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Tubulin polyglutamylation differentially regulates microtubule-interacting proteins

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **15th Aug 2022**

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received comments from three reviewers, which are included below for your information.

As you will see from the reports, the reviewers find the study novel and interesting, while also indicating a number of issues that would have to be addressed and clarified before they can support publication of the manuscript, in particular the potential effect of other tubulin modifications on the observed results (referee #2, point 3 and referee #3, point 1), the discrepancy between in vitro and in vivo data on the effect of tubulin glutamylation on tau and kinesin-1 (referee #1, point 6 and referee #3, point 7), as well as the variability of the presented data (referee #2, point 1). From my side, I find the reviewer comments generally reasonable. Based on these positive assessments, I would like to invite you to address the issues raised by the reviewers in a revised manuscript.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work to discuss the appropriate course of action. Should you foresee a problem in meeting this three-month deadline, please contact us to arrange an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess. Please also see the attached instructions for further guidelines on preparation of the revised manuscript.

Please feel free to contact me if you have any further questions regarding the revision. I would be happy to discuss the revision in more detail via email or phone/videoconferencing. Thank you for the opportunity to consider your work for publication, and I look forward to your revision.

Referee #1:

In this interesting and well-presented study the authors measure the effects of microtubule polyglutamylation on the binding/activity of tau, katanin and kinesin-1 in vitro using purified proteins. This is an important piece of work, one might even consider it a landmark study, because it compares microtubules that have a natural level of post-translational modifications with microtubules that lack two specific types of polyglutamylation. The authors were able to do this for the first time in a very systematic manner, because they have knock-out mice lacking specific modifying enzymes and are able to purify tubulin from brain from these mice in a very reproducible manner, allowing them to perform experiments with well-controlled modifications. Using their approach, they can explicitly test what the effect of natural modification levels are compared to the absence of a specific modification (avoiding the problem of overexpression used in earlier work in this field). They find specific effects on the studied proteins/enzymes: tau binds better to modified microtubules, katanin severs better (in agreement with previous work),

and kinesin-1 is less processive (also mostly in agreement with previous work). The strength of this study is that the quality of the data is high and that it is nicely quantitative. Interestingly, the authors always find that the observed effects are in the range of up to a factor of 2, revealing the likely physiological magnitude of the degree of regulation by these posttranslational modifications. A weakness is that the main statements in the more general parts of the manuscript are only qualitative, almost ignoring the scale of the measured effects.

Some specific minor comments, mostly referring to presentation

1. Language: the style of the abstract is a little flowery ("landscape" (?), "emblematic", "strikingly") and would profit from a more factual style of reporting. Given that the study is nicely quantitative, shouldn't this also be reflected by the statements in the abstract?

2. Introduction: It would be helpful to clearly state previously observed effects on katanin-mediated severing or kinesin-1 motility to allow the non-expert to put the presented results into context from the beginning.

3. Results: ATAT1 is not introduced. The activities of TTLL1 and TTLL7 could be stated more explicitly early on in the text (and not only in Fig. 1a)

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5. Page 8: the term "non-linear regression equation" does not mean much. Please specify which type of function is used.

6. The authors cite a paper stating that the physiological tau concentration is 2 micro-M and conclude that based on their measurement of the Kds of tau binding to GMPCPP microtubules polyglutamylation can regulate tau binding in cells. This argument has two weaknesses: 1st, the Kds have been measured for GMPCPP, and not for GDP microtubules, 2nd, they are both smaller than the reported intracellular concentration, which rather indicates that, given the measured Kds, the level of polyglutamylation will not have a strong effect on tau binding to microtubules in cells.

7. Katanin experiments: Why were now taxol stabilized microtubules used for experiments? Please state the katanin concentration in the main text. Why was this particular concentration chosen? Please state by how much the katanin activity is affected by polyglutamylation.

8. Kinesin-1 experiments: The kymograph in Fig. 4B is not very convincing - one sees many pauses and few processive events. Is this representative? Do the authors also have kinesin-1 motility data for acetylated and deacetylated microtubules? If yes, they could be shown to demonstrate the specificity of the effects induced by (de)polyglutamylation.

9. It was not always clear how the various normalizations have been performed. Please check if sufficient detail is provided in the Methods for the reader to be able to follow the data analysis.

10. The Discussion could profit from comparing the magnitude of the effect of (de)polyglutamylation on the various tested proteins to effects of other posttranslational modifications (such as for example phosphorylations where this has been quantified) and discussing the potential reasons/consequences.

Referee #2:

This manuscript from Genova and colleagues addresses the question of how PTMs impact the interactions and activities of MAPs on their microtubule substrate, particularly MAPs that associate with microtubules in the brain. This is an important question in the microtubule field. Although our knowledge of tubulin PTMs has grown over recent decades, how and whether those PTMs impact the activities of MAPs to ultimately lead to the regulation of microtubule networks has remained understudied. This study focuses on two types of PTMs, acetylation and polyglutamylation, and how these impact three MAPs, Tau, katanin, and kinesin-1/Kif5B. All three MAPs have previously been shown to be sensitive to polyglutamylation, but this study is unique in that it uses tubulin proteins purified from genetically-altered mice that lack the relevant modifying enzymes, which were developed by the authors and described in previous publications. Presumably, the microtubules formed from these tubulin proteins reflect the physiological presence or absence of the modifying enzyme more accurately than previous studied that removed modifications proteolytically or added them through the exogenous addition of recombinant enzymes. This is an advance for the field in terms of faithfully reconstituting a genetically-manipulated microtubule environment and dissecting the roles of tubulin PTMs.

