EMBO reports

Trigger factor is a *bona fide* secretory pathway chaperone that interacts with SecB and the translocase

Jozefien De Geyter, Athina G. Portaliou, Bindu Srinivasu, Srinath Krishnamurthy, Anastassios Economou and Spyridoula Karamanou

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

1st Editorial Decision 22 October 2019

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

As you will see, the referees acknowledge that the findings are potentially interesting but they also have a number of suggestions for how the study should be strengthened. It will be important to exclude artefacts induced by the chemical denaturation of proOmpA and to verify that TF's function depends on its chaperone activity. Furthermore, the stoichiometry of the complex should be clarified (TF monomer vs dimer). Please also address a potential competitive binding between SecB and TF to proOmpA and whether SecB can bind to proOmpA released from TF. Moreover, further evidence is required to exclude the presence of SecB:proOmpA complexes. Finally, the relevance of SecB binding to the C-terminus of SecA should be validated in vivo.

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.

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

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.

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
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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 (<https://www.embopress.org/page/journal/14693178/authorguide>). 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 (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>)

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures 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: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- 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) Appendix/Supplementary information: Please note that all materials and methods must be part of the main manuscript file and may not be part of the Supplementary material. The tables listing strains, plasmids, primers, and buffers may remain part of the Appendix but all other methods need to be part of the main materials and methods section.

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
<https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

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

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can be accessed at the end of the reference. Further instructions are available at <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

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.

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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.

****

REFEREE REPORTS

Referee #2:

The manuscript "Trigger factor is a networked secretory pathway chaperone by Jozefien De Geyter and colleagues studies interplay and function of the chaperones Trigger factor (TF) and SecB in post-translational translocation. They perform growth analysis of chaperone mutants, and a series of in vitro experiments using the outer membrane porin OmpA as the model substrate. By performing ATPase assays, binding experiments, hydrogen deuterium exchange mass spectrometry, isothermal titration calorimetry and translocation assays, they describe a tight interaction of TF with chemically denatured proOmpA that suppresses OmpA aggregation. The TF-proOmpA complex can engage the SecA bound translocon, but translocation is impaired until the association of SecB relieves the inhibitory role of TF.

The study is interesting and experiments are generally of good quality. The finding that TF binds and traps translocation substrates and that SecB is required to overcome the TF-mediated inhibition of OmpA translocation can potentially explain the cold-sensitivity of SecB mutants which can be restored by further deletion of tig. The study is potentially suitable for publication in EMBO reports once the following concerns have been fully addressed by the authors:

1. The authors (like others before) generally use chemically denatured proOmpA for performing the in vitro assays. There is no doubt that proOmpA is translocated post-translationally, however, I wonder whether the conformation of the renatured protein resembles the conformation of in vivo synthesized proOmpA that vectorially emerges from the ribosomal tunnel and is engaged by chaperones. Weissman and Bukau (Oh et al. 2011, Cell) have shown that proOmpA is cotranslationally bound by TF and that TF dissociates before translation is completed. This may

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indicate that in cells, OmpA acquires a conformation that suppresses post-translational TF engagement. It may also indicate that another chaperone engages nascent OmpA once TF has dissociated, for example SecB. In order to support their claims and to exclude an artifact due to chemical denaturation of the translocation substrate, the authors should perform a coupled translation/translocation experiment (similar to Fig. 4F) to analyze translocation of co-translationally folded proOmpA.

2. If the detrimental impact of TF is due to post-translational binding of TF to translocation substrates like proOmpA, a TF mutant that retains the chaperone properties of TF but lacks the affinity for ribosomes should be as toxic in ΔsecB mutants as the wild-type TF. To show this, the authors should include the TF-AAA mutant in the genetic complementation assays shown in Fig. 1A. Expression levels of TF under the conditions used must be tested by Western blotting.

Minor comments:
• Line 448: typo "tis"

Referee #3:

The study of de Geyter et al. elaborates on previous findings from Wickner's lab made in mid-80ties, which showed interactions between the model secretory protein proOmpA and the cytoplasmic chaperone trigger factor (TF). TF:proOmpA interactions together with the observed sensitivity of cells to TF over-expresson implied an interplay of SecB/TF chaperones. Here, de Geyter and co-workers use a range of approaches to investigate the dynamics of the in vitro assembled proOmpA:TF complex and explore the role of SecB:TF interplay in the preprotein targeting. The extensive analysis brings authors to a conclusion that TF plays a role in proOmpA translocation serving as an intermediate holdase/carrier that is able to deliver the substrate to the translocase, but may not able to release it for further translocation in absence of SecB.

The manuscript is well-written and describes the experimental procedures and observations in a detailed manner. However, the significance of the discussed findings is not clear. Presented experiments always focus on a rather linear scenario when the targeting/translocation cycle starts with the proOmpA:TF assembly, and it is proOmpA/OmpA but not TF that guides the targeting to SecA. Further, proOmpA can be released from the complex only in presence of SecB prior it is transferred to the translocase. However, once present in solution, the ubiquitous chaperone SecB would most likely interact with proOmpA directly, thus potentially excluding TF from the pathway. To explore this scenario and a role TF may play in targeting and/or holding, the authors should complement the existing data with the experiment when TF is titrated into pre-assembled proOmpA:SecB complexes, so a putative transfer of proOmpA between two chaperones, or an assembly of the larger ternary complex can be examined.

