EMBO reports

Bacterial FtsZ protein forms phase-separated condensates with its nucleoid-associated inhibitor SlmA

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

1st Editorial Decision 21 February 2018

Thank you for the submission of your manuscript to EMBO reports. I apologize for my delayed response but I have now read and discussed your work with my colleagues here, and I regret to say that we all agree that it is not well suited for our journal.

We appreciate that your study reports that crowding induces the liquid-liquid phase separation of FtsZ. You find that the formation of dynamic FtsZ condensates is enhanced in the presence of DNA-bound SlmA.

We acknowledge that you extend your previous findings on the phase separation of FtsZ by providing evidence that SlmA further promotes the formation of condensates. Clearly, your results will be of interest to researchers working in the field. However, taking these earlier findings into account, we overall feel that the conceptual advance provided is not sufficient for publication here and we have therefore decided not to proceed with in-depth peer review.

Please note that we can only publish a very small fraction of the many manuscripts that are submitted to our journal and that we therefore have to make a rather stringent selection on which ones to send out for peer review. I am sorry to have to disappoint you on this occasion, and hope that this will not prevent you from considering EMBO reports for publication of your work in the future.

Authors' response 9 March 2018

Thank you very much for considering our manuscript and for taking the time to send your comments on our work. We believe that there has been a misunderstanding though, as this manuscript is not an extension to our previous work, as you state in the text justifying your negative decision. In those previous papers we did not find condensates of FtsZ itself, but analyzed the differential distribution

of the protein oligomers and fibers in aqueous two phase systems composed of two polymers, one being PEG and the other one dextran, Ficoll or unspecific DNA, as models of cellular microenvironments possibly occurring in the cytoplasm. This allowed us to test how FtsZ organization and reactivity could be influenced by micro-environments arising from phase transitions of other unrelated molecules.

In the present manuscript we prove the formation of dynamic liquid-like condensates of FtsZ, under crowding conditions, which we observed, unexpectedly, upon addition of SlmA. Condensation is further enhanced when the specific nucleic acid sequence targeted by SlmA is also present in the solutions containing FtsZ and SlmA. We acknowledge that maybe we failed in sending a clear message in our manuscript and therefore we have modified it to better explain the different nature of both processes. We are enclosing a new version of the abstract for your consideration (*).
As we previously pointed out, the existence of these dynamic condensates would constitute a novel element of modulation of FtsZ function along its vital cycle. More importantly, our findings allow extending the concept of liquid-phase biomolecular condensates as organizers of intracellular biochemistry to bacterial systems. We believe that this is of great interest not only to researchers in the bacterial division field, but also for a very broad audience as addressed by EMBO reports.
For the reasons stated above, I would appreciate your reconsidering your negative decision regarding our manuscript.

2nd Editorial Decision 24 April 2018

Thank you for the submission of your research manuscript to our journal. I apologize for the delay in handling your manuscript but we have only recently received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, all referees also point out that important controls are missing and that the biological significance of the FtsZ condensates remains elusive at this stage.

From these comments it is clear that publication of the manuscript in our journal cannot be considered at this stage. On the other hand, given the potential interest of your findings, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. It will be important to strengthen the data on condensate formation, to provide missing controls but also to perform further experiments to show that the observed FtsZ/SlmA condensates are relevant for Z ring positioning.

Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

You can submit the revision either as a Scientific Report or as a Research Article. For Scientific Reports, the revised manuscript can contain up to 5 main figures and 5 Expanded View figures. If the revision leads to a manuscript with more than 5 main figures it will be published as a Research Article. In this case the Results and Discussion section can stay as it is now. If a Scientific Report is submitted, these sections have to be combined. This will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. In either case, all materials and methods should be included in the main manuscript file.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2

etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
(In order to avoid delays later in the publication process please check our figure guidelines before preparing the figures for your manuscript:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf)
- a separate PDF file of any Supplementary information (in its final format)
- all corresponding authors are required to provide an ORCID ID for their name. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (http://embor.embopress.org/authorguide).

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS
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Referee #1:

In this work, the authors show that the bacterial protein FtsZ makes biomolecular condensates in the presence of SlmA, a protein that is antagonistic to FtsZ polymerization, the specific DNA sequence SBS, and suitable crowding agents. These observations build on prior work from some of the authors. In the new work, the authors show that GTP depletion promotes condensate formation, and FtsZ in condensates turn over into FtsZ fibers in the presence of excess (?) GTP.

Overall, the authors present a series of intriguing results that hold tantalizing possibilities for the presence and roles of condensates in bacteria. The in vitro work is impressive if somewhat standard fare at this juncture given all that's been established in the field as of now. The most intriguing and interesting data from this work pertain to the observations regarding the interplay between condensation and polymerization of FtsZ and the GTP dependence of this interplay. The impact of different crowders and the formation of spatially organized condensates is also interesting. These results certainly merit serious consideration of the manuscript because they are sufficiently distinctive and rather compelling. However, the manuscript falls short on three aspects: A fundamental mechanistic understanding of how / why FtsZ makes condensates and a mechanistic understanding of the dependence on SlmA / SBS and crowders does not come through. The proposals based on the GTP dependence are intriguing, but a clearer demonstration of this inside cells would be more compelling. The absence of systematic titrations such as uncovering the [salt] dependence leaves one with a lack of a clear understanding of the interplay amongst depletion mediated interactions, conformational transitions, salting out vs. salting in effects, and the source of multivalency as drivers of phase separation.

The average reader of EMBO Reports will get the following impression: This looks interesting and relevant. It is quite possible, given all that's happening in the eukaryotic world, that FtsZ makes condensates that are biologically relevant. The data presented here point in this direction. However, these data fall short of directly establishing the biological bona fides and / or relevance. The alternative, which would be to uncover the details of the molecular driving forces, also goes missing.

Referee #2:

In this paper, the authors describe a macromolecular crowding induced condensate involving the E. coli division protein FtsZ and a known interaction partner, SlmA. They show convincingly that FtsZ forms condensates with SlmA (and in particular SlmA bound to its binding sequence, SBS). This is demonstrated under several different in vitro conditions, including droplets and in systems using DNA as a crowding agent.

Our major concerns are the tenuous reasoning used to support how this helps regulation of bacterial division, and the general lack of positive controls for bacterial protein condensate formation.

Major comments:

1. The authors preform necessary controls showing that, in the absence of SlmA, FtsZ does not produce condensates, and that in the absence of crowding agents FtsZ and SlmA do not produce condensates. The size of the condensates is larger and formation more robust when SlmA is in the presence of its DNA binding motif, SBS. The condensates appear to be stable.

However, we are not entirely convinced that this biologically relevant phenomenon. Specifically, We were startled by the ability of FtsZ to still form fibers under physiological (2-4mM) concentrations of GTP while in the condensate. This does not seem to be a strong argument for SlmA as a good inhibitor of FtsZ ring formation. FtsZ does not condensate without SlmA or without crowding agents, but will it form fibers in the presence of GTP? How strong are these fibers compared to fiber formation in the presence of SlmA? Supplemental figure 7F address this in the context of the PEG/dextran LLPS system, and it does seem that fiber formation is reduced in the presence of SlmA-SBS.

The authors then repeat the condensate experiments in homogenous crowders in a dual crowder

LLPS system (PEG8/dextran 500), and show that FtsZ forms condensates only in the presence of SlmA, especially with SBS present. In the dual crowder systems, the additional of GTP still causes fiber formation. Thus, we are still confused how this is helpful for the spatial regulation of the Z ring. The authors may wish to should consider moving supplemental figure 7F to the main text and better quantify the ability of fibers to form with or without SlmA.

2. The authors go on to test their system in conditions that more closely resemble the bacterial cell. This culminates in a system with a lipid droplet and using DNA as a crowding agent, an impressive technical achievement. As far as we can tell, these experiments are well done and make a strong case that this phenomenon is possible in vivo. The authors could help their argument by emphasizing that the are using physiologically relevant concentrations of proteins, etc. Also, we are not sure about the importance is of the condensate diameters, as almost all of them are greater the size of the E. coli cell, which normally has a cylindrical diameter (width) of less than 1um. Perhaps condensate diameter indicates the strength of the phase transition due to crowding, but this is not clear from the text.

3. It is mentioned that SlmA is also able to create condensates on its own under the dual crowder LLPS system (shown in Sup Fig 7BC). The authors may wish to discuss the significance of this result. It seems reasonable that SlmA, as a DNA binding protein that can also bind itself, is the more important player in this phenomenon. Does SlmA form condensates in the homogenous crowders when alone? The lack of this experiment highlights what seems to me a general lack of positive controls (i.e., situations in which condensates are sure to be made by various proteins) in the paper. Under what conditions will proteins of this size form condensates in this LLPS system? Can the authors devise as system where FtsZ does form condensates on its own, even if it is not biologically relevant? The data does not clearly indicate if or how SlmA's binding to FtsZ is a critical aspect of the system, though we are led to assume it is. If it is, what about other FtsZ binding proteins such as MinC or ZapA? MinC affects the GTPase activity of FtsZ, but not ZapA, which is thought to help bundle FtsZ.

