nature research

Peer Review Information

Journal: Nature Structural and Molecular Biology
Manuscript Title: Structurally derived universal mechanism for the catalytic cycle of the tail-anchored targeting factor Get3
Corresponding author name(s): Professor William Clemons

Reviewer Comments & Decisions:

Decision Letter, initial version:

3rd Feb 2022

Dear Bil,

Thank you again for submitting your manuscript "Structurally derived universal mechanism for the catalytic cycle of the tail-anchored targeting factor Get3". I apologize for the delay in responding, which resulted from the difficulty in obtaining suitable referee reports. Nevertheless, we now have comments (below) from the 3 reviewers who evaluated your paper. In light of those reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

I hope you will be pleased to see that all reviewers are positive about the quality and interest of the study. However, they make detailed suggestions for additional experiments to strengthen some of the conclusions, and for improving the presentation and discussion of the findings. Please be sure to address/respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file. If you have comments that are intended for editors only, please include those in a separate cover letter.

We expect to see your revised manuscript within 6 weeks. If you cannot send it within this time, please contact us to discuss an extension; we would still consider your revision, provided that no similar work has been accepted for publication at NSMB or published elsewhere.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in our papers are correct and accurate. As such, if there are any changes that should be reported, please submit an updated version of the Reporting Summary along with your revision.

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When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

If there are additional or modified structures presented in the final revision, please submit the corresponding PDB validation reports.

SOURCE DATA: we urge authors to provide, in tabular form, the data underlying the graphical representations used in figures. This is to further increase transparency in data reporting, as detailed in this editorial (http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html). Spreadsheets can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file. When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

Data availability: this journal strongly supports public availability of data. All data used in accepted papers should be available via a public data repository, or alternatively, as Supplementary Information.

If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found below:
https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data

We require deposition of coordinates (and, in the case of crystal structures, structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDB and released upon publication. Deposition and immediate release of NMR chemical shift assignments are highly encouraged. Deposition of deep sequencing and microarray data is mandatory, and the datasets must be released prior to or upon publication. To avoid delays in publication, dataset accession numbers must be supplied with the final accepted manuscript and appropriate release dates must be indicated at the galley proof stage.

While we encourage the use of color in preparing figures, please note that this will incur a charge to partially defray the cost of printing. Information about color charges can be found at http://www.nature.com/nsmb/authors/submit/index.html#costs

Nature Structural & Molecular Biology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as ‘corresponding author’ on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. This applies to primary research papers only. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on ‘Modify my Springer Nature account’. For more information please visit please visit www.springernature.com/orcid.

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Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Kind regards,
Florian

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Associate Editor
Nature Structural & Molecular Biology
ORCID 0000-0002-1153-2040

Referee expertise:

Referee #1: TA pathway

Referee #2: TA pathway, structural biology

Referee #3: TA pathway

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In this manuscript, Fry et al. provide a number of Get3 structures and sheds a light on the mechanism of tail-anchored (TA) protein capture by Get3. Get3 is the conserved ATPase chaperone that post-translationally captures transmembrane domains (TMDs) of TA proteins with the help of other factors (Sgt2 and Get4/5). Earlier studies from the author's lab and other labs have solved many different versions of Get3 including Get3 bound to Get4 or TA protein cargo. Although these studies have provided important insights into how Get3 captures TMDs of TA proteins, it is still debated how Get3 transforms from an empty state to substrate loaded state, and how this is regulated by ATP. These questions were challenging to address since available Get3 structures were obtained from different states from different labs. In the current manuscript, the authors aim to address these questions by using Get3 from Giardia intestinalis. Remarkably, the authors show GiGet3 in nucleotide-free states, ATP bound states and TA protein-bound states. This series of structures not only confirmed previous structural studies but also identify novel conformations that take place in GiGet3 with or without TA protein cargo. For example, in contrast to previous yeast Get3 structures, the authors find helix5 (H5) shields the client binding domain (CBD) in GiGet3 and that is displaced when CBD is occupied with the TMD of TA protein. Overall the manuscript is well organized, and the data are of high quality. The authors should address and/or discuss the below concerns to further strengthen the manuscript.

Major comments:

1. The central finding from this study is that the authors discover H5 of GiGet3 plays an important role in shielding CBD in the apo state as well as shielding the TMD of TA protein. Also, interestingly, compare to other Get3 homologs, GiGet3 has an extended sequence in H5 (126 to 134 amino acids) (Fig S1). This raises the question of whether the function of H5 shown here is unique to GiGet3 or is conserved to other Get3 homologs. It would be good to delete or mutate H5 in GiGet3 and test if it captures inefficiently the TMD of TA protein. The authors can do this by co-expressing GiGet3 mutant and TA protein in E. coli and examining the complex formation as they have done in Fig. S17.

