EMBO reports

Protease protection assays show polypeptide movement into the SecY channel by power-strokes of the SecA ATPase

Marco Catipovic and Tom Rapoport
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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 25th Jun 2020

Dear Tom,

Thank you for the submission of your research manuscript to our journal. We have received the reports from the three referees that were asked to assess it (copied below).

As you will see, the referees judged the broader advance provided by your findings differently. Referee 1 supported revision for EMBO reports but also noted that similar conclusions with a similar approach have been addressed in previous publications of your lab. Referee 3 suggested publication in a more specialized journal in the summary table returned with the report. Given the support from referee 1 and 2 I have discussed this aspect further with the referees. I have meanwhile received feedback from all three and given that also the more critical referee 3 supported a revision for EMBO reports and given the value of your findings for the transport community, we have decided to invite you to revise your study for potential publication in EMBO Reports.

All three referees note a lack of quantification and confirmation using independent biological repeats and it will be essential to address this concern. Referee 1 suggested to extend the findings to a model substrate other than proOmpA. While we agree that this would be potentially interesting, it is not required from our side and the universality of the findings obtained with proOmpA can be discussed. The referees also asked for the display of larger gel sections. Please note that we also encourage authors to upload and publish source data and this would also be a good place to show the full, uncropped gels used to generate the figures (see also point 8 below).

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. 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.

We invite you to submit your manuscript within three months of a request for revision. This would be September 25th your case. Yet, given the current COVID-19 related lockdowns of laboratories, we have extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) Supplementary information. You can have up to 5 Expanded View Figures. These should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).
Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and

instruction on how to label the files are available .

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

10) 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.
IMPORTANT: Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates. If the data rely on a smaller number of replicates, scatter blots showing individual data points are recommended.
- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).
- Please also include scale bars in all microscopy images.

11) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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

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.

Kind regards,
Martina

Martina Rembold, PhD
Editor
EMBO reports

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Referee #1:

The work by Catipovic & Rapoport titled "The power-stroke of the SecA ATPase moves polypeptides into the protein-conducting SecY channel" is a well-written manuscript that tries to settle the ongoing dispute between two alternative modes of operation performed by the AAA ATPase SecA, ratcheting versus power-stroke. Using the reductionist approach and reconstituting secretion (protease protection assay) of a commonly used model substrate (OmpA) in vitro with only the minimally required components (SecYEG proteoliposomes + SecA) Catipovic & Rapoport convincingly demonstrated that
a) wild type SecA drives post-translational translocation of OmpA into proteoliposomes,
b) the transport occurs specifically in presence of swiftly or slowly hydrolyzing ATP or ATPγS,
c) this protein conductance is stalled in presence of ADP or non-hydrolysable ADP•BeFx,
and, most importantly,
d) different SecA mutants in the two-helix finger or the clamp domain compete and interfere with the kinetics of the push-and-slide reaction.
Those are valuable findings that further our understanding of the mechanism used by the bacterial SecA ATPase. While the nature of the experiments is simple, and yet elegant, the manuscript should be improved by some points listed below before publication.

Summary
1. Does this manuscript report a single key finding? YES
The in vitro data speak in favor of the bacterial SecA ATPase catalyzing post-translational preprotein secretion via the SecYEG channel by a power-stroke mechanism.
2. a) Is the reported work of significance? YES
The manuscript sheds a detailed and convincing light on the mechanism used by the monomeric, bacterial SecA ATPase.
2. b) Does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms? YES
Similar conclusions with a similar approach and a similar set of mutants has been addressed in a previous publication by the same lab (Bauer et al. 2014, A "Push and Slide" Mechanism Allows Sequence-Insensitive Translocation of Secretory Proteins by the SecA ATPase, https://doi.org/10.1016/j.cell.2014.03.063)
3. Is it of general interest to the molecular biology community? YES
The paper documents the power of the reconstitution approach, even for non-experts in bacterial protein secretion.
4. Is the single major finding robustly documented using independent lines of experimental evidence? YES and NO (see below points 1, 2, 3, and 10)

Constructive criticism for the authors
Major points:
(1) Regarding point 4 of the Summary "Is the single major finding robustly documented?" the major critique is the lack of any independent, biological repeats. Although the authors use a kinetic approach to demonstrate the transport reactions, the authors - as far as I can tell - do NOT mention or show in the text, figures or the legends that any experiment was repeated.
(2) Was the same batch of reconstituted proteoliposomes used for all the experiments? Can the authors estimate the distribution of the SecYEG orientation in the proteoliposomes? Would that be similar for different preparations?
(3) None of the kinetics is quantified. It would be helpful to either provide quantification for the 30 min value or, preferably, like it was done in a previous publication from the same lab (Bauer et al. 2014, Cell, https://doi.org/10.1016/j.cell.2014.03.063).
(4) For the majority of experiments only a small segment of the gel is shown for the signal(s) of

relevance and usually (excluding Fig. EV3A, B) no molecular weight comparison is given.

