is the first place any classification metric is defined in the text, and there is no clear definition stated of how the "fast vs. slow" proportions were measured in fig 2-4, the reader might be confused (as I was) that the 20nm cutoff was used for not just the "move time vs. velocity" graphs, but also for all of the other data, What this cutoff applies to should be clarified to prevent any confusion.

See response to point 3 above.

c) Why they chose 20nm/sec as a cutoff is unclear, as it currently appears arbitrarily defined: Looking at all of the data in this paper, it is hard to see why 20nm/sec was chosen, as 1) there is no clear bimodality to hint where that cutoff should be, and 2), the 20nm/sec cutoff would cut through (nearly) through the mean (and often the peak) of all of the WT distributions (fig 2B and 5Ai), almost separating the data in half. Thus, the authors should better justify this cutoff, as it is confusing when one examines all the WT velocity data.

See response to point 3 above.

6. The title claims a link from division to chromosome segregation. However, it's clear from the discussion that this is a model, not a definitive conclusion. While many bacterial papers use the model as the title, they might think about rephrasing it, as this is a bit speculative.

We believe our biochemical and genetic data firmly establish a role for FtsK in signaling between FzlA and FtsWI to regulate constriction. The body of literature implicating FtsK in dimer resolution and chromosome translocation, along with our data demonstrating substantial DNA damage when this pathway is disrupted in *Caulobacter*, motivate us to keep the title as is.

7. lines 153-154 state they see switches in the directions of molecules. It is unclear if those "Switches" in direction are occurring only on one side of the cell (next to the coverslip, as in TIRF) or if these are arising from molecules moving

around the cell. I presumed these comments related to motion only on one side of the cell (from TIRF), but this should be clarified to the reader.

We used epifluorescence-illumination mode, not TIRF, for all SMT experiments (Line 708). Because the cell width of Caulobacter (~500 nm) is close to and slightly smaller than the depth of our 100X objective the entire cell (not the bottom half) should be observed in our experiments. We did not apply 3D-SMT and thus are not able to distinguish direction changes from circumferential movement. Because of the limitations discussed above, we have eliminated any conclusions regarding changes in direction.

8. It would be great if the fraction of FtsW tracks that switched directions were calculated, as it might be insightful data. (Not necessary to include but might be useful to the field in the future).

As mentioned in point 7, without 3D-SMT, it is technically challenging to tell whether a molecule is moving around the circumference or changing its direction if the molecule is at the edge of a cell. We decided not to elaborate on this type of data to avoid any mis-interpretation.

9. There are many points in the text where they compare the means of similarities of different distributions. However, these comparisons lack any significance metric, making it hard to understand what is significant and if the different N values of each experiment affect the significance (as the number of samples defines how well the distribution was sampled). Could the authors provide some measure of the significance of the difference between these distributions, so the reader can evaluate their certainty?

We thank the reviewer for this suggestion. Now we performed K-S tests on all distribution comparisons. These are reported in Tab 2 of Table S1.

10. I laud their use of superplots. However, I found it hard to know if all the statistical comparisons shown in ALL the figures were analyzed via the superplot method or if superplots were sued for some statistics and others used analysis of pooled data points. Could this be briefly clarified in the legend, just as we would do when we note what our error bars/box plots indicate?

We used Superplots for situations when we were comparing mean values (e.g. constriction rate) across samples. For the bulk of our single molecule tracking data, we did not assess mean or median values, but distributions, so we did not use Superplot analysis (instead performing KS tests (see response to point 9)). We have made it more clear in the text and legends when Superplots were used.

11. Putting the significance (*) into all panels in Fig 1 B+D and Fig 2G (and p-values in the legend) would be helpful to the reader. For example, it is very hard to understand why the difference in 1B is significant but 1C is not.

We thank the reviewer for this helpful suggestion and have added these significance values to the legend to prevent crowding on the figure.

12. Lines 160-162 - they state the interesting observation that single FtsW molecules change their direction of movement or transition between states of motion and immobility. However, it is unclear if they are referring to the "fast-moving" or "slow-moving" molecules. Could this be clarified?

We have clarified it now in the discussion section: “For both FzlA and FtsW, we observed transitions from the fast- or slow-moving population to the stationary state and vice versa. Though it is difficult to quantify the frequency of these events, this suggests the PG synthase complex can transfer between the FtsZ- and PG-track.” (Lines 548-551) We did not proceed to perform statistical analysis since there are two few of these transition events to be statistically meaningful.

13. Many of their arguments in Figures 1-5 rely on the relative proportions of different motions, and these proportions are central to many of their arguments. A few modifications would make this data, and thus their conclusions, more accessible.

a) They make many comparisons between these proportions in their text with the percentages in brackets. As these comparisons are central to their conclusions, It would be far better to display these fractional comparisons in a table in the main text. What might be better is a small figure next to each figure, which would help the reader grasp the significance of these proportions while also looking at the three other types of data in each experiment.

