Subunit cooperation in the Get1/2 receptor promotes tail-anchored membrane protein insertion

Un Seng Chio, Yumeng Liu, SangYoon Chung, Woo Jun Shim, Sowmya Chandrasekar, Shimon Weiss, and Shu-ou Shan

Corresponding Author(s): Shu-ou Shan, California Institute of Technology

Review Timeline:  
Submission Date: 2021-03-12  
Editorial Decision: 2021-04-16  
Revision Received: 2021-08-03  
Editorial Decision: 2021-08-10  
Revision Received: 2021-08-16

Monitoring Editor: Ulrich Hartl  
Scientific Editor: Dan Simon

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.)

DOI: https://doi.org/10.1083/jcb.202103079
April 16, 2021

Re: JCB manuscript #202103079

Dr. Shu-ou Shan  
California Institute of Technology  
Division of Chemistry and Chemical Engineering  
1200 E. California Bl.  
Pasadena, CA 91125

Dear Dr. Shan,

Thank you for submitting your manuscript entitled "Subunit cooperation in the Get1/2 receptor promotes tail-anchored membrane protein insertion." The manuscript was assessed by expert reviewers, whose comments are appended to this letter. You will see that all reviewers are very enthusiastic about your study and request relatively minor revisions. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

Reviewer 1 asks for additional quantification of the FRET data regarding Get3 conformations. Reviewer 2 asks for a pulse-chase of TA translocation to ER in a get3Δ strain and for at least 3 replicates of these time course experiments in all strains. Reviewer 3 notes that the photo-crosslinking experiment requires an additional control of a Get2 site that does not crosslink to Get3 to establish specificity.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and
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When submitting the revision, please include a cover letter addressing the reviewers’ comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you’ve had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Ulrich Hartl, MD, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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Reviewer #1 (Comments to the Authors (Required)):

The manuscript by Chio et al investigates mechanistic events and quantifies binding parameters in the eukaryotic GET system. The main technique used is single molecule FRET, complemented with bioinformatic analysis and a functional in cell assay. By combining suitable preparations of differently FRET-labeled protein constructs of Get 1/2/3, a model substrate, and other entities, the authors conclude on novel features of the domain interactions within the GET functional mechanism. They discover cooperativity and structural changes in the Get1/2 pair upon interacting with Get3, and they identify two previously undescribed signalling motifs in a flexible linker of Get2 that, albeit with minor effect, are shown to contribute to the overall insertion efficiency of the GET system. The current work uses the mini-Get system, but given that this system is generally used and well established, most of the conclusions drawn can reasonably be expected of functional relevance also in the full WT system.

The work is technically sound, the experiments were selected elegantly and were executed with high care. The manuscript is well written and accessible to readers, even though reporting on complicated experiments. The work does contribute new insights into the GET system and these
fit in my eyes well to the scope and impact of JCB. I recommend publication after addressing the minor points below.

- The data shown in Figure 4 resolve different open and partially closed states of Get3. The interpretation of the data is done however only qualitatively, but it could readily be done (semi-)quantitatively, i.e. by extracting or at least estimating the population levels of the different open/closed/partial open states. For example, the sentence in line 194 "This confirms the results of previous work showing that the TA substrate induces Get3 to sample open conformations." should qualify that these are 10-20% open conformations and 80-90% closed (my estimate). Accordingly, the population levels in all experiments of Fig 4 should be quantified.

- For better accessibility by general readers, I recommend to provide a supplementary Figure that shows the available structural work on the GET system and displays the findings and experiments made in this work on those high-resolution structures or models.

- The cartoon model in Figure 1A shows only one acceptor dye position, but the rest of the Figure has either of two different positions.

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Reviewer #2 (Comments to the Authors (Required)):

The GET pathway is a conserved targeting pathway for C-terminally anchored membrane proteins (TA proteins) to the membrane of the ER. A plethora of structural models guides mechanistic understanding of the different targeting steps. This manuscript affords substantial new insights into the conformational interplay between Get3, the ATPase central to targeting, and the cytosolic domains of the two receptor subunits Get1 and Get2. The advance is in pinpointing cooperativity between these receptor domains. Furthermore, specific helical motifs in the cytosolic domain of Get2 are identified that are important for the effective delivery of TA proteins from Get3 to the membrane. Taken together, the work presented here elucidates an important step in GET-dependent membrane delivery at the molecular level.

The main points are supported by FRET measurements designed to monitor binding or changes in protein conformation. In addition, the role of the helical motifs in the cytosolic domain is supported by site-specific crosslinking to Get3, FRET measurements, and an in-vivo functional assay for TA protein targeting to the ER membrane.