Minor points:
(5) Page 5, paragraph 2: I would change "additional SecA mutations into preformed translocation complexes" to "additional SecA mutations into preformed wild type SecA translocation complexes"
(6) Figure EV2 (Page 4, paragraph 3; Page 5, paragraph 2): I would change the panel order. EV2B should become EV2A (given that the reduced/slower ATP hydrolysis by the WB SecA is introduced first). EV2C should become EV2B (given that the mutations are introduced second) and EV2A should become EV2C (given that the double mutants in the WB SecA background are mentioned last).
(7) Page 5, paragraph 2: The conclusion "indicating they bind to the translocon" is not warranted based on the data presented (Fig. EV2A, only FL band is visible). Best to delete that phrase. Alternatively, flotation or crosslink experiments with mutants carrying different tags could be used to demonstrate interaction of SecYEG and the different mutants.
(8) Page 5, paragraph 3; Legend Fig. 3: The authors mentioned the conserved nature of the Y794, R792, and K797 residues in the in the tip of THF region multiple times. Could they provide a reference or an additional figure panel showing this conservation in different SecA expressing prokaryotes?
(9) Page 7, paragraph 1: "polypeptide fragment increases by ~1 kDa upon ATP binding" This statement is hard to judge without any molecular weight comparison (cf. comment 4).
(10) This work and most of the previous work (Bauer et al. 2014, Cell; Catipovic et al. 2019, EMBO) is based on one (truncated and variably modified) preprotein substrate proOmpA. Could the authors show similar behavior for another secretory precursor or speculate/discuss the "universality" of their findings?
(11) Page 10, paragraph 2 Translocation assay: In the main text the WB SecA is described as a dominant-negative inhibitor of SecA. Why is WB SecA (1 µM) added in fivefold molar excess over WT SecA (200 nM)?
(12) Page 10/11 for example: Sometimes number and corresponding unit are not separated by a space (e.g. 20mM glucose, 250mM imidazole, 5mM ATPγS)
(13) Page 17, Fig. 3 (and page 5, paragraph 3): The authors should put Fig. EV3C left to Fig. 3E to have the direct comparison between Y794G + ATP (Fig. EV3C) and Y794G + ATPγS (Fig. 3E).
(14) Page 18, Legend Fig. 1: Consider changing "translocation can be completed with" to "translocation of the full-length (FL) substrate can be completed with".
(15) Page 20, Fig. EV2: For Fig. EV2A a bigger panel for each of the variants should be shown, so that the IM and FL fractions are visible (cf. Fig. 1B-D, Fig. 3E)
(16) Page 21, headlines to all three EV Figures are numbered as "1".
(17) Page 21, Legend Fig. EV2: Consider changing "WB SecA was added at" to "Different WB SecA variants (WT, Y794G, R342E) were added at".
(I see, I guess this figure answers my previous comment 11. Pardon me.).
(18) If required by EMBO reports, for all gel sections shown the original data/gel they were cutout from should be uploaded as supplementary data.

Overall:
Despite some criticism on my end, I hope the authors can address these points and I would like to see their results being published. Thank you!

Referee #2:

This study aims at providing new insights into the mechanism by which SecA promotes transport of polypeptide segments across the bacterial cytoplasmic membrane via the SecY channel.

Post-translational secretion by the Sec translocon has been extensively studied in the last decades. How the energy harnessed from ATP hydrolysis converts into protein translocation activity remains ill-defined. Recent articles by the team of Rapoport and others support a model according to which transport is promoted by a power-stroke mechanism mediated by SecA. In the present work, Catipovic and Rapoport search for direct experimental evidence that ATP binding by SecA and a movement of the two-helix finger (THF) domain induce a forward step of the substrate polypeptide into the SecY channel. The authors also want to assess whether the clamp domain of SecA contributes to prevent back-sliding prior to ATP hydrolysis.