2. To show the significance of the author's new findings of H5, I was also wondering H5-mediated shielding of CBD or TMD might help to capture even suboptimal (less hydrophobic) TA proteins. It would be good to test a few different TA substrates that vary in hydrophobicity from the list that they identified.

Minor comments:

1. Fig. S11 and Fig. S12 legends ends with helices are numbered as in Fig. ??”

Reviewer #2:
Remarks to the Author:
The manuscript by Fry et al. describes two significant contributions to understanding tail-anchored (TA) protein insertion by the Guided Entry of TA proteins (GET) pathway. Firstly, the authors identify the GET factors in the parasite Giardia intestinalis. Secondly, they use X-ray crystallography and single-particle cryo-EM to determine the conformational landscape of GiGet3. The authors observe that apo Get3 can adopt two conformations in a single crystal lattice, which both differ from a consensus cryo-EM structure. They also determine the structure of a GiGet3 ATPase inactivating mutant with ATP, which assumes a slightly different conformation than other Get3 structures bound to ATP analogs. These conformations inform how intramolecular interactions stabilize mobile elements of Get3 and how Get3 domains move relative to each other. Perhaps the most exciting finding is the cryo-EM structure of Get3 bound to a TA protein in the post-ATP hydrolysis state which shows how Get3 rearranges to chaperone a TA protein. The main caveat of the manuscript lies in the limitation to mostly observational descriptions. However, a lack of experimental tools for the GiGET system, combined with the structures primarily revealing conformational differences, may preclude structure-guided experiments. Overall, the analyses and interpretations are thoughtfully and thoroughly considered in the manuscript and generally support

the authors' claims of identifying the GiGET pathway and establishing the conformational landscape of Get3 in different nucleotide-bound states as it transitions between binding partners.

Main comments:
1. The cryo-EM structure of apo Get3 looks more like the crystal structure of ATP-bound Get3 more than apo1 and apo2 in Fig. 2. Is it possible that the apo1 and apo2 structures arise from Get3 held by crystal contacts in conformations that are otherwise rarely occupied in biological contexts?
2. Based on the local resolution of the post-hydrolysis Get3 bound to TA protein, additional information would strengthen the interpretation related to H4/5. Firstly, enforcing C2 symmetry may introduce artifacts at the axis of symmetry close to the position of H4/5. Can alpha carbons be placed or is this helical element still clear if symmetry is not enforced? Approximately how long is the substrate TMD and does this match the Bos1 TMD? Secondly, it would be useful if the model fitted to map in Fig. 3B was colored and labeled similarly to Fig. 3D to show the continuity that supports the assignment of the purple helix as H4/5 instead of other parts of Get3 such as H8.
3. It should be clearly noted that GiGet3 is overexpressed with a tag for staining in Fig. 1C. The claimed cytosolic vs. ER staining in Fig. S5 is also not obvious to me—the images all look like disperse spotty patterns, although this may be due to the resolution of the file. Since protein dynamics and fine complex structure is not needed to make this point, is it possible that cytosolic vs. ER staining would be more clearly distinguished using a lower-resolution confocal such as spinning disk instead of STED?

Reviewer #3:
Remarks to the Author:
Summary of the key results: The manuscript "Structurally derived universal mechanism for the catalytic cycle of the tail-anchored targeting factor Get3" by Fry and colleagues uncovers components of the guided-entry of tail-anchored proteins (GET) pathway in the pathogenic protist Giardia intestinalis and provides structural insights into Get3 in different states. The identification of homologs of the pre-targeting complex component Get4, the targeting factor Get3 and the ER-bound GET receptor component Get2, support the conservation of the GET targeting pathway to Excavata. Focusing on GiGet3, the authors use crystallography and cryo-EM to generate a series of structures that provide insights into how the conformation of this central GET pathway component changes upon nucleotide binding/hydrolysis and client interaction.
Originality and significance, conclusions: The GET pathway is a key ER targeting route for a significant proportion of membrane proteins, and insights into the conservation of the pathway and mechanistic details are of broad general interest. Structures of C. thermophilum, S. cerevisiae, S. pombe and human Get3 in different nucleotide bound/unbound states have been available for some time and already provide a detailed framework for TA protein targeting by Get3. The key features already identified in other species are largely conserved to G. intestinalis, but the direct comparison of different states of

the same protein in this study is advantageous. What this manuscript adds to the understanding of TA client capture and delivery by Get3 is a more definitive model of how the hydrophobic groove is occluded upon formation of the close conformation, visualization of the architecture of the post-ATP hydrolysis state, and an alternative view of how shielding of the TA protein during targeting is accomplished. Beyond the structural analyses, the second aspect of the manuscript focusing on the conservation of the GET pathway to G. intestinalis is rather preliminary and would benefit from further development.

