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Tail-anchor targeting by a Get3 tetramer: The structure of an archaeal homologue


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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 30 May 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see, the referees are all supportive of publication of the study here after appropriate revision. More specifically, referees 2 and 3 both think that the study does not make a sufficiently strong case for the physiological significance of the structure (yet), and referee 2 puts forward suitable suggestions how this point could be addressed. I would thus like to invite you to submit a revised version of the manuscripts that addresses the referees’ concerns in an adequate manner. I would like to specify that it would be rather important to perform the experiments put forward by referee 2. 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. Please do not hesitate to contact me at any time in case you would like to discuss any aspect of the revision further.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

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Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFeree REPORTS

Referee #1

Get3 is an ATPase that functions in the membrane insertion of tail anchored proteins. Suloway and Clemons report the crystal structure of an archaeal Get3 protein and observe a tetrameric association that displays a large hydrophobic cavity. This structure suggests a mechanism of binding the hydrophobic C-terminal residue of substrate proteins that is fundamentally different from the prevailing models for binding by a Get3 dimer. The structural findings are complemented by mutagenesis, association, and binding studies that support the implications of the crystal structure to the solution state and for generality of the tetramer binding mechanism in yeast as well as in archaea. Overall, the findings in this paper are surprising and change the way we must think about mechanisms of Get3 binding to tail anchor proteins. Moreover the paper is clearly written and, for the most part, nicely illustrated. There are no substantial concerns.

Minor points.

(1) There are a number of instances when "data" is treated as singular rather than plural, as is "data was" rather than "data were".

(2) Page 15. "submits" should be "subunits"

(3) Page 16. "incubation thrombin" should be something like "incubation with ??concentration?? thrombin"

(4) Figures 1EF and Figure S2. I don't understand the orientation/alignment of the two proteins being shown.

(5) Figure 4B legend. State explicitly the species of Get3 used.

(6) Figure 3D. A less heavily loaded gel would be preferable because the migration appears to be distorted, at least slightly, by the excessive amount of protein in each lane.

Referee #2

The manuscript by Suloway and Clemons provide new structural insights into the structure of Get3 and suggests a new model for its binding to tail-anchored substrates. The structural data appear sound and well executed. The major question that is not addressed by the Authors is whether the new structure they determine is physiologically relevant and represents a Get3 complex that is actually capable of membrane targeting?

Major comments.

The authors provide no evidence that the tetrameric form of MjGet3 they identify is competent for facilitating membrane insertion and hence represents a structure that is on a productive pathway. Bozkurt et al (2009) showed that canine microsomes are capable of accepting and integrating a model substrate from C. Therm Get3 and hence this is technically a straightforward experiment to attempt. The homogeneity of the preparations makes their system well suited to addressing this
issue. Indeed their ability to efficiently separate the S. cerevisiae dimeric and tetrameric form of Get3 would allow them to directly compare these two populations when loaded with substrate. Alternatively they might use the MjGet3, which is primarily tetramer anyway. In either case direct evidence that the tetrameric form of Get3 is functional would greatly strengthen their case.

Minor comments.

1. The authors should drop the paragraph starting "The need for a TA-targeting protein, such as Get3, was thought to be unique to eukaryotes...." Firstly Archae are not strictly prokaryotes, and secondly since prokaryotes and archae both have a signal recognition particle, I would disagree with their basic premise about a lack of requirement for targeting in these domains.

2. The authors should include a key control in the supplementary data, namely Sbh1 without a TM does not form complexes with MJGet3.

3. Figure 3B should be better labeled and the identity of the products confirmed by immunoblotting - at present I see a number of unlabeled bands and I can deduce what I think they are.

4. "TkGet3 ..... dominant dimer pool with some protein eluting as a teramer" I think this overstates the case - the amount of tetramer looks much more than for the SCGet3. Rephrase or calculate the estimated area under the two (overlapping peaks).

5. The tetramer is sensitive to detergent consistent with hydrophobic interactions. There are no controls - what happens with the addition of high salt or urea, is the tetramer unaffected?

6. The largely dimeric M196D mutant shown ion Figure 4E could be tested for function (see major point).

7. Page 9: Stoichiometry of ScGet3/TA suggests more than one TA-protein is bound per Get3 tetramer. How - can two TAs fit into the "cage" proposed by the Authors?