The main conclusions of the study are related to the effects of glutamylation on Tau, katanin, and Kif5B. The conclusions for Tau

and katanin are mostly consistent with previous work. The authors report no strong effects for acetylation on Tau binding or katanin activity, and do not test how acetylation effects Kif5B. The design of the study is generally clear; however, there are several key points where the results seem oversimplified, particularly with regard to the high degree of variability in the data. These points are detailed below and should be addressed prior to publication.

MAJOR POINTS

1) What is the source of the high variability in the data for Figures 2 and 3? This variability is particularly clear when evaluating the extended data figures, which show data points from separate experiments measuring Tau binding and katanin severing activities. For Tau, the intensity of bound Tau from experiment to experiment varies by as much as 40% (see Extended Data Figure 2A, comparing wild type vs T1-/-T7-/- microtubules). The trends of wild type vs mutant binding appear similar across experiments, which somewhat mitigates the concern for this experiment, but the potential source of variability should be addressed. It is not clear whether these experimental replicates are biological replicates, with tubulin prepared from brains of different animals, or technical replicates using the same material. If they are biological replicates, this could suggest that there is some variability in the tubulin protein in the animals or perhaps in the in the quality of the protein from different preps. If they are technical replicates, is there some obvious aspect of the experiments that is different; e.g., errors in the measurement of purified Tau-meGFP?

The data from the katanin experiments are even more variable and amplify the concern. Here the Extended Data Figure 3 shows large differences in estimated microtubule half-life across experiments with tubulin from the same genotype. The wild-type control is the most glaring example. In some experiments the median half-life for wild-type microtubules is 27 seconds, while in others the median half-time is 281 seconds. This 10-fold difference is much greater than any differences reported between wild-type tubulin and Ttll-mutant tubulin within an experiment, and this weakens confidence in any conclusions regarding Ttll-related differences. Again, the authors should seek to identify the sources of variability and report them. Are these biological or technical replicates? Is the same purified katanin used in each experiment, or could separate katanin preps yield protein with different levels of activity?

In some cases, the authors acknowledge this variability and present normalized data where results from separate experiments are normalized to a wild-type control or some sample of another genotype. This creates distributions in Figures 2C and 3E that appear to be normal, but it would be better to avoid normalization unless there is a strong justification (i.e., if the authors understand and describe the sources of variability in the data). Showing the data as superplots with differentially-labeled data corresponding to each experiment would also be appropriate here.

2) Related to the prior point, there is no indication of any variability in the data for the kinesin experiments (Figure 4). This is important to show, given the concerns with the data for Tau and katanin. The authors should include an extended data figure showing the data from individual kinesin experiments.

3) The authors make strong claims about the knockout mice affecting only specific PTMs; for example, in the second paragraph on page 6. However, the results supporting this claim are from western blots of one set of samples probed for alpha tubulin, K40 acetylation, polyglutamylation, beta monoglutamylation, and Δ2 tubulin. This represents the 3 PTMs predicted to be altered in the mouse mutants plus one other PTM (Δ2 tubulin). This is hardly exhaustive and does not support the strong statements on page 6 about other potential PTM changes. Whether certain PTMs impact the presence and abundance of other PTMs is an important question in the field. The authors should either test it more rigorously here or refrain from overstating their knowledge of the tubulin PTM state in the brain tubulin from these mice. More rigorous analysis would help strengthen the conclusions of the study.

We suggest the following: first, the authors should quantify the western blot data in Figure 1E and show a chart of that quantification. It appears that acetylated tubulin may be altered in some of the mutants, and densitometry analysis across replicate experiments would make that clear. Second, the authors should use commercially available antibodies to probe for tyrosinated and/or detyrosinated tubulin. This is important because polyglutamylation of the alpha tubulin tail is close to the site of tyrosination and an effect seems plausible, but also because tyrosination has been shown by the Ross lab to alter Tau binding to microtubules. Therefore a change in tyrosination could strongly impact the interpretation of the results here. Third, it would be valuable to provide an extended data figure showing western blot analysis for tubulin prepared from each animal used in the study, to demonstrate the reproducibility of the effected PTM levels.

MINOR CONCERNS:

1) The authors state on page 5 that "no other protein was detected in any of the purified tubulin samples (Figure 1D)." It would be helpful to include similar stained gels for the preps from each animal used in the study. This is a concern because of the unidentified source of variability in the experiments. It seems plausible that some of these preps could contain contaminating proteins that alter the experimental results. Providing stained gels of the SN7 fractions from each prep would address this concern.