Data & methodology: The manuscript is well-presented, the majority of the data is of high quality and the main conclusions are generally well supported. However, the procedures used for indirect immunofluorescent staining raise several questions: The custom-made (?) rat anti-GiGet3 antibody should be characterized. In the Methods section it states that “an anti-HA tag antibody” from Roche was used to detect Pdi2 but nowhere is it stated that the G. intestinalis strain used expresses an HA-tagged Pdi2. And in Fig. S5 GiGet3 is HA-tagged and an anti-Pdi2-antibody is mentioned. The catalogue number for this antibody should be listed. It would be good to create a small Table listing all antibodies and in which experiment they were used. All micrographs (Fig. 1C and Fig. S5) lack size bars. If cells were transfected with tagged constructs for the IF please state in the Legend. The use of statistics is questionable for the IF experiments since it is not stated how often the experiments were repeated and how many cells were analyzed.

Suggested improvements: 1) The identification of Get3 homologues should be presented more informatively. The data behind Fig 1B should be made available as a table because the current visualization does not allow the individual proteins to be identified. Furthermore, the main distinguishing features of clade I and II should be explained to rationalize this categorization. The specific proteins in yeast, humans and G. intestinalis should be marked with arrows in the figure.

2) Based on fractionation experiments, it is suggested that GiGet3 primarily localizes to the cytosol. However, a strong signal for GiGet3 is also detected in the pellet, indicating its association with membranes. This is in line with the co-precipitation of GiGet2 and the microscopy data (Fig. 1C,D) so should be acknowledged. Replica experiments and quantification would enable the proportion of GiGet3 present at the ER compared to the cytosol to be determined.

3) GiGet2 and GiGet4 were identified in pulldown assays followed by mass spectrometry and an Sgt2 homolog was found by structure-based homology searching. However, no homologs of Get1 or Get5 are mentioned. Isolation of complexes via GiGet2 and GiGet4 could reveal these additional homologs as they would be anticipated to form stable complexes with potential GiGet1 and GiGet5 proteins, respectively. The identification of GiGet5 is important as, by analogy to other species, this protein would be expected to mediate hand-over of the TA protein from GiSgt2 to GiGet3. Demonstrating interactions between Gi-pre-targeting complex components, and also between GiGet5 with GiGet3 would consolidate that the GET pathway is organized as in other species.

4) The identification of potential clients of the GiGET pathway is currently based on co-expression of GiGet3 and GiTA proteins in E. coli, an indirect approach that is prone to artefacts. The conclusion that GiGet3 targets endogenous TA proteins would be much better supported by showing mislocalization of

such proteins in cells lacking GiGet3, as has been done in other model organisms. Alternatively, identification of proteins retained with ATPase inactive GiGet3 D53N could provide evidence of TA protein interacting with GiGet3 in a more endogenous context.
Minor points:
Page 2, line 51: should read “WRB/CAML in mammals”
Page 6, Legend to Fig. 1C: Pdi2 does not mark the ER membrane but the organelle by staining its lumen
Fig S8: Where the membrane was cut in half should be indicated. Also, for consistency and to better visualize GiGet3, the image should be inverted to show black band on a white background.

Author Rebuttal to Initial comments

Reviewers' Comments:

Reviewer #1:

In this manuscript, Fry et al. provide a number of Get3 structures and sheds a light on the mechanism of tail-anchored (TA) protein capture by Get3. Get3 is the conserved ATPase chaperone that post-translationally captures transmembrane domains (TMDs) of TA proteins with the help of other factors (Sgt2 and Get4/5). Earlier studies from the author's lab and other labs have solved many different versions of Get3 including Get3 bound to Get4 or TA protein cargo. Although these studies have provided important insights into how Get3 captures TMDs of TA proteins, it is still debated how Get3 transforms from an empty state to substrate loaded state, and how this is regulated by ATP. These questions were challenging to address since available Get3 structures were obtained from different states from different labs. In the current manuscript, the authors aim to address these questions by using Get3 from Giardia intestinalis. Remarkably, the authors show GiGet3 in nucleotide-free states,

ATP bound states and TA protein-bound states. This series of structures not only confirmed previous structural studies but also identify novel conformations that take place in GiGet3 with or without TA protein cargo. For example, in contrast to previous yeast Get3 structures, the authors find helix5 (H5) shields the client binding domain (CBD) in GiGet3 and that is displaced when CBD is occupied with the TMD of TA protein. Overall the manuscript is well organized, and the data are of high quality. The authors should address and/or discuss the below concerns to further strengthen the manuscript.