Because of space constraints, we cannot add another main table. Instead, we added a small table panel to each figure providing the percentage of molecules that are moving (overall), slow-moving, and fast-moving.

b) Importantly, as noted below, they should report each graph's percentage of slow, stationary, and fast molecules. Currently, it appears they are excluding one or the other of the inactive states in every measure of fractional activity they use here.

See response to (a).

c) This work states that the 1) slow-moving molecules are active and that the 2) stationary AND the 3) fast-moving molecules are inactive. However, it does not appear the two inactive states are included in their active/inactive comparisons. Currently, it appears from the text and the table they are comparing the proportions of slow-moving molecules to the stationary ones, neglecting to include the fast-moving molecules in the inactive tally. Conversely, in figure 3B, it appears the fast-moving population is considered the inactive form, with no inclusion of the stationary molecules. To be consistent with their arguments, these relative active/inactive comparisons should include both the stationary and fast molecules as the "inactive" molecules.

We thank the reviewer for drawing our attention to these areas where we can clarify the populations more. In general, we have modified the language throughout to specifically denote whether we are referring to “fast-moving” or “stationary” molecules (or both). We do not specifically tabulate “inactive” molecules, but infer in the text that fast-moving and stationary molecules are both inactive. (e.g. Lines 180-183,196, 254, 259-260).

d) Likewise, the fraction of fast-moving molecules is lacking in the table and most experiments. These should be included.

We have updated all these populations in Table S1 as well as in the main Figures (such as 2D, 3E, and 5D).

14. In figure 5, it is noted a computational approach is used, but no more details are given. Can the method be named or briefly described in the text or legend?

The fitting equation for determination of the two populations and the statistical test to justify the goodness of fit have been detailed in the Methods section (Lines 732-748, 754-764).

15. Lines 197-199 state- FtsW** in the ΔfzlA background appeared to have processive movement similar to FtsW** in the presence of FzlA, suggesting that FzlA does not affect the processivity of PG synthases, but rather the fraction of active PG synthases (Fig. 2 C) "while the processivity argument is clear, the reasoning behind the statement regarding the "fraction of active PG synthases" is hard to grasp as there is no plotting of the differences in the "active vs. inactive" enzymes. Even if this data can be inferred from Table S1, it would be way clearer to the reader if this was shown in the main text as a plot or a table.

Likewise, Plots (or tables) of the fractions in each class should be shown for 203-206 and 216-218.

As the reviewer suggested, we now include a small table with proportions of total moving, slow- (active) and fast-moving (inactive) molecules to make this clearer.

16. It is fascinating that both FzlA and FtsN are activators and that A) FzlA moves with FtsW and B) binds to FtsZ filaments. A few recent findings about this comparison/analogy are missing from their discussion but might be insightful if they wish to include them: 1) The Loose lab showed that FtsN molecules are immobile, binding to treadmilling filaments. This appears to be a substantial difference from FzlA, where at least a subset of the FzlA moves with the filaments. Likewise, the Löwe group showed the acidic FtsN could dimerize 2 FtsA protofilaments, giving a putative mechanism for activation. These differences might be worth mentioning in the discussion if the authors wish.

But I wonder if any insights regarding activation or "stationary vs. moving" in regard to FzlA and FtsN be gained from any structural / domain information within FzlA and FtsN?

We apologize if our discussion of similarities/differences between FzlA and FtsN was misleading. We do not mean to assert that they fill analogous roles – indeed *Caulobacter* has an FtsN homolog that may perform a similar function to *E. coli* FtsN. Rather than causing further confusion by including a deeper comparison, we elected to instead highlight our proposed model in which FzlA and FtsN each contributes as part of independent modules that feed into the regulation of FtsWI activity.

17. The effective resolution of the microscope (with instrument noise) that was used for single molecule localizations should be reported in the methods.

We thank the reviewer for noting this absence and have added additional clarification in the methods for the localization precision of our microscope (see Methods section “Advanced epifluorescence imaging and single-molecule tracking of Halo-FtsW and Halo-FzlA”, lines 729-732). We took our single molecule trajectories, extracted the stationary segments, then analyzed the nearest-neighbor distance between each localization based on the equations developed by Endesfelder et al. (PMID 24522395) for a localization precision of ~23 nm.

18. Lines 239-245 state: "We next assessed the stationary FtsW population in our depletion strain with and without FzlA. Surprisingly, depletion of FzlA in a WT background did not change the proportion of moving FtsW molecules (Table S1, ~50% in both deplete and induced), unlike in the ΔfzlA background (Table S1, 25%). These results indicate that the increase in stationary FtsW** molecules observed in the ΔfzlA background is likely due to binding the PG synthases to a stationary target rather than an inability to move dynamically about the Z-ring in the absence of FzlA".

a) The logic underlying this conclusion is unclear, most especially why this conclusion arises from differences between a depletion vs. a knockout. Can it be explained more overtly?