4. We had trouble believing the hypothesis suggested in the discussion and shown in Figure 7. The authors posit that this condensation is a good strategy for the cell to use to preferentially bias Z ring formation to the midcell. On its face, it seems illogical; why would the cell form condensates of FtsZ in the cytoplasmic space around the nucleoids, potentially sequestering FtsZ away from the midcell, where the Z ring should eventually form? Moreover, why is it possible for FtsZ to still form fibers when bound to SlmA? It may be possible to resolve this dilemma, but we believe it requires a better understanding of FtsZ's propensity to form fiber inside and outside of the condensates. Generally, the nuances of this argument are not clear.

Minor comments:

1. Throughout the work, the authors used fluorescence labelled FtsZ and SlmA to assess the condensation. One important control is to show that the formation of both condensates and filaments is not specific to labelled proteins. In Fig. 1F, the authors showed that the results from turbidity measurement are consistent with fluorescence measurement, so the authors should compare the turbidity of Alexa labelled FtsZ and SlmA sample with non-labelled wild type proteins sample, at least for one assay (for example, FtsZ·SlmA·SBS in crowding condition). The experiments in which alternative labels are used are appreciated.

2. SlmA is not a widely conserved protein in bacteria, which means that the mechanism of SlmA-mediated nucleoid occlusion is specific to some species. Therefore, from the beginning of the Results session, the authors should clearly state that both FtsZ and SlmA are from E. coli.

3. Page 3, line 73, whether bacterial division ring deforms inner membrane is still an open question. So here the author should not state as a fact that division ring constricts the membrane.

4. Some of the sentences in Results session are not clear. The authors should remove them.
- Page 8 line 272-274, the last sentence in this paragraph is not supported by any evidence.
- Page 9 line 318-319, "...likely because of the influence the inhibitory SlmA complex on their size and arrangement". This sentence is again a speculation without any proof.

5. We suggest removing wording like "for the first time" (in abstract), "the first description" (page 4 line 114) and let the readers to evaluate the significance of this work by themselves.

6. Because SlmA is able to form condensates on its own, the authors must test another similarly sized DNA binding protein for its ability to form condensates, or at minimum point to negative results in the literature to show that this phenomenon is uncommon. Otherwise, it is impossible for the reader to asses whether this is a special property of FtsZ and SlmA, or a general property that can happen under these conditions. We would also suggest that they explore other FtsZ binding proteins, specifically ZapA, or justify why they did not consider other regulators.

7. If the authors want to put forth their schematic for how condensate formation can help regulate Z ring positioning, they must include a more thorough quantification of FtsZ fiber formation in the presence of SlmA and without. This can likely be done with their current data, but, to me, is glaringly deficient. If the authors instead want to limit their claims to their experimental system, which is a considerable achievement, this recommendation can be ignored.

8. During revision, the authors should consider breaking long sentences into two shorter sentences to improve readability.

Referee #3:

In this manuscript, Monterroso and co-authors describe the liquid-liquid phase separation of a complex formed by FtsZ, SlmA and SBS under crowded conditions. They found that FtsZ is, under these conditions, still able to polymerize fibers and further analysed the behaviour of these condensates in different compartmentalized systems. Although the initial finding is interesting, the contribution of the various component for the formation of these drops is not analyzed in detail nor is the biological relevance of this finding (e.g., effect on FtsZ fiber formation or stability). The manuscript would also benefit from more quantification.

Major concerns

1. Important controls to support the conclusions are missing. The first message of this manuscript is that FtsZ forms dynamic condensates upon interaction with the inhibitory complex SlmA-SBS under crowding conditions, but the contribution of each of the components for the formation of these drops is not explored. They show that FtsZ alone can not form condensates under crowded condition even at high concentrations of the protein (40 μM). However, no controls for the propensity of SlmA-SBS mixture to form condensates on their own is explored. In the second part (encapsulated/compartimentalized systems) they showed that few small condensates are observed in the SlmA-SBS mixture (e.g., Figure S7B), but only one concentration of these elements is used in these experiments. Showing a titration of the concentrations of the SlmA-SBS complex or of SlmA alone, under equal crowding conditions and similar concentration to that used for FtsZ alone (i.e., 40 μM), is an important control to support their conclusion. In most experiments, the ratio used for the formation of the condensates are 12 μM of FtsZ, 5 μM of SlmA and 1 μM of SBS but no explanation for this ratio or exploration of different ratios is shown.

The second main conclusion of this manuscript is that compartmentalization affects the distribution and localization of the condensates formed by FtsZ and SlmA. I am however not sure which is the data that supports this conclusion or what they are referring with that. The distribution of FtsZ localization in the different compartimentalizations systems (PEG/dextran, PEG/DNA) or encapsulation has already been reported by this group before (Monterroso et al., 2016; Sobrinos-Sanguino et al., 2017). Figure 3 to 6 of the main text contain the same experiments published before but now using FtsZ-SlmA-SBS condensates instead of soluble FtsZ. The localization is similar to that reported already, so I am not sure which main new information these experiments bring. The authors also conclude that liquid condensates of FtsZ and SlmA accumulate at lipid surfaces. They already shown in previous report that encapsulated FtsZ alone accumulates at the lipid surface (Monterroso et al., 2016; Sobrinos-Sanguino et al., 2017), so this result does not seem also that novel as the condensates behave as the FtsZ alone. In summary, I would recommend that they focus on the main message and show strong evidence for that and remove all that experiments that do not add much to the story and are somehow repetitive to previous published data.

2. The conclusions are reach in several occasions based on images that could have better resolution, and more quantification. Examples of this are: the effect of SBS on FTSZ-SlmA condensates formation (Fig. S2) or the effect of SlmA-SBS inhibitory complex on fiber arrangement and localization in LLPS (Fig. 3ED versus Fig. S7F).

3. The biological relevance of their main finding is not explored in detail. Assembly of FtsZ rings over unsegregated nucleoids is prevented by nucleoid occlusion. SlmA is, in E. coli, implicated in this mechanism. To inhibit Z ring formation SlmA must be bound to SBS (SlmA binding site). SBS sites are numerous and scattered through the chromosome with exception of the Ter region. The current mechanistic model is that SlmA works by sequestration and depolymerization of FtsZ in the nucleoid, competing with its binding partner at the membrane, FtsA. Some competition experiments with FtsA, the use of a FtsZ mutants or a more in deep exploration of the effect of this condensate on fiber formation will be needed for some stronger biological relevance. The use of a long DNA sequence with multiple SBS site (BAC) in the encapsulation experiment could also bring interesting data on its sequestration from the lipid bilayer.

Minor concerns

I. Figure 1 contain images with different concentration of FtsZ (25 μM in B and E and 12 μM D and E) and different concentration of each crowder (15%, 8% or 5%). It would be better to use images with the same concentration of protein and percentage of crowder to compare them.

II. Scale bars are of different sizes in different panels of the same figure, suggesting different resolution used for each condition. It would be better to compare images at the same magnification.

III. Concentration of proteins in some panels are missing (e.g., concentration of SlmA in Fig. S5B).

IV. Figure 2A, B and C contain the same experiment (fresh FtsZ incorporation into drops) repeated with 3 different crowders. I would suggest to remove any repetitive information from main figures and move it to supplementary.

V. Fiber formation in Figure 2D can not be appreciated. I would suggest to try to get images with better resolution in general, showing less panels per figure and make them bigger.

VI. Co-localization of SBS and FtsZ in Figure 4BC, compare to SlmA and FtsZ in Figure 4D seems completely different. I would rather say that SlmA and FtsZ do not colocalize in Figure 4D. It would interesting to know if in these conditions (Figure 4D, 6 μM of FtsZ, 3 μM of SlmA and 0.5 μM) but in the absence of GTP condensate form and compare this result with or without GTP to that with double concentration of proteins but keeping the GTP constant.

References

Monterroso, B., Zorrilla, S., Sobrinos-Sanguino, M., Keating, C.D., and Rivas, G. (2016). Microenvironments created by liquid-liquid phase transition control the dynamic distribution of bacterial division FtsZ protein. Scientific reports 6, 35140.

Sobrinos-Sanguino, M., Zorrilla, S., Keating, C.D., Monterroso, B., and Rivas, G. (2017). Encapsulation of a compartmentalized cytoplasm mimic within a lipid membrane by microfluidics. Chemical Communications 53, 4775-4778.

REFEREE #1:

Overall, the authors present a series of intriguing results that hold tantalizing possibilities for the presence and roles of condensates in bacteria. The in vitro work is impressive if somewhat standard fare at this juncture given all that's been established in the field as of now. The most intriguing and interesting data from this work pertain to the observations regarding the interplay between condensation and polymerization of FtsZ and the GTP dependence of this interplay. The impact of different crowders and the formation of spatially organized condensates is also interesting. These results certainly merit serious consideration of the manuscript because they are sufficiently distinctive and rather compelling.

We thank the reviewer for these positive comments.

R1.1: *However, the manuscript falls short on three aspects: A fundamental mechanistic understanding of how/why FtsZ makes condensates and a mechanistic understanding of the dependence on SlmA/SBS and crowders does not come through. The proposals based on the GTP dependence are intriguing, but a clearer demonstration of this inside cells would be more compelling.*

The absence of systematic titrations such as uncovering the [salt] dependence leaves one with a lack of a clear understanding of the interplay amongst depletion mediated interactions, conformational transitions, salting out vs. salting in effects, and the source of multivalency as drivers of phase separation.