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H5 (126 to 134 amino acids) (Fig S1). This raises the question of whether the function of H5 shown here is unique to GiGet3 or is conserved to other Get3 homologs. It would be good to delete or mutate H5 in GiGet3 and test if it captures inefficiently the TMD of TA protein. The authors can do this by co-expressing GiGet3 mutant and TA protein in E. coli and examining the complex formation as they have done in Fig. S17.

The reviewer makes the excellent observation that H5 is longer (9 amino acids) in both the structure and alignment. Due to manuscript length, we were unable to discuss this in detail. A notable observation is that the helix length changes across the crystal forms with the apoA having the longest H5 while in apoB this extension is disordered due to crystal packing. This suggests the helix is flexible consistent with its conformational changes and lack of conservation.

We performed the experiment as requested and have added this as an additional figure (the new Fig. S23). To be specific, we truncated H5 to be similar to the length of the metazoan and yeast H5 to directly address the length question. Deleting this helix completely would be incompatible with the post-hydrolysis structure as the H5 helix additionally contributes to the walls of the hydrophobic groove. In the experiment, the helix is still capable of forming a complex with the TA client consistent with this difference not driving the interaction.

Spurred by the suggestion, we were interested in testing the role of H4/5, the loop that forms the lid in the post-hydrolysis structure, and whether it was required for forming a stable Get3/TA complex, as one would predict from the structure. Deletion of H4/5 resulted in a loss of complex supporting the model that it was critical for complex stability (Fig S23). Of note, previous replacement of the H8 "lid" helix (as referred to in the metazoan pre-targeting structure, Keszei et al. NSMB, 2021) with a linker does not affect client binding or targeting, only client transfer from SGTA to Get3.

2. To show the significance of the author's new findings of H5, I was also wondering H5-mediated shielding of CBD or TMD might help to capture even suboptimal (less hydrophobic) TA proteins. It would be good to test a few different TA substrates that vary in hydrophobicity from the list that they identified.

As suggested from the response to the first point, it doesn't appear that GiGet3 will have different properties from the fungal Get3s and will likely have similar binding rules. In the original draft we had mentioned that we tested a variety of Giardia TA proteins, but only provided data for two of them. We now have provided data for all the variants, Fig. S9, and adjusted our discussion. The putative *Gi*TAs selected represent a range of TMD hydrophobicities (sum of 12.9-31.08 using the TM tendency scale) similar to previous reports of ER-bound TA proteins in metazoans (12.5-27.3) (Guna *et al.*, Science 2018). In the previous report, zebrafish Get3 was unable to capture and insert TA proteins with hydrophobicity values less than 22 in an *in vitro* targeting experiment. The list of *Giardia* TA proteins provided in Table S3 is a putative list complied based on predicted TMDs and includes TA proteins that are likely mitochondria-bound. Localization in *Giardia* is not as well characterized as in yeast and metazoans so confidently selecting TAs that are ER-bound and Get3 substrates is difficult. Our selection of TAs represents the expected range of hydrophobicities for ER-bound TA proteins in *Giardia*. Interestingly, the *Gi*TA proteins that bind Get3 and are likely clients (Fig. S9) have high TMD hydrophobicities (greater than the 22 cutoff determined by Guna and colleagues). While an absence of binding in *E. coli* does not mean there is no binding, it is encouraging that the bound clients are the more hydrophobic TAs. We see no evidence that the longer H5 aids in binding less hydrophobic TA proteins as the strongly bound clients are above the hydrophobic threshold seen in opisthokonts.

Minor comments:

1. Fig. S11 and Fig. S12 legends ends with helices are numbered as in Fig. ??"

We have fixed this. Thank you for identifying this typo.

