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Substrate relay in an Hsp70-cochaperone cascade safeguards tail-anchored membrane protein targeting

Hyunju Cho and Shu-ou Shan

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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 06th April 2018

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two referees whose comments are shown below.

As you will see from the reports, the referees both highlight the significance and quality of the findings reported in your manuscript and support publication here following adequate revision. In particular, you will see that ref #2 makes a number of constructive suggestions and requests for clarification, discussion and additional data that should all help strengthen the manuscript further.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of both reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

REFEREE REPORTS

Referee #1:

In this manuscript the authors investigated the role of yeast Hsp70 Ssa in the targeting of tail-anchored membrane proteins (TAs). Using in vitro biochemical assays the authors showed that Ssa can prevent aggregation of TAs. FRET experiments revealed that the TA substrate is handed over from Ssa to Sgt2 to Get3. Finally they showed that Ssa is also required for efficient TA targeting to the ER in vivo.

This is a very interesting study that is technically sound and very well written. The authors convincingly showed the role of Ssa in a chaperone cascade that routes newly synthesized TAs to the ER membrane and preserves the conformational quality of TAs to be successfully inserted into the membrane. Although it is not too surprising that a Hsp70 supports transport processes - in this

case the Get-mediated pathway for TAs -, this manuscript nicely demonstrates this function and furthermore declines - at least in vitro - the order of events and the hierarchy of factors acting in this pathway. These results will be of high interest for a broad readership.

Referee #2:

Although the steps of the post-translational transfer of tail-anchored (TA) proteins from the co-chaperone Sgt2 to Get3 and thence to the ER membrane have been extensively characterized, the pathway of newly synthesized TA polypeptides from the ribosome to Sgt2 has been less investigated. Because Sgt2 has a TPR domain that interacts with chaperones, it was suggested that it might receive TA substrates from members of the Hsp70 family, but this hypothesis had not been tested. On the contrary, the finding that the deletion of Sgt2's TPR domain does not affect its ability to bind a TA substrate (Wang F. et al., Mol. Cell, 2010) suggested that Hsp70 was not involved. In the submitted manuscript, the authors demonstrate that the major constitutively expressed yeast cytosolic Hsp70, Ssa1, is more effective than Sgt2 in preventing the aggregation of a TA substrate (Bos1) and go on to reconstitute and characterise in a series of elegant cell-free experiments the handover of the TA substrate from Ssa1 to Sgt2 and from Sgt2 to Get3. The in vitro work is complemented by in vivo experiments, which analyse the effect of acute Ssa1 depletion as well as the role of Sgt2's TPR domain in the association of its C-terminal domain with the TA substrate. The submitted paper, which uncovers a previously undescribed role of Ssa1, by adding this new player to the Get pathway, contributes important novel information to the field of TA protein biogenesis. The experiments are beautifully planned and controlled, and the interpretations are in general straightforward. I do, however, have a major conceptual problem with one aspect of the in vivo results, as well as with the discrepancy on the role of Sgt2's TPR domain in comparison to previously published work (Wang et al., 2010).
From Fig. 7D, S6, and S7, we see that acute Ssa1 inactivation has a strong effect on TA protein insertion, comparable to the effect of Get3 deletion. However, Sgt2 deletion has a much smaller effect. If the only role of Ssa1 were to hand over the TA substrate to Sgt2, we would expect deletion of each of these two proteins to have the same effect. Thus, Ssa1 presumably has roles additional to transferring newly synthesized TA substrates to Sgt2. These possible additional roles are indeed indicated by the dashed line of the model of Fig. 9, however, the discrepancy between the effects of Ssa1 inactivation and Sgt2 deletion should be more clearly discussed in the text.
Concerning the discrepancy with the work of Wang et al., who reported no role of Sgt2's TPR domain in TA binding, the authors state that "the defect of Sgt2-TPRmt in TA capture was not detected previously (Wang et al., 2010), likely due to the longer timescale of previous experiments compared to ours (>1 hr vs 10 min) and the more stringent purification of Sgt2•TA used here". I find it unlikely that the differences in the purification procedure could explain the results of Wang et al., since they detected no difference at all in substrate binding to wt Sgt2 and Sgt2 with mutated TPR. The timescale may make a difference, but in the experiment of Fig. 8A, the in vivo expression time of the substrate was longer than 10 min (the Methods section specifies 30 to 90 min). In any case, the hypothesis that kinetics explain the difference between this study and the one of Wang et al. could easily be tested. It would be very interesting to see whether in vivo replacement of Sgt2 with the TPR mutant has the same effect on the time course of TA insertion as Sgt2 deletion. Otherwise, my suspicion is that the difference in the results of the two studies (this one vs Wang et al.) is due to the use of different TA substrates. Different TA proteins are known to have different targeting requirements, so it could be that the substrate used by Wang et al. (Sec22) can bind directly to Sgt2 without the Ssa1 intermediary. To avoid confusion in the field, this possibility should be acknowledged.