The experiments, based on elegant in vitro approaches, are sound and well-illustrated. Most importantly, with their results the authors corroborate the notion that ATP binding results in the stepping forward of the polypeptide (Catipovic et al., EMBO Journal 2019; Bauer et al., Cell 2014) and provide evidence that the grip of the THF domain on the translocating polypeptide contributes to its ATP-dependent movement (Figure 2B and Figure 3D). Several aspects of the experiments assessing the role of the SecA clamp domain need to be clarified:

-Figure 2B shows how the WB SecA variant (low ATPase activity) can maintain the translocating polypeptide segment into the channel. Replacing wild-type SecA with the WB variant pushes the polypeptide forward into the channel. Over time, however, the substrate seems to slide backwards as the signal intensity of the corresponding fragment decreases. A signal quantification from independent experimental repeats will help to appreciate the degree by which the signal fades away. If there is backsliding, the result would suggest that the clamp domain does not suffice to keep the polypeptide in the channel. The authors should clarify if there is backsliding.

-In the same Figure 2B, the authors should show larger portions of the gels below and above the fragment to appreciate whether any backsliding intermediate can be detected, as well as the odd possibility that some of the substrate escapes the disulfide-induced block.

-In Figure 3B (ATP) and 3D (slowly hydrolysable ATP), SecA variants with reduced grip in the THF domain cannot prevent backsliding of the substrate. Thus, in the presence of ATP, the SecA clamp domain is inactive, which is in contrast to one of the main conclusions of the authors (see for instance the last sentence of the Abstract and the discussion part "Furthermore, we show that closure of the SecA clamp is essential for prohibiting substrate sliding before ATP hydrolysis has occurred").

- In general, is not clear if the clamp would prevent backsliding when SecA binds ATP or during ATP hydrolysis. The authors should clarify this point that may be at the origin of their conflicting results on the clamp function. Are the THF mutants able to prevent backsliding of the substrate in the presence of ADPBeF or ADP Pi?

Minor points

-I suggest to show larger portions of gel areas below the IM bands, which may help to appreciate any intermediate of the backsliding reaction in the experiments where proteinase K is used.
-After a short time of incubation (1 min), the WB SecA mutant variant seems quite efficient in promoting full substrate translocation in the presence of ATP and DTT (EV Fig. 2B) with almost half

of the substrate becoming protected from proteinase K, whereas less substrate is translocated after 1 min by wild type SecA (Fig. 1D). How this result reconciles with the idea that WB SecA should perform less power-stroke cycles? Please clarify.
-Fig. 3E: I would assume that the two experiments (top and bottom gels) use similar quantities of pre-formed intermediate as starting material (lane 1) but this seem to be not the case from the shown gels, which have different signal intensities. Please clarify.
-EV Fig. 2 and 3 are mislabeled in the legends.

Referee #3:

The major pathway for post-translational protein translocation across the plasma membrane in bacteria is mediated by SecYEG and SecA. Elucidation of the molecular mechanism of this process is a very important goal. The "Power Stroke Model" and the "Brownian Ratchet Model" have currently being proposed as possible molecular mechanisms. The corresponding author's group had previously reported that protein translocation occurs by the "Power Stroke Model" (Catipovic et al, 2019, EMBO J; Bauer et al., 2014, Cell). In this study, using various SecA mutants and various nucleotides, including nucleotide analogs, the authors performed protease protection assays that are now standard for monitoring the stability of protein translocation intermediates. The results are in support of the "Power Stroke Model". In addition, the tip of the THF domain has been shown to be involved in the prevention of backsliding of the substrate. This paper includes reliable results from mutational analyses and its story is clear. However, the paper does not contain particularly unusual results of high novelty value. Conceivably, data that disprove or question the Brownian ratchet model (Allen et al. 2016, eLife; Corey et al., 2019, eLife) would provide more compelling support of the Power Stroke Model.

Major comments
1. Quantification of the experiments.
In the previous report (Bauer et al., 2014, Cell), the protection assays were quantitatively evaluated. In contrast, the reproducibility of the data in this manuscript is not discussed or demonstrated. For example, the authors should show quantitative data in each figure.
2. The title of the paper is inappropriate.
The experimental approach described in the paper probes protease access to substrate. However, the authors do not demonstrate that the substrate in company with the THF domain is inserted into the channel. A possible title might be: "The power-stroke model of SecA ATPase is supported by protease protection assays." Alternatively, this could be written: "Protease protection assays support the power-stroke model of SecA ATPase."