In response to this reviewer’s point 21, we have sequenced the relevant strains and found two point mutations in the Δ*fzlA* strain that are not in the depletion strain. These could account for the differences we observe in the stationary population, although neither of the mutated genes has a direct role regulating cell wall metabolism or cell division. Alternatively, mutant FtsW** (which is not present in the depletion strain) may have a different affinity for other elements at the division site (e.g. PG) from WT and this difference is alleviated somehow by deletion of FzlA. We have updated the language found in lines 289-295 and note the point mutations in Table S4.

b) "Moving FtsW molecules" should be clarified if this is "all moving molecules," "slow-moving molecules," or "fast-moving molecules."

This has been clarified to indicate “total moving FtsW molecules” in lines 287-288.

19. Likewise, on line 262. Likewise, they say the speed increases, but this appears to be referring to all moving molecules. Please clarify if this is the proportion of slow-moving molecules or the proportion of all moving molecules.

We have changed our statement to clarify that the overproduction of FzlA drove shifted the overall average FtsW speeds to be slower in line 277.

20. Similar to the above point, the text on lines 246-249 appears to be making a logical leap and should be better explained. To me, it uses observations of stationary and slow-moving molecules to make a claim about how fast molecules transition into the slow-moving state. This is a confusing conclusion as there is no measure of the number of fast-moving molecules here. Can the number of fast-moving molecules be measured? Or at least this logic be spelled out more clearly?

Lines 246-249 - "The amount of time FtsW molecules remain stationary decreased when FzlA was depleted in a WT background (Table S1, Fig. 3 C, 8.8 {plus minus} 0.7 s during FzlA depletion, N = 107 vs. 10.9 {plus minus} 0.6 s at WT FzlA levels, N = 244). These tracking data are consistent with FzlA-mediated signaling acting to convert fast-moving, inactive molecules of FtsW into an activated, slow-moving state. "

We thank the reviewer for the opportunity to clarify the language here. We have removed that statement. The statement was a reiteration of the model from the section before and the sentence after was in reference to these new observations in this section. We have also removed discussion of stationary time data as the differences in stationary times were largely found not to be statistically significant with additional analysis.

21. Regarding the conclusions from the comparison of molecules between the FzlA depletion and the knockout, the first thing that comes to mind here is that there may be some other genetic difference between the strains, i.e., a possible adaptative mutation within the knockout strain. Have both strains been sequenced to see if there are any other mutations?

Thanks for this suggestion. As mentioned in response to point 18a, we sequenced the genomes of the relevant strains as suggested, and did find two point mutations in the Δ*fzlA* strain that were not present in the depletion (in *divL* and *ubiB*). We have now indicated that as a possible source for the difference. Since all the other trends between the deletion and depletion relative to +FzlA controls are the same (and the opposite of our observations for FzlA overproduction), we do not think these point mutations otherwise impact our conclusions.

22. Many of the graphs have "speed" on one axis. Do they mean to use speed as the measure (the average frame-to-frame displacement) or the velocity (the sustained movement in one direction, as determined by MSD vs. t?) This should be clarified or the axes corrected.

In this work, we did not use MSD to measure the speed of single molecules since the directed moving portion of the trajectories are identified and segmented. We fit that part of the trajectory linearly. Therefore, the “speed” in the figures is defined as distance/time (not frame-to-frame displacement nor MSD fitted V). The fitting is demonstrated in Figure 2Aiv (dark lines) and detailed in the Methods Yang, et al. 2021.

Reviewer #2 (Comments to the Authors (Required)):

This manuscript describes a novel pathway that allows the coordination of chromosome segregation and septum synthesis in Caulobacter crescentus, a model alpha proteobacterium. The authors elegantly show that in Caulobacter, the FtsZ-interacting protein FzlA, which is specific to alpha proteobacteria, fulfils a key role in mediating the activation of the essential peptidoglycan synthesising proteins FtsW and FtsI. Using a combination of genetics and single molecule tracking they show that FzlA influences the speed of FtsW molecules and promotes the slow movement of FtsW which has been shown to be associated with active PG synthesis. Using in vivo pull-down and two-hybrid interaction studies they further identify a novel interaction partner of FzlA, the essential DNA translocase FtsK. I find this part of the work particular exciting. The authors elegantly show

show that FzlA plays a crucial role in coordinating the licencing of division septum formation with the completion of DNA segregation and that misregulation of this step leads to DNA damage with lethal consequences.

This work is exciting and uncovers a new layer of regulation of cell division, demonstrating the diverse strategies and molecular players bacteria employ to ensure faithful propagation. The presented experimental work is thorough and the experiments are well executed experiments, with appropriated controls and clear data presentation. The manuscript is well written, however in some places a bit dense for none-specialist reader. Perhaps the few minor comments I have is that although this work aims to unravel the FzlA-FtsW/I activation pathway, the molecular basis of this activation is still unclear. For example, for most part of the manuscript, the authors seem to imply that FzlA directly influences FtsW activity/movement speed but there is no evidence that the proteins physically interact and thus, this activation mechanism is probably more complex and may involve additional components. This aspect could probably be made a bit clearer throughout the manuscript.

