nature portfolio

Peer Review File

Ribosomes translocation into the spore of Bacillus subtilis is highly organised and requires peptidoglycan rearrangements.

Corresponding Author: Professor Agata Starosta

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Iwanska et al. examined the localization of the translational machinery during sporulation in B. subtilis. The authors propose that ribosomes migrate from the mother cell to the forespore at later points in the sporulation program. The translocation of these ribosomes appears to be dependent of peptidoglycan remodeling mediated by SpoIIDMP and does not, at least directly, require two of the “feeding tube” components SpoIIIAH and SpoIIQ. The authors propose that the asymmetric septum drives the recruitment of ribosomes, after which peptidoglycan rearrangement is required for the translocation of the ribosomes into the forespore. The major advance in the paper is the observation that ribosomes (unlike the transcription machinery) are unevenly segregated into the daughter cells after asymmetric division during sporulation, which was convincingly demonstrated. However, the major shortcoming of the paper is that the authors’ conclusion on the requirement for peptidoglycan remodeling for ribosome translocation into the forespore is not fully justified by the data because it does not eliminate multiple other possibilities. Deleting spoIID, spoIIM, or spoIIP result in pleiotropic effects, including membrane remodeling defects and a general arrest of sporulation. Thus, it is not clear to me how the authors may conclude that PG remodeling, and not some downstream event that is dependent on engulfment, is responsible for the ribosome translocation defects. The only additional mechanistic insight is that the ‘feeding tube’ components are not required.
Major comments:
1. Fig. 1c. Please indicate the orientation of the cell along the x-axis. Is the forespore on the right or left? I assume forespore is on the left? If so, it is opposite to the orientation shown in the cartoon, which makes this foundational figure very confusing. Also, consider showing a zoomed-in/higher resolution image of the cells indicated with arrows in Fig. 1b. The localization pattern of GFP next to the septum is very difficult to appreciate unless I really zoom in. OPTIONAL: Instead of showing Fig. 1c plots at different time points, perhaps it would be more informative to show cells at the same stage of septation/engulfment so that the reader can more clearly appreciate 1) the ‘bump’ in red fluorescence due to the polar septum, and 2) the ‘bump’ in green fluorescence adjacent to the septum?
2. The authors place a central importance on the asymmetric septum in ribosome placement, but are ribosomes generally recruited to ribsosomes? The SIM images in Fig. 3 indicate that in WT cells, there is an enrichment of RpsB at the medial septum at T1. Does RpsB localize to vegetative septa (which later become poles)? In mutant B. subtilis cells that are disporic (ex:, spoIID/M/P triple mutants or SigE mutant), does RpsB localize to both septa?
3. Fig. 2f. Please indicate in the legend that the plots correspond to the images in Fig. 2d (I believe?). Also consider labeling the graph with the strain genotype in the figure itself.
4. The authors focused on the [delta]spoIID, IIM, and IIP cells based on the mass spec data, but engulfment requires both hydrolysis AND synthesis of PG. How does RpsB localize in a pbpG deletion strain?
5. The role of the feeding tube was ruled out by examining RpsB localization in [delta]spoIIIAH and spoIIQ strains, but deletion of both proteins leads to pleiotropic effects due to multiple functions of both proteins in sporulation. How does deletion of spoIIAG (which has only been ascribed a feeding tube function) affect RpsB localization?
6. There are multiple statements about ‘decreases’ in translation in different mutant backgrounds, but I did not see any data directly quantifying and comparing the OPP experiments in WT and any of the tested mutants. Please include a direct comparison or consider removing statements about ‘decreases’ in translation.

Minor comments:

1. Fig. 1c. Consider labeling the red, green, and blue traces in the figure itself so that the reader need not refer to the legend.
2. Abstract, last phrase ("…novel antibiotic targets…"). This phrase may be excluded. The data are lagely compelling enough for a general audience in this report, especially since the drugability of ribosomes during sporulation has not been explicitly tested in this paper.
3. Fig. 1f, Fig. 2e, Fig. S2. Consider adding a membrane stain to these images or an arrow in the images indicating where the forespore is.
4. Figure 2 a/b: Why are there two blue arrows at T4? In later figures this is used to indicated chromosome movement out of the forespore. Is that also the case here? If so, it needs to be detailed in the caption.
5. Fig. S1. Please indicate in the legend what the arrows are pointing to.
6. Fig. S3. To help a general reader understand the data, consider labeling what ribosome populations the peaks refer to.
7. Fig. S4. Consider adding arrows only to the merged images, since there are neither ribosomes/rpoC or DNA is present in the FM4-64 panel, for example.
8. Fig. S5-6. Consider labeling where the forespore is with arrows.
9. Fig. S5. The title is a bit confusing. Perhaps "Spatial coordination of transcription factors and translation…" instead?

Reviewer #2

(Remarks to the Author)
The very interesting manuscript "Ribosomes translocation into the spore of Bacillus subtilis is highly organised and requires peptidoglycan rearrangements" by Iwanska et al investigates the change of subcellular localization of ribosomes during the cellular developmental process of sporulation, where, after the initiation of an asymmetric cell division, the resulting larger and smaller cells are developing in a highly regulated and orchestrated manner into two fundamentally different cell types. During this cellular differentiation process the larger mother-cell phagocytizes the smaller forespore, while nurturing and fueling its development into a very stable dormant spore with two membranes, carrying a chromosome. Finally, the mother cell is sacrificing itself to release the mature spore, which has the potential to germinate and develop back into a proliferating cell, when nutrients become available again.