8. In the case of mammalian TRC40, a recent study by Leznicki et al (2011) shows the TA can have a 5000 Da PEG attached to it at any one of several locations and this will still bind to TRC40 and become membrane inserted. How does this fit with the tetrameric model proposed by the Authors? Would such the cage suggested accommodate a PEG of this size?

Referee #3

Membrane targeting of hydrophobic transmembrane segments necessitates mechanisms that shield them from the aqueous environment of the cytosol lest they lead to protein aggregation. During cotranslational targeting, this is achieved by direct passage of transmembrane segments from the ribosome to a protein translocation channel and then from the channel to the surrounding lipid bilayer. In the case of tail-anchored (TA) proteins, cotranslational targeting is not possible because their sole transmembrane segment is C-terminal and, thus, occluded by the ribosome until protein synthesis is completed. Instead, Get3, a dimeric ATPase, posttranslationally binds to the transmembrane segment of TA proteins that reside in the secretory pathway of eukaryotic cells and delivers them to the endoplasmic reticulum (ER) for insertion.

Suloway and Clemons provide structural and functional support for a model in which a dimer of Get3 dimers (in other words, a Get3 tetramer) forms a hydrophobic cage that can shield a C-terminal transmembrane segment while excluding adjacent N-terminal hydrophilic regions. This is an interesting structural model that is distinct from the earlier proposal, made by these authors and several other independent groups, in which a Get3 dimer (with a hydrophobic "lid") is sufficient to shield its hydrophobic substrate. Moreover, the Get3 tetramer model, in contrast to the original dimer model, nicely predicts that mutations in helix 6 of Get3 should not disrupt TA protein binding, which indeed they don't.
A weakness of this work is that there is no direct evidence that the tetrameric archeal Get3 homolog is a bona fide TA protein targeting factor. Since archea lack any apparent homologs of the other GET factors (e.g., Get1, which has both fungal and mammalian homologs) needed for TA protein insertion by Get3, it is arguably more parsimonious to conclude that the Get3 homologs carry out a distinct function in these organisms. The fact that overexpressed archeal Get3 homologs bind overexpressed TA proteins in bacteria is an encouraging sign that archea have a functional TA protein targeting pathway but this could also conceivably be a spurious hydrophobic interaction (see controls suggested in minor comments for Figures 3/4). This concern extends to ScGet3/TA protein complexes as well, despite their ability to deliver bound TA proteins for insertion, as shown by others. I am guessing that the authors share some of these concerns based on their speculation that other GET pathway factors (Bag6/ Sgt2/Get4/Get5) that bind Get3 in yeast and mammalian cytoplasms might be needed to load just a single substrate per Get3 tetramer (cf., the presence of additional substrate copies in the ScGet3/TA protein complexes prepared in bacteria; see Figure 5 comments). In my opinion, the authors’ manuscript will be strengthened by a discussion of these issues and by further experiments that address the issues described below.

Figure 3
i) The authors should show data with a control substrate lacking the transmembrane domain (which they mention in the text that they have already done) and with a bona fide ArsA homolog, which should not bind TA proteins.

ii) The authors should point out in the text that their analysis of TA protein sequence accessibility in the Get3/TA protein complex is not inconsistent with the Get3 dimer model for substrate binding.

Figure 4
The authors should monitor the ability of MjGet3 point mutants to bind TA protein by their bacterial coexpression/tandem affinity purification assay. The prediction of their model is that substrate binding should be reduced by mutations that disrupt tetramer formation.

Figure 5
i) Specify the calculated molecular weights of the ScGet3/TA protein complexes based on MALLS data shown.

ii) Compare the isolated dimer and tetramer populations of ScGet3 with the purified ScGet3/TA protein complexes. Comparing MjGet3 (strictly tetrameric) with the purified ScGet3/TA protein complexes, the way it is now, introduces species-specific differences that complicate their analysis.