For this last experiment (Fig. 8) I have two comments: I would really like to see the same time course for a get3 deletion strain. The get2 deletion strain is more strongly affected than the get3 deletion strain in growth. Hence, the depicted differences could simply reflect how well the respective mutants cover the null allele. In order to interpret in terms of the Get3-receptor interplay we would need to see the degree of effect on the glycosylation of the reporter TA protein when Get3 is missing. The reference that establishes the assay shows an effect but not in the same time-resolved manner (one steady-state data point; Fig. 6B of Cho and Shan). The same pulse-chase with all time points should be shown for a get3 deletion in this Figure for comparison. Error bars should not be used with two data points, please show the two points. I would also find it appropriate to show the experiment in Fig. 8 in triplicate.
Reviewer #3 (Comments to the Authors (Required)):

This study uses a combination of biochemical and biophysical analysis to understand how the Get1/2 complex triggers TA protein release from the Get3-TA complex. This is an important process for successful insertion of this class of proteins, and the topic is clearly of interest to this field. The authors use a really nice minimal system using purified Get3-TA complex and a soluble heterodimer of the cytosolic domains (CD) of Get1 and Get2. The key initial discovery is that the "whole is greater than the sum of the parts" - that the Get1/2 heterodimer clearly has functionality beyond what Get1 or Get2 individually do. From this initial finding, the authors go on to identify putative semi-conserved MoRFs in Get2 that seem to influence Get3 conformations and are functionally involved in TA protein release. Overall, the study is an important contribution to this field, and I support its publication. There is one experimental suggestion for improvement, with a number of other suggestions that can all be handled by adjustments to the text.

Major points:

1) The photo-crosslinking experiment would be substantially more convincing if the authors included other sites in the unstructured region of Get2 that did NOT crosslink to Get3. Otherwise, the negative controls shown, while important, are rather trivial. An alternative would be to show specificity of the result is to demonstrate that the crosslink is diminished when the MoRF is mutated. Either way, some type of specificity controls are needed for this experiment for it to be meaningful. This is the only experimental work that I feel is required.

2) Earlier studies by Rome et al showed binding of Get1-CD and Get2-CD to the Get3-TA complex to have Kd values in the micromolar range. In that same study and elsewhere, binding measurements with just Get3 show Kd values of ~50 nM and ~150 nM (e.g., Mariappan et al., 2011, Fig. S8). It might be worth speculating or explaining why there is such a difference.

3) The conservation of the hydrophobic properties of the MoRFs is difficult to appreciate. I think a depiction of conservation combined with the helical wheel and hydrophobicity might be better. For an example of how this can be achieved, see Figure 5, figure supplement 1, of PMID 26158507, which illustrated the same idea for a helix in SRP.

4) Rather than saying this work "suggests an improved model" perhaps it is more impactful to say something like "this work identifies a previously unappreciated and mechanistically important step in the membrane-associated steps...". The wording is up to the authors of course, but I feel the main advance here is the discovery of a new step in the process and explicitly stating this will tell the reader more precisely what has been accomplished.

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Dear editor,

Thank you for your time and efforts in handling our manuscript. We also appreciate the constructive comments from the reviewers. Below are point-by-point responses to the reviewers’ comments, with the comments in black, and the responses in blue. We think the manuscript is significantly improved and hope that it is now suitable for publication.

Reviewer #1 (Comments to the Authors (Required)):

The manuscript by Chio et al investigates mechanistic events and quantifies binding parameters in the eukaryotic GET system. The main technique used is single molecule FRET, complemented with bioinformatic analysis and a functional in cell assay. By combining suitable preparations of differently FRET-labeled protein constructs of Get 1/2/3, a model substrate, and other entities, the authors conclude on novel features of the domain interactions within the GET functional mechanism. They discover cooperativity and structural changes in the Get1/2 pair upon interacting with Get3, and they identify two previously undescribed signalling motifs in a flexible linker of Get2 that, albeit with minor effect, are shown to contribute to the overall insertion efficiency of the GET system. The current work uses the mini-Get system, but given that this system is generally used and well established, most of the conclusions drawn can reasonably be expected of functional relevance also in the full WT system.