Other points:

1. Line 141: The sub-chapter title is misleading, since the described in vivo experiment does not examine ratios of SecB and TF, but rather tests the strain viability in absence/presence of the chaperones.

2. Figure 2A: Presence of proOmpA in the high molecular weight adduct should be confirmed, e.g. via immunoblotting. Also here, and for many other presented gel-based assays, quantification of bands would offer more detailed description of the results.

3. The oligomeric state of TF: Along the manuscript the authors describe TF to be a dimer, and so all concentrations are presented for dimers. However, their own results (line 218) suggest a monomeric complex of proOmpA:TF. If indeed the complex is formed in 1:1 stoichiometry, the dissociation constants should be presented for monomers of TF, not dimers.

4. Line 229: When describing effect of TF on proOmpA solubility, authors probably mean lanes 3-9 in Fig. 2B. Also here it should be noted that the maximum recovery is already reached at proOmpA:TF ratio 0.75, thus supporting the complex to form in 1:1 stoichiometry.

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5. Lines 303-306: The reasoning to exclude the scenario (a) is not convincing, since the affinity of TF to proOmpA is not too high (200 nM), and may be converted into 400 nM when calculated for TF monomers. Also the claimed absence of SecB:proOmpA complex on the Native-PAGE (lines 320-324, Fig. 4C) may be simply due to poor staining of the complex (see the comment below)

6. Figure 4C: Immunoblotting should be employed to test presence of proOmpA or proOmpA:SecB in Native-PAGE, as there is a broad band below 140 kDa visible upon adjusting the intensity levels at the elevated SecB concentrations (lanes 5-8).

7. Figure 4D: The authors should indicate that the apparent mass of the putative ternary complex proOmpA:TF:SecB (~400 kDa) is substantially different from the value provided by the GPC-MALLS analysis (134 kDa). Validating the composition of the adduct on Native-PAGE via mass-spectrometry or immunoblotting would strongly support the authors' hypothesis on the composition of the complex.

Referee #4:

De Geyter and colleagues have used in vitro approaches to address the role of the Trigger Factor (TF) chaperone in Sec-dependent protein export. Especially, this work answers a long lasting question concerning the in vivo antagonism previously observed between TF and SecB, both cytosolic chaperones involved in protein export under certain conditions. Using the E. coli outermembrane protein OmpA as a substrate of TF and SecB for in vitro reconstitution, the authors show that TF forms a tight complex with proOmpA and that it prevents its aggregation. Remarkably, the TF-proOmpA complex (but not TF alone) specifically binds to the SecYEG-SecA translocase but in this case, proOmpA translocation is blocked. Addition of the SecB chaperone very efficiently relieves such a preventive effect of TF on proOmpA translocation, thus nicely reproducing the deleterious effect of TF found in vivo in E. coli when SecB is absent. In addition, this work further shows that TF, proOmpA and SecB can form a soluble quaternary complex and that SecB improves TF-proOmpA binding to the translocase. In this case, SecB likely release proOmpA from TF to allow its translocation, thus possibly acting as both an exchange factor and a chaperone.
-The manuscript is clearly written and the data are novel. They have used elegant in vitro approaches to demonstrate how TF and SecB contribute to both targeting and triggering proOmpA translocation. This clearly pushes forward our understanding of major chaperone networks in bacteria. I only have few comments that need to be addressed.
Comments:
-TF is playing a major role in the folding of cytosolic proteins. The title is thus misleading and should be changed, as for now it gives the impression that TF is only a secretory pathway chaperone. In addition, the important discovery described here does not only concern TF but also SecB and the interplay between these two chaperones.
- Figure 1A only reproduces what has been already published by several groups. Therefore it should be removed from the main manuscript. Furthermore, dilutions stop at -5, which is not enough to detect single colonies on plates.
-The authors propose an attractive model in which SecB binding to the extreme C-ter region of SecA is necessary for the TF-related SecB exchange factor function. Indeed, they show that addition of SecB does not relieve the inhibitory effect of TF in vitro. Yet, there is no in vivo evidence for such model. To strengthen this part of the work, the authors should test whether the SecA C-ter mutant is cold sensitive for growth in the presence of TF (as observed for the secB mutant e.g. Figure 1A) and if overexpression of TF is more toxic in such a strain background when compared to wild-type.
- TF can leave the ribosome while bound to its substrate and SecB was shown to bind nascent protein co-translationally. A possible early complex occurring during translation is missing in Figure 5A. In addition, on the model it seems that SecB has specific affinity for signal sequence, which is not the case. In Figure 5B, the quaternaty SecB-TF-OmpA complex is missing. In addition, it is not clear whether TF would leave Sec before SecB once translocation initiates.
-The data suggest that the observed TF-SecB-SecA interplay might be part of a quality control mechanism to insure protein export and avoid undesired cytosolic protein translocation. Therefore the authors should refer to the work by Eser and Ehrmann (PNAS. 2003, 100(23):13231-4), especially in the discussion related to figure 5.
-There are references to "data not shown" that should be presented in supplementary data, i.e., line

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279 and 321. In addition, there is reference to a manuscript under preparation Krishnamurthy et al., in preparation line 501, with no support available.