1st Revision - authors’ response 24 August 2011

We are submitting our revised manuscript of “Tail-anchor targeting by a Get3 tetramer: The structure of an archaeal homologue”. We are grateful to the referees for their thorough efforts that have made our manuscript better. We have added a significant amount of data based on the referee’s comments. Most notably, we can now show that our tetramer complex can facilitate insertion. We have also added a discussion of a related manuscript that has been published during this revision which we think adds to the archaenal discussion of our story. We briefly list the most significant changes to the manuscript:

• In vitro membrane insertion assay for ScGet3 (Figure 5) and MjGet3 tetramer TA complexes (Figure S9)

• In vitro translation and membrane insertion assay for Sc, Mj, Mm, and TkGet3 (Figure S9)

• Control showing MjGet3 tetramer is stable in high salt and denaturant (Figure S8)

• Control showing the TA-protein TM deletion cannot form a complex with MjGet3 (Figure S7)

• Immunoblot against TA proteins for archaeal Get3/TA protein complexes (Figure S7)

• Improved TA protease protection assay gel using immunoblotting (Figure 3D)

• Comparison of crystal forms (Figure S2)

• Description of how TA proteins could be sequestered in a central cage and how this is
related to biochemical work done by Leznicki et al. 2011

We have addressed all of the comments in detail below. Referee’s comments are in italics. We feel that we have fully addressed the presented concerns and hope that the manuscript is now suitable for publication.

Referee #1

(1) There are a number of instances when "data" is treated as singular rather than plural, as is "data was" rather than "data were". These instances have all been fixed.

(2) Page 15. "submits" should be "subunits"

Fixed.

(3) Page 16. "incubation thrombin" should be something like "incubation with ??concentration?? thrombin"

Changed to “incubation with 2 U of thrombin per ml”.

(4) Figures 1EF and Figure S2. I don't understand the orientation/alignment of the two proteins being shown.

We have re-written the description in the figure legends and adjusted figure 1EF to more clearly reflect that we are showing two half images which is possible because of the two-fold symmetry in the molecule.

(5) Figure 4B legend. State explicitly the species of Get3 used.

Fixed.

(6) Figure 3D. A less heavily loaded gel would be preferable because the migration appears to be distorted, at least slightly, by the excessive amount of protein in each lane.

We have re-run this gel with much less protein and performed a western blot to clearly show the location of the MBP before and after cleavage.

Referee #2

Major comments.

The authors provide no evidence that the tetrameric form of MjGet3 they identify is competent for facilitating membrane insertion and hence represents a structure that is on a productive pathway. Bozkurt et al (2009) showed that canine microsomes are capable of accepting and integrating a model substrate from C. Therm Get3 and hence this is technically a straightforward experiment to attempt...

This is an astute observation and was a problem with our original report. We note in the manuscript that in Bozkurt et al (2009) they demonstrated that their C. therm Get3/TA complex was a tetramer of Get3 further showing that this complex is functional for insertion, even in a heterologous system. We have spent a considerable amount of effort to replicate these experiments and have added authors to reflect that. We have carefully purified our ScGet3/TA tetramer complex along with generating yeast microsomes and can now conclusively show that in conditions similar to those reported in Bozkurt (2009) we can get specific insertion by the tetramer. A Get3/TA dimer complex has never been generated to our knowledge and cannot be used for comparative study. Unfortunately, our MjGet3/TA complex was unable to insert in this assay. We wanted to determine if the archaeal system was capable of insertion. To do this, we used an in vitro translation assay that allowed us to add back Get3 homologues and test for insertion. This worked very well for ScGet3; however, none of the archaeal homologues would insert in this assay. This has all been added to the results in the section “Fungal Get3 tetramer is capable of TA membrane insertion in vitro” with appropriate figures in the main text and supplemental. To summarize, the purified fungal tetramer is capable of insertion. The archaeal homologues we have tested cannot insert in a heterologous system.
Minor comments.

1. The authors should drop the paragraph starting "The need for a TA-targeting protein, such as Get3, was thought to be unique to eukaryotes..." Firstly Archae are not strictly prokaryotes, and secondly since prokaryotes and archae both have a signal recognition particle, I would disagree with their basic premise about a lack of requirement for targeting in these domains.

   We agree with both of the referee’s points. First, archaea certainly are their own kingdom distinct from bacteria; however, we feel the simpler prokaryote description is reasonable in this context since we are making the point about not containing membrane bound organelle. Secondly, as read it was originally misleading to discuss targeting as all membrane proteins must be targeted. We have added “specific targeting” to better explain our point.

2. The authors should include a key control in the supplementary data, namely Sbh1 without a TM does not form complexes with MJGet3.

   We have added this figure (S7A).