We thank this reviewer for their positive comments and thoughtful suggestions for improvement. As described below, we have more clearly stated that FzlA signals to FtsWI through other divisome proteins and does not impact its activity/movement directly. We have more clearly stated this at the outset of the manuscript and throughout.

I have a couple of minor more specific components and comments that should be addressed.

Introduction
It would be beneficial to the reader to briefly provide some more background about the nature/activity associated with the FtsW**/I* variants.

We thank the reviewer for providing the opportunity to give additional background about these variants. Please find the additional information in lines 87-97.

Main text
L169/170: Do FtsW** molecules move at the same rate in the WT and the FtsW**/I* background? Can this be simply assumed or has this been demonstrated elsewhere?

We only measured the FtsW** in the FtsW**/FtsI* background to avoid the confounding factor of having both WT FtsW and Halo-FtsW** present in a WT background. Our point here was to show the more active FtsW** moves slower than WT FtsW, which is similar to the observation in *E. coli*.

Line 175: active FtsW** are more processive than active WT FtsW molecules

We are not sure what the reviewer would like changed about this statement.

Line 182/183: Perhaps tone down this statement as this data does not provide direct evidence that more PG is made.

We have revised the statement in lines 242-244.

How does FzlA affect FtsW mobility? Directly or via FtsZ?

We believe the effects of FzlA on FtsW movement are indirect, and mediated by modulating the active state of FtsW (and thus its ability to move via PG synthesis), as none of our data suggest a direct interaction between FzlA and FtsW. Throughout the manuscript, we now more explicitly state that it is unlikely that FzlA directly influences FtsW dynamics, instead signaling to impact its active state, for example in lines 101 and 397. In addition, we do not think FzlA influences FtsW (solely) via effects on FtsZ because a FzlA mutant that can bind FtsZ but not FtsK does not cause hyperconstriction. This is clarified in lines 374-377.

L195/196: FtsW**/I* can still receive input from FzlA even though it is not required for activation indirect or direct? Unfortunately, this is not further addressed in the manuscript

We rephrased this statement to indicate that FtsW**I* can still receive activating signals from FzlA. With the additional changes to the manuscript clarifying that we believe the activation from FzlA to be indirect, signaling through other divisome proteins, we hope this clarifies this statement.

Line 205-206: Not quite clear to my why authors consider the stationary FtsW complexes as "poised for FzlA activation" and not the fast-moving FtsW complexes.

We have removed discussion of stationary time data as the differences in stationary times were largely found not to be statistically significant with additional statistical analysis (Table S1, Tab 2).

Line 209 and 219: FzlA and FtsWI activation - has it been established that FzlA directly interact with FtsW and FtsI? These proteins do not come up in the co-IP analysis.

FzlA does not appear to have a direct interaction with FtsW or FtsI. We believe FzlA activation is indirect, and that FtsK is the next step in signaling to activate FtsWI. Downstream signals from FtsK may be direct or occur through other components (e.g. FtsQLB). To eliminate this confusion, we have added the explicit statement in line 397 ruling out a direct interaction between FzlA and FtsW or FtsI.

Line 262-263 and Line 273-274: The authors state that overexpression of FzlA does not alter the proportion of moving/stationary FtsW and FtsW** molecules. If stationary FtsW/FtsW** molecules are poised for turning into active complexes, wouldn't access level of FzlA be expected to reduce the number of stationary molecules?

Inactive FtsW molecules can be stationary or fast-moving. It is possible that FzlA converts inactive, fast-moving molecules to slow-moving active molecules. Since we do not know all of the parameters promoting switching of FtsW between slow, fast, and stationary states (e.g. how FtsW is linked to FtsZ filaments in the case of fast-moving molecules, or whether there are different subpopulations of stationary molecules) we hesitate to draw conclusions about why FzlA does not impact the overall stationary/moving %.

Line 280: The use of the computational approach needs a bit more explanation. Are the graphs shown in Figure based on a simulation (text) or a re-analysis of data (as indicated in the figure legend)?

The fitting equation for determination of the two populations and the statistical test to justify the goodness of fit have been detailed in the Methods section now.

Line 294: "solidifying" seems a rather strong expression considering that this data has only been modelled.

We have re-organized this section so this statement was eliminated.

Do the authors have additional SMT data of FtsW molecules in an FtsK depletion background to support their hypothesis that FtsK is involved in activating FtsW/I.

While we agree with the reviewer that this experiment would be interesting and informative, we believe additional SMT experiments with FtsK are outside the scope of this study. Our genetic data implicating FtsK in activation of FtsWI and our functional data demonstrating enhanced DNA damage in the absence of proper FzlA-FtsK-FtsWI signaling are, we believe, consistent with our conclusions that FtsK participates in this pathway downstream of FzlA.

Do the authors envision that FtsK moves with FzlA/active FtsW/I complexes and how would this kind of dynamics affect chromosome segregation dynamics?