In addition to the issues discussed above, several points in the manuscript need to be clarified/corrected, as follows.

1. The authors begin their study by showing that Sgt2 at physiological concentration is much less effective than Ssa1 in preventing aggregation of Bos1. For the other substrate used in this study, Sbh1, they show that Sgt2 at physiological concentrations is completely ineffective, however, I did not see the data on the effect of Ssa1 on preventing aggregation of this substrate, even if they do use the Sbh1-Ssa1 complex in subsequent experiments. The omission of the turbidity assay on Sbh1 in

the presence of Ssa1 is perplexing, and these data should be provided.
2. On pp. 4 and 7 of the manuscript, Mateja et al., 2015 and Shao et al., 2017 are cited as having used super-physiological concentrations of Sgt2 in their studies. This is true for the study of Mateja et al., while Shao et al.'s investigation was in the mammalian system, with SGTA, so is not comparable. Also, the statement on p.17 that direct loading of TA substrates onto Sgt2 tends to result in aggregated and inactive complexes should be somehow supported. The data of the present manuscript tell us that Sgt2 is less effective than Ssa1 in preventing TA aggregation, but say nothing on the activity of the Sgt2-TA complexes that form in the absence of Ssa1.
3. The description of the constructs used in this study is somewhat confusing, even if most are described in the Methods section. For instance, at the beginning of the Results section, it is stated that a non-cleavable SUMO domain fused to the TMD either of Sbh1 or Bos1p was used, but the Strep tag and of the opsin tag are not mentioned. It is not clear what parts of the SytII sequence were included in the Bos1-BirA construct (Fig. 7A); from the Methods section, it seems that not the entire N-terminal domain, but only portions of it, were included; also the sequence of the 6K segment might be specified somewhere, because from the cartoon of Fig. 7A, one might understand this to be an uninterrupted sequence of 6 lysines. It would be useful to include in the figures cartoons of the constructs the first time that they are used, and to indicate the position of the consensus N-glycosylation sequences.
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1st Revision - authors' response 05th May 2018

Dear editor,

Thank you for your time and work handling our manuscript. We also appreciate the reviewers for their valuable and constructive comments, and have addressed specific concerns by revisions to the text, figures, and references. Our point by point responses to the reviewers' comments are described below, with the comments in *italics*, and our responses in regular text.

(***Black Bold italic***: referee comments, Black: our responses)

Referee #1:

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We thank the reviewer for his / her appreciation of this work.

Referee #2:

Although the steps of the post-translational transfer of tail-anchored (TA) proteins from the co-chaperone Sgt2 to Get3 and thence to the ER membrane have been extensively characterized, the pathway of newly synthesized TA polypeptides from the ribosome to Sgt2 has been less investigated. Because Sgt2 has a TPR domain that interacts with chaperones, it was suggested that it might receive TA substrates from members of the Hsp70 family, but this hypothesis had not been tested. On the contrary, the finding that the deletion of Sgt2's TPR domain does not affect its ability to bind a TA substrate (Wang F. et al., Mol. Cell, 2010) suggested that Hsp70 was not involved. In the submitted manuscript, the authors demonstrate that the major constitutively expressed yeast cytosolic Hsp70, Ssa1, is more effective than Sgt2 in preventing the aggregation of a TA substrate (Bos1) and go on to reconstitute and characterise in a series of elegant cell-free experiments the handover of the TA substrate from Ssa1 to Sgt2 and from Sgt2 to Get3. The in vitro work is complemented by in vivo experiments, which analyse the effect of acute Ssa1 depletion as well as the role of Sgt2's TPR domain in the association of its C-terminal domain with the TA substrate.

