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Peer Review Information

Journal: Nature Structural and Molecular Biology
Manuscript Title: Structural basis for polyglutamate chain initiation and elongation by TTLL family enzymes
Corresponding author name(s): Dr. Antonina Roll-Mecak

Reviewer Comments & Decisions:

Decision Letter, initial version:

10th Apr 2020

Dear Antonina,

Thank you again for submitting your manuscript "Structural basis for polyglutamate chain initiation and elongation by TTLL family enzymes". 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, experts in tubulin code (referees #1 and #2), structure and function of microtubule-interacting proteins (referee #2) and structural enzymology (referee #3). 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.

You will see that, while all three reviewers appreciate the insights into the TTLLs catalytic mechanism and substrate specificity, they also have some reservations. Among other points, referee #1 is concerned about the physiological relevance of TTLL6 activity on detyrosinated α- tubulin and both structural biology experts point out to potential issues with the processing/presentation of the crystallograpic data. 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 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.

We usually expect to see revised manuscripts within 6 weeks, but we understand that it may not be possible given the current situation. 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. In that regard, please note that as of Monday, April 13th, I will transfer to our sister journal Nature Communications and will no longer handle the

manuscripts submitted to NSMB. Thus, our chief editor Ines Chen (cc'ed here) will be the editor of this manuscript from then on.

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.

Please follow the links below to download these files:

Reporting Summary:
https://www.nature.com/documents/nr-reporting-summary.pdf

Please note that the form is a dynamic 'smart pdf' and must therefore be downloaded and completed in Adobe Reader.

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

Please note that all key data shown in the main figures as cropped gels or blots should be presented in uncropped form, with molecular weight markers. These data can be aggregated into a single supplementary figure item. While these data can be displayed in a relatively informal style, they must refer back to the relevant figures. These data should be submitted with the final revision, as source data, prior to acceptance, but you may want to start putting it together at this point.

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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[REDACTED]

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.

With best wishes,

Katarzyna

Katarzyna Marcinkiewicz, Ph.D.
Associate Editor
Nature Structural and Molecular Biology

ORCID 0000-0001-8136-4559

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Reviewer's Comments – Mahalingan et al.
In a variety of cleverly designed and depicted experiments that use crytallography as well as mass spectrometry and enzyme kinetics, this paper established important elements of the catalytic mechanism of TTLL enzymes that initiate or those that elongate glutamate chains. As such, the paper represents an important contribution to our knowledge of tubulin posttranslational modification. I do

have some critiques of the interpretation and conclusions of the paper, though:
First, this paper is said to establish 'the molecular basis for regioselectivity for the entire TTLL family' but since the two enzymes compared differ in TWO ways (initiation vs. elongation and α- vs β), this seems an over-reach.
In establishing substrate preference, use of α1B and α1B-Y peptides for comparison seems like an expedient strategy. From these experiments, the authors raise the possibility that TTLL6 could carry out glutamylation on detyrosinated α- tubulin; some predictions for whether or not this might occur in vivo could come from a comparison of the C-terminal sequence/structure with the sequence/structure surrounding E-443 (e.g., number of E residues and residues flanking). However, it begs the of whether the authors or reports in the literature provide any direct evidence that glutamylation on detyrosinated α- tubulin is physiologically significant for TTLL6 or other initiator TTLLs.
The authors demonstrate that TTLL7 recognizes regions of the tubulin polypeptide or lattice outside of those that interact with the TTLL7 catalytic site. Perhaps this is true for other TTLLs, in addition (e.g., for TTLL4 and 6) or instead of (for other TTLLs) the interactions detailed here between catalytic site and tubulin polypeptide. This seems especially important to consider, given the other non-tubulin substrates that the TTLLs have to recognize. In fact, the authors should discuss whether any of the initiator enzymes that prefer either α- or β-tubulin actually have more activity on non-tubulin proteins than do others.
Adducts used for structural determination present an interesting potential for preparing further small molecule inhibitors and making the best derivatives of these, but it is hard to see how these could be made to specifically inhibit one TTLL over another. That is, the initiator analog and its chemical progeny would be predicted to inhibit all initiator TTLLs, which would have questionable use in the many disorders caused by abnormal TTLL expression or sequence. Thus, the promise suggested in the Discussion should be tempered.
The literature on enzymes of tubulin posttranslational modification is rife with claims that studies of catalytic mechanisms will engender the availability of pure populations of microtubules for study of each modification. However greatly the results presented in papers like this advance our knowledge, this claim remains speculative, far-fetched, or impossible. Using purified enzymes to produce micromolar amounts of microtubules for studies of protein-protein interactions seems completely unrealistic, even if the enzymatic reactions could be expected to go to completion. This type of claim in the Discussion should be toned down considerably. Similarly, the authors should tone down their speculation that the TTLL4 and TTLL6 studied here will necessarily offer possibilities of incorporating unnatural amino acids into microtubules.
Minor comments (typographical error):
(monoplr; should be monopolar) in legend to Box 1 on page 48 of the manuscript.

Reviewer #2:
Remarks to the Author:
Tubulin glutamylation and glycylation have been described more than 20 years ago and these modifications are known to play a critical role in correct microtubule function. However, understanding the molecular detail of these modifications has been notoriously difficult, due to an inherent complexity of tubulin (isoforms, multiple modifications, differential expression etc) but also the tools available to study these modifications in vitro. It is only in the recent years that significant progress has been made in understanding the atomic detail of these interactions, a prerequisite for ultimate understanding of the tubulin code. This work is built on previous work mostly by the same authors that over the last years have managed to systematically address the molecular understanding of

tubulin glutamylation and glycylation. Through detailed structural and biochemical analysis, they have been able to address the question of how the interaction between TTLL enzymes and tubulin (microtubules) occurs, as well as define structural elements that distinguish glycylases from glutamylases, or initiating from elongation glycylases. However up until now several important mechanistic questions remain unanswered.