The average reader of EMBO Reports will get the following impression: This looks interesting and relevant. It is quite possible, given all that's happening in the eukaryotic world, that FtsZ makes condensates that are biologically relevant. The data presented here point in this direction. However, these data fall short of directly establishing the biological bona fides and/or relevance. The alternative, which would be to uncover the details of the molecular driving forces, also goes missing.

1) Following the reviewer's suggestions we have now conducted systematic titrations to better understand the factors driving the formation of the condensates. These experiments include turbidity measurements and confocal imaging of samples, in dextran 500 as crowding agent, at i) different concentrations of FtsZ, SlmA and SBS ii) different concentrations of salt (KCl) and iii) different concentrations of the crowder. These results are now included in the revised version of the manuscript (Pages 5-6, and **Fig 2** and **Appendix Fig S5**).

We have found that the number of condensates in solution critically depends on the **concentrations of the three components** (FtsZ, SlmA and SBS). Moreover, in the absence of SBS, even at relatively high concentrations of the two proteins, aggregates are scarce (see **Appendix Figs S3** and **S4A** in the revised MS), as previously shown in the LLPS systems in the initial version of the MS. In the presence of SBS, condensation increases with the concentrations of the three elements in the

solutions, probably because the interactions between FtsZ and SlmA·SBS and the multivalency of the whole system are favored (see **FIGURE A1** at the end of this document, included as **Fig 2A** in the revised MS).

The formation of condensates is also strongly dependent on volume exclusion as clear formation is only observed above ~50-100 g/L dextran (see **FIGURE A2** at the end of this document, included as **Appendix Fig S5A** in the revised MS). This observation further reinforces the idea that **crowding favors condensation** as previously demonstrated for other systems exhibiting this kind of behavior (Walter and Brooks 1995, Banani et al. 2017).

According to the new experiments performed, a decrease in the **ionic strength** of the solution enhances FtsZ·SlmA·SBS condensation (see **FIGURE A3** at the end of this document, included as **Fig 2B** and **Appendix Fig S5B** in the revised MS). Formation of the FtsZ·SlmA·SBS condensates may be facilitated by interactions between the negatively charged FtsZ (pI 4.7), positively charged SlmA (pI 8.8) and negatively charged SBS. These electrostatic interactions would be enhanced at low salt concentration and shielded at higher salt concentrations.

In conclusion, protein/nucleic acid concentration, volume exclusion and low ionic strength are key factors driving the formation of the condensates of FtsZ·SlmA·SBS likely because they promote the self-association of FtsZ in the absence of GTP (Rivas et al. 2000, Rivas et al. 2001) and the interactions of SlmA with the SBS and with FtsZ (Du and Lutkenhaus 2014, Cabre et al. 2015).

2) In order to better understand the "*interplay between condensation and polymerization of FtsZ and the GTP dependence of this interplay"* we have also analyzed the evolution of the condensates into fibers upon **addition of GTP** in more detail by confocal and time-lapse imaging. These results have been included in the revised version of the manuscript (Page 7 and **Figs 3** and **EV1**). We have prepared new samples in dextran 500 containing the FtsZ·SlmA·SBS condensates and followed the evolution with time, after triggering polymerization with GTP. Our results show that FtsZ polymerizes into filaments in which the protein colocalizes with SlmA·SBS. In addition, compared with the control lacking SlmA·SBS, the fibers appear thinner and they disassemble much more rapidly upon GTP depletion (see **FIGURE A4** at the end of this document, included as **Fig 3C** and **Fig EV1A** in the revised MS). We also observed that, after disassembly of the fibers, FtsZ·SlmA·SBS form condensates of bigger diameter than those before FtsZ polymerization.

In addition, we have performed new experiments in which SlmA·SBS complexes are added to preformed FtsZ-GTP fibers in dextran 500 (**FIGURE A5** at the end of this document, included as **Fig EV1B** in the revised MS). These experiments show a reduction in the lifetime of the filaments and the formation of round condensates of FtsZ·SlmA·SBS upon GTP depletion. This indicates that the control of FtsZ fiber formation by SlmA·SBS is not only exerted through condensation with the unassembled form (FtsZ-GDP) but also through interaction with preformed fibers, that will be disassembled by SlmA·SBS and incorporated into condensates.

3) Finally, the potential biological implications of our findings have been extensively discussed in the revised version of the Discussion (Page 13). Please see also the specific answers to questions **R2.1**, **R2.4**, and **R3.3** below.

REFEREE #2:

In this paper, the authors describe a macromolecular crowding induced condensate involving the E. coli division protein FtsZ and a known interaction partner, SlmA. They show convincingly that FtsZ forms condensates with SlmA (and in particular SlmA bound to its binding sequence, SBS). This is demonstrated under several different in vitro conditions, including droplets and in systems using DNA as a crowding agent. Our major concerns are the tenuous reasoning used to support how this helps regulation of bacterial division, and the general lack of positive controls for bacterial protein condensate formation.

We thank the reviewer for the nice comments on the work presented. The potential biological implications of our findings have been extensively discussed in the revised version of the Discussion. Please see also the specific answers to questions **R2.1**, **R2.4**, and **R3.3** below.

Major comments:

R2.1: *The authors perform necessary controls showing that, in the absence of SlmA, FtsZ does not produce condensates, and that in the absence of crowding agents FtsZ and SlmA do not produce condensates. The size of the condensates is larger and formation more robust when SlmA is in the presence of its DNA binding motif, SBS. The condensates appear to be stable.*

However, we are not entirely convinced that this biologically relevant phenomenon. Specifically, we were startled by the ability of FtsZ to still form fibers under physiological (2-4mM) concentrations of GTP while in the condensate. This does not seem to be a strong argument for SlmA as a good inhibitor of FtsZ ring formation. FtsZ does not condensate without SlmA or without crowding agents, but will it form fibers in the presence of GTP? How strong are these fibers compared to fiber formation in the presence of SlmA? Supplemental figure 7F address this in the context of the PEG/dextran LLPS system, and it does seem that fiber formation is reduced in the presence of SlmA-SBS.

The authors then repeat the condensate experiments in homogenous crowders in a dual crowder LLPS system (PEG8/dextran 500), and show that FtsZ forms condensates only in the presence of SlmA, especially with SBS present. In the dual crowder systems, the additional of GTP still causes fiber formation. Thus, we are still confused how this is helpful for the spatial regulation of the Z ring.

The authors may wish to should consider moving supplemental figure 7F to the main text and better quantify the ability of fibers to form with or without SlmA.

The reviewer is right to question why FtsZ can form GTP induced fibers in the presence of an inhibitor of FtsZ polymerization. However, in most of the reports dealing with the effect of SlmA·SBS on FtsZ polymerization, fibers of FtsZ are still observed, depending on the conditions and relative concentrations of the three elements. Indeed, SlmA·SBS complexes accelerate the disassembly of FtsZ fibers resulting in shorter species with the same GTPase activity of the intact

single stranded filaments (Tonthat et al. 2013, Cabre et al. 2015) but do not preclude fiber formation (Tonthat et al. 2011, Tonthat et al. 2013). This is because, in contrast to other proteins that completely block polymerization by sequestration of FtsZ monomer pools (like SulA (Chen et al. 2012), OpgH (Hill et al. 2013) or Kil (Hernandez-Rocamora et al. 2015)), SlmA inhibition only needs to counteract the assembly of some of the cellular FtsZ in the vicinity of the nucleoid, allowing the remaining FtsZ in the cell to be poised for assembly into an FtsZ ring at midcell.

The fact that FtsZ can still form fibers when bound to SlmA may be explained as follows. According to Du and Lutkenhaus (Du and Lutkenhaus 2014), SlmA accesses FtsZ by binding to its C terminus, then severs FtsZ filaments by also binding to the core region of FtsZ. One could imagine that many SlmA molecules may not be always able to achieve both interaction steps, and only interact with the FtsZ C terminus. This would not block FtsZ polymerization. Moreover, if the FtsZ·SlmA·SBS interaction depends upon the multivalency of the FtsZ C terminus as proposed (Schumacher and Zeng 2016), then as FtsZ filaments become shorter, they will be less likely to stay bound by SlmA·SBS. This in turn might result in some limited reassembly of FtsZ filaments, keeping them in a shortened equilibrium state. Also, SlmA effect does not result in total disassembly of FtsZ filaments but only reduces the lifetime, as showed in Cabre et al. (Cabre et al. 2015). The fact that FtsZ mutants D86N and K190V (in the core domain) and K380M (at the C terminus) still form filaments when bound to SlmA indicates that the loss of one charged residue in the target is enough to prevent SlmA from breaking FtsZ fibers. Given that formation of the FtsZ·SlmA·SBS condensates may be facilitated by interactions between the negatively charged FtsZ, positively charged SlmA (pI 8.8) and negatively charged SBS, it is not surprising that changing a charged residue in FtsZ might have a large impact on the system. We have included a comment on this in the revised Discussion (Page 14).