This is an interesting line of thought; we are open to many hypotheses for how FtsK “plugs in” to the dynamics of FtsW. We speculate only that if FzlA moves with FtsWI via its interactions with FtsK, FtsK also likely moves with

active FtsWI, presumably when it is not bound to the chromosome (e.g. lines 612-616). Further clarifying these interactions is an area for future study.

Line 530-531: This statement is slightly confusing because in other places of the manuscript, the authors suggest that stationary FtsW complexes are poised for activation (e.g. Figure 10 legend, results and text below).

We have removed discussion of stationary time data as the differences in stationary times were largely found not to be statistically significant with additional statistical analysis (Table S1, Tab 2). Because of this, we eliminated this statement.

Line 560: But Caulobacter has a FtsN homolog (see sentence above and DOI: 10.1111/j.1365-2958.2009.06706.x).

Yes, we noted this in the discussion as well. We apologize for any confusion here and have clarified our model for how FzlA and FtsN both contribute to FtsWI activation in *Caulobacter*. Our comparison of the two proteins was meant to highlight similar features among independent activators of FtsWI in terms of modulating FtsWI dynamics.

Line 563: Do the authors have any experimental evidence that the highlighted residues in in the FzlA C-terminus are indeed crucial for the interaction with FtsK?

We would like to draw the reviewer's attention to the BTH data in Figure 7C showing that the C-terminal tail mutant FzlA^{D227K} does not interact with full-length FtsK or FtsK's C-domain.

Figure S1, A and B:
What does D227K, NB2 mean?

We would like to draw the reviewer's attention to lines 370-372 which define the D227K and NB2 mutants. We have added these definitions to the legend for Fig S1A-B, as well.

Panel A: FzlA protein levels in induced sample of the WT and the hyp variant - why are FtzLA leves in the WT(+)/diluted sample higher than in the undiluted sample and the hyp(+)/diluted sample?

The undiluted samples are not induced for *fzlA* overexpression, so the FzlA observed is native FzlA plus any leaky expression from the uninduced Pxyl-*fzlA* construct. The signal is slightly less than the 1:20 diluted, induced sample so we estimate >20-fold overproduction of FzlA in the WT background. It is not clear why FzlA was not overproduced to the same extent in the ftsW**I* strain, but since we observe strong toxicity in that background with induction the excess FzlA appears sufficient to cause lethal hyperconstriction.

Figure 10: Please label all the components shown in the figure panels

We have labeled all divisome components in the figure.

Panel B: do black arrows indicate direct interaction?

The black arrows do not indicate direct interaction. They are arrows dictating potential signals. We have made this clarification in the legend.

How does the model explain the influence FzlA has on the different populations of FtsW (static, fast, slow)?

The model shows FzlA-mediated signaling promoting slow-moving, active FtsW. We tried not to over complicate this figure by including all effects on movement.

Does the interaction between FtsK and FtsWI change depending on the movement/activity?

The interaction between FtsK and FtsW that we reference is a genetic interaction. We do not rule out a direct interaction between the two but do not want to overstate any conclusions about movement or activity. We are excited to dig deeper into how interactions among FzlA, FtsK, the chromosome, and FtsWI are regulated and relate to signaling in future work.

Video 1 did not play for me.

We have reshared the video with the editor.

Reviewer #3 (Comments to the Authors (Required)):

This work focuses on FzlA, which is a division protein conserved in alphaproteobacteria. Previous work has shown that FzlA is essential in Caulobacter, but in its absence the divisome is apparently assembled but inactive. The need for FzlA can be suppressed if the strain contains a mutation that is hyperactive for division (W**I*). Knowing that the divisome appears fully assembled in the absence of FzlA, along with knowing it can be bypassed by a hyperactive mutant (a W**I* mutant has a wild type length in the absence of FzlA), suggests that FzlA is required for activation of the divisome. In a sense it mimics the activation mutations). What is examined in this paper is how FzlA activates the divisome, i.e. how does it contribute to the activation of FtsWI. The mechanism is not clear and it is likely that FzlA acts very indirectly.

We agree with the reviewer that FzlA is part of a signaling pathway that ultimately impinges on FtsWI activity, but that FzlA does not activate FtsWI directly. We have further clarified our model to this effect throughout the manuscript.

The authors show that increasing FzlA enhances constriction speed and FzlA is observed to be mostly present in slow moving complexes in a W**I* strain (indicative of it moving with an active complex). Also, FzlA overexpression in the W**I* strain leads to the death of an FtsK deletion mutant. This is likely due to DNA getting in the way of the accelerated constriction leading to cell death by a combination of DNA damage and cell lysis. Eliminating the ability of FzlA to interact with FtsZ or FtsK does away with the overexpression killing indicating FzlA has to interact with both of these division proteins to have an effect. Also, FzlA appears to interact with the C-terminus of FtsK. Overall there is good correlation between the speed of WI and active vs inactive complexes that indicates that FzlA is an activator. However, the mechanism is far from clear and is likely to be very indirect with layers of regulation. For example, initiation of constriction is not earlier its just that septation is faster as the first section of the results show. Also, according to Goley et al, 2011 FzlA arrives very eary in assembly of the divisome (20 min before start of constriction) whereas in E. coli FtsN (thought to be the trigger) arrives last (expected of a trigger). In Caulobacter FtsK appears to arrive more closely to the time of initiation of constriction and could be the trigger. Perhaps FslA enhances the recruitment of FtsK (since it interacts with FtsK and FtsZ) and that is what's going on. Also, Caulobacter has FtsN which is essential.