2) Why do the authors use GMPCPP-stabilized microtubules for the Tau-binding experiments in Figure 2B-E, and taxol-stabilized microtubules in other experiments? This is particularly concerning because work from the Odde lab (PMID 33294790) has shown that Tau has a ~4X tighter affinity on GDP-microtubules vs GMPCPP-microtubules. Thus, the GMPCPP-microtubules used here are a poor substrate for Tau, and may mask the effects of PTMs. It would be better to use GDP microtubules or taxol-stabilized GDP-microtubules, which are used to test lower concentrations of Tau. At minimum, the authors should comment in the manuscript on how GMPCPP impacts Tau binding.

3) Figure 3. The descriptions of the measurement methods for microtubule severing and half-life calculation are difficult to understand. These should be more clearly described in the methods.

4) Figure 4. The results here show that Kif5B has long run length when Ttll1 is absent. Could this be due to differences in microtubule length in these experiments? Microtubule length is never reported, and could be simply measured from the IRM images and reported to address this concern.

5) Figure 4. Several details are missing. Do these experiments use mixtures of wild type and mutant microtubules on the same coverslip? The text results suggests so, but the graphic in Figure 4A seems to indicate homogenous microtubules on the coverslip. Also, the authors should state whether these are taxol-stabilized or GMPCPP-stabilized microtubules.

6) Figure 4. It is not clear why the authors do not test whether acetylation impacts Kif5B, but did test the effect of acetylation on Tau and katanin.

7) The authors should refrain from including discussion in the results section of the manuscript; for example, paragraph 3 on page 10, and the last two paragraphs of the results.

Referee #3:

The manuscript by Genova et al examines the influence of tubulin polyglutamylation on three different microtubule associated proteins. This is a timely and important report as we still have very little understanding of how tubulin PTMs impact microtubule associates proteins and thereby microtubule function. The authors utilize mice lacking the polyglutamylation enzymes TTLL1 and TTLL7 to obtain tubulin preps that lacks specific glutamylation modifications. This is an important contribution as the control situation (tubulin from wildtype mice) contains the PTMs that a microtubule associated protein would see in a cell. The authors assemble microtubules from these tubulins and carry out TIRF microscopy-based assays to examine the activity of Tau, katanin, and kinesin-1 on these microtubules. The data are rigorous and the writing is clear, however, several controls are missing and additional information is needed.

1. In Figure 1 and Figure S1 the authors show the purity and PTM state of their tubulins obtained from the different mouse lines. What is missing is the western blot of detyrosinated tubulin. This is particularly important as kinesin-1 has been shown to be sensitive to the detyrosination state. In addition, like the tubulin purity (Figure S1), the state of the PTMs should be shown across multiple preps and the PTM state should be quantified with western blots that are in the linear range. There seems to be an inverse relationship between alpha-tubulin acetylation and alpha-tubulin polyE (Figure 1E). Is this true across preps?

2. It would be nice to know the state of the polyglutamylation for each tubulin prep by mass spectrometry. For example, in the Ttll-/- mice, there is no alpha tubulin glutamylation by western blotting but is this just below the level of detection? And are there compensatory changes in the length of polyglutamylation on beta tubulin?

3. For the kinesin experiments, it looks like there is an increase in the number of motility events on the Ttll7-/- and Ttll1-/-Ttll7-/- tubulins. For microtubule associated proteins, tubulin PTMs are most likely to affect the affinity for the microtubule although this parameter is often ignored in the literature. This could be measured as either a landing rate (obtained for the existing motility data if equal amounts of motor were added under all conditions) or an affinity measurement via microtubule cosedimentation experiments.

4. With respect to affinity, it is ciear in Figure 2 that tau has a higher affinity for the wild type tubulin. What about katanin? The authors show changes in severing activity in Figure 3 but is this because katanin's binding has changed or its activity?

5. Also for the kinesin data, what is the biological significance of the interaction time versus the run length? They seem very similar in terms of polygluamylation impact. Probably one of them could be moved to the supplemental data and replaced by the more biologically-relevant landing rate or microtubule affinity data. I also do not understand the statistics tables.

6. For the Introduction, another way used in the literature to test the impact of polygutamylation is to compare kinesin activity on brain vs HeLa tubulin. Please add the Lessard et al 2019 reference which shows that polyglutamylation increases the landing rate of KIF1A (a kinesin-3 motor).

7. A most critical aspect of the entire manuscript lies in the relevance to the in vivo situation. The authors acknowledge this in the Discussion p. 13-15 where they provide several examples of where their in vitro data do not match the in vivo data including kinesin-1 sensitivity to polyglutamylation (Bodakuntla et al 2020 but also Maas 2009), katanin sensitivity to acetylation (Sudo and Baas 2010), and kinesin-1 sensitivity to acetylation (Reed et al 2006 but also Cai et al 2009, Guardia et al 2016, Tas et al 2017). The authors indicate the complexity of the cell as a reason for discrepancies between their in-vitro and previous cell-based observations. An alternative explanation is just that the in vitro experiments cannot replicate what is happening inside a cell. The purification of tubulin and its reassembly in in vitro assays likely fails to replicate the microtubule architecture in cells where a microtubule is first assembled and then modified by PTM enzymes, MAPs, motors walking, plus tip proteins, etc. While both cellular and reconstitution experiments are important, the inability to replicate in vitro what is seen in a cellular environment should be considered a big red flag rather than a problem with the cellular experiments.