Minor comments
P4 lines 8-10: The explanation concerning FV1 is a leap in logic. One possible interpretation is that SecA folding becomes strong by ATP analog binding. Then SecA may protect the substrate. Please rephrase the notation.
P21: The numbers of the EV figures are wrong.
Material and method: Protein is not DNA. Sentences, like that protein A was cloned into a pET30b, are wrong. Please rephrase these sentences.

Dear Martina,

Thank you very much for the reports of the reviewers and your own comments. We were pleased to see that all reviewers recommended publication and that you invited us to submit a revised manuscript. The main point of all reviewers was the request for quantification of the data, which we have now done. We also provide the original gels in the Source Data, which show larger sections than in the actual figures. We have added an important experiment (Fig EV1) showing that protease protection is solely due to the membrane, not to SecA. This new data provides strong evidence that the changes in the sizes of protected fragments are indeed caused by movement of the polypeptide into the SecY channel.

We went through the 11 points that you listed and hope that the paper is now consistent with the requirements of EMBO Reports.

Below is a point-by-point response to the reviewer's comments (our response is in red):

Referee #1:

The work by Catipovic & Rapoport titled "The power-stroke of the SecA ATPase moves polypeptides into the protein-conducting SecY channel" is a well-written manuscript that tries to settle the ongoing dispute between two alternative modes of operation performed by the AAA ATPase SecA, ratcheting versus power-stroke. Using the reductionist approach and reconstituting secretion (protease protection assay) of a commonly used model substrate (OmpA) in vitro with only the minimally required components (SecYEG proteoliposomes + SecA) Catipovic & Rapoport convincingly demonstrated that
a) wild type SecA drives post-translational translocation of OmpA into proteoliposomes,
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and, most importantly,
d) different SecA mutants in the two-helix finger or the clamp domain compete and interfere with the kinetics of the push-and-slide reaction.
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2. a) Is the reported work of significance? YES
The manuscript sheds a detailed and convincing light on the mechanism used by the monomeric, bacterial SecA ATPase.
2. b) Does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms? YES
Similar conclusions with a similar approach and a similar set of mutants has been addressed in a

previous publication by the same lab (Bauer et al. 2014, A "Push and Slide" Mechanism Allows Sequence-Insensitive Translocation of Secretory Proteins by the SecA ATPase, https://doi.org/10.1016/j.cell.2014.03.063)
3. Is it of general interest to the molecular biology community? YES
The paper documents the power of the reconstitution approach, even for non-experts in bacterial protein secretion.
4. Is the single major finding robustly documented using independent lines of experimental evidence? YES and NO (see below points 1, 2, 3, and 10)

Constructive criticism for the authors
Major points:
(1) Regarding point 4 of the Summary "Is the single major finding robustly documented?" the major critique is the lack of any independent, biological repeats. Although the authors use a kinetic approach to demonstrate the transport reactions, the authors - as far as I can tell - do NOT mention or show in the text, figures or the legends that any experiment was repeated.

Most experiments were performed in at least two replicates, which is now stated in the figure legends. We have added figures that show the quantification of each experiment.

(2) Was the same batch of reconstituted proteoliposomes used for all the experiments? Can the authors estimate the distribution of the SecYEG orientation in the proteoliposomes? Would that be similar for different preparations?

Different proteoliposomes batches were used for each experiment. We did not independently test the topology of the SecYEG complexes in each liposome reconstitution. Previous experiments have established that SecYEG reconstituted in liposomes shows no orientation bias: roughly half the complexes are oriented with their cytosolic face outwards (Allen et al., 2016, Fig4 – figure supplement 1D). We have clarified this and included the relevant citations in the methods section (page 11 line 2-5).

(3) None of the kinetics is quantified. It would be helpful to either provide quantification for the 30 min value or, preferably, like it was done in a previous publication from the same lab (Bauer et al. 2014, Cell, https://doi.org/10.1016/j.cell.2014.03.063).

We have now provided quantifications for both the backsliding of intermediates, and the completion of translocation in the presence of different nucleotides and mutants based on the gels shown and their replicates.