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Although FRET histograms are typically fit by sums of Gaussian distributions to extract the population of molecules in each FRET state, this practice is based on the assumption that molecules in discrete conformational states do not exchange on the timescale of the measurement. As noted in our earlier work (Chio et al, PNAS 2017) and in this work, the sub-millisecond dynamic exchange of Get3 between open and closed states invalidated this assumption. It is therefore not appropriate to fit the Get3 FRET histograms with Gaussian distributions, as the intermediate FRET bins likely arise from conformational averaging rather than being a discrete state. Nevertheless, we report a semi-quantitative estimate of the degree of Get3 opening as suggested by the reviewer, by taking the ratio of Get3 populated in low (E* = 0.3-0.5) vs high (E* = 0.7-0.9) FRET states. These are added as new Figures 4E and S2G. The quantification in Figure 5G was also updated.
- For better accessibility by general readers, I recommend to provide a supplementary Figure that shows the available structural work on the GET system and displays the findings and experiments made in this work on those high-resolution structures or models.

Thank you for the suggestion. We have added Figures S2A and S2B, which depict structure of the Get1CD-Get3 and Get2CD-Get3 complexes, respectively. The position of the dyes and location of the Get1(NR) and Get2(RERR) mutations are also depicted.

- The cartoon model in Figure 1A shows only one acceptor dye position, but the rest of the Figure has either of two different positions.

We have revised Figure 1A accordingly.

- typo in line 173; "femo" - > "femto"

Corrected.

- Figure 7A is called before Figure 6.

Corrected.

Reviewer #2 (Comments to the Authors (Required)):

The GET pathway is a conserved targeting pathway for C-terminally anchored membrane proteins (TA proteins) to the membrane of the ER. A plethora of structural models guides mechanistic understanding of the different targeting steps. This manuscript affords substantial new insights into the conformational interplay between Get3, the ATPase central to targeting, and the cytosolic domains of the two receptor subunits Get1 and Get2. The advance is in pinpointing cooperativity between these receptor domains. Furthermore, specific helical motifs in the cytosolic domain of Get2 are identified that are important for the effective delivery of TA proteins from Get3 to the membrane. Taken together, the work presented here elucidates an important step in GET-dependent membrane delivery at the molecular level.

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comparison. Error bars should not be used with two data points, please show the two points. I would also find it appropriate to show the experiment in Fig. 8 in triplicate.

The pulse-chase experiment for TA insertion in Δget3 strain was reported as Fig. EV4 in the Cho et al, EMBO 2018 paper and copied below for the reviewer’s reference.

![Pulse-chase experiment](image)

We repeated pulse-chase measurements for WT Get2 and the Get2 mutants in side-by-side measurements (n = 5-8 combining the original and the new measurements). Summaries of the pulse-chase data are reported in new Figure 8, and gels for all the replicates are shown in Figure S5. It turns out that TA insertion in the GET2-FLAG strain is slower and more variable than in the WT curve shown above; thus, differences with Get2 H1 and H2 mutants are smaller. Statistical analysis only supported a modest delay in translocation efficiency at the earliest (1 min) time point. We have revised the description in the text accordingly.

Reviewer #3 (Comments to the Authors (Required)):

This study uses a combination of biochemical and biophysical analysis to understand how the Get1/2 complex triggers TA protein release from the Get3-TA complex. This is an important process for successful insertion of this class of proteins, and the topic is clearly of interest to this field. The authors use a really nice minimal system using purified Get3-TA complex and a soluble heterodimer of the cytosolic domains (CD) of Get1 and Get2. The key initial discovery is that the "whole is greater than the sum of the parts" - that the Get1/2 heterodimer clearly has functionality beyond what Get1 or Get2 individually do. From this initial finding, the authors go on to identify putative semi-conserved MoRFs in Get2 that seem to influence Get3 conformations and are functionally involved in TA protein release. Overall, the study is an important contribution to this field, and I support its publication. There is one experimental suggestion for improvement, with a number of other suggestions that can all be handled by adjustments to the text.

Major points:

1) The photo-crosslinking experiment would be substantially more convincing if the authors included other sites in the unstructured region of Get2 that did NOT crosslink to Get3. Otherwise, the negative controls shown, while important, are rather trivial. An alternative would be to show specificity of the result is to demonstrate that the crosslink is diminished when the MoRF is mutated. Either way, some type of specificity controls are needed for this experiment for it to be meaningful. This is the only experimental work that I feel is required.
We performed BpA crosslinking with the Get2ΔH1ΔH2 mutant, as suggested by the reviewer. The results show that the mutations in H1 and H2 significantly reduced Get3 crosslinking to the Bpa moiety at A85 and S111, in agreement with an interaction of these motifs with Get3 (new Figure 7).

2) Earlier studies by Rome et al showed binding of Get1-CD and Get2-CD to the Get3-TA complex to have Kd values in the micromolar range. In that same study and elsewhere, binding measurements with just Get3 show Kd values of ~50 nM and ~150 nM (e.g., Mariappan et al., 2011, Fig. S8). It might be worth speculating or explaining why there is such a difference.