3. Figure 3B should be better labeled and the identity of the products confirmed by immunoblotting - at present I see a number of unlabeled bands and I can deduce what I think they are.

   We have fixed the labeling and added a figure to the supplemental showing the corresponding western blot of the substrate (S7B).

4. "TkGet3 ...... dominant dimer pool with some protein eluting as a teramer" I think this overstates the case - the amount of tetramer looks much more than for the SCGet3. Rephrase or calculate the estimated area under the two (overlapping peaks).

   We agree with the observation. We have calculated the area under the peaks and it is clear that the dimer pool is not dominant. As these numbers are somewhat subjective to the model used in making the estimate, we have not added this as data. We have adjusted the text to better reflect what we see “TkGet3, which lacks the coordinating cysteines, is similar to ScGet3 in that the protein elutes as both a tetramer and a dimer”

5. The tetramer is sensitive to detergent consistent with hydrophobic interactions. There are no controls - what happens with the addition of high salt or urea, is the tetramer unaffected?

   We added these controls to the supplemental material (Fig. S8). The tetramer is stable in both conditions.

6. The largely dimeric M196D mutant shown ion Figure 4E could be tested for function (see major point).

   This experiment was not possible as we were unable to demonstrate that wild-type MjGet3 could facilitate insertion into yeast microsomes. The related experiment has been done for the equivalent ScGet3 mutation by Mateja et al (2009) and that mutant was deficient in TA binding.

7. Page 9: Stoichiometry of ScGet3/TA suggests more than one TA-protein is bound per Get3 tetramer. How - can two TAs fit into the "cage" proposed by the Authors?

   We have added text in the results and discussion to discuss this. This is coupled to the question posed in 8 below.

8. In the case of mammalian TRC40, a recent study by Leznicki et al (2011) shows the TA can have a 5000 Da PEG attached to it at any one of several locations and this will still bind to TRC40 and become membrane inserted. How does this fit with the tetrameric model proposed by the Authors? Would such the cage suggested accommodate a PEG of this size?

   We have added text to address our ideas about the chamber in the discussion along with addressing Leznicki et al. We don’t think the chamber would necessarily need to accommodate the PEG to fit in this model, in fact, in some ways the tetramer model might better fit the results they presented.

Referee #3

A weakness of this work is that there is no direct evidence that the tetrameric archeal Get3 homolog is a bona fide TA protein targeting factor. Since archea lack any apparent homologs of the other GET factors (e.g., Get1, which has both fungal and mammalian homologs) needed for TA protein
insertion by Get3, it is arguably more parsimonious to conclude that the Get3 homologs carry out a
distinct function in these organisms. The fact that overexpressed archaean Get3 homologs bind
overexpressed TA proteins in bacteria is an encouraging sign that archaean have a functional TA
protein targeting pathway but this could also conceivably be a spurious hydrophobic interaction
(see controls suggested in minor comments for Figures 3/4). This concern extends to ScGet3/TA
protein complexes as well, despite their ability to deliver bound TA proteins for insertion, as shown
by others. I am guessing that the authors share some of these concerns based on their speculation
that other GET pathway factors (Bag6/Sgt2/Get4/Get5) that bind Get3 in yeast and mammalian
cytoplasms might be needed to load just a single substrate per Get3 tetramer (cf., the presence of
additional substrate copies in the ScGet3/TA protein complexes prepared in bacteria; see Figure 5
comments). In my opinion, the authors' manuscript will be strengthened by a discussion of these
issues and by further experiments that address the issues described below.

We concur that there is no direct evidence that the archaeal Get3 homologue is a TA-
targeting factor and have softened our discussion towards that end. The structural comparisons
remain surprising and enticing but a true functional study in archaean is beyond the scope of this
work.

We have now taken the purified ScGet3 tetramer and can show specific insertion into yeast
microsomes demonstrating that they are functional. We go further in our discussion to address the
chamber and binding implications for TA substrates.

Figure 3
i) The authors should show data with a control substrate lacking the transmembrane domain (which
they mention in the text that they have already done) and with a bona fide ArsA homolog, which
should not bind TA proteins.

We have performed the control experiment of binding to MjGet3 in the absence of a TA
domain and provide it in the supplemental (Fig. S7A). We endeavored to get an ArsA homologue
for testing; however, the process of obtaining it in a reasonable time frame for resubmission was not
possible.

ii) The authors should point out in the text that their analysis of TA protein sequence accessibility in
the Get3/TA protein complex is not inconsistent with the Get3 dimer model for substrate binding.