Reviewer #2:

The manuscript by Fry et al. describes two significant contributions to understanding tail-anchored (TA) protein insertion by the Guided Entry of TA proteins (GET) pathway. Firstly, the authors identify the GET factors in the parasite Giardia intestinalis. Secondly, they use X-ray crystallography and single-particle cryo-EM to determine the conformational landscape of GiGet3. The authors observe that apo Get3 can adopt two conformations in a single crystal lattice, which both differ from a consensus cryo-EM structure. They also determine the structure of a GiGet3 ATPase inactivating mutant with ATP, which assumes a slightly different conformation than other Get3 structures bound to ATP analogs. These

conformations inform how intramolecular interactions stabilize mobile elements of Get3 and how Get3 domains move relative to each other. Perhaps the most exciting finding is the cryo-EM structure of Get3 bound to a TA protein in the post-ATP hydrolysis state which shows how Get3

rearranges to chaperone a TA protein. The main caveat of the manuscript lies in the

limitation to mostly observational descriptions. However, a lack of experimental tools for the GiGET system, combined with the structures primarily revealing conformational differences, may preclude structure-guided experiments. Overall, the analyses and interpretations are thoughtfully and thoroughly considered in the manuscript and generally support the authors' claims of identifying the GiGET pathway and establishing the conformational landscape of Get3 in different nucleotide-bound states as it transitions between binding partners.

Main comments:

1. The cryo-EM structure of apo Get3 looks more like the crystal structure of ATP-bound Get3 more than apo1 and apo2 in Fig. 2. Is it possible that the apo1 and apo2 structures arise from Get3 held by crystal contacts in conformations that are otherwise rarely occupied in biological contexts?

Crystal structures represent a likely conformational state seen in solution. The crystal contacts in our Apo crystal likely capture states that are only transiently occupied in solution. Our structures represent the range of conformations apo Get3 can adopt as predicted by single molecule FRET experiments (Chio *et al*. PNAS, 2017). Based on the number of picked particles and the final number of particles that resulted in the cryo-EM reconstruction of apo *Gi*Get3 we can estimate that approximately 1.34% of the total number of picked particles used for 3D classification are in the closed state. This is after some filtering by 2D and 3D classification and the total number of particles should reflect particles and not noise or contaminants. While a small percentage, the closed apo conformation is likely the most common conformation of apo Get3. Our work further demonstrates how ATP binding regulates the Get4 interface.

2. Based on the local resolution of the post-hydrolysis Get3 bound to TA protein, additional information would strengthen the interpretation related to H4/5. Firstly, enforcing C2 symmetry may introduce artifacts at the axis of symmetry close to the position of H4/5. Can alpha carbons be placed or is this helical element still clear if symmetry is not enforced?

Symmetry was imposed at the final step. Without symmetry constraints the reconstruction refined to 3.87 Å resolution with sharpening as outlined in the Methods section (lines 680-681). New representative images of the map without symmetry have been added to Fig S20 panels F and G. H4/5 is present in this map and the density reflects a helix (Fig S20G), although it is important to note that we could not accurately build into this density.

Approximately how long is the substrate TMD and does this match the Bos1 TMD?

The density corresponding to client in the groove is sufficiently long that we estimate it could accommodate ~22 residues built into a helix, more than sufficient to accommodate the Bos1 TMD that is 18 residues long. It is important to note that we are unable to build accurately into this density. This is likely due to binding of the TMD is based on hydrophobicity and does not result in a fixed orientation. Reconstructions from single particle cryo-EM are the result of averaging across thousands of particles. It is likely that the TMD binds each dimer in slightly different orientations and registries.

Secondly, it would be useful if the model fitted to map in Fig. 3B was colored and labeled similarly to Fig. 3D to show the continuity that supports the assignment of the purple helix as H4/5 instead of other parts of Get3 such as H8.

We have edited Fig 3D to make the coloring and labels clearer.

. It should be clearly noted that GiGet3 is overexpressed with a tag for staining in Fig. 1C.

There appears to be confusion based on our unclear description. GiGet3 is not

overexpressed in the data shown in Fig. 1C&E where endogenous promotors were used. In both the immunoprecipitation with the BAP-tag and the STED microscopy the protein was overexpressed. The methods and figure legend have been adjusted to make this more explicit.

The claimed cytosolic vs. ER staining in Fig. S5 is also not obvious to me—the images all look like disperse spotty patterns, although this may be due to the resolution of the file. Since protein dynamics and fine complex structure is not needed to make this point, is it possible that cytosolic vs. ER staining would be more clearly distinguished using a lower-resolution confocal such as spinning disk instead of STED?

The intention of the microscopy analysis was to visualize the colocalization of GiGet3 and GiGet2. For this reason, both proteins were overexpressed with tags that are more suitable for STED imaging. Unfortunately, STED analysis enhances membrane localization over the soluble cytosolic one. The cytosolic localization of GiGet3 is thus better demonstrated by the use of the antibody against the endogenous protein on the western blot of the cellular fractions.