This latest manuscript adds new and important molecular detail to the tubulin code, which is absolutely required in order to understand the detailed functions of modified tubulin species. The authors provide biochemical evidence for distinct initiation and elongation activities of glutamylases, analogous to what had been previously reported for glycylases (Garnham et al, PNAS, 2017). They also determine the nature of the linkage of glutamate chains for different TTLLs. Although it has been known for some time that both alpha linked and gamma linked chains exist (using antibodies that can distinguish these linkages), no direct biochemical and structural characterization of the latter has been reported. Using a combination of structural biochemistry and enzymology the authors have been able to unambiguously determine that TTLL6 preferentially elongates a-linked glutamate chains and has an elongation activity, contrary to TTLL4 that shows an initiation activity. Careful analysis of the active site has allowed to find determinants for regioselectivity for all TTLL enzymes. This is the first unambiguous demonstration of the reaction mechanism of TTLL enzymes and paves the way for a mechanistic comprehension of tubulin code generation, specificities and precise functions of individual enzymes. Moreover, the tools developed in this study (the analogs) will be useful for future studies and will contribute towards a better understanding of these complex enzymes. This is solid work from a technical point of view and adds new information from a scientific point of view. It is an important contribution to the field, and I thus recommend this paper to be published in NSMB after addressing some minor comments I have detailed below.

Comments and points to address (by paragraph):

TTLL6 adds long glutamate chains to the alpha-tubulin

This paragraph clearly demonstrates that TTLL6 has an elongation activity with a strong preference towards the exposed alpha-carboxyl groups of free glutamates rather than the gamma-carboxyl group of main chain glutamates.

Please mention:

- How the brain and recombinant tubulin used was produced/purchased. This should be described or referred to in Protein expression and purification (Materials and Methods).

- Why the choice of recombinant alphaIB/betaIII? Please explain the rationale behind those isoforms.

NMR shows glutamate chains catalysed by TTLL6 are alpha-linked

The authors used NMR to unambiguously attribute the connectivity on glutamate chains via alpha linkages generated by TTLL6.

No comments

TTLL6 is preferentially inhibited by a phosphinate alpha-elongation analog

No comments.

Structures with tetrahedral intermediate analogs reveal molecular basis for a-linked glutamate chain elongation

In the main text when the authors enumerate the different structures obtained, it is a bit cumbersome to have both resolution and Rfree (without R that should also be mentioned) (maybe refer to the table for the R factors).

For Figure 4, it would be nice to have the chemical structures of Figure 3a next to the images of the different complexes. It would make the Figure easier to "read". In fact as the first paragraph describes the overall structure and the ATP binding only, I would suggest to make a first paragraph/Figure (3) with this information only and then move on to everything related to the TTLL-analog complexes (in a new Figure 4). In this case the activity assays would have to be split to correspond to each.

In Figure 3c and 3d, the ATP and other ligands are quite difficult to see, maybe increase stick width or use ball and sticks.

G181 is not labelled in Figure 3e

Last sentence p.12 and first sentence p.13: the Supplementary Figure 7 only looks at TTLLs not TTL. Please add the corresponding amino acids sites for TTL in Figure S7.

Active site molecular determinants for glutamate chain elongation versus initiation

Very elegant demonstration of initiation vs elongation activities and the responsible amino acids.

Lines 4-5 of the first paragraph: TTLL7 is not mentioned here. Please add.

Molecular engineering switches TTLL6 preference from elongation to initiation

No comments

Structures of engineered mutants reveal mechanism of regioselectivity switch

Figure 7 should be mentioned at the same time as Supplementary Figure 10 b-d.

In Figure 7 (as for Figure 4) it would be easier to have the chemical structures of the analogues next to each close-up view.

In general, the figures contain a lot of information and are very dense. It would be nice to have a summarizing schematic of the active site that could highlight the regions responsible for selectivity in initiase vs elongase, alpha vs gamma and those that distinguish glycylase vs glutamylase, combined with the prediction of which TTLL would fall in which of these categories (schematically representing the information found in Supplementary Figure 7 and in the last two paragraphs). This would greatly improve the visibility of the main findings and be a quick reference for those who cannot read/remember all the details of the paper.

In the Materials and Method section, the crystallography contains a lot of information that is also found in Table 1. This information could be removed from M&M and only kept in the Table where it is much easier to read.

Please show all the electron densities for all the ligands in a supplementary figure. They are only partially represented.

For the validation reports of the X-ray structures:

-The poly-Ala chains appear as separate chains with very poor quality: please make sure that modelling these is justified
-please state more clearly in the materials and methods that these partial, poorly visible chains do not affect the interpretation of the work presented.
-for some of the models the percentile ranks for Rfree are quite high, I assume this is due to the partially poor modelling of the C-ter. Please check this and maybe make a general sentence in M&M to explain this.

Reviewer #3:
Remarks to the Author:
Mahalingan et al. present a series of biophysical experiments and structures of TTLL6, an enzyme involved in post-translational glutamylation of tubulin tails.