The submitted paper, which uncovers a previously undescribed role of Ssa1, by adding this new player to the Get pathway, contributes important novel information to the field of TA protein biogenesis. The experiments are beautifully planned and controlled, and the interpretations are in general straightforward. I do, however, have a major conceptual problem with one aspect of the in vivo results, as well as with the discrepancy on the role of Sgt2's TPR domain in comparison to previously published work (Wang et al., 2010).

We thank the reviewer for his / her constructive comments on the quality and impact of this work.

From Fig. 7D, S6, and S7, we see that acute Ssa1 inactivation has a strong effect on TA protein insertion, comparable to the effect of Get3 deletion. However, Sgt2 deletion has a much smaller effect. If the only role of Ssa1 were to hand over the TA substrate to Sgt2, we would expect deletion of each of these two proteins to have the same effect. Thus, Ssa1 presumably has roles additional to transferring newly synthesized TA substrates to Sgt2. These possible additional roles are indeed indicated by the dashed line of the model of Fig. 9, however, the discrepancy between the effects of Ssa1 inactivation and Sgt2 deletion should be more clearly discussed in the text.

We agree with the reviewer that if the Ssa1-Sgt2-Get3 cascade is the only route to target TAs in yeast, inactivation of Ssa1 and deletion of Sgt2 should have a similar phenotype. However, several recent studies have uncovered multiple alternative pathways for targeting TAs to the ER that could be mediated by the SND components (Aviram *et al*, 2016) or the EMC complex (Guna *et al*, 2018). As discussed in the Discussion section, deletion of Sgt2 could allow nascent TAs to be re-routed to these alternative pathways before TAs are committed to the GET pathway (new Fig 8, step 7). These redundant targeting pathways likely contribute to the weak phenotype or targeting defect of *Δsgt2* cells reported in this and multiple other studies (Kiktev *et al*, 2012, Kohl *et al*, 2011, Yeh *et al*, 2014), This hypothesis is supported by the observation that a model TA, Sbh1, is efficiently inserted in Δ*sgt2*Δ*get2* cells (apparently bypassing the GET pathway), whereas it forms cytosolic aggregates with cytosolic GET proteins in Δ*get2* cells (Kiktev et al, 2012). On the other hand, it is probable that Ssa1 functions not only in the GET pathway but also in alternative TA targeting routes (new Fig 8, step 7), and hence its inactivation leads to a larger defect in TA insertion than deletion of Sgt2. These information has been incorporated into the Discussion (p20-21).

Concerning the discrepancy with the work of Wang et al., who reported no role of Sgt2's TPR domain in TA binding, the authors state that "the defect of Sgt2-TPRmt in TA capture was not detected previously (Wang et al., 2010), likely due to the longer timescale of previous experiments compared to ours (>1 hr vs 10 min) and the more stringent purification of Sgt2•TA used here". I find it unlikely that the differences in the purification procedure could explain the results of Wang et al., since they detected no difference at all in substrate binding to wt Sgt2 and Sgt2 with mutated TPR. The timescale may make a difference, but in the experiment of Fig. 8A, the in vivo expression time of the substrate was longer than 10 min (the Methods section specifies 30 to 90 min). … Otherwise, my suspicion is that the difference in the results of the two studies (this one vs Wang et al.) is due to the use of different TA substrates. Different TA proteins are known to have different targeting requirements, so it could be that the substrate used by Wang et al. (Sec22) can bind directly to Sgt2 without the Ssa1 intermediary. To avoid confusion in the field, this possibility should be acknowledged.

We agree with the reviewer that the discrepancy between two studies could result from the different TA substrates used. In fact, we observed that transfer of Sbh1 is less strictly dependent on Ssa1-Sgt2 interaction than Bos1 (Figure 4A). As shown in Table1-response, both the hydrophobicity (GRAVY score) and helical content (Agadir score) of Sec22-TMD are lower than Bos1 and Sbh1, and their values lie between the model substrates, 5AG and 6AG, that define the boundary between GET-dependent and GET-independent substrates in a previous study (Rao *et al*, 2016). The lower hydrophobicity of Sec22-TMD could render it much less dependent on a concerted substrate transfer mechanism. This is incorporated into the text to replace the discussion on purification procedure.