(4) For the majority of experiments only a small segment of the gel is shown for the signal(s) of relevance and usually (excluding Fig. EV3A, B) no molecular weight comparison is given.

Full autoradiographic images, from which each figure has been cropped, are now shown as Source Data. The figures also show molecular weight markers derived from gels analyzed in parallel by Coomassie blue staining.

Minor points:

(5) Page 5, paragraph 2: I would change "additional SecA mutations into preformed translocation complexes" to "additional SecA mutations into preformed wild type SecA translocation complexes"

We have made the suggested textual change (page 5 line 9-10).

(6) Figure EV2 (Page 4, paragraph 3; Page 5, paragraph 2): I would change the panel order. EV2B should become EV2A (given that the reduced/slower ATP hydrolysis by the WB SecA is introduced first). EV2C should become EV2B (given that the mutations are introduced second) and EV2A should become EV2C (given that the double mutants in the WB SecA background are mentioned last).

We have rearranged the panels as the reviewer suggested.

(7) Page 5, paragraph 2: The conclusion "indicating they bind to the translocon" is not warranted based on the data presented (Fig. EV2A, only FL band is visible). Best to delete that phrase. Alternatively, flotation or crosslink experiments with mutants carrying different tags could be used to demonstrate interaction of SecYEG and the different mutants.

Only the FL band would be expected in the experiment pictured in Fig EV2A (now EV2D). In this experiment, substrate was incubated with a mixture of WT SecA, along with the indicted concentration of the WB SecA mutants in the presence of ATP and DTT. Increasing concentrations of WB SecA saturates the SecY binding sites and prohibits the WT SecA from initiating and completing the translocation of the substrate. We have edited the text to clarify this point (page 5 line 13-14). However, the reviewer is correct in pointing out that this experiment does not directly demonstrate the interaction of the WB SecA proteins with SecY. We have therefore softened the language relating to this claim, and now say the experiment "suggests they still bind to the translocon" (page 5 line 15).

(8) Page 5, paragraph 3; Legend Fig. 3: The authors mentioned the conserved nature of the Y794, R792, and K797 residues in the in the tip of THF region multiple times. Could they provide a reference or an additional figure panel showing this conservation in different SecA expressing prokaryotes?

We have provided references in the text (page 6 line 9): Erlandson et. al., 2008a, Supplementary Table 1; Bauer et. al., 2014, Supplementary Figure 7E.

(9) Page 7, paragraph 1: "polypeptide fragment increases by ~1 kDa upon ATP binding" This statement is hard to judge without any molecular weight comparison (cf. comment 4).

We have added MW markers to gels throughout the manuscript. See the response to point #4.

(10) This work and most of the previous work (Bauer et al. 2014, Cell; Catipovic et al. 2019, EMBO) is based on one (truncated and variably modified) preprotein substrate proOmpA. Could the authors show similar behavior for another secretory precursor or speculate/discuss the "universality" of their findings?

We agree with the editor that these experiments would be beyond the scope of the current paper. However, we have added a sentence to the discussion to make limitations of these experiments with a single substrate explicit (page 7 line 11-13).

(11) Page 10, paragraph 2 Translocation assay: In the main text the WB SecA is described as a dominant-negative inhibitor of SecA. Why is WB SecA (1 µM) added in fivefold molar excess over WT SecA (200 nM)?

WB SecA serves as a dominant negative inhibitor since it binds irreversibly to the SecYEG translocon. However, when competing with wild-type protein, which is already bound, the release kinetics of WT SecA, as well as its ability to rebind before the WB mutant, limits the efficacy of competition at equimolar ratios. We therefore supply it in a moderate excess to WT SecA in our replacement experiments.

(12) Page 10/11 for example: Sometimes number and corresponding unit are not separated by a space (e.g. 20mM glucose, 250mM imidazole, 5mM ATPγS)

We have corrected these errors.

(13) Page 17, Fig. 3 (and page 5, paragraph 3): The authors should put Fig. EV3C left to Fig. 3E to have the direct comparison between Y794G + ATP (Fig. EV3C) and Y794G + ATPγS (Fig. 3E).

We respectfully request to leave Fig EV3C in the supplement. We do not believe that the experiment is essential to understanding the conclusions drawn in the paper, and Fig 3 is quite busy already with the introduction of gel quantifications.

(14) Page 18, Legend Fig. 1: Consider changing "translocation can be completed with" to "translocation of the full-length (FL) substrate can be completed with".