The authors used fluorescence microscopy to follow the subcellular localization of ribosomes (RpsB-GFP) and translation (OPP), transcription by RNA-Pol (RpoC-GFP) together with specific fluorescent dyes for staining the membrane (FM4-64) and DNA (DAPI) during the course of sporulation (Fig 1& S1). In these experiments they observe that after asymmetric cell division and at the beginning of engulfment by the mother cell, an increased amount of mother cell ribosomes localizes to the forming asymmetric septum with the just formed two membranes (T2-T3). And most interestingly these ribosomes appear to become translocated into the fore spore compartment independent of the chromosome and RNA Polymerase during the continuing and finishing engulfment. Interestingly, translation visualized by OPP coincides with the observed localization of ribosomes to the mother cell side of double membrane and their subsequent transport in the forespore (Fig 1& S1).
The authors designed a mass spectrometry-based experiment to identify interaction partners which might support or facilitate this transport of ribosomes into the forespore. For this they developed a scheme to lyse sporulating and control cells with digitonin with and without DSP crosslinker with subsequent 70S Ribosome enrichment with sucrose gradient ultra-centrifugation. In the text they describe possible candidates, including proteins of membrane secretion system and of a peptidoglycan remodelling complex, which they somehow could detect with this approach (Fig S3 text l143-167p6, Supplementary data sheet). Based on these candidates and also the known literature of these protein complexes playing a role during engulfment, they went on to show with their fluorescence microscopy experiments that the SpoIIDMP peptidoglycan remodelling complex SpoIIDMP plays a direct role in the ribosome transport over the asymmetric membrane but the secretion membrane complex SpoIIQIIIA does not (Fig 2 & 3).
This very interesting manuscript shows with solid evidence that ribosomes can be sorted to the forespore and those can engage on translation. However, the molecular mechanism of this process is not completely clear, suggesting that peptidoglycan remodelling is important to it.

Comments
- The supplementary table containing the MS data is confusing as it lacks any legends which would enable to understand the experiments, for example a description of which comparisons are being performed and which data column would be relevant. For example, it could be assumed that the "DSP" data is the crosslinked sample and the "D" is the not crosslinked?

- Visualization of the MS data could be achieved by, for example, volcano plots in which proteins named on the text are highlighted. Also, a functional annotation analysis could be useful to see if proteins involved in peptidoglycan remodelling are enriched.

- Was the data of non-crosslinked samples and or exponentially growing cells used for deciding which proteins are relatively enriched?

- the supplementary video files have no comments or explanation. Maybe an extra file with a series of snapshots with arrows and comments would be helpful to understand this data/video

More specific comments:
- In Figure 1, it will be easier to understand if in the model the forespore is aligned to the left side as in the plots

- In Figure 2, plots showing the distribution of blue, green and red channel intensities through cell length (as in Fig 1) would also be useful to understand the results of the mutants better

- In Line 106, the authors mention OPP-bound ribosomes. However, it has been showed that peptidyl-OPP is released from the ribosome (https://doi.org/10.7554/eLife.60303). The OPP labelled peptides could nevertheless form closeby aggregates indicating thereby the translation activity.

- Time is inconsistently displayed in Fig 1 and 2, being in hh:mm in the former and fraction of hours in the latter

- In line 479, authors mention they used SynaptoRed for membrane staining but in figure legends is mentioned as FM4-64

- Figure 3d is not mentioned in the text, and the model could be explained in more detail in the figure itself and in the figure legend. The model is only for wt cells but could also include the effect of the spoIID mutation on ribosome localization.

- In the graphical abstract, the authors show that a spoIID deletion mutant will have most of their ribosomes facing the mother cell side of the septum. Is there evidence supporting this?

-Abstract L33 p2 delete “construction of”

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revised manuscript by Iwanska et al. has been largely improved from its original submission. The added experiments provide a clearer idea of what the mechanism of ribosome translocation may be, and certainly provide some data that should permit lots of follow up stories.

Major comment:
I am a bit disappointed that the authors were unable to perform the requested experiment in the disporic mutant background, which was not meant to be an onerous request. The authors state that the [delta]sigE strain, for example, was difficult to obtain and that the Stock Center did not get back to them. I’m not sure that this is a valid excuse, especially since dozens of B. subtilis labs across the world likely have this strain. The experiments that they provided in the reviews were not as convincing as they could be, given the relatively few disporic cells they were able to visualize, and the authors did not include these images in the revised version of the manuscript. I am not a fan of multiple rounds of reviews, so I will leave this as an editorial decision, especially since the authors are requesting the latitude to explore this in a subsequent report.

Minor comments:
Line 22: consider rewording to read, “…during sporulation in wild type and mutant Bacillus subtilis.”
Line 35: consider deleting, “in sporulating bacteria”.
Line 84: delete “the” before “chromosome translocation”.

Reviewer #2

(Remarks to the Author)
All our comments and questions were addressed in the revised version of this very interesting Manuskript. During the discussion of the revision with my co-reviewer a comment concerning Fig 1 came up.

-Results Fig 1 b&c Text in results on p4 l85-89

"Interestingly, we observed that during asymmetric septation and chromosome translocation (T2-T3), the ribosomes that were previously at the cell pole are restricted from the forespore rather than trapped in the divided cytoplasm. It appears that the ribosomes gather and wait at the asymmetric septum at the mother cell side and during or very shortly after the engulfment by the mother cell, the ribosomes are translocated into the spore (T4)."

This description concerning the dynamic of the ribosomes should actually refer to T1-T2-T3, since the ribosomes at the poles are clearly visible at T1. Then at T2 the forespore has already formed, and less ribosomes are detected (by fluorecence) in this new morphological structure, especially when compared to the ribosomes at the pole at T1. Then at T3 a significant amount of Ribosomes become detected in the mothercell between the forespore membrane and the mothercell

chromosome wich is next to another peak of ribosome at the opposite (right) pole of the mothercell. At T4 the translocation of these adjacent ribosomes into the forespore has already started .
This dynamic process can also be observed at higher time resolution in the Suppl Fig 1 & 3 (Movie S1).

To state that "the ribosomes that were previously at the cell pole are restricted from the forespore rather than trapped in the divided cytoplasm" appears to be speculative at this stage and could be addressed in more detail in the discussion.

-Discussion (p11 l 320-1)
In the first statement (2nd sentence) in the discussion on this topic, I would not use the term "belief" and replace it by other terms such as "models".
One could consider to further discuss different models, possible mechanisms or experimental observations for this interesting developmental process in more detail.