Peptide and protein glutamylation assay using mass spectrometry and radiolabelling approaches with various relevant substrates showed TTLL6 to be an elongase and were used to elucidate its substrate preference. NMR was used to show that the amide bond TTLL6 forms links through the alpha (main chain) carbonyl carbon. Accordingly, the phosphinate inhibitor analogue that mimics the intermediate with a tetrahedral main chain carbonyl was the most potent of the three inhibitors. The three inhibitors were used in crystallographic studies which in total produced 4 co-complex structures of ligands bound to a construct containing wild type residues 51-502 of TTLL6. Interestingly, the electron density maps reveal phosphotransfer has occurred to all three inhibitors. The structures provided detailed views of relevant complexes and suggested that the specificity determinant for elongation vs initiation TTLLs to be a Gln180 and proximal residues. This assertion was supported with conservation analysis, biochemistry on the initiator TTLL4, mutagenesis of TTLL6 and a structural study of a triple mutant TTLL6.

Overall, this a very nice and well performed study, and will be of interest to the readership of NSMB.

Specific points:
Molecular replacement / Rfree set: All of the structures with analogues bound (6 of 7 total structures presented) appear to essentially be of the same crystal form (Tables 1 and 2). From the description in the Methods, the structure determination for the three wild type co-complexes appear to have been performed in parallel for using TTLL7 as a search model. Is that correct, or was one structure determined, and then that used for phasing the other wild type structures?
The mutant structures are stated as having been phased by MR using one or more wt TTLL6 model. Why was MR for these structures used and not simple rigid body refinement? Is the Rfree set designation transferred from the wt data set(s), or is a new one generated (as is the default) in MR? The Rfree will be biased if a new set was generated during MR using a structure from essentially the same crystal form. Removing the models of residues around the active site would not compensate for this.

Figure 1a & c: In the figure legend or in the text where these panels are called, please add a very short description of the assay used. The reader shouldn't have to go to the methods to find these are LC-MS assay and radio-labeled glutamate incorporation assay.

"The TTLL6 core is elongated… similar to the closely related glutamylase TTLL7 6, the glycylase TTLL3 40 and the tubulin tyrosine ligase TTL".
Please add % identity & % similarity numbers to the main text, and add a supplemental figure comparing the overall structures of TTLL7, TTLL3 and TTL.

4a and supplemental Fig 5: Please add Chemdraw schematics of phosphinate analogues / complexes obtained.

Author Rebuttal to Initial comments

We thank the reviewers for their careful reading of our manuscript and for their supportive comments regarding the importance and high technical quality of our work. We have addressed all their comments/suggestions in our revised manuscript through textual and figure changes. These are detailed in a point-by-point response below. None of the reviewers requested any additional experiments.

Reviewer #1:
"In a variety of cleverly designed and depicted experiments that use crytallography as well as mass spectrometry and enzyme kinetics, this paper established important elements of the catalytic mechanism of TTLL enzymes that initiate or those that elongate glutamate chains. As such, the paper represents an important contribution to our knowledge of tubulin posttranslational modification."
We thank the reviewer for his/her support.

"I do have some critiques of the interpretation and conclusions of the paper, though:
First, this paper is said to establish 'the molecular basis for regioselectivity for the entire TTLL family' but

since the two enzymes compared differ in TWO ways (initiation vs. elongation and α- vs β), this seems an over-reach."

Given the nature of the reaction, TTLL glutamylases and glycylases can only be initiases or elongases. Our study resolves the mechanistic basis for this segregation in activity and finds the signature residues that are critical for elongation and initiation in both glutamylases and glycylases. Thus we think that our statement in our introduction and discussion that we resolve the mechanistic basis for regioselectivity (alpha vs. gamma glutamate) for the entire family is justified. We do not claim to resolve the molecular specificity of □- vs □ tubulin recognition.

"In establishing substrate preference, use of α1B and α1B-Y peptides for comparison seems like an expedient strategy. From these experiments, the authors raise the possibility that TTLL6 could carry out glutamylation on detyrosinated α- tubulin; some predictions for whether or not this might occur in vivo could come from a comparison of the C-terminal sequence/structure with the sequence/structure surrounding E-443 (e.g., number of E residues and residues flanking). However, it begs the of whether the authors or reports in the literature provide any direct evidence that glutamylation on detyrosinated α-tubulin is physiologically significant for TTLL6 or other initiator TTLLs."

We are not quite sure what the reviewer means by *"However, it begs the of whether the authors or reports in the literature provide any direct evidence that glutamylation on detyrosinated α- tubulin is physiologically significant for TTLL6 or other initiator TTLLs."* We performed activity assays with peptides which have branched glutamates at two positions that were reported to be glutamylated in vivo based on MS-MS experiments (Figure 1). The assay with the detyrosinated peptide was an additional assay that provided support for the fact that a free □-carboxylate is important for the robust activity of TTLL6. Moreover, to show that the glutamylation of a detyrosinated tubulin tail can happen in the context of an assembled microtubule, we performed assays with recombinant tyrosinated and detyrosinated microtubules. These, showed that indeed, detyrosinated microtubules are very efficiently modified by TTLL6, unlike the tyrosinated microtubules (Supplementary Fig. 3). Based on this we state: **"Long glutamate chains have so far been detected only originating from internal glutamates in the tubulin tails, but the high activity of TTLL6 with detyrosinated microtubules raises the interesting possibility that long glutamate chains could also be added to the carboxy-terminus of detyrosinated □-tubulin *in vivo*. "**

We are not clear how looking at sequence context around E443 is going to shed light on whether detyrosinated tubulin is glutamylated *in vivo*. However, we have experimentally demonstrated that detyrosinated microtubules are glutamylated in vitro at much higher rates than tyrosinated ones by TTLL6 (Supplementary Fig. 3) which we would argue is more powerful than looking at sequences *in silico*. We do not claim that we established the physiological significance of glutamylation of detyrosinated □-tubulin and we clearly state that so far only polyglutamylation at internal glutamates sites has been described. However, our work raises the interesting possibility that glutamylation of detyrosinated tubulin at its C-terminus can occur *in vivo* and thus serves as the impetus for future experiments to

address this question, hence our statement "... raises the interesting possibility that …" [emphasis added, see above for context]. Thus, we think our statement is appropriate.