1st Revision - authors' response 21 January 2020

Referee #2:

"…The study is interesting and experiments are generally of good quality. The finding that TF binds and traps translocation substrates and that SecB is required to overcome the TF-mediated inhibition of OmpA translocation can potentially explain the cold-sensitivity of SecB mutants which can be restored by further deletion of tig."
Response:
We thank the reviewer for the positive feedback.

Comments:
#1
1. "The authors (like others before) generally use chemically denatured proOmpA for performing the in vitro assays. There is no doubt that proOmpA is translocated post-translationally, however, I wonder whether the conformation of the renatured protein resembles the conformation of in vivo synthesized proOmpA that vectorially emerges from the ribosomal tunnel and is engaged by chaperones…."
and
"…In order to support their claims and to exclude an artifact due to chemical denaturation of the translocation substrate, the authors should perform a coupled translation/translocation experiment (similar to Fig. 4F) to analyze translocation of co-translationally folded proOmpA."
Response:
It is difficult to compare directly/accurately the conformation of nascent proOmpA synthesized *in vivo* with proOmpA refolding *in vitro* after dilution out of a chaotrope. Nevertheless, proOmpA derived from chaotrope has all the properties consistent with a physiologically relevant translocation reaction, i.e. binds to chaperones, to the translocase with high affinity and is translocation-competent.
Action taken:
We have synthesized [^{35}S]-proOmpA in the absence of urea and shown similar *in vitro* translocation, the TF inhibitory effect and the relieve from SecB (Appendix Figure S6B).

#2
"…Weissman and Bukau (Oh et al. 2011, Cell) have shown that proOmpA is cotranslationally bound by TF and that TF dissociates before translation is completed. This may indicate that in cells, OmpA acquires a conformation that suppresses post-translational TF engagement…."
Response:
Ribosome profiling was done under cellular levels of production of proOmpA and using antibody pull-downs. By definition then the proOmpA that comes down with anti-TF antibodies is only the one that is trapped on the ribosomes with nanomolar affinity. These nanoM interactions may be short-lived and only serve to scan for intense aggregators. But we know from *ex vivo* pull-downs of cytoplasmic tagged TF that non-ribosomal TF: exported protein complexes do exist (Crooke and Wickner 1987, PNAS; Martinez-Hackert and Hendrickson 2009, Cell). In profiling experiments such free-standing proOmpA:TF complexes (as they are low to high micromolar affinity) or membrane-bound TF:proOmpA (as membranes are not included) are lost and cannot be evaluated. Upon dilution, micromolar affinity complexes will dissociate.
Action taken:
Experiments in Fig. 1A have now been expanded using a ribosome binding deficient and client binding deficient TF derivatives. The TF-mediated inhibition of secretion we describe is not related to ribosome-bound TF but to client binding.

#3
"…It may also indicate that another chaperone engages nascent OmpA once TF has dissociated, for example SecB...."
Response:

This is a fair possibility and a possible parallel route that other reviewers also bring up. This would be the "traditional:" way SecB is expected to work by holding preproteins in solution. However, the new exciting development here is that our data demonstrate the formation of a new, previously unsuspected TF:proOmpA:$SecB_4$ nanomolar affinity super-complex. Formation of this supercomplex is favoured by our current data because:

1. In the cell, SecB is understoichiometric to TF, i.e. ~40 µM TF dimer = ~80 µM functional monomer, while SecB is at ~5 µM functional tetramer.
2. The affinity of TF for proOmpA is 7-15 times higher to that of SecB for proOmpA.
3. In size exclusion experiments a single super-complex population is formed. This would not be seen if extensive release of proOmpA occurred.
4. TF:proOmpA complexes are targeted to the translocase and proOmpA cannot be translocated indicative of no release. Only when SecB is added release-translocation can resume.
5. We performed ATPase and *in vitro* translocation experiments with pre-bound TF:proOmpA complexes to which SecB was added afterwards and proOmpA is successfully released and allowed to stimulate ATPase activity/translocate. This observation cannot completely exclude the possibility that proOmpA is indeed released to solution and then SecB captures it but it does demonstrate that TF:proOmpA can exist in a SecA-bound complex on which SecB can act.

Action taken:
We have better explained these points in the text and present additional data.

#4
*2. "If the detrimental impact of TF is due to post-translational binding of TF to translocation substrates like proOmpA,.....a TF mutant that retains the chaperone properties of TF but lacks the affinity for ribosomes should be as toxic in Δ*secB* mutants as the wild-type TF. To show this, the authors should include the TF-AAA mutant in the genetic complementation assays shown in Fig. 1A."*
Response:
We thank the reviewer for the suggested insightful experiment.
Action taken:
We generated:

a. the TF(3A) mutant (F44A, R45A, K46A) that is severely defective in ribosome association (Kramer 2002, Nature),
b. the TF(4A) mutant (M374A/Y378A/V384A/F387A) that is severely affected in client binding (1)
c. TF(4A,3A), a combination of both.