Reply to the reviewer's comment to Genova et al.:

We would like to express our gratitude to all referees for their thoughtful and constructive comments. These suggestions were an invaluable help in the revision of our manuscript. We believe that the revised manuscript is now significantly improved, and hopefully meets the referees' expectations.

Our answers to the original referees' comments are in blue for easier reading. In the manuscript text, we have highlighted changes introduced during the revision process in red.

Reviewers' Comments:

Referee #1:

In this interesting and well-presented study the authors measure the effects of microtubule polyglutamylation on the binding/activity of tau, katanin and kinesin-1 in vitro using purified proteins. This is an important piece of work, one might even consider it a landmark study, because it compares microtubules that have a natural level of post-translational modifications with microtubules that lack two specific types of polyglutamylation. The authors were able to do this for the first time in a very systematic manner, because they have knock-out mice lacking specific modifying enzymes and are able to purify tubulin from brain from these mice in a very reproducible manner, allowing them to perform experiments with well-controlled modifications. Using their approach, they can explicitly test what the effect of natural modification levels are compared to the absence of a specific modification (avoiding the problem of overexpression used in earlier work in this field). They find specific effects on the studied proteins/enzymes: tau binds better to modified microtubules, katanin severs better (in agreement with previous work), and kinesin-1 is less processive (also mostly in agreement with previous work). The strength of this study is that the quality of the data is high and that it is nicely quantitative. Interestingly, the authors always find that the observed effects are in the range of up to a factor of 2, revealing the likely physiological magnitude of the degree of regulation by these posttranslational modifications. A weakness is that the main statements in the more general parts of the manuscript are only qualitative, almost ignoring the scale of the measured effects.

Some specific minor comments, mostly referring to presentation

1. Language: the style of the abstract is a little flowery ("landscape" (?), "emblematic", "strikingly") and would profit from a more factual style of reporting. Given that the study is nicely quantitative, shouldn't this also be reflected by the statements in the abstract?

We would like to thank the referee for having pointed this out, and we re-wrote the abstract following the referee's suggestions. We also added the notion of quantitative measurements. However, the limitations in abstract size did not allow us to fully present our detailed results.

2. Introduction: It would be helpful to clearly state previously observed effects on katanin-mediated severing or kinesin-1 motility to allow the non-expert to put the presented results into context from the beginning.

In the introduction (p.3-4) we report on previous *in-vitro* experiments that measure the effect of glutamylated microtubules on kinesin-1 and kinesin-3 motility (Sirajuddin *et al*, 2014; Lessard *et al*, 2019). The effect of polyglutamylation on katanin was not assessed *in vitro* to our knowledge, but it was proposed based on structural similarities with spastin (Shin *et al*, 2019). In the discussion on p.14 we also comment on prior results obtained in cells for katanin and kinesin-1 regulation by acetylation.

3. Results: ATAT1 is not introduced. The activities of TTLL1 and TTLL7 could be stated more explicitly early on in the text (and not only in Fig. 1a)

We would like to thank the referee for having spotted this. We now describe in the introduction that TTLL1 and TTLL7 have specific activities for α- and β-tubulin, and we also introduce ATAT1 and acetylation.

4. Figure 1: in the presence of 70 nM tau, tau binding is reduced to half for microtubules lacking the modifications added by TTLL1 and TTLL7. If this concentration is smaller than than the Kds, then the Kds are expected to also differ by a factor of 2. If the concentration is in the range of the Kds (as it seems to be the case), then the Kds should differ by more than a factor of two. However they differ by less than a factor of 2 which is inconsistent. Please explain.

The concentration used in the binding experiments of Fig 2B, C and D is smaller than the K_d values determined in the binding curve (Fig 2E). This concentration is even slightly lower than the lowest point we used to determine the K_d in the binding curve, which is 78 nM. In the curve at this concentration the difference in binding of Tau to wild type and *Ttll1*$^{-/-}$/*Ttll7*$^{-/-}$ microtubules is within a factor of ~ 1.7, which we believe is consistent with the expected 2-fold difference within the experimental error. We now further point out that even at saturating concentrations of Tau there is a difference of its fluorescent intensity on the wild-type and the *Ttll1*$^{-/-}$/*Ttll7*$^{-/-}$ microtubules, suggesting that besides the affinity the presence or absence of tubulin polyglutamylation can also change Tau's occupancy on microtubules.

5. Page 8: the term "non-linear regression equation" does not mean much. Please specify which type of function is used.

The precise model used is now specified in the Results: "Fitting these intensities with a one-site-specific binding model (GraphPad Prism 9)…" and further explained in the Methods: "To calculate the dissociation constants (K_d) of Tau, the experimental data was fitted with an built-in model for one-site specific binding using Prism 9 software (GraphPad) based on the following formula:

$$D = \frac{\text{Dmax} \times [\text{Tau}]}{\text{Kd} + [\text{Tau}]},$$

where D is the corrected integrated fluorescence signal density of Tau on the microtubule (calculated as described above), D_{max} is the estimated maximum value of D and [Tau] is the concentration of Tau.".