Reviewer #3:

Summary of the key results: The manuscript "Structurally derived universal mechanism for the catalytic cycle of the tail-anchored targeting factor Get3" by Fry and colleagues uncovers components of the guided-entry of tail-anchored proteins (GET) pathway in the pathogenic protist Giardia intestinalis and provides structural insights into Get3 in different states. The identification of homologs of the pre-targeting complex component Get4, the targeting factor Get3 and the ER-bound GET receptor component Get2, support the conservation of the GET targeting pathway to Excavata. Focusing on GiGet3, the authors use crystallography and cryo-EM to generate a series of structures that provide insights into how the conformation of this central GET pathway component changes upon nucleotide binding/hydrolysis and client interaction.

Originality and significance, conclusions: The GET pathway is a key ER targeting route for a significant proportion of membrane proteins, and insights into the conservation of the pathway and mechanistic details are of broad general interest. Structures of C. thermophilum, S. cerevisiae, S. pombe and human Get3 in different nucleotide bound/unbound states have been available for some time and already provide a detailed framework for TA protein targeting by Get3. The key features already identified in other species are largely conserved to G. intestinalis, but the direct comparison of different states of the same protein in this study is advantageous. What this manuscript adds to the understanding of TA client capture and delivery by Get3 is a more definitive model of how the hydrophobic groove is occluded upon formation of the close conformation, visualization of the architecture of the post-ATP hydrolysis state, and an alternative view of how shielding of the TA protein during targeting is accomplished. Beyond the structural analyses, the second aspect of the manuscript focusing on the conservation of the GET pathway to G. intestinalis is rather preliminary and would benefit from further development.

Data & methodology: The manuscript is well-presented, the majority of the data is of high quality and the main conclusions are generally well supported. However, the procedures used for indirect immunofluorescent staining raise several questions: The custom-made (?) rat anti-GiGet3 antibody should be characterized.

We have added the following sentence to the Methods section (Lines 450-454):

"Purified GiGet3 was used as an antigen for in-house production of a polyclonal antibody in rats. The antibody was validated by western against purified *Gi*Get3 and recognized the same band as a commercial anti-BAP antibody when both were probed against the BAP-tagged *Gi*Get3."

In the Methods section it states that "an anti-HA tag antibody" from Roche was used to detect Pdi2 but nowhere is it stated that the G. intestinalis strain used expresses an HA-tagged Pdi2. And in Fig. S5 GiGet3 is HA-tagged and an anti-Pdi2-antibody is mentioned. The catalogue number for this antibody should be listed. It would be good to create a small Table listing all antibodies and in which experiment they were used. All micrographs (Fig. 1C and Fig. S5) lack size bars. If cells were transfected with tagged constructs for the IF please state in the Legend. The use of statistics is questionable for the IF experiments since it is not stated how often the experiments were repeated and how many cells were analyzed.

We modified the Methods sections on the immunofluorescence and STED microscopy to clarify the cell lines and antibodies used for protein detection. We added scale bars to all image series. We have not performed a statistical analysis on the protein localization. Protein localization was uniform in all confocal and STED images. Representative images are shown in an updated Figure S6.

Suggested improvements: 1) The identification of Get3 homologues should be presented more informatively. The data behind Fig 1B should be made available as a table because the current visualization does not allow the individual proteins to be identified. Furthermore, the main distinguishing features of clade I and II should be explained to rationalize this categorization. The specific proteins in yeast, humans and G. intestinalis should be marked with arrows in the figure.

We added a table containing the accession numbers and affiliation of the sequences to a particular clade (Table S1). The categorization is based on the phylogenetic analysis of all sequences included into the dataset. The grouping of proteins into two clades is based

on the mutual relationship among the sequences and reflects similarities or differences present in the initial protein sequence alignment. The presence of two Get3 clades has also been noted in Xing *et al*. PNAS, 2017. Neither analysis identified any motifs specific to either clade. The differences on the primary sequence level are spread across the entire sequence which is demonstrated in a new figure (Fig S1).

2) Based on fractionation experiments, it is suggested that GiGet3 primarily localizes to the cytosol. However, a strong signal for GiGet3 is also detected in the pellet, indicating its association with membranes. This is in line with the co-precipitation of GiGet2 and the microscopy data (Fig. 1C,D) so should be acknowledged. Replica experiments and quantification would enable the proportion of GiGet3 present at the ER compared to the cytosol to be determined.

We thank the reviewer for suggesting the quantification experiment. Indeed, we agree that while much of the protein is present in the cytosol, there is a considerable amount of *Gi*Get3 associated with the ER membrane, which agrees with the protein function. We have addressed this in the manuscript (Line 130-132).