- Discussion p12 l351-353 include "might" : ...ΔspoIID, this MIGHT suggest that ribosomes are not synthesised de novo in the forespore and are “inherited” from the mother cell.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript by Iwanska et al. examined the localization of the translational machinery during sporulation in *B. subtilis*. The authors propose that ribosomes migrate from the mother cell to the forespore at later points in the sporulation program. The translocation of these ribosomes appears to be dependent of peptidoglycan remodeling mediated by SpoIIDMP and does not, at least directly, require two of the "feeding tube" components SpoIIIAH and SpoIIQ. The authors propose that the asymmetric septum drives the recruitment of ribosomes, after which peptidoglycan rearrangement is required for the translocation of the ribosomes into the forespore. The major advance in the paper is the observation that ribosomes (unlike the transcription machinery) are unevenly segregated into the daughter cells after asymmetric division during sporulation, which was convincingly demonstrated. However, the major shortcoming of the paper is that the authors' conclusion on the requirement for peptidoglycan remodeling for ribosome translocation into the forespore is not fully justified by the data because it does not eliminate multiple other possibilities. Deleting spoIID, spoIIM, or spoIIP result in pleiotropic effects, including membrane remodeling defects and a general arrest of sporulation. Thus, it is not clear to me how the authors may conclude that PG remodeling, and not some downstream event that is dependent on engulfment, is responsible for the ribosome translocation defects. The only additional mechanistic insight is that the 'feeding tube' components are not required.

Major comments:

1. Fig. 1c. Please indicate the orientation of the cell along the x-axis. Is the forespore on the right or left? I assume forespore is on the left? If so, it is opposite to the orientation shown in the cartoon, which makes this foundational figure very confusing.

- We have now changed the orientation of the spore in the cartoon and added a description of cell orientation in the figure caption:

Fig. 1c and e: The cell lengths were normalised and fluorescence was measured along a line drawn across the cell long axis. The cells are oriented so that the spore is located to the left.

Also, consider showing a zoomed-in/higher resolution image of the cells indicated with arrows in Fig. 1b. The localization pattern of GFP next to the septum is very difficult to appreciate unless I really zoom in. OPTIONAL: Instead of showing Fig. 1c plots at different time points, perhaps it would be more informative to show cells at the same stage of septation/engulfment so that the reader can more clearly appreciate 1) the 'bump' in red fluorescence due to the polar septum, and 2) the 'bump' in green fluorescence adjacent to the septum?

- We have now added additional panels with zoomed-in cells at consecutive timepoints and with the same spore orientation as in the cartoon. We amended the figure caption as follows:

Fig 1c and e: Example cells and plots of mean GFP fluorescence intensity (green) across the cells show subcellular localisation of ribosomes (n>100) (**c**) or RNA polymerase (n>80) (**e**) during sporulation in relation to the chromosome (blue) and cell membrane and asymmetric septum (red).

2. The authors place a central importance on the asymmetric septum in ribosome placement, but are ribosomes generally recruited to ribsosomes? The SIM images in Fig. 3 indicate that in WT cells, there is an enrichment of RpsB at the medial septum at T1. Does RpsB localize to vegetative septa (which later become poles)? In mutant B. subtilis cells that are disporic (ex:, spoIID/M/P triple mutants or SigE mutant), does RpsB localize to both septa?

- This is indeed a very interesting question, and we were keen to explore this. We did construct several Δ*sigE* strains to investigate this, however, the mutants kept proving to be unstable by keeping the selection cassette in the *sigE* locus and recombining the *sigE* gene elsewhere in the genome. Since we struggled with mutant construction, we ordered the Δ*sigE* strain from the Bacillus Genetic Stock Center repository, but unfortunately our emails and orders remain unanswered.

  Therefore, we decided to look at the disporic cells in the data that we already have. Since the asymmetric septum is produced very early in the sporulation process at both poles, and then the cell decides which one to keep, we did in fact observe a number of cells with two spores between two and three hours post sporulation induction, in all genetic backgrounds. We observed two phenotypes of disporic cells. The first is a less frequently observed phenotype (mostly in *ΔspoIIIAH* and *ΔspoIIQ* strains), in which a cell has two spores equal in size, both exhibiting low GFP fluorescence signal. In this phenotype, the ribosomes are evenly distributed in the mother cell, are not gathered at either septum, and the mother cell is devoid of the chromosome (Fig. R1. a). This is a disporic phenotype observed in Δ*sigE* strain[1].

  The more frequent phenotype (observed in all genetic backgrounds) is a disporic cell in which there is a disproportion in the spore size, and the smaller spore shows higher GFP fluorescence signal compared to the bigger one (Fig. R1. b). Also, the smaller spore is not enriched in the blue signal corresponding to the chromosome. This is a transiently disporic state, in which the secondary septum is not maintained by the cell as the sporulation progresses. Thus, we hypothesize that in the transiently disporic cells (in other words during normal development) the two asymmetric septa differ in composition and the ribosomes are recruited to the primary asymmetric septum. This is most likely mediated via asymmetric septum related proteins of the SigE regulon. In the in Δ*sigE*-like disporic phenotype however, these septum associated protein factors appear to be missing, rendering ribosomes lacking specific subcellular localisation. The mechanism of ribosome recruitment to the asymmetric septum, together with the detailed mechanism of ribosome translocation into the developing spore, is definitely a subject which we would like to explore in the near future.

  In the vegetatively developing cells ribosomes are generally recruited to cell poles as the cell grows and divides. Analogously, we envision that during cell division the ribosomes are recruited to the division septum by specific protein machineries (perhaps associated with the Z-ring or bacterial cytoskeletal homologs), rather than simply being non-specifically associated with the cell membrane.