6. The authors cite a paper stating that the physiological tau concentration is 2 micro-M and conclude that based on their measurement of the Kds of tau binding to GMPCPP microtubules polyglutamylation can regulate tau binding in cells. This argument has two weaknesses: 1st, the Kds have been measured for GMPCPP, and not for GDP microtubules, 2nd, they are both smaller than the

reported intracellular concentration, which rather indicates that, given the measured Kds, the level of polyglutamylation will not have a strong effect on tau binding to microtubules in cells.

Following this comment, we went back to the original publication in which the physiological tau concentration was determined, and which we had cited (Ksiezak-Reding et al, 1988). In this paper, the tau concentration was determined by quantitative immuno blot with antibodies which we know now are phosphorylation sensitive. So perhaps these data are less precise as we initially thought. We thus removed this citation and the related statement from our manuscript.

Notwithstanding the Tau concentrations in cells, we show that there is a difference in microtubule binding up to 5 µM of Tau, suggesting that up until this concentration in cells, Tau will be sensitive to microtubule polyglutamylation (Fig 2E).

Concerning the GMPCCP related question: In Extended Fig 2E, we show that the same relative differences in Tau binding are found with Taxol-stabilised microtubules. This strongly indicates that the impact of tubulin polyglutamylation on Tau binding is independent of the state of the microtubules.

7. Katanin experiments: Why were now taxol stabilized microtubules used for experiments? Please state the katanin concentration in the main text. Why was this particular concentration chosen? Please state by how much the katanin activity is affected by polyglutamylation.

For almost all our experiments Taxol-stabilised microtubules were used as the most suitable choice due to their resemblance of the GDP microtubule lattice. The exception are the experiments where we measure the diffusive Tau binding (Fig 2), because the rapid formation of Tau envelopes on Taxol-stabilised microtubules makes further analyses more difficult, especially for the assays where we determine the binding curve of Tau. We nevertheless show in presence of low Tau concentration (18 nM), that the difference in Tau binding between wild-type and *Ttll1*$^{-/-}$/*Ttll7*$^{-/-}$ microtubules is retained on Taxol-stabilised filaments, as addressed in the comment above.

Regarding the concentration of katanin, it was essential to choose a concentration at which the severing happens at a measurable pace during the experiment. If the concentration is higher, the severing is too fast, and cannot be measured, while at lower concentrations, it takes too long for reliable observations. Thus, for experimental reasons, we are bound to work with an empirically chosen concentration.

Concerning the question by how much katanin activity is affected: we now added the numbers directly in the text.

8. Kinesin-1 experiments: The kymograph in Fig 4B is not very convincing - one sees many pauses and few processive events. Is this representative? Do the authors also have kinesin-1 motility data for acetylated and deacetylated microtubules? If yes, they could be shown to demonstrate the specificity of the effects induced by (de)polyglutamylation.

The percentage of non-motile events was less than 5% and did not differ from our previous experiments with Kif5B (e.g. Henrichs et al. 2020). These events are always present in motility experiments, and are typically attributed to misfolded or non-functional molecules and are not included in the motility parameter analysis. The numbers of pausing motors in our experiments were also low, which is reflected in the dwell time and run length survival plots, which show qualitatively identical results, i.e. *Ttll1*$^{-/-}$ and wild-type traces colocalize, and *Ttll7*$^{-/-}$ and *Ttll1*$^{-/-}$*Ttll7*$^{-/-}$ traces

colocalize in both plots. If the motor would pause very often for example on the *Ttll1*$^{-/-}$*Ttll7*$^{-/-}$ microtubules, we would expect the dwell time to be identical to wild type, while its run length decreasing drastically. This is clearly visible in the supplementary videos, and in the revised manuscript we mention it explicitly in the Results section.

As kinesin motility on acetylated and deacetylated microtubules was characterised in great detail by two independent studies before, we chose not to repeat these experiments.

9. It was not always clear how the various normalizations have been performed. Please check if sufficient detail is provided in the Methods for the reader to be able to follow the data analysis.

We carefully checked the methods and we think that the normalisation procedure is now described in sufficient detail. For tau, we write: "*In Fig 2C the value D for each wild-type and mutant microtubule was normalised to the mean wild-type D in the same channel of the microscopy chamber. Each experiment was performed with wild-type and one differentially modified type of microtubules. For representation purposes, one normalised wild-type data set was shown in the scatter plot for each experiment.*"

For katanin, we explain how severing rates are determined, and how we normalise those to the wild-type values. We have now added a sentence to explain how we normalised assays that do not contain wild-type microtubules.

Kinesin experiments were not normalised as the absolute values did not visibly vary between the experiments.

10. The Discussion could profit from comparing the magnitude of the effect of (de)polyglutamylation on the various tested proteins to effects of other posttranslational modifications (such as for example phosphorylations where this has been quantified) and discussing the potential reasons/consequences.

We have now expanded the last chapter of the discussion by underpinning the importance of gradual regulation of microtubule-based processes.