In order to analyze the ability of fibers to form with or without SlmA in greater detail, as suggested by the reviewer, we have conducted additional measurements of the polymerization of FtsZ in the presence of SlmA·SBS in dextran 500 (see **R2.11** below and **FIGURE A4** and **A5** at the end of this document). Original supplemental **Fig 7F** has been moved to **Fig EV2C**.

R2.2: *The authors go on to test their system in conditions that more closely resemble the bacterial cell. This culminates in a system with a lipid droplet and using DNA as a crowding agent, an impressive technical achievement. As far as we can tell, these experiments are well done and make a strong case that this phenomenon is possible in vivo. The authors could help their argument by emphasizing that they are using physiologically relevant concentrations of proteins, etc. Also, we are not sure about the importance is of the condensate diameters, as almost all of them are greater the size of the E. coli cell, which normally has a cylindrical diameter (width) of less than 1um. Perhaps condensate diameter indicates the strength of the phase transition due to crowding, but this is not clear from the text.*

We thank the reviewer for appreciating these aspects of the work.
About the diameters of the condensates, we agree that they may not be physiologically meaningful, and hence we have reduced the description of these results and shifted the graph with the distributions to the Appendix. Nevertheless, they are similar (or a bit smaller) to the diameters

reported in the literature for eukaryotic condensates *in vitro*, and the sizes greater than the bacterial cell size can be due to the effect of the crowders, as the reviewer says, and/or to diffusion of material from the whole sample, particularly in the case of the bulk systems. Indeed, when encapsulated, condensates showed a smaller size.

R2.3: *It is mentioned that SlmA is also able to create condensates on its own under the dual crowder LLPS system (shown in Sup Fig 7BC). The authors may wish to discuss the significance of this result. It seems reasonable that SlmA, as a DNA binding protein that can also bind itself, is the more important player in this phenomenon. Does SlmA form condensates in the homogenous crowders when alone? The lack of this experiment highlights what seems to me a general lack of positive controls (i.e., situations in which condensates are sure to be made by various proteins) in the paper. Under what conditions will proteins of this size form condensates in this LLPS system?*

The experiments showing the behavior of SlmA and SlmA·SBS in homogeneous crowders suggested by the reviewer have been conducted and included in the revised manuscript. Confocal imaging of samples in dextran and Ficoll show that SlmA forms scarce round condensates both in the absence and presence of SBS (see **FIGURE A6B** at the end of this document, included as **Appendix Fig S4B** in the revised MS), similarly to that found in the PEG/dextran LLPS system. The turbidity measured in dextran for samples containing SlmA with or without the SBS is much lower than when FtsZ is present (see **FIGURE A6A** at the end of this document, included as **Appendix Fig S4A** in the revised MS). In PEG, we observe large structures of SlmA, a bit more abundant than in the other two crowders and less round, which may be related with the low tendency of SlmA to partition into this phase in the LLPS systems in which PEG coexists with dextran or DNA.

These experiments reinforce the idea that it is the presence of the three elements that clearly promotes condensation, as the ternary complexes exhibit three typical features associated with crowding-driven condensation, namely multivalency (FtsZ self-associates and SlmA binds to the SBS with a stoichiometry exceeding 1:1), binding of nucleic acids (through SlmA) and partially unstructured regions (FtsZ linker).

Can the authors devise as system where FtsZ does form condensates on its own, even if it is not biologically relevant?

We were not able to find conditions under which FtsZ forms condensates on its own, even increasing its concentration to 40 μM, far beyond the physiological one, or decreasing salt to 100 mM. However, we cannot rule out the possibility that this protein, given its multivalency and the presence of an unstructured region, could form condensates by itself under certain conditions. We have included a comment in the revised version of the manuscript to acknowledge this possibility (Page 12).

The data does not clearly indicate if or how SlmA's binding to FtsZ is a critical aspect of the system, though we are led to assume it is. If it is, what about other FtsZ binding proteins such as MinC or ZapA? MinC affects the GTPase activity of FtsZ, but not ZapA, which is thought to help bundle FtsZ.

This is a very interesting question. We have performed preliminary turbidity and confocal imaging experiments using MinC, a protein we have previously purified and characterized in the lab, and we have detected significant turbidity (0.031±0.004), that increases upon addition of FtsZ (0.137±0.006), in 150 g/L dextran 500. In the images, large structures are profusely observed both in the absence and presence of FtsZ (**FIGURE A7** at the end of this document), some of which resemble the condensates observed for FtsZ·SlmA·SBS. At this point, we cannot confirm or discard the formation of condensates by MinC. This would require a full study out of the scope of the current manuscript, involving different ratios and concentrations of the proteins, different crowders, different combinations of labels, different buffer conditions, removing the His-tag from the preparation of MinC used, etc. We have included a comment in the revised version of the manuscript on the possibility that other proteins interacting with FtsZ could lead to condensation and that this fact could deserve further investigation in the field (Page 12).

R2.4: *We had trouble believing the hypothesis suggested in the discussion and shown in Figure 7. The authors posit that this condensation is a good strategy for the cell to use to preferentially bias Z ring formation to the midcell. On its face, it seems illogical; why would the cell form condensates of FtsZ in the cytoplasmic space around the nucleoids, potentially sequestering FtsZ away from the midcell, where the Z ring should eventually form? Moreover, why is it possible for FtsZ to still form fibers when bound to SlmA? It may be possible to resolve this dilemma, but we believe it requires a better understanding of FtsZ's propensity to form fiber inside and outside of the condensates. Generally, the nuances of this argument are not clear.*

We have made some changes in **Figure 7** (which is now **Fig 6** in the revised manuscript) to clarify the proposed mechanism. We did not intend to propose that condensation could be "*a good strategy for the cell to use to preferentially bias Z ring formation to the midcell*", but rather that the FtsZ·SlmA·SBS condensates would harbor less polymeric forms of FtsZ, suppressing the ability of FtsZ in the nucleoid regions to self-assemble into aligned filaments at the membrane. As a result, we propose that FtsZ·SlmA·SBS condensates would not sequester all the FtsZ. In fact, the nature of the condensates means that a significant fraction of FtsZ is still outside the condensate and available to assemble into fibers on the membrane at midcell. Of course, it is important to emphasize that inhibition of FtsZ assembly by SlmA acts as a backup system, as inactivation of SlmA has no detectable phenotype in cells with normal chromosome replication and segregation. We have explained in detail the potential biological implications of our results, illustrated in Figure 6, in the discussion section of the revised MS (Page 13).

Minor comments:

R2.5: *Throughout the work, the authors used fluorescence labelled FtsZ and SlmA to assess the condensation. One important control is to show that the formation of both condensates and filaments is not specific to labelled proteins. In Fig. 1F, the authors showed that the results from turbidity measurement are consistent with fluorescence measurement, so the authors should compare the*

turbidity of Alexa labelled FtsZ and SlmA sample with non-labelled wild type proteins sample, at least for one assay (for example, FtsZ·SlmA·SBS in crowding condition). The experiments in which alternative labels are used are appreciated.

We have conducted the control measurement suggested by the reviewer by including Alexa 488 labeled FtsZ in samples containing FtsZ·SlmA·SBS (12 µM/5 µM/ 1 µM) in working buffer (300 mM KCl) and 150 g/L dextran 500. The turbidity obtained for the unlabeled proteins was 0.20±0.02, a value equal, within the error, to that obtained in the presence of FtsZ-Alexa 488 (0.184±0.003). Likewise, we have imaged the FtsZ·SlmA·SBS condensates without labeled protein or SBS in working buffer (300 mM KCl) and 150 g/L dextran 500. Transmitted images clearly show the condensates (see **FIGURE A8** at the end of this document).

R2.6: *SlmA is not a widely conserved protein in bacteria, which means that the mechanism of SlmA-mediated nucleoid occlusion is specific to some species. Therefore, from the beginning of the Results session, the authors should clearly state that both FtsZ and SlmA are from E. coli.*

The statement has been included in results section (Page 5 of the revised MS) and in the introduction (Page 3 of the revised MS).

R2.7: *Page 3, line 73, whether bacterial division ring deforms inner membrane is still an open question. So here the author should not state as a fact that division ring constricts the membrane.*

This statement has been removed.

R2.8: *Some of the sentences in Results session are not clear. The authors should remove them.*
- Page 8 line 272-274, the last sentence in this paragraph is not supported by any evidence.
- Page 9 line 318-319, "...likely because of the influence the inhibitory SlmA complex on their size and arrangement". This sentence is again a speculation without any proof.

These sentences have been modified/removed.

R2.9: *We suggest removing wording like "for the first time" (in abstract), "the first description" (page 4 line 114) and let the readers to evaluate the significance of this work by themselves.*

Sentences have been removed/toned-down.

R2.10: *Because SlmA is able to form condensates on its own, the authors must test another similarly sized DNA binding protein for its ability to form condensates, or at minimum point to negative results in the literature to show that this phenomenon is uncommon. Otherwise, it is impossible for the reader to assess whether this is a special property of FtsZ and SlmA, or a general property that can happen under these conditions. We would also suggest that they explore other FtsZ binding proteins, specifically ZapA, or justify why they did not consider other regulators.*

We have tested the ability of MatP, a protein of similar size as SlmA, to form condensates in the presence of its specific target DNA sequence (*matS*) in 150 g/L dextran. We observed aggregates that may resemble those in the SlmA·SBS samples, but these were rare (see **FIGURE A9** at the end of this document). Their low abundance is also reflected in the low turbidity of these samples, which is virtually 0 (-0.004±0.01).