We thank the reviewer for recognizing several important aspects of our model and previous supporting observations, and for providing us the opportunity to clarify several points. First, we do not believe FzlA and FtsN play analogous roles in *Caulobacter*. *Caulobacter* has an FtsN homolog that is essential for division, as the reviewer notes. It does not arrive last in *Caulobacter* however, and we have no evidence that FtsN is “the” trigger for constriction in *Caulobacter*. As we describe in the Discussion, we propose that FtsN-dependent signaling couples amidase activity to constriction while the FzlA-dependent pathway couples chromosome segregation and FtsZ to constriction. Each is a critical control point and we think it is likely that they both feed into FtsQLB-mediated direct activation of FtsWI. In addition, we do not propose that FzlA acts to regulate initiation of constriction and we explicitly say that, e.g., FzlA overproduction does not impact time to initiation of constriction. We have further clarified this point in the discussion, proposing that FzlA acts as a “pacer” of constriction rather than a “trigger” (lines 612-618). To address the final point raised here, we have visualized FtsK in Δ*fzlA* cells and find that FtsK still localizes in the absence of FzlA (Supp Fig. S3B). We also show that FtsK localizes independent of FtsW (Supp Fig. 3C) and we previously showed that FtsW localization is independent of FzlA (Goley et al 2011).

FzlA is interesting since it binds to both FtsZ and FtsK. Thus, one would think FzlA should be either stationary or being propelled by FtsZ (like fast moving WI complexes). In cells with W**I* most FzlA is slow moving indicating it is likely moving with W**I* meaning it is no longer with FtsZ. Is it with FtsK? Is FtsK with W**I*? Where is FzlA when FtsK or FtsW are depleted.

Indeed we observe stationary, slow-moving, and fast-moving molecules of FzlA as depicted in Figure 5. Depletion of FtsK or FtsW causes filamentation. In these filamentous cells, we now show that FzlA still localizes to rings and foci, suggesting it does not require these factors to localize. This is consistent with our prior work demonstrating a direct interaction between FzlA and FtsZ (Supp Fig. S3A, Goley et al, 2010). Monitoring the movement dynamics of FtsK under various conditions is an exciting area for future work, but it is beyond the scope of the current study.

Overall some aspects of this work are clear, e.g. FzlA activates FtsWI by somehow converting fast moving WI (inactive) to slow moving WI (active). Consistent with this W**I* bypasses FzlA. However, when it comes to a mechanistic understanding of what FzlA does and how it converts fast moving complexes to slow ones, it is not so clear. Since it binds FtsZ and FtsK, perhaps it stabilizes the Z ring in some way that it is easier for WI to be activated. Zap mutants e.g., which have a less coherent Z ring, have a hard time activating WI for example. As I said perhaps it helps recruit FtsK.

We have clarified throughout the manuscript that we believe FzlA signals through other divisome proteins, including FtsK, to convert FtsWI to an active state, i.e. that it is an indirect regulator of FtsWI activity. In *Caulobacter*, deletion of *zapA* has a very modest effect on cell length (Woldemeskel et al., 2019) and is distinct from the filamentation observed with depletion of FzlA. In addition, loss of *zapA* causes obvious distortion of Z-ring structure even by epifluorescence imaging (Woldemeskel et al., 2019), whereas depletion of FzlA does not (Goley et al., 2010; Lariviere et al., 2018). Our observation that overexpression of a *fzlA* mutant that interacts with FtsZ in a manner indistinguishable from WT in vitro ($FzlA^{D227K}$, Lariviere et al., 2018) does not hyperactivate FtsWI is not consistent with the effects of FzlA on FtsWI active state being mediated by modulation of the Z-ring (Fig 6 B). We have added a statement to this effect in lines 374-377. Finally, as stated above, FtsK localizes normally in Δ*fzlA* cells.

Line 265 to 274 and again around 290. I have a question about terminology and the effect of FzlA, especially in the W**I* background. The terms are activation and also hyperactivaiton. W**I* suppresses the loss of FzlA and cell length is said to be normal. Thus, activation is not needed in this case- W**I* are active. When FzlA is added the average speed is slower but there is no change in processivity or the proportion of moving molecules - lines 269-271. The authors then state W**I* are primed for activation, but as stated they can be active without FzlA - some other term needs to be used - even hyperactivation is not clear. Also, around 292 it is stated that a greater proportion of W**I* is moving slower. In the discussion it is stated FtsW** is primed for activation.