"The authors demonstrate that TTLL7 recognizes regions of the tubulin polypeptide or lattice outside of those that interact with the TTLL7 catalytic site. Perhaps this is true for other TTLLs, in addition (e.g., for TTLL4 and 6) or instead of (for other TTLLs) the interactions detailed here between catalytic site and tubulin polypeptide. This seems especially important to consider, given the other non-tubulin substrates that the TTLLs have to recognize. In fact, the authors should discuss whether any of the initiator enzymes that prefer either α- or β-tubulin actually have more activity on non-tubulin proteins than do others."
We assume that the reviewer is referring to our previous work on TTLL7 which shows that this enzyme uses a microtubule binding domain (MTBD) to position itself on the microtubule to optimally recognize the □-tubulin tail (Garnham et al., Cell 2015). In the same work we showed that a MTBD can be identified bioinformatically in all autonomous TTLLs and that it is important for their glutamylation activity, including for TTLL4 and 6. Our TTLL6 structure presented in the current manuscript indeed shows that this enzyme also has an MTBD. But unlike TTLL7, which does not discriminate between alpha and beta-tubulin tails in isolation (our work in Garnham et al., as well as that from the Setou lab, Mukai et al., Biochemistry 2009), we show in our current manuscript that TTLL4 and 6 do discriminate. TTLL4 shows preference for the beta tubulin tail in isolation, while TTLL6 prefers the alpha tubulin tail. Thus, the active site selectivity of these enzymes is higher than that of TTLL7. This does not rule out that the MTBD is also used for microtubule contacts. We are not sure what the reviewer would like us to discuss about the other tubulin substrates here. While we agree with the reviewer that this is an interesting topic, we think that any discussion about the recognition of non-tubulin substrates is premature at this point given that there are no kinetic studies with purified non-tubulin substrates for any TTLLs and very little mapping of their exact modification sites. Also, this would go well beyond the scope of this manuscript which addresses the initiation vs elongation activity of TTLL family enzymes.

"Adducts used for structural determination present an interesting potential for preparing further small molecule inhibitors and making the best derivatives of these, but it is hard to see how these could be made to specifically inhibit one TTLL over another. That is, the initiator analog and its chemical progeny would be predicted to inhibit all initiator TTLLs, which would have questionable use in the many disorders caused by abnormal TTLL expression or sequence. Thus, the promise suggested in the Discussion should be tempered."
We never state that these exact analogs can be used to specifically inhibit TTLLs. We simply state **"Last, but not least, our structures will serve as a springboard for the design of inhibitors for therapeutic intervention in neurodegenerative disorders characterized by tubulin hyper-glutamylation or modulation of the innate immune response by cGAS glutamylation."** As this reviewer himself/herself states, it is common in inhibitor design to start from an inhibitor with limited specificity like ours and then based on structural data of co-complexes, like the ones we present in our manuscript, change/add functional groups to the inhibitor to improve its specificity and binding affinity. Thus, we think our

statement in Discussion is appropriate and backed up by decades of structure-guided design of small molecule inhibitors.

"The literature on enzymes of tubulin posttranslational modification is rife with claims that studies of catalytic mechanisms will engender the availability of pure populations of microtubules for study of each modification. However greatly the results presented in papers like this advance our knowledge, this claim remains speculative, far-fetched, or impossible. Using purified enzymes to produce micromolar amounts of microtubules for studies of protein-protein interactions seems completely unrealistic, even if the enzymatic reactions could be expected to go to completion. This type of claim in the Discussion should be toned down considerably. Similarly, the authors should tone down their speculation that the TTLL4 and TTLL6 studied here will necessarily offer possibilities of incorporating unnatural amino acids into microtubules."

We respectfully but strongly disagree with this reviewer on several points he/she raises here.

(1) Regarding the impossibility of using these enzymes to make differentially modified microtubules for biochemical studies. This has already been accomplished. Please see Valenstein and Roll-Mecak, Cell 2016 for examples of biochemical amounts of modified microtubules produced with such techniques. These differentially modified microtubules were used both for microscopy-based assays and bulk biochemical assays such as ATPase assays which require mg amounts of differentially modified microtubules.

(2) Regarding the use of TTLL family enzymes to incorporate unnatural amino acids in microtubules. This is a feasible future goal and in fact has been accomplished for TTL by several groups already. See for example Schumacher et al., Angew Chem Int Ed, 2015. Versatile and Efficient Site-Specific Protein Functionalization by Tubulin Tyrosine Ligase or Banerjee et al., ACS Chem Biol., Site-specific orthogonal labeling of the carboxy terminus of alpha-tubulin. We cite these studies now in our Discussion. We state: "**Enzymes in the ATP-grasp superfamily to which TTLL enzymes belong are known to use non-protogenic amino acids as substrates [47]. Our structures enable the future rational engineering of TTLL active sites to be compatible with amino acid analogs, similar to the compensatory mutations made for kinases [47] that have revolutionized studies into their function. These analogs could also be functionalized for fluorescence or used for click chemistry for imaging in cells as has been recently accomplished for TTL [48,49]"**

"Minor comments (typographical error):
(monoplr; should be monopolar) in legend to Box 1 on page 48 of the manuscript."
We thank the reviewer for this careful reading. We think the reviewer means here "multiple" glutamates and not monopolar. We have corrected the typo.