The number of examples of proteins displaying condensation in eukaryotes has increased exponentially in recent years so it is possible that such condensation is more prevalent than expected. However, it seems that condensation is dependent on several special conditions including nucleic acid binding, multivalency and the presence of unstructured regions. Although the mere presence of these conditions may not suffice to form condensates depending on each particular case, so far reported cases (all eukaryotic) present some/all of them and these features are also found in the FtsZ·SlmA·SBS complexes (see answer to **R2.3**). This is the reason why we chose SlmA for this study. Among the modulators of FtsZ assembly, SlmA is the one that interacts directly and simultaneously with FtsZ and nucleic acids. Analysis of the formation of condensates in the presence of other regulators may represent an interesting line of future investigation in this field (see answer to **R2.3**).

R2.11: *If the authors want to put forth their schematic for how condensate formation can help regulate Z ring positioning, they must include a more thorough quantification of FtsZ fiber formation in the presence of SlmA and without. This can likely be done with their current data, but, to me, is glaringly deficient. If the authors instead want to limit their claims to their experimental system, which is a considerable achievement, this recommendation can be ignored.*

Following the reviewer's suggestion, we have conducted a more thorough analysis of the interplay between fibers and condensates in single crowding conditions to illustrate this point. We have analyzed in more detail the evolution of the condensates into fibers upon addition of GTP by confocal and time-lapse imaging, and included these results in the revised version of the manuscript (Page 7 and **Figs 3** and **EV1**). We have prepared new samples in dextran 500 containing the FtsZ·SlmA·SBS condensates and followed the evolution with time, after triggering polymerization with GTP. Our results show that FtsZ polymerizes into filaments in which the protein colocalizes with SlmA·SBS. In addition, compared with the control lacking SlmA·SBS, the fibers appear thinner and they disassemble much more rapidly upon GTP depletion (see **FIGURE A4** at the end of this document, included as **Figs 3C** and **EV1A** in the revised MS). We also observed that, after disassembly of the filaments, FtsZ·SlmA·SBS form condensates of larger diameter than those before FtsZ polymerization.

In addition, we have performed new experiments in which SlmA·SBS complexes are added on preformed FtsZ-GTP fibers in dextran 500 (see answer to **R1.1** and **FIGURE A5** at the end of this document, included as **Fig EV1B** in the revised MS). These experiments show a reduction in the lifetime of the filaments and the formation of round condensates of FtsZ·SlmA·SBS upon GTP depletion. This indicates that the control of FtsZ fiber formation by SlmA·SBS is not only exerted through condensation with the unassembled form (FtsZ-GDP) but also through interaction with preformed fibers, that will be disassembled by SlmA·SBS and incorporated into condensates.

R2.12: *During revision, the authors should consider breaking long sentences into two shorter sentences to improve readability.*

We have significantly edited the text in the revised manuscript.

REFEREE #3:

In this manuscript, Monterroso and co-authors describe the liquid-liquid phase separation of a complex formed by FtsZ, SlmA and SBS under crowded conditions. They found that FtsZ is, under these conditions, still able to polymerize fibers and further analysed the behaviour of these condensates in different compartmentalized systems. Although the initial finding is interesting, the contribution of the various component for the formation of these drops is not analyzed in detail nor is the biological relevance of this finding (e.g., effect on FtsZ fiber formation or stability). The manuscript would also benefit from more quantification.

Major concerns

R3.1: *Important controls to support the conclusions are missing. The first message of this manuscript is that FtsZ forms dynamic condensates upon interaction with the inhibitory complex SlmA-SBS under crowding conditions, but the contribution of each of the components for the formation of these drops is not explored.*

Following the suggestions of this and other reviewers we have now conducted systematic titrations to better understand the factors driving the formation of the condensates. These experiments include turbidity and confocal imaging of samples, in dextran 500 as crowding agent, at 1) different concentrations of FtsZ, SlmA and SBS, 2) different concentrations of salt (KCl) and 3) different concentrations of the crowder. These results are now included in the revised version of the manuscript (Pages 5-6 and **Fig 2** and **Appendix Fig S5**).

We have found that the number of condensates in the solution critically depends on the concentrations of the three components (FtsZ, SlmA and SBS). Moreover, in the absence of SBS, even at relatively high concentrations of the two proteins, scarce aggregates are found (see Appendix **Figs S3** and **S4A** in the revised MS), as previously shown in the LLPS systems in the initial version of the MS. In the presence of the SBS, condensation increases with the concentrations of the three elements in the solutions, probably because the interactions between FtsZ and SlmA·SBS and the multivalency of the whole system are favored (see **FIGURE A1** at the end of this document, included as **Fig 2A** in the revised MS). In addition we have found that volume exclusion and low ionic strength are key factors driving the formation of the condensates of FtsZ·SlmA·SBS (see **FIGURES A2** and **A3** at the end of this document) likely because they promote the self-association of FtsZ in the absence of GTP (Rivas et al. 2000, Rivas et al. 2001) and the interactions of SlmA with the SBS and with FtsZ (Du and Lutkenhaus 2014, Cabre et al. 2015).

They show that FtsZ alone cannot form condensates under crowded condition even at high concentrations of the protein (40 μM). However, no controls for the propensity of SlmA-SBS mixture to form condensates on their own is explored.
In the second part (encapsulated/compartimentalized systems) they showed that few small condensates are observed in the SlmA-SBS mixture (e.g., Figure S7B), but only one concentration of these elements is used in these experiments.

We have also explored the ability of SlmA with and without SBS to form the condensates in different crowding conditions and at different concentrations, and the information is now included in the Appendix of the manuscript (**Appendix Fig S4**). Confocal imaging of samples in dextran and Ficoll show that SlmA forms scarce round condensates both in the absence and presence of SBS (see **FIGURE A6B** at the end of this document, included as **Appendix Fig S4B** in the revised MS), similarly to that found in the PEG/dextran LLPS system. The turbidity measured in dextran for samples containing SlmA with or without the SBS is much lower than when FtsZ is present (see **FIGURE A6A** at the end of this document, included as **Appendix Fig S4A** in the revised MS). In PEG, we observe large structures of SlmA, a bit more abundant than in the other two crowders and less round, which may be related with the low tendency of SlmA to partition into this phase in the LLPS systems in which PEG coexists with dextran or DNA.

Showing a titration of the concentrations of the SlmA-SBS complex or of SlmA alone, under equal crowding conditions and similar concentration to that used for FtsZ alone (i.e., 40 μM), is an important control to support their conclusion.

We have conducted the control suggested by the reviewer using SlmA at 40 μM in 150 g/L dextran 500. At this concentration, we find several aggregates both in the absence and presence of the SBS (see **FIGURE A10** at the end of this document). We believe that some of these aggregates could be in fact the condensates we are studying but this may be an artifact due to the high concentration of SlmA, largely exceeding the one used in any published study on the protein. We have included this experiment in the revised version of the manuscript (see **Appendix Fig S4C**).

In most experiments, the ratio used for the formation of the condensates are 12 μM of FtsZ, 5 μM of SlmA and 1 μM of SBS but no explanation for this ratio or exploration of different ratios is shown.

Following referee's suggestions, we have performed additional experiments at different concentrations of the three elements (see above). The concentrations are in the order of those typically employed in other studies on FtsZ and on SlmA·SBS. When choosing the concentrations we have essentially kept a 1:5 SBS:SlmA molar ratio to ensure the formation of the 1:4 SBS·SlmA complex previously described in solution (Tonthat et al. 2013, Cabre et al. 2015).

R3.2: *The second main conclusion of this manuscript is that compartmentalization affects the distribution and localization of the condensates formed by FtsZ and SlmA. I am however not sure which is the data that supports this conclusion or what they are referring with that. The distribution of FtsZ localization in the different compartimentalization systems (PEG/dextran, PEG/DNA) or encapsulation has already been reported by this group before (Monterroso et al., 2016; Sobrinos-Sanguino et al., 2017). Figure 3 to 6 of the main text contain the same experiments published before but now using FtsZ-SlmA-SBS condensates instead of soluble FtsZ. The localization is similar to that reported already, so I am not sure which main new information these experiments bring.*

We conclude that compartmentalization affects the distribution and localization of the condensates formed by FtsZ·SlmA·SBS, because in the model LLPS systems studied these condensates

preferentially partition into one of the phases, instead of being homogeneously distributed within both crowder solutions.

The reviewer is correct that there is no significant difference in the preferential distribution among the two phases of the LLPS systems when comparing FtsZ alone, which we previously reported, and its condensates with SlmA·SBS, which we report here . We have found here that SlmA and the SBS preferentially partition in these systems into the same phase as FtsZ, what was not foreseeable given their properties are very different from those of FtsZ. If FtsZ, SlmA and the SBS would have distributed differently in the LLPS systems their interaction would have been disfavored, thus reducing or even precluding condensate formation. As understanding this is not the focus of the manuscript, part of this information has been shifted to the Appendix and the corresponding description in the results section has been considerably shortened.