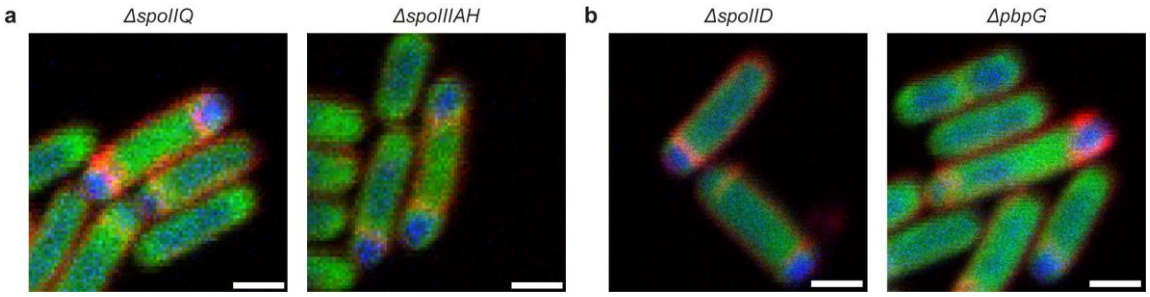

Fig. R1. (**a**) Example disporic cells in the *ΔspoIIQ* and *ΔspoIIIAH* backgrounds. The two prespores show limited GFP signal corresponding to the ribosome (RpsB-GFP). The mother cell is devoid of the chromosome (stained with DAPI, blue) and the ribosomes are localised homogenously in the mother cell. (**b**) Example disporic cells in the *ΔspoIID* and *ΔpbpG* backgrounds. The two prespores are not symmetrical with the primary prespore showing low GFP fluorescence signal and high DAPI fluorescence due to the presence of the chromosome. The secondary prespore is devoid of the chromosome, but not the ribosomes, which suggests a potential involvement of recruiting and translocating protein machinery the at the primary asymmetric septum. The cells express GFP-tagged ribosomal protein RpsB and are stained with DAPI to visualise chromosome and a membrane strain FM4-64. Scale bar is 1 µm.

3. Fig. 2f. Please indicate in the legend that the plots correspond to the images in Fig. 2d (I believe?). Also consider labeling the graph with the strain genotype in the figure itself.

- We have edited Fig. 2. which in the corrected version of the manuscript is Fig. 3 now. This was done to include zoomed in cells as well as plots of fluorescence intensities requested by Reviewer 2. We have also labelled graphs with strain genotypes and clarified figure legends.

4. The authors focused on the [delta]spoIID, IIM, and IIP cells based on the mass spec data, but engulfment requires both hydrolysis AND synthesis of PG. How does RpsB localize in a pbpG deletion strain?

- We performed fluorescence microscopy for Δ*pbpG* strain with GFP-labelled ribosome (GFP-labelled ribosomal protein RpsB), and the results are presented in Fig. R2. The mutant exhibited the expected phenotype of elongated jellybean-like spores, however, we did not observe a pronounced phenotype regarding ribosome translocation into the spore. Thus, it appears that translocation of ribosomes into the spores of Δ*pbpG* was not considerably affected. We hypothesize that a single deletion might have been insufficient for interference in the peptidoglycan synthesis machinery to a high enough degree, and perhaps a deletion of an additional gene (e.g. *spoIIIM*, as illustrated by Mohamed et al. (2021)[2]) might result in a phenotype affecting the ribosomal localisation. We agree with the Reviewer that peptidoglycan remodelling including both hydrolysis and synthesis is essential for engulfment. Based on our results presented in this manuscript, we further hypothesize that the cell may perhaps utilize the septal pore to translocate ribosomes into the spore, sometime after the chromosome translocation and loss of SpoIIIE focal localization.
- We have now added this to the discussion section:

LINES 328-331: Peptidoglycan rearrangement by SpoIIDMP is crucial for ribosome packing into the spore after the chromosome translocation and we hypothesize that this transfer is mediated by mother cell membrane or cell wall proteins, possibly via the septal pore, after SpoIIIE loss of focal localisation and before the spore peptidoglycan synthesis.

This fits nicely into the timeline of chromosome and ribosomes translocation into the spore, and also gives some insight into why lack of a single gene involved in the coordination of septal pore closure (*pbpG*) did not significantly affect ribosomal translocation. Hence, we propose that balanced peptidoglycan rearrangement lays at the foundation of the mechanism of ribosome translocation into the spore and elucidating the details, which we envision to involve septal pore closure, is a part of the research question that we would definitely like to pursue in the very near future.

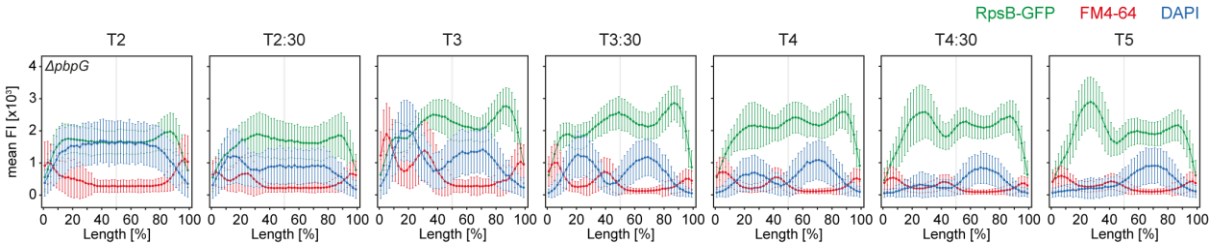

Figure R2. Mean fluorescence intensity plots in Δ*pbpG* mutant strain. The subcellular localisation of ribosomes is shown in green (RpsB-GFP), chromosome in blue (DAPI) and cell membrane in red (FM4-64). Cell lengths were normalised and fluorescence intensity was measured along the cell long axis. Plots were prepared based on n>100 measurements per timepoint.

5. The role of the feeding tube was ruled out by examining RpsB localization in [delta]spoIIIAH and spoIIQ strains, but deletion of both proteins leads to pleiotropic effects due to multiple functions of both proteins in sporulation. How does deletion of spoIIAG (which has only been ascribed a feeding tube function) affect RpsB localization?

- We performed fluorescence microscopy for Δ*spoIIIAG* strain with GFP-tagged ribosomal protein RpsB, and measured the fluorescence intensities along the cells (Fig. R3). We observed the expected phenotype of the mutant strain – small spores – however, the ribosomal translocation into the developing spores was not affected. We have now added this information to the manuscript and Supplementary Fig. 9.

LINES 228-233: The feeding tube channel has been shown to transport small molecules including arginine[30], however, we also investigated the effect of deletion of the ring-forming complex of the channel[31], *ΔspoIIIAG*, on the ribosome localisation. The mutant showed the expected small spores phenotype, and the ribosomal translocation into the forespore was not affected suggesting that the feeding tube channel does not take part in ribosome translocation into the developing spore (Supplementary Fig. 9).