Reviewer #2:

"Tubulin glutamylation and glycylation have been described more than 20 years ago and these modifications are known to play a critical role in correct microtubule function. However, understanding

the molecular detail of these modifications has been notoriously difficult, due to an inherent complexity of tubulin (isoforms, multiple modifications, differential expression etc) but also the tools available to study these modifications in vitro. It is only in the recent years that significant progress has been made in understanding the atomic detail of these interactions, a prerequisite for ultimate understanding of the tubulin code. This work is built on previous work mostly by the same authors that over the last years have managed to systematically address the molecular understanding of tubulin glutamylation and glycylation. Through detailed structural and biochemical analysis, they have been able to address the question of how the interaction between TTLL enzymes and tubulin(microtubules) occurs, as well as define structural elements that distinguish glycylases from glutamylases, or initiating from elongation glycylases. However up until now several important mechanistic questions remain unanswered.

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We thank the reviewer for his/her strong support of our work.

"*Comments and points to address (by paragraph):*

TTLL6 adds long glutamate chains to the alpha-tubulin
This paragraph clearly demonstrates that TTLL6 has an elongation activity with a strong preference towards the exposed alpha-carboxyl groups of free glutamates rather than the gamma-carboxyl group of main chain glutamates."
"Please mention:

- How the brain and recombinant tubulin used was produced/purchased. This should be described or referred to in Protein expression and purification (Materials and Methods)."
The recombinant tubulin was purified as previously described in Vemu et al., JBC 2016. We have now included more detail about this in our Methods section on page 23. Brain tubulin was purchased from Cytoskeleton. This information has also been included in the Methods section, page 25 .

"Why the choice of recombinant alphaIB/betaIII? Please explain the rationale behind those isoforms."
We used these isoforms because they are the ones we routinely use in the lab and for which we have published a purification procedure. We also note that we had a typo in the Methods – the isoforms used are alpha1A/betaIII. We have also characterized these tubulin isotypes structurally and biochemically (please see Vemu et al., JBC 2016).

"NMR shows glutamate chains catalysed by TTLL6 are alpha-linked
The authors used NMR to unambiguously attribute the connectivity on glutamate chains via alpha linkages generated by TTLL6"
No comments"

"TTLL6 is preferentially inhibited by a phosphinate alpha-elongation analog
No comments."

"Structures with tetrahedral intermediate analogs reveal molecular basis for a-linked glutamate chain elongation
In the main text when the authors enumerate the different structures obtained, it is a bit cumbersome to have both resolution and Rfree (without R that should also be mentioned) (maybe refer to the table for the R factors)."
We have taken the R_{free} values out and refer the reader to Table 1 which has this information.

"For Figure 4, it would be nice to have the chemical structures of Figure 3a next to the images of the different complexes. It would make the Figure easier to "read".
This is a great suggestion. We have now modified Figures 4 and 7 to present the chemical structures of the phosphorylated inhibitors.

"In fact as the first paragraph describes the overall structure and the ATP binding only, I would suggest to make a first paragraph/Figure (3) with this information only and then move on to everything related to the TTLL-analog complexes (in a new Figure 4). In this case the activity assays would have to be split to correspond to each."
Unfortunately, given the high density of data in our paper (7 structures and functional assays), we cannot see an efficient way of splitting this figure and making a new Figure 4 while still maintaining

overall figure dimensions that would respect NSMB guidelines. We hope the reviewer will be understanding of this.

"In Figure 3c and 3d, the ATP and other ligands are quite difficult to see, maybe increase stick width or use ball and sticks."
We have changed the stick width in Figures 3c and d as per the reviewer's suggestion so that the ligands are more easily visible.

"G181 is not labelled in Figure 3e"
Thank you for picking this up. It is labeled now.

"Last sentence p.12 and first sentence p.13: the Supplementary Figure 7 only looks at TTLLs not TTL. Please add the corresponding amino acids sites for TTL in Figure S7."
Thank you for this suggestion We have added now a column for TTL in a revised Figure S7.

"Active site molecular determinants for glutamate chain elongation versus initiation
Very elegant demonstration of initiation vs elongation activities and the responsible amino acids.

Lines 4-5 of the first paragraph: TTLL7 is not mentioned here. Please add."
We have now added TTLL7 to this list. We now state: "**The conservation patterns of this residue among TTLLs glutamylases suggests a segregation between initiases and elongases with TTLL1, 6, 9 ,11, and 13 as elongases, and TTLL2, 4, 5 and 12 as initiases (Supplementary Fig. 7a). TTLL7 likely acts efficiently as both an initiase and elongase, however the nature of the glutamate chain linkage is unclear."**

"Molecular engineering switches TTLL6 preference from elongation to initiation
No comments"

"Structures of engineered mutants reveal mechanism of regioselectivity switch"
Figure 7 should be mentioned at the same time as Supplementary Figure 10 b-d."
Done

"In Figure 7 (as for Figure 4) it would be easier to have the chemical structures of the analogues next to each close-up view."
We have added these in our revised Figure 7.