Referee #2:

This manuscript from Genova and colleagues addresses the question of how PTMs impact the interactions and activities of MAPs on their microtubule substrate, particularly MAPs that associate with microtubules in the brain. This is an important question in the microtubule field. Although our knowledge of tubulin PTMs has grown over recent decades, how and whether those PTMs impact the activities of MAPs to ultimately lead to the regulation of microtubule networks has remained understudied. This study focuses on two types of PTMs, acetylation and polyglutamylation, and how these impact three MAPs, Tau, katanin, and kinesin-1/Kif5B. All three MAPs have previously been shown to be sensitive to polyglutamylation, but this study is unique in that it uses tubulin proteins purified from genetically-altered mice that lack the relevant modifying enzymes, which were developed by the authors and described in previous publications. Presumably, the microtubules formed from these tubulin proteins reflect the physiological presence or absence of the modifying enzyme more accurately than previous studied that removed modifications proteolytically or added them through the exogenous addition of recombinant enzymes. This is an advance for the field in terms of faithfully reconstituting a genetically-manipulated microtubule environment and dissecting the roles of tubulin PTMs.

The main conclusions of the study are related to the effects of glutamylation on Tau, katanin, and Kif5B. The conclusions for Tau and katanin are mostly consistent with previous work. The authors report no strong effects for acetylation on Tau binding or katanin activity, and do not test how acetylation effects Kif5B. The design of the study is generally clear; however, there are several key points where the results seem oversimplified, particularly with regard to the high degree of variability in the data. These points are detailed below and should be addressed prior to publication.

MAJOR POINTS

1) What is the source of the high variability in the data for Figures 2 and 3? This variability is particularly clear when evaluating the extended data figures, which show data points from separate experiments measuring Tau binding and katanin severing activities.

This is a very important point, and we would thank the referee for highlighting it. We are aware of this variability, and we aimed at being very transparent about it in the manuscript.

Importantly, we do not think that the variability originates in different PTM levels or purification quality in between different batches of tubulin. To support this notion, we now present the analysed three independent batches of each microtubule type used in our study, at the PTM level (by immunoblot), as well as at the protein level (by Coomassie-brilliant blue staining).

Consequently, we attribute the observed variance in absolute values of Tau binding and katanin severing activities to technical difficulties in controlling the protein concentrations in the measurement chamber. In our assays, the range of concentration providing meaningful, analysable results is rather narrow, typically in the 0.1 - 100 nM range. At these concentrations, depletion of proteins from solution becomes an important parameter that can lead to varying levels of protein concentration in the measurement chamber. Protein depletion is dependent on various factors, such as the total amount of microtubules in the measurement chamber, the volume of the chamber and variations in the hydrophobicity of the coverslips. These factors are, however, rather challenging to control precisely.

Importantly, since we cannot fully control these (technical) factors, all our experiments were designed such that we always directly compare two different types of microtubules next to each other in one

measurement chamber. Thus, all relative values were acquired under absolutely identical conditions, including identical protein concentrations - which then allows us to calculate reliable relative values.

While protein depletion affected Tau binding and katanin severing, kinesin stepping was largely unaffected. This is expected, as kinesin motility parameters, such as velocity and run length are within a large range independent of the concentration of kinesin in the measurement chamber.

For Tau, the intensity of bound Tau from experiment to experiment varies by as much as 40% (see Extended Data Figure 2A, comparing wild type vs T1-/-T7-/- microtubules). The trends of wild type vs mutant binding appear similar across experiments, which somewhat mitigates the concern for this experiment, but the potential source of variability should be addressed. It is not clear whether these experimental replicates are biological replicates, with tubulin prepared from brains of different animals, or technical replicates using the same material. If they are biological replicates, this could suggest that there is some variability in the tubulin protein in the animals or perhaps in the in the quality of the protein from different preps. If they are technical replicates, is there some obvious aspect of the experiments that is different; e.g., errors in the measurement of purified Tau-meGFP?

Please see above, where we described challenges that affect the binding of Tau and the intensities detected. We solve this issue by always directly comparing two different types of microtubules, in one measurement chamber, under identical conditions, providing reliable relative values.

The data from the katanin experiments are even more variable and amplify the concern. Here the Extended Data Figure 3 shows large differences in estimated microtubule half-life across experiments with tubulin from the same genotype. The wild-type control is the most glaring example. In some experiments the median half-life for wild-type microtubules is 27 seconds, while in others the median half-time is 281 seconds. This 10-fold difference is much greater than any differences reported between wild-type tubulin and Ttll-mutant tubulin within an experiment, and this weakens confidence in any conclusions regarding Ttll-related differences. Again, the authors should seek to identify the sources of variability and report them. Are these biological or technical replicates? Is the same purified katanin used in each experiment, or could separate katanin preps yield protein with different levels of activity?

Above we described challenges that can cause variations between microtubule-severing rates of katanin between different experiments. This effect is further amplified because microtubule severing is, in contrast to MAP binding to microtubules, a catalytic process (a single severing event is amplified by the induced microtubule depolymerisation). Consequently, the variations in the katanin experiments are much stronger than in the above-discussed Tau binding. Nevertheless, upon normalisation of the data, the impact of polyglutamylation on the severing is very obvious. We have now discussed this in more detail in the legend of Fig EV3.