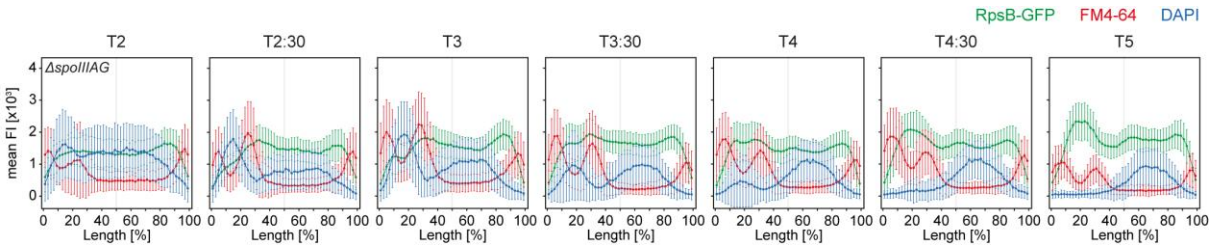

Figure R3. Mean fluorescence intensity plots in Δ*spoIIIAG* mutant. The localisation of ribosomes is shown in green (RpsB-GFP), chromosome in blue (DAPI) and cell membrane in red (FM4-64). Cell lengths were normalised and fluorescence intensity was measured along the cell long axis. Plots were prepared based on n>100 measurements per timepoint (Supplementary Fig. 9).

6. There are multiple statements about 'decreases' in translation in different mutant backgrounds, but I did not see any data directly quantifying and comparing the OPP experiments in WT and any of the tested mutants. Please include a direct comparison or consider removing statements about 'decreases' in translation.

- We agree that OPP-Alexa 488 fluorescence data would be challenging to quantify and compare between the strains directly and rather, a difference in the fluorescence ratios along the cells should be compared. In the manuscript, we could only identify the use of 'decrease' twice, referring to both translation (LINE 269) and RNA polymerase (LINE 284). We therefore propose the following change in the wording:

LINE 268: In the light of SpoIIIE protein stripping activity during chromosome translocation discussed above, a ~~substantial~~ decrease of OPP-Alexa 488 fluorescence intensity in the forespore at T3 (Fig. 3d), ~~translation~~ together with the presence of RNA polymerase in the forespore led us to further investigate the ribosomal localisation during sporulation.

- We have removed the statement in LINE 222: "…low levels of translation in the *ΔspoIIQ* strain (Supplementary Fig. 5a and b)…"

Minor comments:

1. Fig. 1c. Consider labeling the red, green, and blue traces in the figure itself so that the reader need not refer to the legend.

- We have added labels to Figures 1 and 3 describing the red, blue and green plots as membrane, chromosome and GFP-tagged protein respectively. We also added labels for the OPP experiment explaining the blue and greed plots (Fig. 1g and 3d).

2. Abstract, last phrase ("…novel antibiotic targets…"). This phrase may be excluded. The data are lagely compelling enough for a general audience in this report, especially since the drugability of ribosomes during sporulation has not been explicitly tested in this paper.

- We have now excluded the fragment about novel antibiotic targets from the abstract.

3. Fig. 1f, Fig. 2e, Fig. S2. Consider adding a membrane stain to these images or an arrow in the images indicating where the forespore is.

- Due to the assay specificity, the cells have to be fixed and permeabilised in order to stain them with Alexa 488 dye. Hence, unfortunately a membrane stain FM4-64 (which is a standard membrane stain used in our lab) cannot be used here, as it is only used for live staining. We have now added arrowheads pointing to the selected forespores (for image clarity) in timepoints T3 – T5, in Fig. 1f and Fig. S2. Fig. 2 has now been changed completely, and only single zoomed-in cells are now shown, which hopefully improved clarity. Images from Fig. 2 have been moved to Supplementary Fig. 7 and forespores were also labelled with white arrowheads in timepoints T3-T5.

4. Figure 2 a/b: Why are there two blue arrows at T4? In later figures this is used to indicated chromosome movement out of the forespore. Is that also the case here? If so, it needs to be detailed in the caption.

- We have edited Fig. 2 (currently Fig. 3) and the microscopic images from Fig. 2 are now in Supplementary Fig. 7. We have carefully re-examined the data and made adjustments to the figure. The blue arrowheads in the previous version of Fig. 2 (now Fig. 3) have been removed, as further experimental results clarified the localisation patterns. This was a direct result of introducing an additional control, which was missing in the previous version of the manuscript. In the *ΔspoIIQ* and *ΔspoIIIAH* strains the chromosome does not back translocate into the mother cell, but in fact, remains in the spore. We have removed the relevant fragments from the manuscript and amended Supplementary Fig. 7 (previously Fig. 2) accordingly.

5. Fig. S1. Please indicate in the legend what the arrows are pointing to.

- We have now added a description of what the arrowheads are pointing to in the figure legend:

Samples were taken two hours after sporulation induction (T2) and every twenty minutes of the sporulation process (T2:20-T4). The green arrowheads indicate the movement of the ribosomes in the cell, highlighting the dynamic changes in ribosomal localisation during sporulation.

6. Fig. S3. To help a general reader understand the data, consider labeling what ribosome populations the peaks refer to.

- We have now amended Fig. S3 and figure legend and added arrowheads and labels to the polysome profiles and indicated which fractions were analysed by MS. We also added PCA plot for all samples to show relationship between the samples.

7. Fig. S4. Consider adding arrows only to the merged images, since there are neither ribosomes/rpoC or DNA is present in the FM4-64 panel, for example.

- We have removed Fig. S4. from the submission, as discussed above. Additional control experiments showed that in fact, the chromosome does not translocate back into the mother cell in the delta mutants of the 'feeding tube' channel components.

8. Fig. S5-6. Consider labeling where the forespore is with arrows.

- We have added the arrowheads pointing to the selected forespores (for image clarity) and included this information in the figure captions.

9. Fig. S5. The title is a bit confusing. Perhaps "Spatial coordination of transcription factors and translation…" instead?