"In general, the figures contain a lot of information and are very dense. It would be nice to have a summarizing schematic of the active site that could highlight the regions responsible for selectivity in initiase vs elongase, alpha vs gamma and those that distinguish glycylase vs glutamylase, combined with

the prediction of which TTLL would fall in which of these categories (schematically representing the information found in Supplementary Figure 7 and in the last two paragraphs). This would greatly improve the visibility of the main findings and be a quick reference for those who cannot read/remember all the details of the paper."
We thank the reviewer for this great suggestion. We added in Supplementary Figure 7 an additional panel d that shows the key interactions with the acceptor and donor glutamates for TTLL6.

We also added an additional panel in Figure 7 that summarizes our findings for all TTL and TTLL family enzymes.

"In the Materials and Method section, the crystallography contains a lot of information that is also found in Table 1. This information could be removed from M&M and only kept in the Table where it is much easier to read."
We decided to keep this information in the Methods as some readers still refer to this section for this information even though it is available in Table 1 also.

"Please show all the electron densities for all the ligands in a supplementary figure. They are only partially represented."
We have revised our figures to show now the complete density for the ligands. This can now be seen in revised Figures 3h, Supplementary Figs. 6c and d and Supplementary Figs. 10b, c and d.

"For the validation reports of the X-ray structures:
-The poly-Ala chains appear as separate chains with very poor quality: please make sure that modelling these is justified"
Yes, helix □14 is flexible and the map in this region is of poorer quality than for the rest of the structure. However, the polarity of the helix is very clear, especially in the higher resolution structures and thus we are certain of the poly-Ala build. We are reasonably certain of the register also, but since we are not 100% sure we decided to keep it as poly-Ala out of an abundance of caution. The connectivity of the helix is also compatible given the sequence. We note that this helix is far away from the active site and does not affect the interpretation of our structures.

"-please state more clearly in the materials and methods that these partial, poorly visible chains do not affect the interpretation of the work presented."
This is now stated explicitly in our revised manuscript. Our Methods section now states on pages 30-31:
"**The density for the C-terminal helix was not of sufficient quality to assign register unambiguously and was left as a poly-alanine model, which likely contributes to a slightly higher R_{free} value. This helix is far away from the active site of the protein (helix □14 in Figure 3c) and does not affect the interpretation of our structures.**"

"-for some of the models the percentile ranks for Rfree are quite high, I assume this is due to the partially poor modelling of the C-ter. Please check this and maybe make a general sentence in M&M to explain this.
We state this now explicitly in the Methods section (please see above)

Reviewer #3:
"*Mahalingan et al. present a series of biophysical experiments and structures of TTLL6, an enzyme involved in post-translational glutamylation of tubulin tails. Peptide and protein glutamylation assay using mass spectrometry and radiolabelling approaches with various relevant substrates showed TTLL6 to be an elongase and were used to elucidate its substrate preference. NMR was used to show that the amide bond TTLL6 forms links through the alpha (main chain) carbonyl carbon. Accordingly, the phosphinate inhibitor analogue that mimics the intermediate with a tetrahedral main chain carbonyl was the most potent of the three inhibitors. The three inhibitors were used in crystallographic studies which in total produced 4 co-complex structures of ligands bound to a construct containing wild type residues 51-502 of TTLL6. Interestingly, the electron density maps reveal phosphotransfer has occurred to all three inhibitors. The structures provided detailed views of relevant complexes and suggested that the specificity determinant for elongation vs initiation TTLLs to be a Gln180 and proximal residues. This assertion was supported with conservation analysis, biochemistry on the initiator TTLL4, mutagenesis of TTLL6 and a structural study of a triple mutant TTLL6."*

"Overall, this a very nice and well performed study, and will be of interest to the readership of NSMB."

We thank the reviewer for his/her strong support.

"Specific points:
Molecular replacement / Rfree set: All of the structures with analogues bound (6 of 7 total structures presented) appear to essentially be of the same crystal form (Tables 1 and 2). From the description in the Methods, the structure determination for the three wild type co-complexes appear to have been performed in parallel for using TTLL7 as a search model. Is that correct, or was one structure determined, and then that used for phasing the other wild type structures?"

The mutant structures are stated as having been phased by MR using one or more wt TTLL6 model. Why was MR for these structures used and not simple rigid body refinement? Is the Rfree set designation transferred from the wt data set(s), or is a new one generated (as is the default) in MR? The Rfree will be biased if a new set was generated during MR using a structure from essentially the same crystal form. Removing the models of residues around the active site would not compensate for this."
We need to clarify here that we did not use the wild-type complex structures to do MR for the mutant complexes. We used the TTLL7 core (PDB ID:4YL; Garnham et al., 2015), with ligand and active site loops

deleted as the molecular replacement search model for all our wild-type structures (ATP and complexed with the three analogs). Each structure was solved by MR independently. This was followed by rigid body refinement and multiple rounds of simulated annealing (~ 20 rounds for each structure) to remove any model bias. Moreover, we calculated simulated anneal-omit maps that confirmed our builds. For the mutant TTLL6 structures with the three analogs, we used the TTLL6 core structure with the ATP and active site loops removed as the MR search model. The TTLL6-ATP crystal form is different than the crystal form of the inhibitor complexes. Each mutant structure was solved by MR independently. This was followed by rigid body refinement and multiple rounds of simulated annealing (~ 20 rounds) to remove any model bias. Moreover, we calculated simulated anneal omit maps that confirmed our builds and showed rearrangement of active sites residues and the presence of the phosphorylated inhibitors. Thus, we are certain that model bias is not a concern. This is now all stated in our Methods section on pages 30 and 33.