We have made the suggested textual change (page 18 line 11).

(15) Page 20, Fig. EV2: For Fig. EV2A a bigger panel for each of the variants should be shown, so that the IM and FL fractions are visible (cf. Fig. 1B-D, Fig. 3E)

We have included the full gels from which are figures are cropped as Source Data. Fig. EV2A (now EV2D) has no other fragments as the experiments are performed in the presence of DTT. We have amended the figure legend for clarification (page 21 line 23).

(16) Page 21, headlines to all three EV Figures are numbered as "1".

We have corrected this typographical error.

(17) Page 21, Legend Fig. EV2: Consider changing "WB SecA was added at" to "Different WB SecA variants (WT, Y794G, R342E) were added at".

We have changed the text (page 21 line 21).

(I see, I guess this figure answers my previous comment 11. Pardon me.).
(18) If required by EMBO reports, for all gel sections shown the original data/gel they were cutout from should be uploaded as supplementary data.

Full gels have been supplied as Source Data.

Overall:
Despite some criticism on my end, I hope the authors can address these points and I would like to see their results being published. Thank you!

We appreciate the overall positive review of our paper.

Referee #2:

This study aims at providing new insights into the mechanism by which SecA promotes transport of polypeptide segments across the bacterial cytoplasmic membrane via the SecY channel.

Post-translational secretion by the Sec translocon has been extensively studied in the last decades. How the energy harnessed from ATP hydrolysis converts into protein translocation activity remains ill-defined. Recent articles by the team of Rapoport and others support a model according to which transport is promoted by a power-stroke mechanism mediated by SecA. In the present work, Catipovic and Rapoport search for direct experimental evidence that ATP binding by SecA and a movement of the two-helix finger (THF) domain induce a forward step of the substrate polypeptide into the SecY channel. The authors also want to assess whether the clamp domain of SecA contributes to prevent back-sliding prior to ATP hydrolysis.

The experiments, based on elegant in vitro approaches, are sound and well-illustrated. Most importantly, with their results the authors corroborate the notion that ATP binding results in the stepping forward of the polypeptide (Catipovic et al., EMBO Journal 2019; Bauer et al., Cell 2014) and provide evidence that the grip of the THF domain on the translocating polypeptide contributes to its ATP-dependent movement (Figure 2B and Figure 3D).

Thank you for the overall positive review of our paper.

Several aspects of the experiments assessing the role of the SecA clamp domain need to be clarified:

-Figure 2B shows how the WB SecA variant (low ATPase activity) can maintain the translocating polypeptide segment into the channel. Replacing wild-type SecA with the WB variant pushes the polypeptide forward into the channel. Over time, however, the substrate seems to slide backwards as the signal intensity of the corresponding fragment decreases. A signal quantification from independent experimental repeats will help to appreciate the degree by which

the signal fades away. If there is backsliding, the result would suggest that the clamp domain does not suffice to keep the polypeptide in the channel. The authors should clarify if there is backsliding.

We have now provided quantifications of our replicates to assess the degree that substrates backslide or complete translocation in different conditions. We see only slight backsliding in the presence of ATPgS/ADP•BeF_x/WB mutants, much less than seen in the absence of ATP or with clamp mutants. This backsliding occurs over timescales of tens of minutes, and would be unlikely to play a significant role in translocation *in vivo*. We have added a sentence to mention the stability of the intermediate (page 4 line 8-9).

-In the same Figure 2B, the authors should show larger portions of the gels below and above the fragment to appreciate whether any backsliding intermediate can be detected, as well as the odd possibility that some of the substrate escapes the disulfide-induced block.

We now show complete gels as Source Data.

-In Figure 3B (ATP) and 3D (slowly hydrolysable ATP), SecA variants with reduced grip in the THF domain cannot prevent backsliding of the substrate. Thus, in the presence of ATP, the SecA clamp domain is inactive, which is in contrast to one of the main conclusions of the authors (see for instance the last sentence of the Abstract and the discussion part "Furthermore, we show that closure of the SecA clamp is essential for prohibiting substrate sliding before ATP hydrolysis has occurred").