Gene	TMD	GRAVY	Agadir
Bos1	LVFWIALILLIIGIYYVL	2.81	13.19
2AG	LVAGIALILLIIGIYYVL	2.78	11.48
3AG	LVAGIALIGLIIGIYYVL	2.55	0.77
4AG	LVAGIALIGAIIGIYYVL	2.44	0.48
5AG	LVAGIALIGAIIGAYYVL	2.29	0.46
6AG	LVAGGALIGAIIGAYYVL	2.02	0.27
Sbh1	PLVVLFLAVGFIFSVVALHVI	2.64	0.98
Sec22	QYAPIVIVAFFFVFLFWWIFL	2.19	0.42
Fis1	VVVAGGVLAGAVAVASFFL	2.39	0.28

Table 1-response. Properties of the TMDs of TAs. Both GRAVY (Grand Average of Hydropathy) and Agadir scores were adapted from the previous study (Rao *et al*, 2016).

In any case, the hypothesis that kinetics explain the difference between this study and the one of Wang et al. could easily be tested. It would be very interesting to see whether in vivo replacement of Sgt2 with the TPR mutant has the same effect on the time course of TA insertion as Sgt2 deletion.

As discussed above, *Δsgt2* already have a fairly weak phenotype, likely due to redundant pathways. The phenotype of two point mutations on Sgt2 is expected to be even weaker. This was what we observed: Sgt2 TRP mutation does not significantly alter Bos1 insertion *in vivo* (Figure1-repsonse). This lack of phenotype can in part be attributed to alternative pathways, because when we further deleted a component of the SND pathway (Aviram *et al*, 2016), a minor defect in Bos1 insertion was observed with the Sgt2 TPR mutant (*Sgt2TPRmtΔsnd1* and *Sgt2TPRmtΔsnd2*) compared to the corresponding cells harboring wild-type Sgt2 (Figure1-repsonse). Given that *SND1* and *SND2* appear to be synthetic, and at least an additional EMC-mediated pathway can also insert TAs (Aviram *et al*, 2016, Guna *et al*, 2018), how many pathways are responsible for Bos1 targeting in yeast remains to be deciphered in future work.

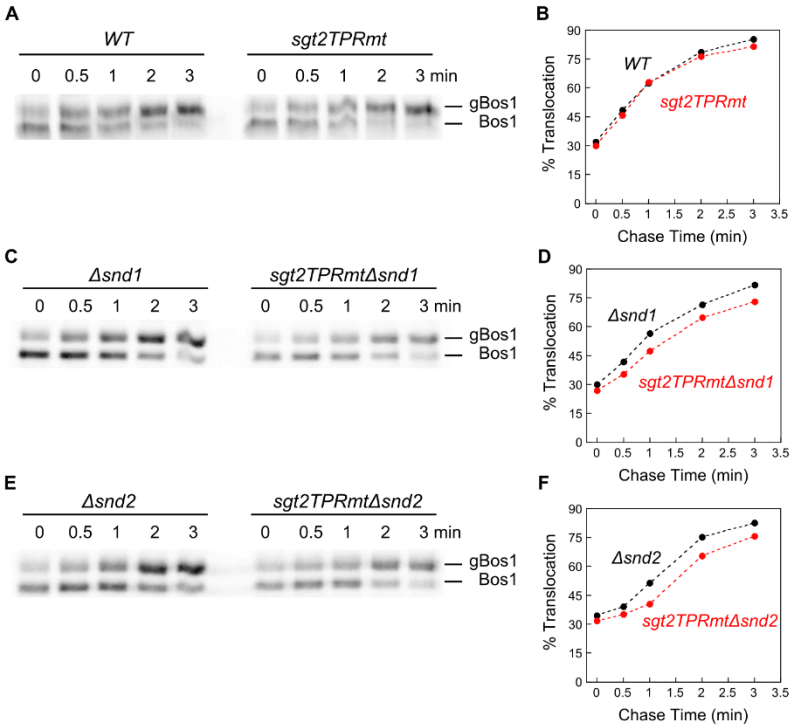

Figure 1-response. Pulse-chase analysis of the translocation of metabolically-labeled BirA-Bos1 in various WT and Sgt2TPRmt cells. Representative autoradiograms for pulse-chase analysis of the translocation of Bos1 was shown in (A) for *WT* and *sgt2TPRmt* cells, (C) for Δ*snd1* and *sgt2TPRmt*Δ*snd1* cells, and (E) for Δ*snd2* and *sgt2TPRmt*Δ*snd2* (E) cells, and the quantifications of translocation efficiencies were shown in (B), (D), (F), respectively.