The authors also conclude that liquid condensates of FtsZ and SlmA accumulate at lipid surfaces. They already shown in previous report that encapsulated FtsZ alone accumulates at the lipid surface (Monterroso et al., 2016; Sobrinos-Sanguino et al., 2017), so this result does not seem also that novel as the condensates behave as the FtsZ alone.

It is true that FtsZ alone does in fact interact with the lipid boundary, to a greater or lesser extent depending on its association state (FtsZ-GTP fibers accumulate much more than FtsZ-GDP oligomers). However, condensates formed upon interaction of FtsZ-GDP with SlmA·SBS are prone to locate almost exclusively at the membrane, strongly suggesting that the tendency of FtsZ is greatly enhanced due to the final properties acquired upon condensation. Moreover, microfluidic encapsulation experiments contribute to show the formation of FtsZ·SlmA·SBS condensates under confined conditions mimicking the cellular environment.

In summary, I would recommend that they focus on the main message and show strong evidence for that and remove all that experiments that do not add much to the story and are somehow repetitive to previous published data.

Following the reviewer's suggestion, we have significantly reduced the text in these sections to focus on the main points of the study and shifted some of the associated figures to supplementary information.

R3.2: *The conclusions are reach in several occasions based on images that could have better resolution, and more quantification. Examples of this are: the effect of SBS on FTSZ-SlmA condensates formation (Fig. S2) or the effect of SlmA-SBS inhibitory complex on fiber arrangement and localization in LLPS (Fig. 3ED versus Fig. S7F).*

We have generally improved the images in the revised version of the manuscript, principally those pertaining to the effect of SlmA·SBS on FtsZ fibers (see **Figs 3**, **EV1 and EV2 and Appendix Fig S3** in the revised MS). In particular, the two figures mentioned have been replaced by better ones.

R3.3: *The biological relevance of their main finding is not explored in detail. Assembly of FtsZ rings over unsegregated nucleoids is prevented by nucleoid occlusion. SlmA is, in E. coli, implicated in this mechanism. To inhibit Z ring formation SlmA must be bound to SBS (SlmA binding site). SBS sites are numerous and scattered through the chromosome with exception of the Ter region. The current mechanistic model is that SlmA works by sequestration and depolymerization of FtsZ in the nucleoid, competing with its binding partner at the membrane, FtsA. Some competition experiments with FtsA, the use of a FtsZ mutants or a more in deep exploration of the effect of this condensate on fiber formation will be needed for some stronger biological relevance. The use of a long DNA sequence with multiple SBS site (BAC) in the encapsulation experiment could also bring interesting data on its sequestration from the lipid bilayer.*

Following the reviewer's recommendation, we have used confocal and time-lapse imaging to analyze in more detail the evolution of the condensates into fibers upon addition of GTP. These results have been included in the revised version of the manuscript (Page 7 and **Fig. 3** and **EV1** of revised MS).

We have prepared new samples in dextran 500 containing the FtsZ·SlmA·SBS condensates and followed the evolution with time, after triggering polymerization with GTP (see **FIGURE A4** at the end of this document). Our results show that, compared with the control lacking SlmA·SBS the fibers appear thinner and they disassemble much more rapidly. This is in line with the observation that SlmA·SBS reduces the lifetime of FtsZ filaments (Cabre et al. 2015). We also observe that, upon disassembly of the fibers, the condensates of FtsZ·SlmA·SBS reassemble into condensates with larger diameters than those before FtsZ polymerization.

In addition, we have performed new experiments in which SlmA·SBS complexes are added to preformed FtsZ-GTP fibers in dextran 500 (see **FIGURE A5** at the end of this document, included as and **Fig EV1B** in the revised MS). These experiments show a reduction in the lifetime of the polymers and the formation of round condensates of FtsZ·SlmA·SBS upon GTP depletion. This indicates that the control of FtsZ fiber formation by SlmA·SBS is not only exerted through condensation with the unassembled form (FtsZ-GDP) but also through interaction with preformed fibers, that are disassembled by SlmA·SBS and incorporated into condensates.

The suggested analysis of FtsZ mutants would certainly contribute to a detailed description of the mechanism, but we believe it is out of the scope of the current work as it would involve a careful selection of the mutants based on the structure of FtsZ and their thorough characterization in terms of their oligomerization in the absence of GTP, GTP induced polymerization and interactions with SlmA·SBS.

We agree that as FtsA and ZipA also bind the FtsZ C terminus, SlmA may be able to antagonize the ability of these two membrane anchors to recruit FtsZ to the membrane. However, the ability of SlmA·SBS to shorten FtsZ filaments *in vitro* suggests that competition is not the major effect of SlmA on FtsZ assembly. Given how little is known about the interplay between FtsA and ZipA in the membrane attachment of FtsZ filaments *in vivo* or *in vitro*, it would be beyond the scope of this initial study to include them in evaluating their effects on the condensates. However, this is certainly

an excellent future direction to take this line of investigation. We have included a comment on this in the revised version of the MS (Page 14).

Minor concerns

R3.4: *Figure 1 contain images with different concentration of FtsZ (25 µM in B and E and 12 µM D and E) and different concentration of each crowder (15%, 8% or 5%). It would be better to use images with the same concentration of protein and percentage of crowder to compare them.*

Images at the same concentration of different crowders and of FtsZ·SlmA·SBS have been included in **Fig 1** in the revised version of the manuscript. In the interest of clarity, images of condensates in PEG have been moved to the Appendix.

R3.5: *Scale bars are of different sizes in different panels of the same figure, suggesting different resolution used for each condition. It would be better to compare images at the same magnification.*

We have included images with similar scale bar sizes in the revised version of the manuscript, when possible.

R3.6: *Concentration of proteins in some panels are missing (e.g., concentration of SlmA in Fig. S5B).*

We have added a statement in the Materials and Methods section indicating that the concentrations of FtsZ, SlmA and SBS, when present, are 12, 5 and 1 µM, respectively (standard concentrations), unless otherwise indicated (Page 15 of the revised MS). In addition, we have specified the concentrations different from these values defined as "standard" in the corresponding figure legends. Moreover, to avoid confusion, in most cases, even when using the "standard concentrations" we have actually specified them in the figure legends. The concentration of SlmA in original figure S5B, **Appendix Fig S10B** of the revised version, was 5 µM. This has now been stated in the figure caption.

R3.7: *Figure 2A, B and C contain the same experiment (fresh FtsZ incorporation into drops) repeated with 3 different crowders. I would suggest to remove any repetitive information from main figures and move it to supplementary.*

We have shifted some figures to the Appendix.

R3.8: *Fiber formation in Figure 2D cannot be appreciated. I would suggest to try to get images with better resolution in general, showing less panels per figure and make them bigger.*

We have repeated the experiment, using both FtsZ and SBS labeled, and included images with enhanced resolution to facilitate the visualization of the fibers and condensates (see **FIGURE A4A** at the end of this document, included as **Fig 3C** in the revised MS).

R3.9: *Co-localization of SBS and FtsZ in Figure 4BC, compare to SlmA and FtsZ in Figure 4D seems completely different. I would rather say that SlmA and FtsZ do not colocalize in Figure 4D. It would interesting to know if in these conditions (Figure 4D, 6 μM of FtsZ, 3 μM of SlmA and 0.5 μM) but in the absence of GTP condensate form and compare this result with or without GTP to that with double concentration of proteins but keeping the GTP constant.*

The appearance of SlmA as fibers in **Fig 4D** actually confirms its interaction with FtsZ, as the latter is the only one with the ability to form fibers. The fact that, as the reviewer points out, those regions with higher amount of SlmA present a weaker signal of FtsZ, is probably due to the inhibitory effect of SlmA·SBS on FtsZ polymerization, exerted on the lateral interactions of the filaments to form the bundles (Tonthat et al. 2013); confocal microscopy can only detect FtsZ bundles but not single protofilaments. This pattern of colocalization is also observed in samples containing double concentration of the proteins (see **FIGURE A4A** at the end of this document, included as **Fig 3C** in the revised MS).

Regarding the formation of condensates at 6 μM of FtsZ, 3 μM of SlmA and 0.5 μM SBS, we have performed these experiments and confirmed that the condensates form at these lower concentrations (see turbidity measurements in **FIGURE A1** at the end of this document, included as **Fig 2A** of the revised MS, and **FIGURE A11** at the end of this document).

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Figures

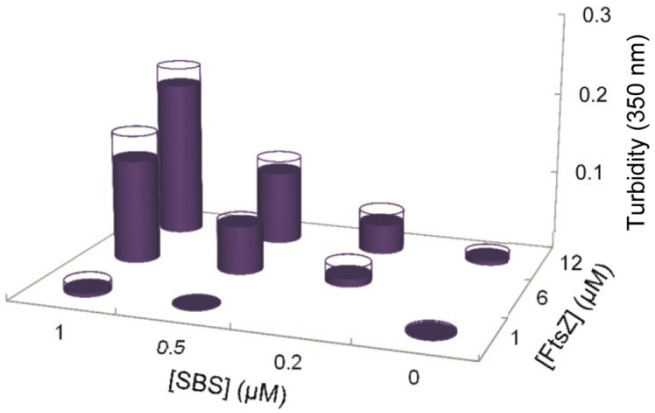


Figure A1: Turbidity measurements at different SlmA/FtsZ/SBS concentrations measured in 150 g/L dextran. SlmA concentration was fivefold that of SBS (except at 0.5 μM SBS, that SlmA concentration was 3 μM). In working buffer (300 mM KCl). Errors (SD), symmetrical, are depicted as white discs.