The different affinities of Get1/2 CDs for Get3-TA vs Get3 can be explained by conformational selection of open Get3 by the receptor complex. As indicated by the smFRET data here and by previous structural analyses, Get1 and (now) Get2 prefer the open conformation of Get3. Under most conditions (except for when Get3 is ATP- and Get4/5-bound), TA drives Get3 closing. Compared to Get3, Get3/Ta complexes are more populated with closed conformations that are disfavored by the receptor. The Kd values in a bulk measurement is a statistical average of the contributions from the different populations; hence Get3/Ta exhibits weaker binding compared to Get3.

3) The conservation of the hydrophobic properties of the MoRFs is difficult to appreciate. I think a depiction of conservation combined with the helical wheel and hydrophobicity might be better. For an example of how this can be achieved, see Figure 5, figure supplement 1, of PMID 26158507, which illustrated the same idea for a helix in SRP.

Thank you for the suggestion. We have remade the figure (new Figure 7A) as suggested.

4) Rather than saying this work "suggests an improved model" perhaps it is more impactful to say something like "this work identifies a previously unappreciated and mechanistically important step in the membrane-associated steps...". The wording is up to the authors of course, but I feel the main advance here is the discovery of a new step in the process and explicitly stating this will tell the reader more precisely what has been accomplished.

Thanks. We have incorporated the suggestions in the text (lines 321-324; 392).

5) The authors might wish to be slightly more cautious in interpreting changes in FRET solely as changes in distance. My understanding is that FRET can also be influenced by local environment and orientation effects, and inserting a caveat or two might be prudent.

The reviewer is correct that FRET efficiency can be influenced by changes in the photophysics of dyes. However, we would like to emphasize that the observed FRET shifts due to binding with Get1CD and Get2CD are noticeably large (e.g. FRET peak was shifted from ~0.8 to ~0.35 upon Get1CD binding, please see Fig. 3B). It is unlikely that such large FRET changes were caused solely by the photophysical changes of dyes without changes in distance. In addition, we investigated the effects of Get1CD and Get2CD on the photophysics of fluorophores labeled on Get3, using analysis of the peak photon count rates of bursts belonging to Get3 molecules labeled with only the donor or acceptor dye. Our analyses showed that the fluo of both donor and
acceptor dyes on Get3 are only marginally affected by Get1CD and Get2CD (please see the reported mean peak count rates in Figure S2C). Therefore, the observed changes in FRET efficiency are primarily attributed to changes in Get3 conformation. We added the peak photon count rate analysis to the main text (materials and methods) and Figure S2C, accordingly.

Minor issues relating to references:

Line 50 - The earliest studies showing there is a multi-step cascade were PMID 20850366 and 20676083.

Line 53 - should probably cite 21835666 in addition to Mariappan & Stefer papers.

Line 77 - worth noting that Get1 CD induces release TA release, but that Get2 CD does not (also shown pretty clearly in Mariappan et al., 2011, which seems worth citing here).

We have corrected the references as suggested by the reviewer. We note that the Get2CD construct tested in Mariappan et al., 2011 contains residues 1–106, which does not include the complete H2 motif. Hence, the inability of Get2CD(1-106) to stimulate TA release does not contradict the data reported here.

Line 89 - This description seems a bit incomplete. The study showed that the mini-Get1-2 heterodimer not only interacts better, but is more potent at releasing TA proteins than Get1-CD. I think rather than being dismissive of the avidity idea, it might be better to say something like "this observation illustrated that Get1 and Get2 cooperate, but the molecular basis for this cooperation remained a matter of speculation."

We have revised the description here, as suggested.
Dear Dr. Shan,

Thank you for submitting your revised manuscript entitled "Subunit cooperation in the Get1/2 receptor promotes tail-anchored membrane protein insertion." We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add MW markers to gels in Figures 8B and S5.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Materials and methods: Should be comprehensive and not simply reference a previous
publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

6) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators. Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary.

7) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
   a. Make and model of microscope
   b. Type, magnification, and numerical aperture of the objective lenses
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   e. Fluorochromes
   f. Camera make and model
   g. Acquisition software
   h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

8) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed. JCB does not allow supplementary references, please remove this section and add any non-duplicate references to the main reference list.

9) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures and 10 videos. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section. Please include one brief sentence per item. There is no need for a separate cover page for supplementary material.

10) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

11) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

12) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).
13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB’s Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

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Monitoring Editor
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Scientific Editor
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