In addition to the issues discussed above, several points in the manuscript need to be clarified/corrected, as follows.

1. The authors begin their study by showing that Sgt2 at physiological concentration is much less effective than Ssa1 in preventing aggregation of Bos1. For the other substrate used in this study, Sbh1, they show that Sgt2 at physiological concentrations is completely ineffective, however, I did not see the data on the effect of Ssa1 on preventing aggregation of this substrate, even if they do use the Sbh1-Ssa1 complex in subsequent experiments. The omission of the turbidity assay on Sbh1 in the presence of Ssa1 is perplexing, and these data should be provided.

We added the data for Sbh1 aggregation in the presence of Ssa1 to Figure EV1F and G. Ssa1 prevented Sbh1 aggregation in a concentration-dependent manner, whereas Sgt2 did not prevent Sbh1 aggregation (Figure 2A).

2. On pp. 4 and 7 of the manuscript, Mateja et al., 2015 and Shao et al., 2017 are cited as having used super-physiological concentrations of Sgt2 in their studies. This is true for the study of Mateja et al., while Shao et al.'s investigation was in the mammalian system, with SGTA, so is not comparable.

We removed the reference (Shao *et al.*) from the corresponding sentences. Thank you.

Also, the statement on p.17 that direct loading of TA substrates onto Sgt2 tends to result in aggregated and inactive complexes should be somehow supported. The data of the present manuscript tell us that Sgt2 is less effective than Ssa1 in preventing TA aggregation, but say nothing on the activity of the Sgt2-TA complexes that form in the absence of Ssa1.

To address whether Sgt2•TA generated by direct loading is less active than that generated via transfer from Ssa1, we prepared the Sgt2•Bos1 complexes with or without preloading and transfer from Ssa1 (Appendix Figure S2A) and compared their efficiencies of transferring the TA to Get3 (with ATP and Get4/5 present) via the on-bead transfer assay (Figure 5A and B). The Sgt2•Bos1 complex generated by direct loading transferred ~9% Bos1 to Get3, whereas over 80% Bos1 was aggregated and irreversibly stuck on the resin (new Figure 5G and H). In contrast, Sgt2•Bos1 complex prepared via transfer from Ssa1 exhibits more efficient Bos1 transfer from Sgt2 to Get3 (~47%). These new data strongly suggest that direct loading of TA substrates onto Sgt2 results in aggregated and inactive complexes, whereas loading of substrates via Hsp70 generates a soluble and functionally competent Sgt2•TA complex.

3. The description of the constructs used in this study is somewhat confusing, even if most are described in the Methods section. For instance, at the beginning of the Results section, it is stated that a non-cleavable SUMO domain fused to the TMD either of Sbh1 or Bos1p was used, but the Strep tag and of the opsin tag are not mentioned. It is not clear what parts of the SytII sequence were included in the Bos1-BirA construct (Fig. 7A); from the Methods section, it seems that not the entire N-terminal domain, but only portions of it, were included; also the sequence of the 6K segment might be specified somewhere, because from the cartoon of Fig. 7A, one might understand this to be an uninterrupted sequence of 6 lysines. It would be useful to include in the figures cartoons of the constructs the first time that they are used, and to indicate the position of the consensus N-glycosylation sequences.

We added a new figure for the model substrates used in *in vitro* assays (new Figure EV1A). The figure and figure legend contain the detailed sequence information for truncated Bos1 and Sbh1. In addition, we edited Figure 7A (new Figure 6A) to provide more information about the model substrates used in the *in vivo* assay. Finally, additional sequence information for the 6 lysine mutations in Bos1-BirA was added in the Methods section.

4. In Fig. S4, how was release of in vitro translated Bos1 from Sgt2 measured?

We added this information in the figure legend (new Figure EV3). Briefly, $Bos1^{CM}$ was synthesized in the presence of His_6-$Sgt2^{BFL}$ using the S30 *in vitro* translation (IVT) system coupled to amber suppression (Rao et al, 2016). The resulting His_6-$Sgt2^{BFL}$•$Bos1^{CM}$ complex was affinity purified (Rao et al, 2016) and chased with superactive cpSRP43 (Figure 4E), and the kinetics of restoration of CM fluorescence due to loss of FRET was monitored over time.