- The title was indeed confusing. It has now been changed as follows:

Supplementary Figure 5. Spatial coordination of transcription factors and translation ~~translational~~ in *ΔspoIIQ* strain during sporulation

Reviewer #2 (Remarks to the Author):

The very interesting manuscript “Ribosomes translocation into the spore of Bacillus subtilis is highly organised and requires peptidoglycan rearrangements” by Iwanska et al investigates the change of subcellular localization of ribosomes during the cellular developmental process of sporulation, where, after the initiation of an asymmetric cell division, the resulting larger and smaller cells are developing in a highly regulated and orchestrated manner into two fundamentally different cell types. During this cellular differentiation process the larger mother-cell phagocytizes the smaller forespore, while nurturing and fueling its development into a very stable dormant spore with two membranes, carrying a chromosome. Finally, the mother cell is sacrificing itself to release the mature spore, which has the potential to germinate and develop back into a proliferating cell, when nutrients become available again.

The authors used fluorescence microscopy to follow the subcellular localization of ribosomes (RpsB-GFP) and translation (OPP), transcription by RNA-Pol (RpoC-GFP) together with specific fluorescent dyes for staining the membrane (FM4-64) and DNA (DAPI) during the course of sporulation (Fig 1& S1). In these experiments they observe that after asymmetric cell division and at the beginning of engulfment by the mother cell, an increased amount of mother cell ribosomes localizes to the forming asymmetric septum with the just formed two membranes (T2-T3). And most interestingly these ribosomes appear to become translocated into the fore spore compartment independent of the chromosome and RNA Polymerase during the continuing and finishing engulfment. Interestingly, translation visualized by OPP coincides with the observed localization of ribosomes to the mother cell side of double membrane and their subsequent transport in the forespore (Fig 1& S1). The authors designed a mass spectrometry-based experiment to identify interaction partners which might support or facilitate this transport of ribosomes into the forespore. For this they developed a scheme to lyse sporulating and control cells with digitonin with and without DSP crosslinker with subsequent 70S Ribosome enrichment with sucrose gradient ultra-centrifugation. In the text they describe possible candidates, including proteins of membrane secretion system and of a peptidoglycan remodelling complex, which they somehow could detect with this approach (Fig S3 text l143-167p6, Supplementary data sheet). Based on these candidates and also the known literature of these protein complexes playing a role during engulfment, they went on to show with their fluorescence microscopy experiments that the SpoIIDMP peptidoglycan remodelling complex SpoIIDMP plays a direct role in the ribosome transport over the asymmetric membrane but the secretion membrane complex SpoIIQIIIA does not (Fig 2 & 3).

This very interesting manuscript shows with solid evidence that ribosomes can be sorted to the forespore and those can engage on translation. However, the molecular mechanism of this process is not completely clear, suggesting that peptidoglycan remodelling is important to it.

Comments

The supplementary table containing the MS data is confusing as it lacks any legends which would enable to understand the experiments, for example a description of which comparisons are being performed and which data column would be relevant. For example, it could be assumed that the "DSP" data is the crosslinked sample and the "D" is the not crosslinked?

- We have now added a legend explaining the origin of samples, as well as a short description of what comparisons were made and the rationale behind the analysis. This is now in Supplementary Data 1.

Visualization of the MS data could be achieved by, for example, volcano plots in which proteins named on the text are highlighted. Also, a functional annotation analysis could be useful to see if proteins involved in peptidoglycan remodelling are enriched.

- Indeed, the manuscript benefited from MS data analysis, which may have seemed incomplete in the previous version – thank you for this comment. We have now added an extended description of the MS data analysis including data visualisation in the form of volcano plots of the comparisons for the cross-linked vs non-cross-linked samples with color-coded enriched proteins based on functionality. This is now in lines 155-157, 163-174 and 180-183 (in red) and in Figure 2. The list of significantly enriched proteins ($p>0.05$, log2 fold change>1) with assigned functionalities is now also provided in Supplementary Data 1. We have added names of proteins from the two investigated protein complexes to the volcano plots. This was done for clarity reasons, as adding more labels unfortunately rendered the graphs indecipherable. The MS data analysis description was also added to Materials and Methods, subsection "Ribosome purification and mass spectrometry" (lines 658-662).

Was the data of non-crosslinked samples and or exponentially growing cells used for deciding which proteins are relatively enriched?

- The log2 fold change calculations were performed for crosslinked vs non-crosslinked samples to investigate the enriched proteins. Both samples were lysed with digitonin to allow for solubilisation of membrane-bound proteins. We also performed log2 fold change calculations for all samples versus flash-frozen pulverised sporulating cells (P). Pulverisation of flash-frozen cells is a well-established method in our lab, yielding replicable results and providing an adequate snapshot of the active translation. The rationale of introducing this sample as a control was to compare the detergent-based lysis method in terms of lysis efficiency and preservation of native ribosomes. The exponentially growing cells were used as an additional control to verify whether we could differentiate between sporulating and non-sporulating ribosomal samples. We have now added volcano plots with annotated functionalities of the enriched proteins for the control comparisons (all samples vs P) in Supplementary Fig. 6. We have also added the PCA plot of log2 normalised reporter intensities for all samples (Supplementary Fig. 5) to visualise data relationship. We can observe a clear grouping of the datasets based on the lysis method and developmental stage. PC1 describes data variability mostly based on the native ribosome preservation (lysis-dependent), whereas PC2 differentiates primarily based on the growth phase (sporulating vs logarithmic).

The supplementary video files have no comments or explanation. Maybe an extra file with a series of snapshots with arrows and comments would be helpful to understand this data/video.

- We have now added two new supplementary figures (3 and 4) of the selected snapshots from the time-lapse videos with comments describing the entire sporulation process and arrowheads pointing to most important events.

More specific comments:

In Figure 1, it will be easier to understand if in the model the forespore is aligned to the left side as in the plots

- We have now changed the orientation of the forespore in the cartoon.

In Figure 2, plots showing the distribution of blue, green and red channel intensities through cell length (as in Fig 1) would also be useful to understand the results of the mutants better.

- We have changed Fig.2 (which is now Fig.3) to improve data presentation and clarity. We added the plots of mean fluorescence intensity, as well as single zoomed-in cells and cartoons of the mutant cells used in the study to better illustrate the differences between the mutant strains. The microscopic images that were used in previous version of Fig.2 are now in Supplementary Fig. 7.

In Line 106, the authors mention OPP-bound ribosomes. However, it has been showed that peptidyl-OPP is released from the ribosome (https://doi.org/10.7554/eLife.60303). The OPP labelled peptides could nevertheless form closeby aggregates indicating thereby the translation activity.