Complementation results were added (Fig. 1A, lanes 7-9).
TF(4A,3A) (lane 7) or TF(4A) (lane 8) do not inhibit growth of MC4100Δ*tig*Δ*secB* any longer. In contrast, TF(3A) still inhibits growth (lane 9). Previously, *a* TF derivative completely lacking the N-terminal ribosome binding domain (2) did not show any inhibition of growth of MC4100Δ*tig*Δ*secB* at 16°C. We attribute the observed difference between the TF(3A) and the previous TFDribosome binding domain result to the deletion having a more wide-ranging effect that compromises TF client binding.

#5
"Expression levels of TF under the conditions used must be tested by Western blotting."
Action taken:
Done. TF levels are not altered (Fig. EV1A).

#6
Minor comments:
1. "Line 448: typo "tis""
Response:
Corrected

Referee #3:

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"… The manuscript is well-written and describes the experimental procedures and observations in a detailed manner"

Response:

We thank the reviewer for the positive comment.

Comments:

#1

1. "However, the significance of the discussed findings is not clear. Presented experiments always focus on a rather linear scenario when the targeting/translocation cycle starts with the proOmpA:TF assembly, and it is proOmpA/OmpA but not TF that guides the targeting to SecA…"

Response:

We did not have any preconceived notions about the mechanism and order of events. However, our results show that proOmpA and SecB are self-targeted to the translocase with a high affinity, while TF alone is not. Therefore, TF will only be membrane targeted if proOmpA or SecB are also bound to the translocase and we can exclude that TF can be targeted alone to any significant extent. The above conclusion does not exclude that TF has its own recognition elements for SecA (as expected from its binding to SecA mutant derivatives). However, such recognition elements would not be readily available in the dimeric TF apoprotein state.

Action taken:

We present parallel possibilities that can be considered in Fig. 7A.

#2

"…Further, proOmpA can be released from the complex only in presence of SecB prior it is transferred to the translocase…"

Response:

We refer to our reply to the comment **#3** of referee #2. Release must. Happen from SecA-bound TF:proOmpA but not substantially from cytoplasmic TF:proOmpA complexes.

#3

"…However, once present in solution, the ubiquitous chaperone SecB would most likely interact with proOmpA directly, thus potentially excluding TF from the pathway…"

Response:

1. SecB is understoichiometric to TF, i.e. 80 μM functional monomer, while SecB is estimated to exist at 5 μM functional tetramer. So, in mass action terms SecB is not competitive.
2. The affinity of TF for proOmpA is 7-15 times better to that of SecB for proOmpA.

Based on the two points above, SecB has less opportunity to form preferential complexes with proOmpA when TF is also present. Therefore, it is less likely that SecB alone can exclude TF from the secretion pathway. In fact, when SecB complexes soluble TF:proOmpA, it would secure that TF remains in the secretory pathway.

Moreover, the proposed scenario goes against the observed data. SecB forms tight nanomolar complexes with TF:proOmpA while it does not bind to TF alone and binds less strongly to proOmpA. Therefore, there is no reason to assume that SecB causes proficient proOmpA release from TF in conditions other than when it itself is regulated by the SecA C-tail at the membrane.

#4

"…To explore this scenario and a role TF may play in targeting and/or holding, the authors should complement the existing data with the experiment when TF is titrated into pre-assembled proOmpA:SecB complexes, so a putative transfer of proOmpA between two chaperones, or an assembly of the larger ternary complex can be examined."

Response:

We have changed the order of addition of TF and SecB but this does not alter the experimental outcome in solution. This is because complexes between SecB and proOmpA are weak and proOmpA would dissociate to bind with a better affinity to TF or SecB:proOmpA would bind to TF to form a tight supercomplex.

At the membrane, though, once SecB and proOmpA are bound on Sec translocase, TF has no longer measurable affinity for the membrane. This could be due to the fact, that at the membrane, once proOmpA is bound it is conformationally altered, or TF-binding domains are hidden.

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Other points:

#5

1. "Line 141: The sub-chapter title is misleading, since the described in vivo experiment does not examine ratios of SecB and TF, but rather tests the strain viability in absence/presence of the chaperones."

Response:

We considered this suggestion but lean towards a different view. Our main purpose was not to systematically titrate the amounts of the chaperones. Nevertheless, we can still deduce that their relative ratio is important to prevent lethality. This is deduced from the growth defect in the absence of SecB combined with the observation that this phenotype is restored when TF is also absent. In line with this observation, over-expression of TF in a WT cell also results in a growth defect.

#6

2. "Figure 2A: Presence of proOmpA in the high molecular weight adduct should be confirmed, e.g. via immunoblotting."

Action taken:

The high molecular weight adduct has now been confirmed unequivocally by Native mass spectrometry to contain TF1:proOmpA1:SecB4 (Fig. 5A and B and EV3), Gel permeation chromatography coupled to MALS (Fig. 5C) and native-PAGE with both coomassie blue and immunoblotting (Fig. EV4B).

#7

"…Also here, and for many other presented gel-based assays, quantification of bands would offer more detailed description of the results…."