5. Fig. S3A: His6-Sgt2 was immobilized on beads; this should be indicated in the scheme as well as in the figure legend.

Thank you for the suggestion. We modified the figure (new Figure EV2A) and figure legend accordingly.

6. Figures 5 and 6 should be combined. Indeed, the data of Fig. 6A and B would fit better together with those of Fig. 5. Indeed, fig. 6B shows a kinetic analysis by FRET of an experiment similar to the one of Fig 5B analysed by pulldown and western blot; and Fig. 5D shows a FRET analysis, from which a possible backtransfer to Ssa1 is excluded. This should be shown after the analysis of Fig. 6B, which shows that transfer to Get3 depends on the presence of Get4/5. The difference in the acceptor fluorophore on Get3 in Figs. 5 and 6 is irrelevant.

We combined the old Figure 5 and 6 as suggested (new Figure 5) and edited the main text accordingly.

7. Discussion, p.18. The very interesting funneling concept has been expressed for the transfer of TA substrates from SGTA to TRC40 in the mammalian system by Shao et al (2017). This might be mentioned.

We added the reference (Shao et al, 2017) in the corresponding sentence.

8. Discussion, p. 19, 6th line: as far as I know, neither Sec62 nor Sec63 contain TPR domains, so the term Sec62/63 complex should be deleted from the sentence.

We meant that the TPR domain-containing Sec71/72, which form a tetrameric complex with Sec62/63 and play a role in post-translational translocation. We revised this sentence to avoid any confusion.

9. Methods, p. 24. The sentence "unconjugated dye was washed with buffer B" should be replaced with "unconjugated dye was removed and the resin was washed with buffer B". In the second paragraph of the same page, the term "peptide" should be replaced with "compound" or "conjugate".

We edited those sentences as suggested. Thank you.

10. Methods: on p. 27 the symbols in equations 4, 5 and 6 are not displayed correctly in the pdf.

We will watch out for this problem in the next submission.

11. The references must be re-formatted. Multi-author papers are cited in some cases with first author et al., in other cases with the first and second author et al. In yet another case, numbers within square brackets, instead of authors, are given (p. 19).

We re-formatted the references according to the Journal guideline.

12. References: in the Introduction (p. 3), a review by Chauduri and Paul is cited in relation to dysfunctional chaperones as causative agents of diseases. The review of Chauduri and Paul is instead focussed on the use of chaperones as therapeutic agents and is thus not relevant here. The citation Yabal et al is of an abstract. The full-length article (J. Biol. Chem, 2003) should be cited.

We correctted the references. Thank you.

References

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2nd Editorial Decision 22nd May 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by one of the original referees whose comments are shown below. As you will see this person finds that all criticisms have been sufficiently addressed and recommend the manuscript for publication.

REFEREE REPORTS

Referee #2:

The authors have addressed my concerns in a satisfactory manner, and the revised manuscript now constitutes a valid contribution to EMBO J. In the revised version, the legends to Appendix Figs. S1 and 2 seem to be missing.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Shu-ou Shan
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-99264

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- 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 $\chi 2$ 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?	Both technical and biological replicates were performed to calculate the mean and the standard deviation of the mean (SD). Repetitions stopped when the SD was sufficient to provide a high level of confidence for the presence (or lack) of a difference between samples.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not applicable.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No.
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable.
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.	No.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable.
5. For every figure, are statistical tests justified as appropriate?	Yes. Standard statistics were used for the type of data reported in this work.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	This work does not involve a population analysis, so this information is not available. In general, two biochemical samples are directly compared in parallel, well controlled experiments. Error bars are small and justifies the interpretation (the presence of a difference or lack thereof).
Is there an estimate of variation within each group of data?	In all cases, quantified data are reported as mean ± SD.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

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

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	The variance information is reported by the SD value for each reported parameter for all samples under comparison.

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).	This information is provided in the Materials and Methods under "Western blot analysis".
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	This information is provided in the Materials and Methods under "Strains and plasmids".

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Not applicable
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not applicable
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Not applicable

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable

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	Not applicable
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).	Not applicable
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).	Not applicable
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable

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.	No.