In some cases, the authors acknowledge this variability and present normalized data where results from separate experiments are normalized to a wild-type control or some sample of another genotype. This creates distributions in Figures 2C and 3E that appear to be normal, but it would be better to avoid normalization unless there is a strong justification (i.e., if the authors understand and describe

the sources of variability in the data). Showing the data as superplots with differentially-labeled data corresponding to each experiment would also be appropriate here.

We agree with the reviewer and use normalisation only when necessary. As we explained, the variations are purely technical, and not related to biological variations. We thus kept the normalisation in the Tau and katanin experiments. As the kinesin stepping parameters are independent of the concentration in solution (please see above), we thus could analyse these data without any normalisation, as suggested by the referee.

To present the data as transparently as possible, we chose to show non-normalised data from all individual experiments as separate plots in EV2 for Tau, EV3 for katanin, and a new Appendix Fig S2 for kinesin-1. Due to high number of replicates in some cases, we believe this presentation is clearer than showing the colour-coded superplots.

2) Related to the prior point, there is no indication of any variability in the data for the kinesin experiments (Figure 4). This is important to show, given the concerns with the data for Tau and katanin. The authors should include an extended data figure showing the data from individual kinesin experiments.

We thank the referee for pointing this out and we have now shown all single kinesin experiments in a new Appendix Fig S2. Due to the amount of data it was not possible to include them in Fig EV4.

3) The authors make strong claims about the knockout mice affecting only specific PTMs; for example, in the second paragraph on page 6. However, the results supporting this claim are from western blots of one set of samples probed for alpha tubulin, K40 acetylation, polyglutamylation, beta monoglutamylation, and Δ2 tubulin. This represents the 3 PTMs predicted to be altered in the mouse mutants plus one other PTM (Δ2 tubulin). This is hardly exhaustive and does not support the strong statements on page 6 about other potential PTM changes. Whether certain PTMs impact the presence and abundance of other PTMs is an important question in the field. The authors should either test it more rigorously here or refrain from overstating their knowledge of the tubulin PTM state in the brain tubulin from these mice. More rigorous analysis would help strengthen the conclusions of the study.

This is an important point which we now addressed by adding another key-PTM of brain tubulin, detyrosination, to our analysis. We also performed the western blot analyses for three independent batches of tubulin to demonstrate the reproducibility of the tubulin purification method, and the according PTM patterns. Surprisingly we found that in mice lacking TTLL1 and TTLL7, i.e. with brain tubulin devoid of polyglutamylation, acetylation is increased. This is an intriguing observation as acetylation is not physically close to the sites of glutamylation on the tubulin molecule. However, given that all microtubule interactors tested in our study are insensitive to acetylation, the increase of this PTM could not have affected the measured values. This is now discussed in the text. We also adapted the text to avoid overstatements.

We suggest the following: first, the authors should quantify the western blot data in Figure 1E and show a chart of that quantification. It appears that acetylated tubulin may be altered in some of the mutants, and densitometry analysis across replicate experiments would make that clear.

We would like to refrain from quantifying western blots for two reasons: First, our past experience shows that quantification of western blots has many caveats and can easily lead to incorrect data.

Second, and most importantly, the message of our experiments is that very obvious, strong changes of tubulin PTMs (basically presence vs. absence of a PTM) have only gradual effects on the measured microtubule interactions. Quantifying the levels of the tubulin PTMs would suggest that we can correlate PTM precise levels the measured microtubule interactions, which is, given the above-discussed intrinsic variations of our experiments, not true. Therefore, quantifying the western blot signals would transmit a misleading message to the reader.

Second, the authors should use commercially available antibodies to probe for tyrosinated and/or detyrosinated tubulin. This is important because polyglutamylation of the alpha tubulin tail is close to the site of tyrosination and an effect seems plausible, but also because tyrosination has been shown by the Ross lab to alter Tau binding to microtubules. Therefore a change in tyrosination could strongly impact the interpretation of the results here. Third, it would be valuable to provide an extended data figure showing western blot analysis for tubulin prepared from each animal used in the study, to demonstrate the reproducibility of the effected PTM levels.

This has been added as mentioned above. We now show that detyrosination as well as Δ2-tubulin levels do not change when polyglutamylation or acetylation are altered, thus excluding the possibility discussed by this referee.

MINOR CONCERNS:

1) The authors state on page 5 that "no other protein was detected in any of the purified tubulin samples (Figure 1D)." It would be helpful to include similar stained gels for the preps from each animal used in the study. This is a concern because of the unidentified source of variability in the experiments. It seems plausible that some of these preps could contain contaminating proteins that alter the experimental results. Providing stained gels of the SN7 fractions from each prep would address this concern.

This is a key point and we how now added Coomassie-stained gels for all tubulin subtypes for three batches. This is now part of a new Fig EV1.