Response:

Quantifications of all gel-based assays were added (Appendix Figure S3).

#8

3. "The oligomeric state of TF: Along the manuscript the authors describe TF to be a dimer, and so all concentrations are presented for dimers. However, their own results (line 218) suggest a monomeric complex of proOmpA:TF. If indeed the complex is formed in 1:1 stoichiometry, the dissociation constants should be presented for monomers of TF, not dimers."

Response:

This is a valid point.

Action taken:

1. TF is measured to be dimeric by native MS (Fig. 2B; Appendix Fig. S2) and MALS (Appendix Fig. S1).
2. Dimer to monomer dissociation constants are presented for TF (Appendix Fig. S1).
3. In all experiments TF concentration was estimated as for TF being dimeric, therefore we consider it more relevant to refer to TF as "TF_2" since we refer to the amount added in each reaction and not to the complex stoichiometry.

#9

4. *"Line 229: When describing effect of TF on proOmpA solubility, authors probably mean lanes 3-9 in Fig. 2B…"*

Action taken:

Indeed, should refer to lanes 4 to 9. We changed 7-9 to 4-9.

#10

"…Also here it should be noted that the maximum recovery is already reached at proOmpA:TF ratio 0.75, thus supporting the complex to form in 1:1 stoichiometry."

Response:

TF was presented here as a dimer, in case of a 1:1 stoichiometry (TF_1:$proOmpA_1$), one would expect saturation at ratio 1:0.5 ($proOmpA_1$:TF_2).

#11

5. "Lines 303-306: The reasoning to exclude the scenario (a) is not convincing, since the affinity of TF to proOmpA is not too high (200 nM), and may be converted into 400 nM when calculated for TF monomers"

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Response:
TF still binds proOmpA 7-15 times better than SecB does and exists in the cell at a 16 fold molar excess.
Action taken:
We still think that scenario (a) is less likely but have now toned down this statement (lines 320-322).

#12
6. "Also the claimed absence of SecB:proOmpA complex on the Native-PAGE (lines 320-324, Fig. 4C) may be simply due to poor staining of the complex (see the comment below). Figure 4C: Immunoblotting should be employed to test presence of proOmpA or proOmpA:SecB in Native-PAGE, as there is a broad band below 140 kDa visible upon adjusting the intensity levels at the elevated SecB concentrations (lanes 5-8)."
Response:
The reviewer is right.
Action taken:
After immunostaining with α-SecB and α-OmpA, a small SecB:proOmpA population was detectable (Fig. 4C, middle and right). We also identified the SecB:proOmpA complex by Native MS analysis (Fig. 5A and appendix Fig. S5).

#13
7. "Figure 4D: The authors should indicate that the apparent mass of the putative ternary complex proOmpA:TF:SecB (~400 kDa) is substantially different from the value provided by the GPC-MALLS analysis (134 kDa)..."
Response:
Accurate protein masses cannot be truly determined by Clear Native PAGE because the migration is influenced by charge, shape and mass. In fact, migration of this complex varied between runs. Biophysical methods of high sensitivity and accuracy are superior.
Action taken:
During revision experiments, we managed to stabilize and have been able to determine the mass of the super-complex with high accuracy using native MS, an "absolute" single Dalton-resolution method (Fig. 5A and B; EV3). We therefore took the native page experiments to the extended view figures (Fig. EV4B).

#14
"…Validating the composition of the adduct on Native-PAGE via mass-spectrometry or immunoblotting would strongly support the authors' hypothesis on the composition of the complex."
Response:
Native MS was successfully run (Fig. 5A and B; EV3). The complex is ~157 kDa, consistent with a TF_1:$ProOmpA_1$:$SecB_4$ stoichiometry.

Referee #4:

"…The manuscript is clearly written and the data are novel. They have used elegant *in vitro* approaches to demonstrate how TF and SecB contribute to both targeting and triggering proOmpA translocation. This clearly pushes forward our understanding of major chaperone networks in bacteria."
Response:
We thank the reviewer for the encouraging comment.

Comments:
#1
1. "…TF is playing a major role in the folding of cytosolic proteins. The title is thus misleading and should be changed, as for now it gives the impression that TF is only a secretory pathway chaperone…."
Response:
The reviewer makes a valid point. However, that TF is an important factor for cytosolic folding is well established. Our data do not negate this role. They rather extend the role of TF to the post-translational secretory pathway.

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and

"…In addition, the important discovery described here does not only concern TF but also SecB and the interplay between these two chaperones."

Response:

The reviewer is right. Indeed, the interaction of TF with SecB and the translocase was previously unknown.

Action taken:

We have changed the title to:

"Trigger Factor is a *bona fide* secretory pathway chaperone that interacts with SecB and the translocase".

#2

2. "Figure 1A only reproduces what has been already published by several groups. Therefore it should be removed from the main manuscript…"

Response:

We would prefer to keep it in the main figures, because:

a. It is an important starting *in vivo* observation that corroborates previous results from others before the *in vitro* dissection.
b. Lethality caused by overexpressing TF in wild-type MC4100 cells was not shown before.
c. In the revised MS Fig. 1A also contains the new experiments with the non-ribosome binding and non-client binding TF derivatives.