- We have now changed the wording of this sentence to the following:

LINE 106: ~~Fluorescent labelling of~~ Fluorescently labelled nascent chains ~~OPP-bound ribosomes~~ allow for visualisation and relative quantification of the incorporated OPP, or in other words, of the active translation[21].

- The puromycylated peptides may indeed be released from the ribosomes, however, the release and migration of the OPP-labelled peptides appear to be dependent on the cell type, as shown by Anadolu et al. (2024)[3] for neuronal cells. In case of bacterial cell, our microscopic data suggests that puromycylated peptides indeed aggregate at the foci of active translation: 1) the localisation of OPP-labelled peptides is very similar to the localisation of the GFP-labelled ribosomes, as shown in Fig. 1 in the manuscript; 2) we have also performed a control experiment, analogous to OPP-labelling, in which we labelled nascent polypeptide chains with noncanonical methionine derivative (an azide-bearing azidohomoalanine (AHA)), which was then chemoselectively tagged with Alexa-488 using click-chemistry. AHA incorporation into the nascent chain does not terminate translation – AHA-Alexa 488 labelled peptides are folded into proteins, reach their cellular localisations and may be visualised microscopically. From the fluorescence intensity plots (Fig. R4) it can be seen that the AHA-labelled proteins loose the pronounced polar/septal localisation of the OPP-labelled prematurely terminated peptides, suggesting that the latter may be considered as adequate proxy for active translation localisation in the *B. subtilis* cell.

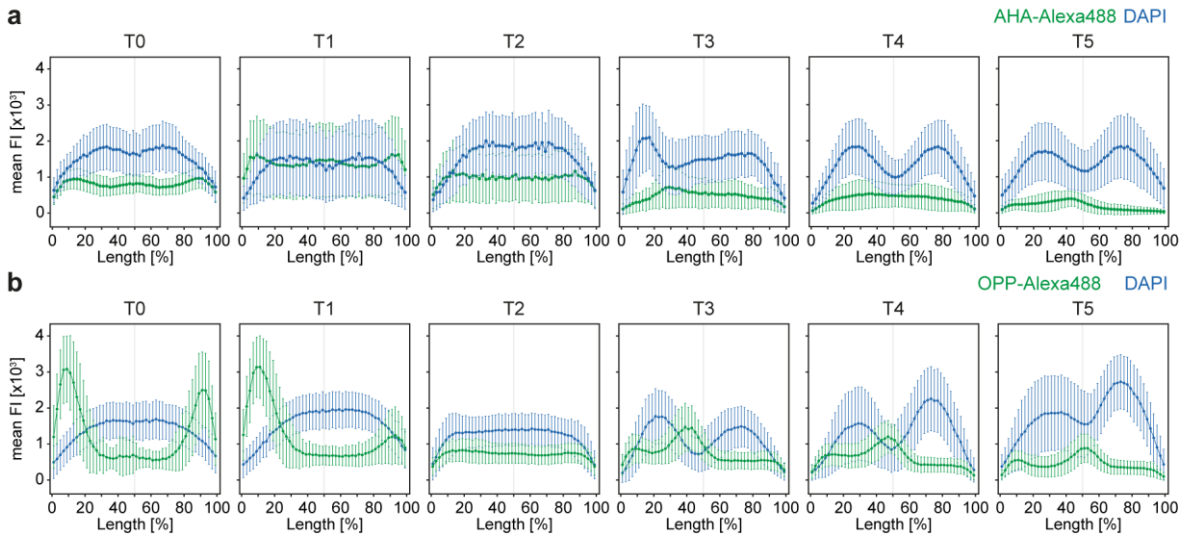

Fig. R4. (**a**) Plots of the mean AHA-Alexa 488 fluorescence intensity (green) across the cell showing relative amount and localisation of newly synthesized proteins in the sporulating *B. subtilis* at one hour intervals (T0 – logarithmic growth, prior to sporulation induction to T5 – five hours post sporulation induction), in relation to to chromosome (DAPI, blue). (**b**) Plots of the mean OPP-Alexa 488 fluorescence intensity (green) across the cell showing localisation and intensity of active translation during sporulation, in relation to the chromosome (DAPI, blue). The cell lengths were normalised and fluorescence was measured along the cell long axis.

Time is inconsistently displayed in Fig 1 and 2, being in hh:mm in the former and fraction of hours in the latter.

- We have repeated the observations and time descriptors have now been changed to hh:mm in all figures. For consistency reasons, we have also provided photographs from T0/T2 to T5 in Fig. 1 and Fig. 3 (previously Fig. 2).

In line 479, authors mention they used SynaptoRed for membrane staining but in figure legends is mentioned as FM4-64

- This has been corrected as follows:

LINE 540: Unless stated otherwise, the cells were visualised with FM4-64~~SynaptoRed~~ dye at a final concentration of 10 µg ml^{-1} and 4',6-diamidino-2-phenylindole (DAPI) dye at a final concentration of 5 µg ml^{-1}.

Figure 3d is not mentioned in the text, and the model could be explained in more detail in the figure itself and in the figure legend. The model is only for wt cells but could also include the effect of the spoIID mutation on ribosome localization.

- We have moved Figure 3d from Results section to the Discussion section (it is now Figure 5), where it has been mentioned in the text and is indeed more fitting. We have also included the model for Δ*spoIID* mutant.

In the graphical abstract, the authors show that a spoIID deletion mutant will have most of their ribosomes facing the mother cell side of the septum. Is there evidence supporting this?

- In this manuscript, we propose that the ribosomes translocate into the developing spore after chromosome translocation, and that the spore peptidoglycan rearrangements are crucial for the ribosome packing into the spore. In Δ*spoIID* mutant spore peptidoglycan hydrolysis is inefficient, preventing the spore from acquiring ribosomes and maturing. We have now added fluorescence intensity plots showing the localisation of the ribosomes relative to the asymmetric septum and bacterial DNA (Fig. 3b). The plots indicate that majority of the ribosomes is located at the mother cell side of the septum, as the red peak corresponding to the asymmetric septum is located at the 20th percentile of cell length while the green peak corresponding to the ribosomes is located more to the right, at approximately 40th percentile of the cell's length (at T4). Also, low levels of translation in the spore of Δ*spoIID* mutant, measured by OPP-Alexa 488 fluorescence intensity (Fig. 3d), suggest that the ribosomes are mostly gathered at the mother cell side of the spore.