We thank the reviewer for this chance to clarify our language. We generally refer to “activation” as conversion of FtsWI from an inactive to active state, and use “hyperactivation/hyperactive” to refer to conditions that increase constriction rate relative to wildtype (e.g. FtsW**I* or overproduction of FzlA). We have removed references to FtsW being “poised for activation” (except in reference to E. coli data from Yang et al, 2021), in part because we removed stationary time data from the results. We have also updated the introduction to explain a bit more about the hyperactivating mutations in FtsW and/or FtsI (Lines 87-97).

Fig. 7 and S5. Does FzlA interact with E. coli FtsZ? Does FtsK interact with. E. coli FtsZ or FtsK. If they do they might localize in E. coli and affect the BACTH results.

We addressed this possibility by visualizing YFP-FzlA produced in *E. coli.* As now shown in Supp Fig. S5B, FzlA is completely diffuse, with no midcell enrichment in *E. coli.* Since both FzlA and FtsK would need to be recruited to midcell by *E. coli* divisome proteins to achieve a false positive BTH result, we believe this further strengthens our conclusion that FzlA and FtsK directly interact.

In the results the author use SEM to analyze the results of the speed of the various constructs. Is it known why there is such wide variation - the scale in most figures go from ~3 to 90 nm/s?

This is an interesting point. Virtually all studies that have monitored single molecule movement of PG synthases in diverse bacteria report a wide spread of speeds (Garner, 2011, Cho, 2016, Bisson, 2017, Yang, 2017, Perez, 2019).

In *Caulobacter* and *E.* coli, this stems, at least partly, from the bimodal distribution of molecule speeds. In addition, intrinsic stochastic behavior at single molecule level, noise from single molecule localization, and noise in the segmentation and speed estimation may also contribute to the variability. To compare different conditions (strains), we used the mean speed as an estimator and its SEM to justify the difference. To differentiate the wide distributions, we also carried out K-S test to confirm the difference is significant (now included in Table S1, Tab 2). Generally, the underlying physical reason for the wide distribution is unknown and beyond the scope of the work.

How can one accurately measure the time of constriction initiation? I assume this is from phase microscopy?

This is indeed from using phase contrast microscopy, as we have previously demonstrated (e.g. Lariviere et al 2018). We used synchrony of *Caulobacter*, a well-defined and useful aspect of *Caulobacter* as an experimental model, to measure time to constriction initiation. Synchrony isolates recently divided swarmer cells from stalked and predivisional cells. By using the time of synchrony as t=0, we can calculate time to constriction initiation. Importantly, unlike in organisms that build a true septum, the earliest stages of constriction are evident as small dips in the outline of *Caulobacter* cells at the division site since *Caulobacter* exhibits a more gradual constriction rather than a septum. These are detected automatically by MicrobeJ and the time they appear is defined as the constriction initiation point. We have clarified this in the text and methods in lines 667-680.

I do not understand why stationary molecules represent. The authors suggest they are poised for activation (lines 180-1)? I thought the fast moving molecules are poised for activation, since one is arguing that activation involves molecules going from fast moving to slow.

We have rephrased our language throughout to indicate that FzlA-dependent signaling converts FtsWI to a slow-moving, active state without indicating whether it is converting from fast- to slow-moving or from stationary to slow-moving since either is possible with the data available. There is a larger stationary fraction of FtsW molecules when we treat with Fosfomycin to inhibit cell wall synthesis indicating that they are likely not active for PG synthesis.

Line 67. This should be Table S1.

This has been changed.

Line 144. What do you mean by more frequently? Do you mean more FtsWs are active?

Yes, we suggest that FzlA could cause a larger fraction of FtsWI molecules to be active at any given time.

Line 182. What is the greater proportion of active W**I* vs WT? Give numbers in text.

The total % moving and the % slow- or fast-moving are now presented in tables in each figure, as well as in Table S1.

Line 197 and entire paragraph. It is argued that FzlA does not activate FtsW**I* but causes more to be moving.(i.e. line 205-6; it is stated that W (referring to W**I*) is waing to be activated by FzlA.

We apologize for the confusion here. As noted in response to Reviewers 1 and 2, we have now removed discussion of stationary lifetimes, because differences were largely not statistically significant with additional statistical tests. We hope the revised paragraph without reference to those data is clearer. We conclude that FzlA does not regulate processivitiy of FtsW movement, but does regulate the active population (i.e. fraction that is slow-moving).

Line 218-21. Conclude that FzlA mains associated with active, slow moving. However, the fast moving population is there. Is fast moving FzlA associated with FtsZ but maybe not with FtsW?

The reviewer is correct in identifying that fast-moving FzlA are likely associated with FtsZ. This point is supported by our new data assessing the effects of GTPase-deficient FtsZ on FtsW dynamics. We clarify that fast-moving molecules are likely associated with FtsZ (Lines 336-339).

How does FzlA act as a switch? FtsN (Ecoli) is only associated with slow moving.