#3

"...Furthermore, dilutions stop at -5, which is not enough to detect single colonies on plates."

Response:

The reviewer is right. There is some variability in the intensity of the spots in these complementation experiments depending on the density of the starting culture. Nevertheless, the effects are strong and in all cases of compromised growth clear single colonies are seen. Therefore, the *in vivo* role of the proteins under study is properly conveyed under the dilutions used.

#4

3. "The authors propose an attractive model in which SecB binding to the extreme C-ter region of SecA is necessary for the TF-related SecB exchange factor function. Indeed, they show that addition of SecB does not relieve the inhibitory effect of TF in vitro…"

Response:

To clarify: we show that addition of SecB does not relieve the inhibitory effect of TF *in vitro* when the SecAdeltaC-tail is used.

"…Yet, there is no in vivo evidence for such model. To strengthen this part of the work, the authors should test whether the SecA C-ter mutant is cold sensitive for growth in the presence of TF (as observed for the secB mutant e.g. Figure 1A) and if overexpression of TF is more toxic in such a strain background when compared to wild-type."

Response:

Complex mechanistic questions cannot be dissected in *in vivo* reactions where the concentrations of multiple factors and the growth conditions change, the factors participate in multiple interaction equilibria etc. There are specific technical problems in doing the proposed experiments *in vivo*:

SecA is essential for viability and so knockout mutants cannot be generated. To test the cold sensitivity idea with a cloned SecA(noC-tail) mutant it would have to be introduced into a chromosomal background that is also cold sensitive for SecA and at the same time ΔTFΔ*secB*. Then, to this *secA*cs,ΔTFΔ*secB* strain, cloned *secA(noC-tail)* or *secA* wild-type would be introduced with or without cloned *tig and* with or without *secB* on a separate plasmid or under a separate promoter. The genetics/plasmid/antibiotic resistance/promoters of doing such combinations pose a severe challenge. In addition, at low temperature the chromosomally-encoded SecAcs will always be non-functional, non-synthesized or even degraded. So, it will not be possible to interpret such results as the cell will die at low temperature both because SecAcs is non-functional and because TF is deleterious. So, such an experiment cannot really be readily performed.

Action taken:

a. Given the above, we resorted to doing a different experiment to partially address the reviewer's concern. We took advantage of the inhibitory role of TF when over-expressed even in a wild-type strain. The cloned SecA(noC-tail) mutant was introduced into a

chromosomal background that is thermosensitive for SecA (BL21.19)(5) and the growth of the strain was monitored as a function of time. Then, to this *secA*ts strain, the cloned *secA(noC-tail)* was introduced and expressed (arabinose promoter; gentamycin antibiotic resistance; p15 origin) with or without cloned *tig* (tetracycline promoter; ampicillin antibiotic resistance; pCol E1 origin) on a separate plasmid. As shown before (6), *secA(noC-tail)* fully overcomes the *ts* phenotype. However, over-expression of TF compromises growth of the strain significantly (Appendix Fig. S6A), indicating that it precedes SecB-caused release and also recapitulating the *in vitro* effect of SecA(noC-tail) (current Fig. 6A). We could not conclusively evaluate whether inhibition was stronger than that seen for wild-type *secA*.

b. We also determined in a new *in vitro* experiment that in the SecADC-tail background even the SecB chaperone inhibits proOmpA translocation ATPase at high concentrations (Fig. 6B). Presumably, SecB mimics the effect of TF, and the holdase role of SecB overcomes its role in facilitating secretion by preventing proOmpA from being released.

#6

4. "TF can leave the ribosome while bound to its substrate and SecB was shown to bind nascent protein co-translationally. A possible early complex occurring during translation is missing in Figure 5A..."

Response:

To the best of our knowledge SecB has only been shown to bind to nascent substrates of ~200 amino acids length (7) but - unlike SecA, TF and SRP - does not bind to the ribosome directly(8, 9). SecB was proposed to interact with ribosome-bound SecA and this interaction would facilitate SecB-nascent chain interaction (10).

Action taken:

We extended the model in Figure 7A (previously: Figure 5A) to better reflect the complexity of possible interactions.

#7

"...In addition, on the model it seems that SecB has specific affinity for signal sequence, which is not the case..."

Response:

A traditional majority consensus was that SecB does not bind to signal peptides(7, 11-14) opposing a minority view from Blobel's group (15, 16). Nevertheless, the most recent high resolution NMR structure from Kalodimos' group showed unequivocal SecB:proPhoA signal peptide interaction. Line 486-488 mentions that all three chaperones can recognize signal peptides and provides relevant references.

#8

"...In Figure 5B, the quaternaty SecB-TF-OmpA complex is missing..."

Action taken:

Added

"...In addition, it is not clear whether TF would leave Sec before SecB once translocation initiates."

Response:

The reviewer raises an interesting mechanistic point that we cannot technically address with the currently available tools. Single molecule studies will be needed.

#9

5. "The data suggest that the observed TF-SecB-SecA interplay might be part of a quality control mechanism to insure protein export and avoid undesired cytosolic protein translocation. Therefore the authors should refer to the work by Eser and Ehrmann (PNAS. 2003, 100(23):13231-4), especially in the discussion related to figure 5."