"Figure 1a & c: In the figure legend or in the text where these panels are called, please add a very short description of the assay used. The reader shouldn't have to go to the methods to find these are LC-MS assay and radio-labeled glutamate incorporation assay."
We have added this information in our revised legend to Figure 1.

"The TTLL6 core is elongated… similar to the closely related glutamylase TTLL7 6, the glycylase TTLL3 40 and the tubulin tyrosine ligase TTL".
Please add % identity & % similarity numbers to the main text, and add a supplemental figure comparing the overall structures of TTLL7, TTLL3 and TTL."
We provide now this information in the text. We now state: "**The TTLL6 core is elongated (~ 80 x 50 x 47 Å^3) and consists of N- (res. 57-117), central (res. 118-206), and C- (res. 207-460) domains (Fig. 3c), similar to the closely related glutamylase TTLL7 [6] (41% identity, 60% similarity), the glycylase TTLL3 [40] (25% identity, 44% similarity) and the tubulin tyrosine ligase TTL [34,35] (27% identity, 45% similarity; Supplementary Fig. 6a)."**

We also included ribbon diagrams for TTL, TTLL7 and 3 in Supplementary Fig. 6a as per the reviewer's suggestion. This panel now shows the three-domain core shared by all enzymes.

"4a and supplemental Fig 5: Please add Chemdraw schematics of phosphinate analogues / complexes obtained."
Thank you for this suggestion. The phosphinate analogs are presented in Figure 3a. We have now revised Figures 4 and 7 to include Chemdraw structures of the phosphorylated analogs next to the active site views.

Decision Letter, first revision:

18th May 2020

Dear Antonina,

Thank you again for submitting your manuscript "Structural basis for polyglutamate chain initiation and elongation by TTLL family enzymes". The comments from one of the original referees are below, and we are happy to accept your paper, in principle, for publication as an Article in Nature Structural & Molecular Biology, on the condition that you revise your manuscript in response to our editorial requirements.

The text and figures require revisions. Note that, within a few days, we will send you detailed instructions for the final revision, along with information on editorial and formatting requirements. We recommend that you do not start revising the manuscript until you receive this additional information.

Data availability: this journal strongly supports public availability of data. Please place the data used in your paper into a public data repository, or alternatively, present the data 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:
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We hope that you will support this initiative and supply the required information. Should you have any query or comments, please do not hesitate to contact me.

If you have any questions, please do not hesitate to contact me directly.
Sincerely,

Ines Chen, Ph.D.
Chief Editor
Nature Structural & Molecular Biology

ORCID 0000-0002-1405-9703

Reviewer #2 (Remarks to the Author):

The authors addressed all points raised satisfactorily, and I now fully support the manuscript for publication.

22nd May 2020

Dear Antonina,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Structural & Molecular Biology manuscript, "Structural basis for polyglutamate chain initiation and elongation by TTLL family enzymes" (NSMB-A42958A). Please follow the instructions provided here and in the attached files (sent separately), as the formal acceptance of your manuscript will be delayed if these issues are not addressed.

SCIENTIFIC ISSUES:
1. While reviewer 2 found the revision and response appropriate, we do feel that reviewer 1's suggestions to avoid overstatements about the immediate impact of the work should be considered, and advise toning down a few sentences (e.g., last sentence in introduction; last paragraph of discussion). We also ask our authors to avoid statements on the work being the "first"; at most, this should be tempered with "to the best of our knowledge".

POLICY ISSUES:

2. Data availability: this journal strongly supports public availability of data. Please place the data used in your paper into a public data repository, or alternatively, present the data 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.

3. DATA DEPOSITION: We require deposition of coordinates (and structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). EM maps must be deposited in the EMDB. Please make sure accession codes are listed in the structural tables; entries must be accessible or HPUB.

4. Nature Research is taking an active approach to improving our transparency standards and increasing the reproducibility of all of our published results. Detailed information on experimental design and reagents is now collected on our Life Sciences Reporting Summary, which will be published alongside your paper. Please provide an updated version of the Reporting Summary (which will be published with the paper) with your final files.
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GENERAL FORMATTING:
5. The manuscript is currently 5543 words (main text; Introduction, 857 words; Results: 3812 words; Discussion: 874 words). Our standard word limit is 4,000 words for main text, we have some flexibility, but I ask that you revise it to under 5,000 words. I find that the discussion could be streamlined (no need to describe the results in detail), and the results section could also be tightened up a bit.

6. Current title: Structural basis for polyglutamate chain initiation and elongation by TTLL family enzymes (12 words, 77 characters, spaces included). That's fine.

7. Your abstract is currently 149 words. That's good too.

8. The Online methods section is currently 4174 words. We do not have a strict limit for this section

but we suggest 3000 words as a target. The simplest way to do this would be to move some subsections (e.g., protein expression/purification and peptide synthesis; or the crystallization methods) to a Supplementary Note, and have a short summary in methods, saying "additional details are provided in Supplementary Note 1). Please note that it is not necessary to list parameters of the crystals that are provided in the table.

9. References: the current manuscript has 60 references in the main text and methods sections. That's fine but Methods-only references should be placed after the Methods section, following the numbering of the main reference list (i.e. do not start at 1). Please make sure all references are cited in numerical order.

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10. There are currently 7 Figures and 1 Table in main article. That's fine. However, we usually do not allow boxes in research articles; you can move it to an Extended Data Figure or make it part of Figure 1 (please resize to fit into one page and ensure legibility).