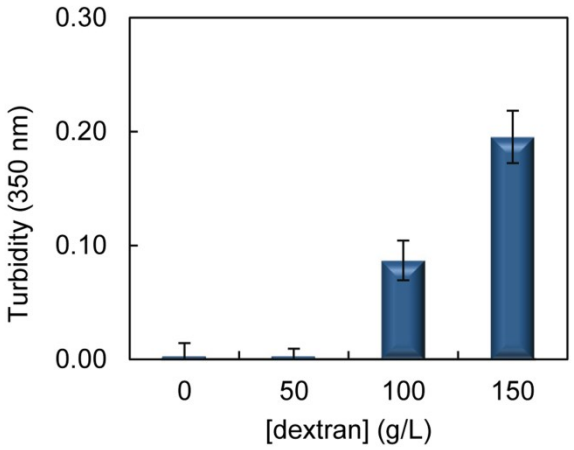


Figure A2: Dependence of the formation of FtsZ·SlmA·SBS condensates on dextran 500 concentration. In working buffer (300 mM KCl). FtsZ, SlmA and SBS concentrations were 12, 5 and 1 μM, respectively.

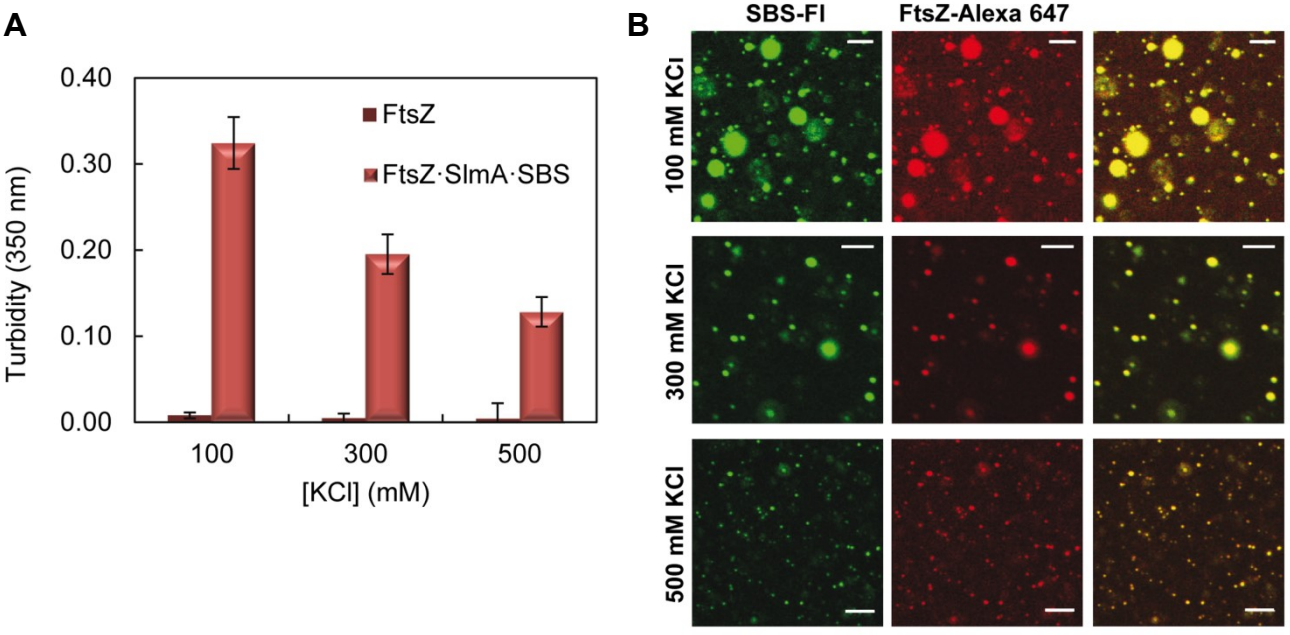


Figure A3: Dependence of the formation of FtsZ·SlmA·SBS condensates on KCl concentration, in 150 g/L dextran determined by turbidity (A) and confocal imaging (B). FtsZ, SlmA and SBS concentrations were 12, 5 and 1 μM, respectively. Scale bars: 5 μm.

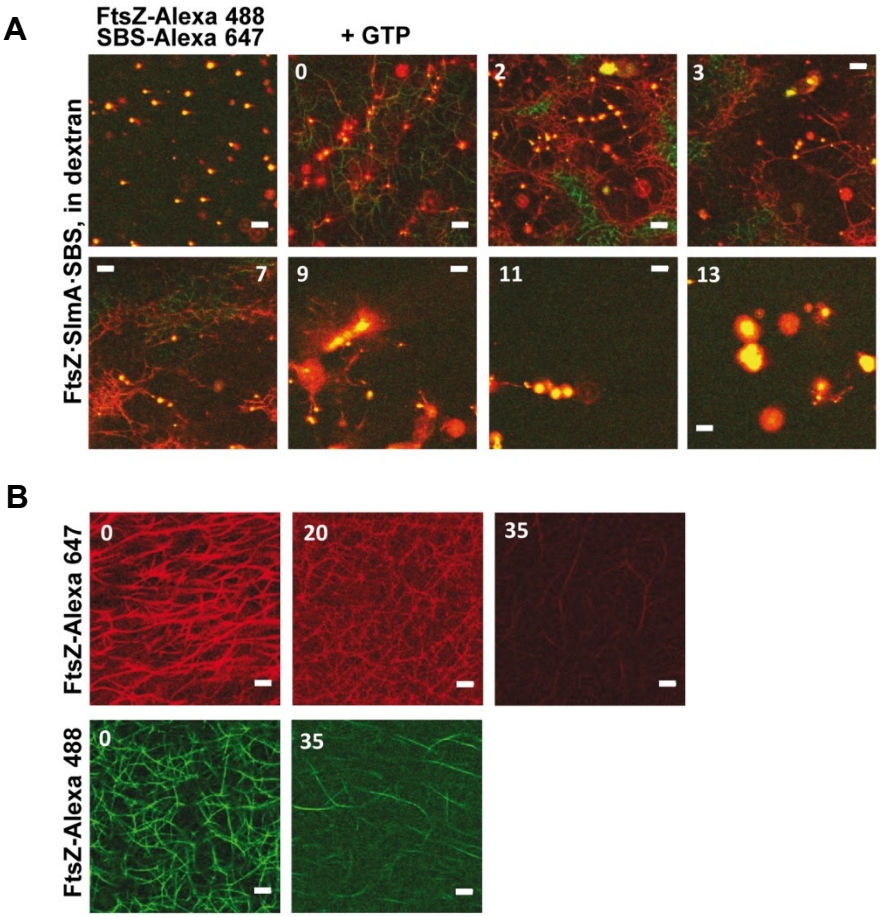


Figure A4: GTP triggered polymerization of FtsZ with and without SlmA·SBS in dextran 500 and monitoring of the disassembly with time, due to GTP depletion. FtsZ fibers in samples containing FtsZ·SlmA·SBS condensates (A), or only FtsZ (B). When present, the concentrations of FtsZ, SlmA and SBS were 12, 5 and 1 μM, respectively. Time in minutes (zero, GTP addition) indicated in each panel. 150 g/L dextran and 0.5 mM GTP. Scale bars: 5 μm.

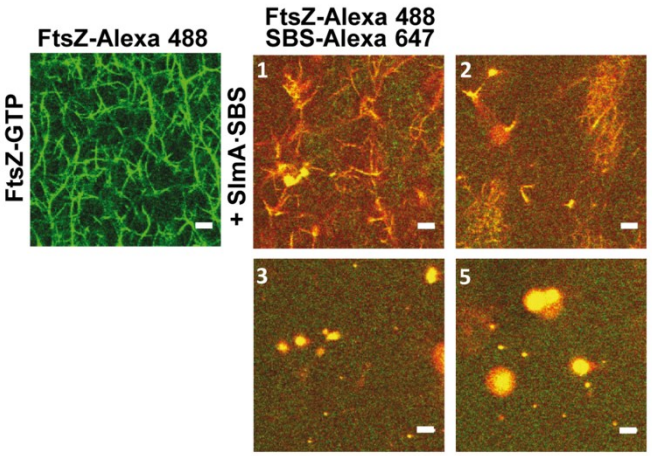


Figure A5. Effect of SlmA·SBS on FtsZ fibers. Addition of SlmA·SBS on preformed FtsZ fibers induced with 0.5 mM GTP in 150 g/L dextran. Indicated times in minutes from SlmA·SBS addition. Concentrations of FtsZ, SlmA and SBS were 12, 5 and 1 μM, respectively. Scale bars: 5 μm.