We do not suggest that FzlA acts as a "switch" (or "trigger"). However, we aren't clear on what the reviewer is proposing with respect to FtsN vs FzlA movement. FtsN can be stationary (bound to PG, for example) or slow-moving with PG synthases. FzlA can be stationary (treadmilling with FtsZ or, rarely, moving fast with FtsZ filament ends) or slow-moving with PG synthases. Our model is that FzlA can bind FtsK and signal to activate FtsWI when FtsK is not actively engaged in translocating DNA. The likelihood of this interaction would increase as the bulk of the chromosome is cleared from the division plane. In this way, FzlA-FtsK could act as a pacer of constriction, for example, rather than an on-off switch. This is clarified in the text and in Figure 10.

Line 248 or so. Do you see stationary FzlA? If you don't what does this mean?

We do see stationary FzlA and actually most of the FzlA we measured was stationary (see Table S1). We have added a statement about the frequency and likely cause of stationary FzlA to the text. Lines 344-345.

Line 259-60. Should you say the 'average' speed?

Yes, thanks, we have changed this.

Line 263-274. What keeps FzlA from having an effect?

Our results suggest that FzlA functions to promote a shift of FtsWI to an active state, perhaps from the fast-moving state since it does not affect the stationary proportion, but once shifted FzlA does not impact how long FtsWI is active.

What happens to FzlA movement when a FtsI inhibitor is added or FtsW is depleted?
Unfortunately we do not have a good, specific inhibitor of FtsI in *Caulobacter* (cephalexin inhibits a range of PBPs in this organism, for example). In response to reviewer 1, we have added substantial new data (Fig. 2) looking at how perturbations to PG synthesis or FtsZ impact FtsW movement. Based on those data, as well as our observations of changes in FzlA movement when FtsWI is hyperactivated, we believe our conclusions about FzlA movement are supported.

Line 270.5 How can overexpresed FzlA impact the W**I* background if there is no change in processivity or proportion of stationary W.

Overproduction of FzlA results in more active (slow-moving) FtsW**I* of those FtsW that are moving at midcell. This could occur through a shift from inactive, fast-moving, to active, slow-moving molecules.

Line 277. Cluster movement. Why not say treadmilling?

Both are accurate, but we have edited to "treadmilling" to save on word count. Thanks for the suggestion.

Line 304. This is a nice section
We'd like to thank the reviewer for the kind words.

Line 387-91. I am not sure that a failure to interact in one orientation is a sign of reduced affinity. The mutant still interacts in one orientation.

Fair enough. We have changed the language in line 434-435 to simply reflect reduced interactions observed.

Line 394-7. Perhaps mention that both conditions are lethal just that the terminal phenotypes are different.

We thank the reviewer for requesting this clarification. We have updated the Lines 440-442 to reflect this change.

November 3, 2023

RE: JCB Manuscript #202211026R

Dr. Erin Goley
Johns Hopkins University
725 N Wolfe St 520 WBSB
Baltimore, MD 21205

Dear Erin,

Thank you for submitting your revised manuscript entitled "Integration of cell wall synthesis and chromosome segregation during cell division in Caulobacter." the manuscript has been re-assessed by all of the original reviewers and we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figure formatting: Articles may have up to 10 main text figures. Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add lines to indicate the precise location of the MW markers for all blots in Figure S1.

Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Please, indicate whether 'n' refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments). If independent experiments with multiple biological replicates have been performed, we recommend using distribution-reproducibility SuperPlots (please see Lord et al., JCB 2020) to better display the distribution of the entire dataset, and report statistics (such as means, error bars, and P values) that address the reproducibility of the findings.

Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

5) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate). Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. If antibodies are not commercial, please add a reference citation if possible.

6) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope

b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures and 10 videos. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section. Please include one brief sentence per item.

9) Video legends: Should describe what is being shown, the cell type or tissue being viewed (including relevant cell treatments, concentration and duration, or transfection), the imaging method (e.g., time-lapse epifluorescence microscopy), what each color represents, how often frames were collected, the frames/second display rate, and the number of any figure that has related video stills or images.

10) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

11) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

12) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. Please note that ORCID IDs are required for all authors. At resubmission of your final files, please be sure to provide your ORCID ID and those of all co-authors.

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Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

15) Journal of Cell Biology now requires a data availability statement for all research article submissions. These statements will be published in the article directly above the Acknowledgments. The statement should address all data underlying the research presented in the manuscript. Please visit the JCB instructions for authors for guidelines and examples of statements at (https://rupress.org/jcb/pages/editorial-policies#data-availability-statement).

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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Please contact the journal office with any questions at cellbio@rockefeller.edu.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Jodi Nunnari, PhD
Editor-in-Chief
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have thoroughly addressed all my concerns, and I believe this manuscript should be published in JCB.

Reviewer #2 (Comments to the Authors (Required)):

I think the authors have done an excellent job in revising the manuscript. All my comments have been addressed. Thank you!

Reviewer #3 (Comments to the Authors (Required)):

I find the revision has responded satisfactorily to the criticisms. It appears that Cc is more dependent upon FtsK for DNA segregation than E. coli as W** bypasses FtsK for initiating constriction but has trouble completing the process.