This point is well taken. We agree that if the clamp alone prevents backsliding, then there should be less backsliding in the presence of the more slowly hydrolyzing ATPγS when compared to ATP, even with the THF mutants. But this is not the case: sliding with THF mutants is equally rapid in the presence of ATPγS and ATP. As both the clamp and THF are engaged with the polypeptide when SecA is bound with ATPγS, it is likely that both domains are needed to block all backsliding. While our data do show that “the SecA clamp is essential for prohibiting substrate sliding,” our wording did not imply a possible role for the THF in preventing backsliding. We have therefore changed the text in the introduction, results, and the discussion to include this nuance (page 3 line 12; page 6 line 19-24; page 7 line 8-10, page 7 line 30).

- In general, is not clear if the clamp would prevent backsliding when SecA binds ATP or during ATP hydrolysis. The authors should clarify this point that may be at the origin of their conflicting results on the clamp function. Are the THF mutants able to prevent backsliding of the substrate in the presence of ADPBeF or ADP Pi?

We tried the proposed experiment with ADP•BeF_x. However, we found that the initial SecA dN20::H6 used to generate the intermediates remained bound to, or even rebound, the translocation complexes after the addition of imidazole in the presence of ADP•BeF_x. Thus, it was impossible to separate polypeptide movements caused by the mutant SecA from those caused by SecA dN20::H6 in the presence of this nucleotide.

Minor points

-I suggest to show larger portions of gel areas below the IM bands, which may help to appreciate any intermediate of the backsliding reaction in the experiments where proteinase K is used.

We now show complete gels as Source Data.

-After a short time of incubation (1 min), the WB SecA mutant variant seems quite efficient in promoting full substrate translocation in the presence of ATP and DTT (EV Fig. 2B) with almost half of the substrate becoming protected from proteinase K, whereas less substrate is translocated after 1 min by wild type SecA (Fig. 1D). How this result reconciles with the idea that WB SecA should perform less power-stroke cycles? Please clarify.

The WB replacement of wild-type SecA is not instantaneous. As the intermediates are established with wild-type SecA, much of that protein is still bound to the translocation complexes upon introduction of the WB SecA and DTT. Some of this bound wild-type SecA can complete translocation before it is replaced by the WB mutant. We attribute the initial rapid completion of translocation to this bound wild-type pool, and the subsequent slower increase in FL substrate to the hydrolysis activity of the WB mutant. We have now clarified this point in the text (page 5 line 3-7).

-Fig. 3E: I would assume that the two experiments (top and bottom gels) use similar quantities of pre-formed intermediate as starting material (lane 1) but this seem to be not the case from the shown gels, which have different signal intensities. Please clarify.

The initial material used in this experiment (lane 1) is the product of a 10 min incubation with imidazole and the indicated mutant in the presence of ATP. During that time, there is more backsliding with the THF mutant than with WT SecA (see Fig 3B,C), making the amount of intermediate remaining different before the addition of DTT and ATPγS. We have amended Fig 3G (see flowchart) to make the incubation protocol more explicit. As the quantifications are normalized to the amount of material in the first lane, the difference in starting material amounts should not affect the conclusions.

-EV Fig. 2 and 3 are mislabeled in the legends.

We have corrected this typographical error.

Referee #3:

The major pathway for post-translational protein translocation across the plasma membrane in bacteria is mediated by SecYEG and SecA. Elucidation of the molecular mechanism of this process is a very important goal. The "Power Stroke Model" and the "Brownian Ratchet Model" have currently being proposed as possible molecular mechanisms. The corresponding author's group had previously reported that protein translocation occurs by the "Power Stroke Model" (Catipovic et al, 2019, EMBO J; Bauer et al., 2014, Cell). In this study, using various SecA mutants and various nucleotides, including nucleotide analogs, the authors performed protease protection assays that are now standard for monitoring the stability of protein translocation

intermediates. The results are in support of the "Power Stroke Model". In addition, the tip of the THF domain has been shown to be involved in the prevention of backsliding of the substrate. This paper includes reliable results from mutational analyses and its story is clear. However, the paper does not contain particularly unusual results of high novelty value. Conceivably, data that disprove or question the Brownian ratchet model (Allen et al. 2016, eLife; Corey et al., 2019, eLife) would provide more compelling support of the Power Stroke Model.

We appreciate the statement that our results are clear. However, we do believe that our results are novel, as they provide strong evidence for a Power Stroke model and argue against the proposed Brownian Ratchet model.

Major comments
1. Quantification of the experiments.
In the previous report (Bauer et al., 2014, Cell), the protection assays were quantitatively evaluated. In contrast, the reproducibility of the data in this manuscript is not discussed or demonstrated. For example, the authors should show quantitative data in each figure.