We have added the sentence “This result could also be consistent with the dimer model
where the groove covers only the TA.”

Figure 4
The authors should monitor the ability of MjGet3 point mutants to bind TA protein by their bacterial
coexpression/tandem affinity purification assay. The prediction of their model is that substrate
binding should be reduced by mutations that disrupt tetramer formation.

This is a great suggestion. In considering the amount of additional data needed to be
generated, we decided to defer to related work that had been exhaustively done using point
mutations in ScGet3 (Mateja et al 2009; Suloway et al 2009). We presume that using MjGet3
mutants would at best support these results.

Figure 5
i) Specify the calculated molecular weights of the ScGet3/TA protein complexes based on MALLS
data shown.

These numbers are provided in Table 1.

ii) Compare the isolated dimer and tetramer populations of ScGet3 with the purified ScGet3/TA
protein complexes. Comparing MjGet3 (strictly tetrameric) with the purified ScGet3/TA protein
complexes, the way it is now, introduces species-specific differences that complicate their analysis.

We concur that this complicates our analysis of the MALLS data to some extent; however,
we feel that the bioSAXS data strongly corroborates our interpretation. The ScGet3 tetramer was
hard to get in quantities that give good MALLS data. As noted, we hypothesize that the ScGet3
tetramer complex contains E. coli peptides making it hard to draw strong conclusions.
The EMBO Journal Peer Review Process File - EMBO-2011-78078

2nd Editorial Decision 23 September 2011

Thank you for sending us your revised manuscript. Our original referees 2 and 3 have now seen it again, and you will be pleased to learn that both referees now support publication. Still, both referees put forward a number of points (see below) that should be addressed/responded to in amended version of the manuscript.

Furthermore there are two editorial issues that need further attention:

At this point I need to ask you to include the full PDB accession numbers into the acknowledgements section.

We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Please let us have a suitably amended manuscript as soon as possible. I will then formally accept the manuscript.

Thank you for your kind cooperation.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #2

My major concern with the previous version of this work was the absence of any direct evidence that a tetrameric form of Get3 was functional. The authors now use a well established assay to confirm that the tetrameric scGet3 can deliver a TA protein to the ER in a form where it can be inserted and hence have addressed this issue.

All of the minor points raised have been adequately explained or addressed.

Other comments:

1. Page 9, line 4. Should this read ... opposing subunit (Figure 4C) rather than 4D?

2. Figure 4E. Both on the screen and printed page the difference in color between red, purple and orange for the various mutants is not obvious - I am sure it can be improved.

3. The authors should include Reference to the two recent publications defining the Get1/2 receptor (Stefer et al 2011, Science and Mariappan et al., 2011 Nature). These could be included in addition to the existing Schuldiner el al 2008 reference on page 3.
Referee #3

In the revised manuscript Suloway et al. provide new functional data to support the main novelty of their work: structural and functional evidence suggesting that Get3 encapsulates TA proteins as a tetramer and delivers them for insertion by Get1/2 in the ER membrane.

My original concern about this study was that even though recombinant, tetrameric ScGet3-TA protein complex generated in bacteria CAN insert TA proteins to some extent (as previously shown in the literature and recapitulated in the revised version), the authors don't demonstrate that this DOES happen under physiological conditions (more formally, the specific membrane insertion activity of recombinant and native ScGet3-TA protein complexes is never measured side by side).

As I said before, I am happy to put this concern aside because I appreciate the value of the authors' Get3 tetrameric structural model in explaining previous mutagenesis data that are harder to reconcile by the prevailing, dimeric model.