Abstract L33 p2 delete "construction of"

- This has been corrected as follows:

LINES 32- 35: We anticipate our findings to be a starting point for ~~construction of~~ more sophisticated structural and mechanistic models of ribosome organisation during bacterial cell development ~~and to play a role in search for novel antibiotic targets~~ in sporulating bacteria.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

References

1. Eichenberger, P., Fawcett, P. & Losick, R. A three-protein inhibitor of polar septation during sporulation in Bacillus subtilis. *Mol. Microbiol.* **42**, 1147–1162 (2001).

2. Mohamed, A. *et al.* Chromosome Segregation and Peptidoglycan Remodeling Are Coordinated at a Highly Stabilized Septal Pore to Maintain Bacterial Spore Development. *Dev. Cell* **56**, 36-51.e5 (2021).

3. Anadolu, M. N. *et al.* Puromycin reveals a distinct conformation of neuronal ribosomes. *Proc. Natl. Acad. Sci. U. S. A.* **121**, e2306993121 (2024).

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The revised manuscript by Iwanska et al. has been largely improved from its original submission. The added experiments provide a clearer idea of what the mechanism of ribosome translocation may be, and certainly provide some data that should permit lots of follow up stories.

Major comment:

I am a bit disappointed that the authors were unable to perform the requested experiment in the disporic mutant background, which was not meant to be an onerous request. The authors state that the [delta]sigE strain, for example, was difficult to obtain and that the Stock Center did not get back to them. I'm not sure that this is a valid excuse, especially since dozens of B. subtilis labs across the world likely have this strain. The experiments that they provided in the reviews were not as convincing as they could be, given the relatively few disporic cells they were able to visualize, and the authors did not include these images in the revised version of the manuscript. I am not a fan of multiple rounds of reviews, so I will leave this as an editorial decision, especially since the authors are requesting the latitude to explore this in a subsequent report.

We are also disappointed that we were unable to perform the requested experiment in the disporic mutant background. We did contact other laboratories, posted a request for strain on Twitter with a 'subtiwiki' hashtag on Sept 18th, and hence, asked the Editors for some extra time. We did in the end receive the strains, but only a few days ago (on the 28th of November), at which point the editorial decision had been made.

Minor comments:

Line 22: consider rewording to read, "…during sporulation in wild type and mutant Bacillus subtilis."

We have changed the wording of this sentence as follows:

LINE 22: "localisation of ribosomes during sporulation in wild type and mutant *Bacillus subtilis.*"

Line 35: consider deleting, "in sporulating bacteria".

We have deleted the above, resulting in:

LINE 32: "of ribosome organisation during bacterial cell development."

Line 84: delete "the" before "chromosome translocation".

We have deleted "the" before "chromosome translocation":

LINE 81: "this is accompanied by chromosome translocation resulting in forespore inflation".

Reviewer #2 (Remarks to the Author):

All our comments and questions were addressed in the revised version of this very interesting Manuskript. During the discussion of the revision with my co-reviewer a comment concerning Fig 1 came up.

-Results Fig 1 b&c Text in results on p4 l85-89

"Interestingly, we observed that during asymmetric septation and chromosome translocation (T2-T3), the ribosomes that were previously at the cell pole are restricted from the forespore rather than trapped in the divided cytoplasm. It appears that the ribosomes gather and wait at the asymmetric septum at the mother cell side and during or very shortly after the engulfment by the mother cell, the ribosomes are translocated into the spore (T4)."

This description concerning the dynamic of the ribosomes should actually refer to T1-T2-T3, since the ribosomes at the poles are clearly visible at T1. Then at T2 the forespore has already formed, and less ribosomes are detected (by fluorecence) in this new morphological structure, especially when compared to the ribosomes at the pole at T1. Then at T3 a significant amount of Ribosomes become detected in the mothercell between the forespore membrane and the mothercell chromosome wich is next to another peak of ribosome at the opposite (right) pole of the mothercell. At T4 the translocation of these adjacent ribosomes into the forespore has already started .

This dynamic process can also be observed at higher time resolution in the Suppl Fig 1 & 3 (Movie S1).

To state that "the ribosomes that were previously at the cell pole are restricted from the forespore rather than trapped in the divided cytoplasm" appears to be speculative at this stage and could be addressed in more detail in the discussion.

We have now referred to T1-T2-T3 timepoints in the main text and described the results as suggested by the Reviewer 2, as follows:

LINES 82-84: Interestingly, we observed that during asymmetric septation and chromosome translocation (T2-T3), the fluorescence signal corresponding to ribosomes at the cell pole (T1) decreases, suggesting that less ribosomes are detected in the developing forespore.

We have moved the following statement to the Discussion section:

LINES 306-310: Our data strongly suggests that ribosomes are restricted from the developing forespore rather than trapped in the divided cytoplasm. The ribosomes move away from the pole where the asymmetric septation will take place, and only a small proportion of membrane associated ribosomes is included in the developing forespore.

-Discussion (p11 l 320-1)

In the first statement (2nd sentence) in the discussion on this topic, I would not use the term "belief" and replace it by other terms such as "models".

We have now changed this sentence as follows:

LINES 303-304: “Contrary to the current model, we propose that the ribosomes are not already present in the forespore and displaced to the forespore periphery during chromosome translocation.”

One could consider to further discuss different models, possible mechanisms or experimental observations for this interesting developmental process in more detail.

Given the data presented in this manuscript, we feel that the discussion provided addresses the observations made in the course of the experimental work, and we would like to refrain from more speculative discussion and also keep the manuscript concise.

- Discussion p12 l351-353 include "might" : ...ΔspoIID, this MIGHT suggest that ribosomes are not synthesised de novo in the forespore and are “inherited” from the mother cell.

We have changed this sentence as follows:

LINES 334-336: Since we did not observe ribosome biogenesis factors in the MS data nor the GFP signal in the bulged membrane of *ΔspoIID*, this might suggest that ribosomes are not synthesised *de novo* in the forespore and are “inherited” from the mother cell.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.