Response:

We thank the reviewer for this excellent suggestion that we have now incorporated (lines 517-518).

#10

6. "There are references to "data not shown" that should be presented in supplementary data, i.e., line 279 and 321."

Action taken:

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We have added all 'data not shown' in the Appendix or, where possible, in the main figures.

1. "However, TF neither displays a generic preference for slightly hydrophobic outer membrane porin β-barrels nor commonly associates with nascent porins [<3% of tis interactors; (17) (18)] co- or post-translationally (e.g. OmpX; Appendix Figure S3) (17, 19)." (line 494)

2. "Moreover, if proOmpA is incubated for 10 min at 37°C prior to its addition to SecYEG-SecA, a 70-80% loss of stimulation was detected (lane 3). Aggregation, that could justify this loss of activity, was not observed (Appendix Figure S1B)". (line 252)

3. "TF alone displayed no measurable binding to SecYEG-IMVs, in the presence or absence of SecA (Fig. 3B). In contrast, TF:proOmpA bound to SecYEG-SecA with a K_d of 1.79 (± 0.58) μM (Fig. 3C). We obtained identical results with either TF:[^{35}S]-proOmpA or [^{35}S]-TF:proOmpA (Fig. 3C), suggesting that TF remains complexed with proOmpA." (line 293)

4. "However, in contrast to TF:proOmpA (Fig. 2A), SecB:proOmpA (Fig. 4C) or SecB:proOmpA* (Appendix Figure S3) complexes could not be detected on native-PAGE, suggesting these might have a high K_{off} and dissociate rapidly. In that case, it seems unlikely that our first hypothesis can explain the fast kinetics of SecB relief of the TF-mediated inhibition." (line 335)

5. "Our inability to detect TF interaction with SecA(noC-tail) in solution (ITC; Appendix Figure S4C) suggested that SecA(noC-tail) becomes primed for its interaction with TF by its prior binding to SecYEG." (line 401)

#11

7. "In addition, there is reference to a manuscript under preparation Krishnamurthy et al., in preparation line 501, with no support available"

Response:

The cited work is still in preparation and cannot be presented in any more detail. We can remove the citation pending the editor's advice.

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14. Weiss JB, Ray PH, & Bassford PJ, Jr. (1988) Purified secB protein of Escherichia coli retards folding and promotes membrane translocation of the maltose-binding protein in vitro. *Proc Natl Acad Sci U S A* 85(23):8978-8982.
15. Watanabe M & Blobel G (1995) High-affinity binding of Escherichia coli SecB to the signal sequence region of a presecretory protein. *Proc Natl Acad Sci U S A* 92(22):10133-10136.
16. Watanabe M & Blobel G (1989) SecB functions as a cytosolic signal recognition factor for protein export in E. coli. *Cell* 58(4):695-705.
17. Loos M*, et al.* (2019) Structural basis of the sub-cellular topology landscape of Escherichia coli. *Front Microbiol*.
18. Oh E*, et al.* (2011) Selective ribosome profiling reveals the cotranslational chaperone action of trigger factor in vivo. *Cell* 147(6):1295-1308.
19. Arifuzzaman M*, et al.* (2006) Large-scale identification of protein-protein interaction of Escherichia coli K-12. *Genome Res* 16(5):686-691.

2nd Editorial Decision 4 March 2020

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees are very positive about the study and support publication after some changes to clarify text and figures. Please provide a point-by-point response to the remaining concerns.

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

REFEREE REPORTS

Referee #2:

I am quite pleased with the revision of the manuscript (Manuscript Number: EMBOR-2019-49054V2). The authors performed the requested experiment and addressed my concerns to complete satisfaction. I now fully support publication.

There is one typo in lane 236: prOmpA

Referee #3:

In the revised version of the manuscript de Geyter and co-workers have adequatelly addressed the comments and complemented the study with additional experimental data. Before recommending the manuscript for publication in EMBO Reports, the following points should be considered though.

Line 92: The statement that TF solubilizes 19 aggregation-prone *secretory* proteins is clearly wrong, as the referred study of Niwa et al. (ref. 30) examined the effect of chaperones on general protein solubility, and barely any secretory protein was found among the 19 identified targets of TF.

The review by Tsirigotaki is extensively cited through the manuscript, especially when addressing the abundance and affinities of chaperones and ribosomes in the cell, e.g. lines 85, 93 and 94. However, those experiemntally derived values are not easy to find within the review, as they are

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provided in the Supplementary table. Thus, I would advise authors to refer to the original publications.

Line 504: I strongly disagree with the statement that TF does not commonly associate with nascent porins. Moreover, the cited publication by Bukau and co-workers (ref. 68) clearly states that outer membrane proteins are critical targets and the most prominent substrates of TF.

Figure 1A: The contrast in the newly added panel is poor.

Figure 5C: Could it be that the labelling of blue and green profiles are swapped, as both the elution volume and the MALS-derived molecular mass are higher for the blue trace (currently SecB, 62 kDa)?

Figure 7A: The newly introduced scheme of pathways is too dense, barely informative, and can be omitted.