3) GiGet2 and GiGet4 were identified in pulldown assays followed by mass spectrometry and an Sgt2 homolog was found by structure-based homology searching. However, no homologs of Get1 or Get5 are mentioned. Isolation of complexes via GiGet2 and GiGet4 could reveal these additional homologs as they would be anticipated to form stable complexes with potential GiGet1 and GiGet5 proteins, respectively. The identification of GiGet5 is important as, by analogy to other species, this protein would be expected to mediate hand-over of the TA protein from GiSgt2 to GiGet3. Demonstrating interactions between Gi-pre-targeting complex components, and also between GiGet5 with GiGet3 would consolidate that the GET pathway is organized as in other species.

We agree that a full analysis of the GET pathway components in *Giardia* will be valuable and an important next step is to identify Get1 and Get5 homologs, if there are any. While we have found some candidate homologs of Get1 and Get5 in either the genome analysis or our proteomic data, these are not as clear as the other components. Before making any additional comments on other components we will need to establish functional studies to identify the role of these proteins. These are complicated experiments and beyond the scope of this current manuscript. We are excited to continue these experiments in future studies.

4) The identification of potential clients of the GiGET pathway is currently based on co-expression of GiGet3 and GiTA proteins in E. coli, an indirect approach that is prone to artefacts. The conclusion that GiGet3 targets endogenous TA proteins would be much better supported by showing mislocalization of such proteins in cells lacking GiGet3, as has been done in other model organisms. Alternatively, identification of proteins retained with ATPase inactive GiGet3 D53N could provide evidence of TA protein interacting with GiGet3 in a more endogenous context.

This is indeed one of the crucial experiments. In fact, we have recently established a CRISPR/Cas9 gene deletion approach in *G. intestinalis*. Unfortunately, we were not able to obtain a viable *Gi*Get3 knockout strain, indicating that the gene might be, in fact, essential in the protist. We are currently developing an alternative inducible approach. The strategy of pulling down the D53N mutant would not guarantee positive results as we do not see client proteins to be stably bound by *Gi*Get3 in the proteomic analysis.

Minor points:

Page 2, line 51: should read "WRB/CAML in mammals"

The human gene for WRB has been renamed by EMBL-EBI as Get1 to be more consistent with its role in the pathway. We have adopted the new nomenclature.

GENE SYMBOL: GET1

GENE NAME: guided entry of tail-anchored proteins factor 1

SYNONYMS: WRB, CHD5, GET1

Page 6, Legend to Fig. 1C: Pdi2 does not mark the ER membrane but the organelle by staining its lumen

We have adjusted the legend to reflect this.

Fig S8: Where the membrane was cut in half should be indicated. Also, for consistency and to better visualize GiGet3, the image should be inverted to show black band on a white background.

We have inverted the images and indicated where the membrane was cut.

Decision Letter, first revision:

19th Apr 2022

Dear Bil,

Thank you for submitting your revised manuscript "Structurally derived universal mechanism for the catalytic cycle of the tail-anchored targeting factor Get3" (NSMB-A45727B). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Structural & Molecular Biology, pending minor revisions to satisfy the referees' final requests regarding the experiment shown in Fig. S23, and to comply with our editorial and formatting guidelines. Regarding the former, we leave it up to you if you want to repeat the experiment with the requested controls or if you want to remove the data from the paper.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

To facilitate our work at this stage, we would appreciate if you could send us the main text as a word file. Please make sure to copy the NSMB account (cc'ed above).

Thank you again for your interest in Nature Structural & Molecular Biology Please do not hesitate to contact me if you have any questions.

Kind regards,
Florian

Florian Ullrich, Ph.D.
Associate Editor
Nature Structural & Molecular Biology
ORCID 0000-0002-1153-2040

Reviewer #1 (Remarks to the Author):

The authors have addressed my concerns. However, Fig. S23 is not convincing since the absence of the Get3 signal in dH4/5 could be explained by the low yield of His BRIL Bos1 TMD (bottom) compared to

the trH5 sample. The authors should redo the experiment to obtain a similar recovery of His BRIL Bos1 TMD for both samples. Alternatively, the authors can remove the data and adjust their conclusion accordingly.