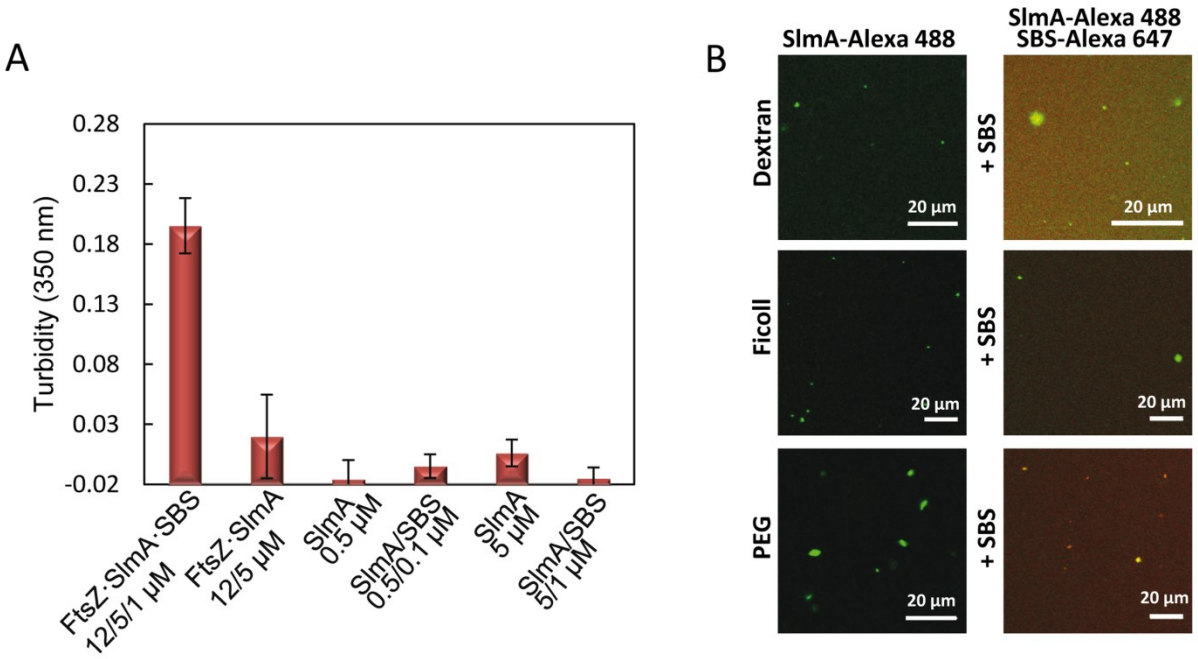


Figure A6: Formation of condensates by SlmA±SBS. (A) Measurements of the turbidity of samples containing SlmA±SBS. Samples containing FtsZ·SlmA±SBS at 12, 5, 1 µM, respectively are included as a reference. In working buffer with 300 mM KCl. (B) SlmA condensates in the absence and presence of SBS in 150 g/L dextran or Ficoll or 50 g/L PEG. Concentrations of SlmA and SBS were 5 and 1 µM, respectively.

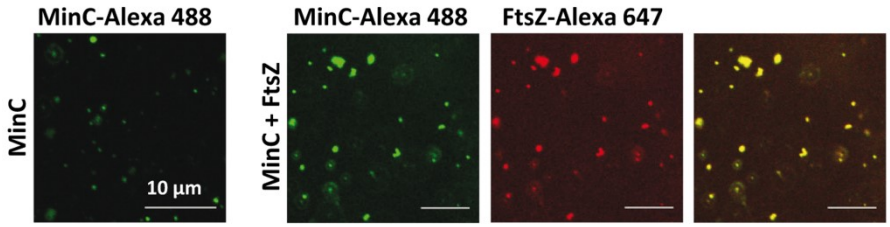


Figure A7: Confocal fluorescence images of MinC (5 µM) in the presence and absence of FtsZ (12 µM) in working buffer with 300 mM KCl.

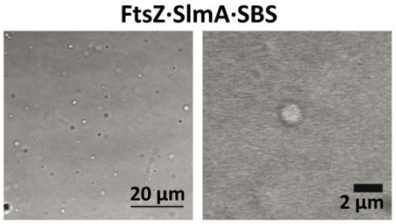


Figure A8: Transmitted images of FtsZ·SlmA·SBS (12 µM/5 µM/1 µM) in working buffer (300 mM KCl) and 150 g/L dextran 500.

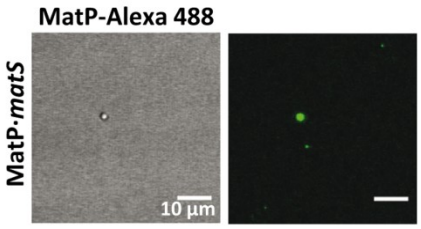
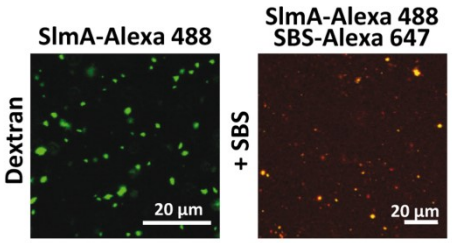


Figure A9: Transmitted and confocal images of MatP·*matS* (5 µM/1 µM) in 150 g/L dextran. In working buffer (300 mM KCl).

Figure A10. SlmA condensates in the absence (left) and presence of SBS (right) in 150 g/L dextran. Concentrations of SlmA and SBS were 40 and 8 µM, respectively. In working buffer with 300 mM KCl.



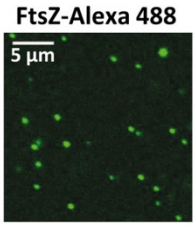


Figure A11: Condensate formation by FtsZ·SlmA·SBS in 150 g/L dextran. Concentrations of FtsZ, SlmA and SBS were 6, 3 and 0.5 µM, respectively. In working buffer with 300 mM KCl.

3rd Editorial Decision 19 October 2018

Thank you for the submission of your revised manuscript to EMBO reports. I apologize for the unusual delay in handling your manuscript, which was caused by difficulties to reach the referees during the summer season. Unfortunately, referee 3 was in the end not available anymore to review the revised version.

As you will see from the reports below, both referee 1 and 2 support publication of your manuscript in EMBO reports after some minor modifications of text and figures. Please address these concerns in the final revision.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

REFEREE REPORTS

****

Referee #1:

I have reviewed the changes made by the authors including the new data and the responses to the reviewers' comments. Overall, the manuscript has improved significantly and the additional data provide a compelling narrative in favor of the hypothesis that crowding drives FtsZ to undergo condensation. The polymeric status of FtsZ within condensates remains unclear, but this, one would presume, will be the subject of intense scrutiny. At this juncture, there are no further revisions to request and the MS, in its current form, seems to be suitable for publication.

Referee #2:

The authors made a genuine effort to perform the controls suggested by us and the other reviewers, and appropriately discussed other concerns which fall outside the scope of this study. Readability and story telling are also improved.
I appreciate the deeper discussion of the size of fibers in relation to action by SlmA. The control experiments showing (weak) SlmA condensation strengthen their argument about the importance for all three components to be present (FtsZ, SlmA, and SBS), for this phenomenon. The time-lapse imaging showing the reduction in fiber lifetime in the presence of SlmA are particularly helpful. Though we did not suggest it, the more systematic measurement of condensation across species concentrations (Fig. 2) is a welcomed addition.
In the end, I am more convinced by argument they put forth in the discussion about the role of these complexes in regulating division (or at least that it warrants more in vivo studies). They also use citations well to support their possible explanation in their rewritten discussion. In either case, I think judgement should be left to the reader. I believe the manuscript deserves publication in its current form as it does establish a new phenomenon in vitro, goes a good way in assessing its robustness, and prompts intriguing in vivo studies for the future.

Other comments are:
1. I suggest the authors mention the control results regarding R2.5 at least in Methods and Materials, and add the transmission light images to the appendix.
2. The words selforganize (line 3) and selfassembles (line 25) need to at least be hyphenated.
3. In Figures EV2, EV3 and S14, the transmission light images need to be labeled. In addition, the details of transmission light microscopy need to be mentioned in Methods and Materials. E.g. Was DIC or brighterfield used? Were the transmission light images taken simultaneously with the fluorescence images?

REFEREE #2

1. I suggest the authors mention the control results regarding R2.5 at least in Methods and Materials, and add the transmission light images to the appendix.
The transmission light images have been included in the manuscript as Appendix Fig S16, and referenced in the Methods and Materials section.

2. The words selforganize (line 3) and selfassembles (line 25) need to at least be hyphenated.

3. In Figures EV2, EV3 and S14, the transmission light images need to be labeled. In addition, the details of transmission light microscopy need to be mentioned in Methods and Materials. E.g. Was DIC or brightfield used? Were the transmission light images taken simultaneously with the fluorescence images?
All these changes have been implemented in the revised version of the manuscript.

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Corresponding Author Name: Dr. Germán Rivas
Journal Submitted to: Embo Reports
Manuscript Number: EMBOR-2018-45946V2-Q

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Each experiment was repeated 2-6 times, with new independent samples prepared for each particular experiment. Sample sizes correspond to those usual in the field.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
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For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Experiments of the same kind were conducted and evaluated by different authors
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5. For every figure, are statistical tests justified as appropriate?	N/A
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	N/A
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Is the variance similar between the groups that are being statistically compared?	N/A

C- Reagents

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http://www.ncbi.nlm.nih.gov/gap

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	N/A
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
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