I am disappointed, however, to see that the revised manuscript does not put the tetrameric model through further biochemical tests. For example, if there were mutations that blocked tetramerization but didn't block TA protein binding (and the resulting complex was now dimeric and still functional), it would raise concerns about the validity of the tetrameric model. My suspicion is that the authors dismissed this possibility ("we decided to defer to related work..."; this work, however, doesn't directly address the concern that I raised) because they see tetramer formation as intimately dependent on substrate binding (i.e., even when they express Get3 in E. coli without the TA protein, they postulate hypothetical native peptides stabilizing the Get3 tetramer population). In other words, they probably intuit that mutations that block tetramerization will also block substrate binding. This very well might be the case but it is not obvious to me how this would distinguish between the tetramer and dimer models. In other words, there is no additional biochemical evidence (e.g., stoichiometry analysis of native ScGet3-TA protein complexes) in the revised manuscript that would falsify the dimer model in favor of the tetrameric model. This burden of proof seems particularly high to me in light of the recently published Get1/2 structural studies showing that there is theoretically very little space between the disrupted Get3 dimer hydrophobic groove and the lipid bilayer when Get3 is in a complex with the cytosolic domain of Get1. Perhaps the tetramer model will be right when all is said and done but I will remain agnostic until then.

2nd Revision - authors' response 04 November 2011

We have addressed the referees in below. Referee's remarks are in italics.

Referee #2

My major concern with the previous version of this work was the absence of any direct evidence that a tetrameric form of Get3 was functional. The authors now use a well-established assay to confirm that the tetrameric scGet3 can deliver a TA protein to the ER in a form where it can be inserted and hence have addressed this issue.

All of the minor points raised have been adequately explained or addressed.

Other comments:

1. Page 9, line 4. Should this read ... opposing subunit (Figure 4C) rather than 4D?

Fixed.

2. Figure 4E. Both on the screen and printed page the difference in color between red, purple and orange for the various mutants is not obvious - I am sure it can be improved.

The colors have been changed from the previous color scheme to a easier to distinguish black/red/blue/green/tan color scheme in Figure 4C & E.
3. The authors should include Reference to the two recent publications defining the Get1/2 receptor (Stefer et al 2011, Science and Mariappan et al., 2011 Nature). These could be included in addition to the existing Schuldiner el al 2008 reference on page 3.

Citations and discussions for these two papers have been added to the discussion.

Referee #3

In the revised manuscript Suloway et al. provide new functional data to support the main novelty of their work: structural and functional evidence suggesting that Get3 encapsulates TA proteins as a tetramer and delivers them for insertion by Get1/2 in the ER membrane. My original concern about this study was that even though recombinant, tetrameric ScGet3-TA protein complex generated in bacteria CAN insert TA proteins to some extent (as previously shown in the literature and recapitulated in the revised version), the authors don’t demonstrate that this DOES happen under physiological conditions (more formally, the specific membrane insertion activity of recombinant and native ScGet3-TA protein complexes is never measured side by side). As I said before, I am happy to put this concern aside because I appreciate the value of the authors’ Get3 tetrameric structural model in explaining previous mutagenesis data that are harder to reconcile by the prevailing, dimeric model. I am disappointed, however, to see that the revised manuscript does not put the tetrameric model through further biochemical tests. For example, if there were mutations that blocked tetramerization but didn’t block TA protein binding (and the resulting complex was now dimeric and still functional), it would raise concerns about the validity of the tetrameric model. My suspicion is that the authors dismissed this possibility (“we decided to defer to related work...”; this work, however, doesn’t directly address the concern that I raised) because they see tetramer formation as intimately dependent on substrate binding (i.e., even when they express Get3 in E. coli without the TA protein, they postulate hypothetical native peptides stabilizing the Get3 tetramer population). In other words, they probably intuit that mutations that block tetramerization will also block substrate binding. This very well might be the case but it is not obvious to me how this would distinguish between the tetramer and dimer models. In other words, there is no additional biochemical evidence (e.g., stoichiometry analysis of native ScGet3-TA protein complexes) in the revised manuscript that would falsify the dimer model in favor of the tetramer model. This burden of proof seems particularly high to me in light of the recently published Get1/2 structural studies showing that there is theoretically very little space between the disrupted Get3 dimer hydrophobic groove and the lipid bilayer when Get3 is in a complex with the cytosolic domain of Get1. Perhaps the tetramer model will be right when all is said and done but I will remain agnostic until then.

We agree with the points Referee #3 made in their remarks. The nature for the interaction of Get3 with TA proteins in vivo is a question that still needs to be addressed. It is very difficult to conceive experiments that strongly argue in favor of the tetramer instead of the dimer. While we will continue to work towards this, it will be the subject of future studies. Furthermore, as we now discuss in the manuscript, the Get1/Get2 structures are not incompatible with a tetramer model. In fact, we strongly think they support it.