Figure EV4B: A typo in "Coommasie Blue"

Referee #4:

The authors have carefully addressed the comments and as a result the manuscript has been significantly improved.

2nd Revision - authors' response 9 March 2020

Referee#2 and #4:

Response:
We thank the reviewers for their comments.

Referee#3:

#1: *In the revised version of the manuscript de Geyter and co-workers have adequately addressed the comments and complemented the study with additional experimental data.*
Response:
We thank the reviewer.

#2: *Line 92: The statement that TF solubilizes 19 aggregation-prone *secretory* proteins is clearly wrong, as the referred study of Niwa et al. (ref. 30) examined the effect of chaperones on general protein solubility, and barely any secretory protein was found among the 19 identified targets of TF.*
Response/ Action taken:
Our statement was misunderstood. In the text we mentioned:
"TF patrols the ribosome exit tunnel and contributes, together with other ribosome-bound factors, to the folding and sorting of cytoplasmic proteins [*Niwa et al*] and the sorting of co- and post-translationally targeted secretory proteins [Bornemann T et al, Patzelt H et al, Ariosa A et al, Singh R et al] through an interplay with the signal recognition particle [Bornemann T et al, Singh R et al] and with ribosome-bound SecA [Huber D et]."
Therefore, the "Niwa et al" citation refers specifically to the TF foldase activity on cytoplasmic proteins. For the TF- holdase activity on secretory proteins we refer to Bornemann T et al, Patzelt H et al, Ariosa A et al, Singh R et al Huber et al.

#3: *The review by Tsirigotaki is extensively cited through the manuscript, especially when addressing the abundance and affinities of chaperones and ribosomes in the cell, e.g. lines 85, 93 and 94. However, those experimentally derived values are not easy to find within the review, as they*

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are provided in the Supplementary table. Thus, I would advise authors to refer to the original publications.

Response:

In the original Tsirigotaki review we put a lot of effort collecting and incorporating loose data and different ways of depicting information from the 30+ papers in the literature and databases specifically. We aimed at specifically helping the reader make direct comparisons. So, we would like to retain referencing only to the Tsirigotaki review Supplementary table.

Action taken:

To help the reader we direct to the supplement of the Tsirigotaki et al review the first time we reference this review in the present manuscript (for the specific purpose of referring to the supplementary table with the Kds).

#4: *Line 504: I strongly disagree with the statement that TF does not commonly associate with nascent porins. Moreover, the cited publication by Bukau and co-workers (ref. 68) clearly states that outer membrane proteins are critical targets and the most prominent substrates of TF.*

Response:

There is a misunderstanding here stemming from our poor phrasing of the statement. Apologies. The data for TF association with OMPs is strong. However, we wanted to point out that as shown in Bukau et al, the secretory proteins that are part of the interactome of TF are not mainly outer membrane porins. OMPs are a minority population.

To be precise, Bukau et al, find 799 nascent TF interactors (Loos et al., 2019, Supplementary Table S16). Of these:

799 total TF interactors

548 cytoplasmic (>65% of TF interactome; 19.6% of total cytoplasmome)

143 IM proteins

108 secretory (19.7% of total secretome) of which only 21 OM proteins (i.e. OMPs are only ~3% of all TF interactors; these constitute only ~20% of all the OMPs in the cell).

Action taken:

We have rephrased the statement in the text:

“TF does not seem to display a generic preference for slightly hydrophobic outer membrane porin b-barrels and associates with ~20% of all nascent outer membrane proteins, that constitute <3% of all its interactors”. In Loos et al (2019) we have done a systematic re-evaluation of all the interaction data and have derived a statistically sound, trustworthy sub-set.

#5: *Figure 1A: The contrast in the newly added panel is poor*

Response/ Action taken:

Contrast improved.

#6: *Figure 5C: Could it be that the labelling of blue and green profiles are swapped, as both the elution volume and the MALS-derived molecular mass are higher for the blue trace (currently SecB, 62 kDa)?*

Response/ Action taken:

Indeed, the colours were mixed in Fig. 5C. Thank you for noticing this. Now corrected.

#7: *Figure 7A: The newly introduced scheme of pathways is too dense, barely informative, and can be omitted.*

Response:

We changed and added more details in our model (Fig. 7A) after suggestions from Reviewer #4. The model provides a bird’s eye view of the different pathways TF is involved inside the cell.

Action taken:

Therefore, we would still like to include the model. However, we made some changes in Fig. 7A to improve clarity and highlight the TF branch of the pathway.

#9: *Figure EV4B: A typo in "Coommasie Blue"*

Action taken:

Corrected.

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Accepted 19 March 2020

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

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Anastassios Economou
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-49054V1

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:

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- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
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Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
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  - 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;
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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 size was chozen such as to secure enough data points to derive good fits to the data and minimal error flactuations. Sample numbers contained enough redudancy such that occasional outliers could be disregarded without affecting data quality.
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Is there an estimate of variation within each group of data?	yes
Is the variance similar between the groups that are being statistically compared?	yes

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).	Antibodies used have been generated in house and were all previously presented
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

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E- Human Subjects

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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	All raw data used for figures and expanded view figures are provided as source data files
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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).	N/A
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G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A