Table 1, please merge the 2 parts; you can fit it on one page by changing the orientation of that page. If you have difficulties with this, I can fix it later. Tables should be pasted into Word files as editable tables, not as images; place them after figure legends. I think you are already using our template, just make sure to include the PDB accession codes on top of each column (in parentheses, below the description).

11. Please make sure all figures and tables, including Extended Data Figures, are cited in the text in numerical order.

12. SUPPLEMENTARY INFORMATION

All Supplementary Information must be submitted in accordance with the instructions in the attached Inventory of Supporting Information, and should fit into one of three categories:

1. EXTENDED DATA FIGURES: please relabel Supplementary Figures 1-10 as Extended Data Figures 1-10 (10 is our limit, so this is fine; make sure to follow sizing and format requirements below). The legend for Extended Data Figures should be placed in the inventory.

Extended Data Figures are an integral part of the paper and only data that directly contribute to the main message should be presented. These figures will be integrated into the full-text HTML version of your paper and will be appended to the online PDF. There is a limit of 10 Extended Data Figures, and each must be referred to in the main text. Each Extended Data Figure should be of the same quality as the main figures, and should be supplied at a size that will allow both the figure and legend to be presented on a single legal-sized page. Each figure should be submitted as an individual .jpg, .tif or .eps file with a maximum size of 10 MB each. All Extended Data figure legends must be provided in the attached Inventory of Accessory Information, not in the figure files themselves.
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Supplementary Information is material that is essential background to the study but which is not practical to include in the printed version of the paper (for example, large figures, video files, large data sets and calculations). Each item must be referred to in the main manuscript and detailed in the attached Inventory of Accessory Information. Supplementary Tables containing large data sets should be in Excel format, with the table number and title included within the body of the table. All textual information and any additional Supplementary Figures (which should be presented with the legends directly below each figure) should be provided as a single, combined PDF. Please note that we cannot accept resupplies of Supplementary Information after the paper has been formally accepted unless there has been a critical scientific error.

Supplementary items (such as Supplementary Tables, Videos, Notes, and additional Supplementary Figures if permitted), should be numbered and called out in main article, as Supplementary Figure 1 (not SI1) and so on.

3. SOURCE DATA: these should be listed in section 3 of the inventory.
We encourage you to provide source data for your figures. Statistical source data (i.e., data behind graphs) should be provided in Excel format, one file for each relevant figure, with the linked figure noted directly in the file.

If you prefer, you can also aggregate data for multiple figures/graphs into one Excel file (Supplementary Table 2, in Excel format, listed in section 2B of inventory).

Source data should be cited in the legend text (eg. "Data for graphs in d-f are available as source data or in Supplementary Table 2").

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17. Data Availability statement: This should be placed after Reporting Summary statement (before Methods-only references). We suggest that you list in this order:
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We ask that you aim to return your revised paper within 7 days. If you have any further questions, please feel free to contact me.

Best regards,

Ines Chen, Ph.D.
Chief Editor
Nature Structural & Molecular Biology

ORCID 0000-0002-1405-9703

Reviewer #2:
Remarks to the Author:
The authors addressed all points raised satisfactorily, and I now fully support the manuscript for publication.

Author Rebuttal, first revision:

Dear Ines:

We have revised our manuscript “Structural basis for polyglutamate chain initiation and elongation by TTLL family enzymes” as per your instructions.
(1) We addressed the concerns from Reviewer 1 and have toned down our wording as per your instructions both in the Introduction and last paragraph of the Discussion. We have also qualified our priority of discover statement in the discussion by adding “to the best of our knowledge”
(2) A data availability statement is included in the manuscript.
(3) Coordinates for all 7 structures have been deposited to the PDB and their accession codes are given in the manuscript.
(4) Reporting Summary is included.
(5) We have streamlined the manuscript as per your instructions and it now is 5101 words. I hope this is OK because further streamlining would negatively affect clarity given the amount of data in the manuscript.
(6) Title was OK
(7) Abstract was OK
(8) We moved part of our Methods to Supplementary Note 1 and now our Methods section is below 3000 words.
(9) References are OK.
(10) We converted Box 1 to Figure 1 as per our email exchange. The ms has 8 figures and one table. We also reformatted the table as per your instructions.
(11) All references are cited in order.
(12) Supplementary Information has been formatted as per your instructions. Replicate information is given everywhere
(13) Statistical information is now given in all legends
(14) Competing interest statement provided
(15) Reporting Summary Statement provided
(16) Data availability statement provided
(17) I do wish to participate in transparent peer review
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(19) ORCID provided

We look forward to seeing this manuscript in print. I remain,

Yours sincerely,
Antonina Roll-Mecak
Senior Investigator and Chief
Cell Biology and Biophysics Unit
National Institutes of Health

Final Decision Letter:

12th Jun 2020

Dear Antonina,

We are now happy to accept your revised paper "Structural basis for polyglutamate chain initiation and elongation by TTLL family enzymes" 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.

Before the manuscript is sent to the printers, we shall make any detailed changes in the text that may be necessary either to make it conform with house style or to make it intelligible to a wider readership. If the changes are extensive, we will ask for your approval before the manuscript is laid out for production. Once your manuscript is typeset you will receive a link to your electronic proof via email within 20 working days, with a request to make any corrections within 48 hours. Please read proofs with great care to make sure that the sense has not been altered. If you have queries at any point during the production process then please contact the production team at rjsproduction@springernature.com. Once your paper has been scheduled for online publication, the Nature press office will be in touch to confirm the details.

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Ines

Ines Chen, Ph.D.
Chief Editor
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ORCID 0000-0002-1405-9703