Reviewer #2 (Remarks to the Author):

The revised manuscript by Fry et al. addresses my original points. My only remaining comment relates to Fig. S23, which was added to address a point concerning the importance of the H4/5 loop versus the length of H5 raised by Reviewer 1. The experiment shown lacks several controls important for interpretation. It would be useful to see the levels of the Get3 and TA proteins in the input samples and how recovery of the GiGet3 mutants compares to wildtype. In addition, although the authors pull on the same TA protein when testing both mutants, the recovery of the TA protein seems to differ by >10-fold between the two samples. This confounds how much of the difference in GiGet3 recovery can be attributed to the specific mutants analyzed. From what is shown, I would conclude that the trH5 can interact with TA protein but would not be confident about conclusions related to the H4/5 loop without additional information. Finally, the Western blot image looks more transparent than expected for the size of the bands (perhaps during figure formatting?) and appears to be missing from the source data file. Otherwise, I support publication.

Author Rebuttal, first revision:

Reviewer #1 (Remarks to the Author):

The authors have addressed my concerns. However, Fig. S23 is not convincing since the absence of the Get3 signal in dH4/5 could be explained by the low yield of His BRIL Bos1 TMD (bottom) compared to the trH5 sample. The authors should redo the experiment to obtain a similar recovery of His BRIL Bos1 TMD for both samples. Alternatively, the authors can remove the data and adjust their conclusion accordingly.

We agree with the reviewer and have removed this data and the corresponding analysis from our manuscript.

Reviewer #2 (Remarks to the Author):

The revised manuscript by Fry et al. addresses my original points. My only remaining comment relates to Fig. S23, which was added to address a point concerning the importance of the H4/5 loop versus the

length of H5 raised by Reviewer 1. The experiment shown lacks several controls important for interpretation. It would be useful to see the levels of the Get3 and TA proteins in the input samples and how recovery of the GiGet3 mutants compares to wildtype. In addition, although the authors pull on the same TA protein when testing both mutants, the recovery of the TA protein seems to differ by >10-fold between the two samples. This confounds how much of the difference in GiGet3 recovery can be attributed to the specific mutants analyzed. From what is shown, I would conclude that the trH5 can interact with TA protein but would not be confident about conclusions related to the H4/5 loop without additional information. Finally, the Western blot image looks more transparent than expected for the size of the bands (perhaps during figure formatting?) and appears to be missing from the source data file. Otherwise, I support publication.

As with Reviewer #1, we agree with the reviewer and have removed this data and corresponding analysis from our manuscript. The source data file was mislabeled FigS20 instead of FigS23. As seen in the unprocessed image, there is no manipulation of the western blot as the image was taken directly form the blot.

Final Decision Letter:

Dear Dr Fry,

Please find below a copy of the decision letter for your manuscript "Structurally derived universal mechanism for the catalytic cycle of the tail-anchored targeting factor Get3" [NSMB-A45727C], which has just been accepted for publication in Nature Structural & Molecular Biology.

The exact publication date will be communicated to the corresponding author. Please note that until publication, the content of your paper remains under embargo (to determine when the paper can be discussed with the media, please consult our embargo policy at http://www.nature.com/authors/editorial_policies/embargo.html).

To assist our authors in disseminating their research to the broader community, our SharedIt initiative provides all co-authors with the ability to generate a unique shareable link that will allow anyone (with or without a subscription) to read the published article. Recipients of the link with a subscription will also be able to download and print the PDF.

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You can now use a single sign-on for all your accounts, view the status of all your manuscript submissions and reviews, access usage statistics for your published articles and download a record of your refereeing activity for the Nature journals.

Sincerely,
Florian Ullrich, Ph.D.
Associate Editor
Nature Structural & Molecular Biology
ORCID 0000-0002-1153-2040

Subject: Decision on Nature Structural & Molecular Biology submission NSMB-A45727C

26th May 2022

Dear Bil,

We are now happy to accept your revised paper "Structurally derived universal mechanism for the catalytic cycle of the tail-anchored targeting factor Get3" for publication as a Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Structural & Molecular Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

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You will not receive your proofs until the publishing agreement has been received through our system.

Due to the importance of these deadlines, we ask that you please let us know now whether you will be difficult to contact over the next month. If this is the case, we ask you provide us with the contact information (email, phone and fax) of someone who will be able to check the proofs on your behalf, and who will be available to address any last-minute problems.

To assist our authors in disseminating their research to the broader community, our SharedIt initiative provides all co-authors with the ability to generate a unique shareable link that will allow anyone (with or without a subscription) to read the published article. Recipients of the link with a subscription will also be able to download and print the PDF.

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Note the policy of the journal on data deposition: http://www.nature.com/authors/policies/availability.html.

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they might be interested in promoting its publication. This will allow them time to prepare an accurate and satisfactory press release. Include your manuscript tracking number (NSMB-A45727C) and our journal name, which they will need when they contact our press office.

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Kind regards,
Florian

Florian Ullrich, Ph.D.
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