We have now provided quantifications for both the backsliding of intermediates and completion of translocation in the presence of different nucleotides and mutants based on the gels shown and their replicates.

2. The title of the paper is inappropriate.
The experimental approach described in the paper probes protease access to substrate. However, the authors do not demonstrate that the substrate in company with the THF domain is inserted into the channel. A possible title might be: "The power-stroke model of SecA ATPase is supported by protease protection assays." Alternatively, this could be written: "Protease protection assays support the power-stroke model of SecA ATPase."

We now provide additional evidence that protease protection of the translocating polypeptide is entirely due to protection by the membrane (new Fig. EV1); the size changes of the protected fragments are not due to protection by SecA, as SecA is rapidly degraded, while the substrate fragments remain protected. These results show that protease protection indeed reports on polypeptide movement into the SecY channel. We have amended the text to mention the new data and clarify this point (page 4, line 9-12).

As requested by the reviewer, we have changed the title to indicate that the conclusions are based on protease protection assays.

Minor comments
P4 lines 8-10: The explanation concerning FV1 is a leap in logic. One possible interpretation is that SecA folding becomes strong by ATP analog binding. Then SecA may protect the substrate. Please rephrase the notation.

See the response to the point above. We now have more conclusive data to support this statement.

P21: The numbers of the EV figures are wrong.

We have corrected this typographical error.

Material and method: Protein is not DNA. Sentences, like that protein A was cloned into a pET30b, are wrong. Please rephrase these sentences.

We have made the suggested textual changes.

1st Revision - Editorial Decision 27th Aug 2020

Manuscript number: EMBOR-2020-50905V2
Title: Protease protection assays show polypeptide movement into the SecY channel by power-strokes of the SecA ATPase
Author(s): Marco Catipovic and Tom Rapoport

Dear Tom,

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

- We can only accommodate up to 5 keywords (you currently have 6). I suggest to remove "E. coli" from the list.

- Reference list: please list the first 10 authors followed by 'et al'

- EV figures: Please note the nomenclature 'Expanded View" instead of "Extended View", which is currently used for the EV figure legends.

- Source data: please merge the source data into one file per figure, i.e., combine the data for Fig. 1B, 1C, 1E into one pdf file and the quantification for 1D and 1F into one file, etc.

- The image shown as source data for Fig. EV1A appears 'empty', i.e., blank white when I open it with the Adobe reader. Could you please double-check?

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-600 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

Once you have made these minor revisions, please use the following link to submit your corrected manuscript:

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If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

Kind regards,
Martina

Martina Rembold, PhD
Editor
EMBO reports

****

Referee #1:

I very much appreciate the detailed explanations given by the authors and the changes they made to the manuscript as part of the revision. I am fully satisfied with the new version and endorse publication of the data in EMBO Reports.

Referee #2:

The authors have carefully revised the manuscript following the suggestions of the reviewers. They have provided an important control experiment for their protease protection assay, replicate data for most experiments and quantifications. I would have preferred to see longer lane crops of the gels in the main figures, anyhow unprocessed crops including full gel lanes are provided as source data. Also, they have clarified several points in the text. My request to clarify the role of the clamp has been fulfilled. The clamp appears to be important (but not sufficient) in preventing substrate backsliding. I look forward to seeing this work published by Embo Reports.

Referee #3:

According to the reviewer's comments, the authors have properly amended the manuscript. The revised version that includes the reproducibility of quantitative analysis would be enough for publication.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 1st Sep 2020

Dr. Tom Rapoport
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United States

Dear Tom,

Thank you for submitting your revised manuscript. I am now very pleased to accept it for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Kind regards,

Martina

Martina Rembold, PhD
Editor
EMBO reports

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Corresponding Author Name: Tom A. Rapoport
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-50905V1

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 iustified
- 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.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- 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?	Sample sizes were not pre-determined.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Data was not excluded from analysis. All replicates were used.
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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.	NA
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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	No statistical tests were used.
Is there an estimate of variation within each group of data?	Raw data points are plotted.

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Is the variance similar between the groups that are being statistically compared?	There are no statistical comparisons.

C- Reagents

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).	NA
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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	A data availability section has been included.
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).	There are no large datasets associated with the study. Raw data has been provided as "Source Data"
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
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