2) Why do the authors use GMPCPP-stabilized microtubules for the Tau-binding experiments in Figure 2B-E, and taxol-stabilized microtubules in other experiments? This is particularly concerning because work from the Odde lab (PMID 33294790) has shown that Tau has a ~4X tighter affinity on GDP-microtubules vs GMPCPP-microtubules. Thus, the GMPCPP-microtubules used here are a poor substrate for Tau, and may mask the effects of PTMs. It would be better to use GDP microtubules or taxol-stabilized GDP-microtubules, which are used to test lower concentrations of Tau. At minimum, the authors should comment in the manuscript on how GMPCPP impacts Tau binding.

We thank the referee for having pointed this out. The reason we decided to use GMPCPP microtubules for the Tau experiments is that the formation of tau envelopes on Taxol-stabilised microtubules makes the precise determination of Tau binding in outside envelopes difficult. The experiment is possible, and we have shown in Fig EV2E that we obtain similar results with both types of microtubules. We have now explicitly mentioned this in the text.

3) Figure 3. The descriptions of the measurement methods for microtubule severing and half-life calculation are difficult to understand. These should be more clearly described in the methods.

The description of this assay has been improved in the method section. Moreover, to make the method section easier to read, we re-structured it into single assays for Tau, katanin and kinesin.

4) Figure 4. The results here show that Kif5B has long run length when Ttll1 is absent. Could this be due to differences in microtubule length in these experiments? Microtubule length is never reported, and could be simply measured from the IRM images and reported to address this concern.

Finite microtubule length is an important point when quantifying run lengths of molecular motors, and is indeed often neglected leading to misinterpretation of these results. For this reason, we use the Kaplan-Meyer statistics to describe the run lengths and dwell times. This analysis minimizes the effect of finite microtubule lengths (in detail described in Ruhnow et al. Challenges in Estimating the Motility Parameters of Single Processive Motor Proteins, Biophys J 113, 2433–2443 2017).

In the revised version of our manuscript, we have also quantified microtubule length distribution as well as kinesin landing rates for a set of representative experiments, and added this as a new figure (Fig EV4). Additionally, we added a detailed description of the Kaplan-Meyer analysis we used for the kinesin data into the method section to provide a rationale why this type of analyses excludes the impact of microtubule length on our data. These two additions should clearly demonstrate that microtubule length had no impact on our data.

5) Figure 4. Several details are missing. Do these experiments use mixtures of wild type and mutant microtubules on the same coverslip? The text results suggests so, but the graphic in Figure 4A seems to indicate homogenous microtubules on the coverslip. Also, the authors should state whether these are taxol-stabilized or GMPCPP-stabilized microtubules.

We thank the referee for having spotted our mistake in the schematic representation Fig 4A. Indeed, all experiments have been performed in pairs of two different types of microtubules in one measurement chamber, which is now visualised in the corrected figure panel. It is now also clear to the reader from the single experiments we show in Appendix Fig S2. To obtain a single kymograph, we selected one microtubule on the slide, representing one PTM state, which is then shown as a single kymograph in Fig 4B. This is now clarified in the figure legend. We also added the information that we use Taxol-stabilised microtubules.

6) Figure 4. It is not clear why the authors do not test whether acetylation impacts Kif5B, but did test the effect of acetylation on Tau and katanin.

In contrast to Tau or Katanin, two highly convincing and coherent studies, available in the literature, demonstrate that Kinesin-1 is not affected by acetylation. We thus decided to not perform these experiments in our study, but rather cite the literature.

7) The authors should refrain from including discussion in the results section of the manuscript; for example, paragraph 3 on page 10, and the last two paragraphs of the results.

We prefer to discuss technical aspects, as well as direct interpretations of the described experiments right in the result section, as it allows the reader to right away understand potential limitations of the

experiments. From our point of view, this facilitates the reading of the manuscript. We reserved the Discussion chapter for a more general discussion of our results, and their broader implications. We believe that both ways of writing a manuscript are acceptable.

Referee #3:

The manuscript by Genova et al examines the influence of tubulin polyglutamylation on three different microtubule associated proteins. This is a timely and important report as we still have very little understanding of how tubulin PTMs impact microtubule associates proteins and thereby microtubule function. The authors utilize mice lacking the polyglutamylation enzymes TTLL1 and TTLL7 to obtain tubulin preps that lacks specific glutamylation modifications. This is an important contribution as the control situation (tubulin from wildtype mice) contains the PTMs that a microtubule associated protein would see in a cell. The authors assemble microtubules from these tubulins and carry out TIRF microscopy-based assays to examine the activity of Tau, katanin, and kinesin-1 on these microtubules. The data are rigorous and the writing is clear, however, several controls are missing and additional information is needed.

1. In Figure 1 and Figure S1 the authors show the purity and PTM state of their tubulins obtained from the different mouse lines. What is missing is the western blot of detyrosinated tubulin. This is particularly important as kinesin-1 has been shown to be sensitive to the detyrosination state.

We thank the referee for this suggestion, and we have now added blots for detyrosinated tubulin. These blots show, together with the Δ2-tubulin blots, that the modification state of the very C-terminus of α-tubulin is not altered.

In addition, like the tubulin purity (Figure S1), the state of the PTMs should be shown across multiple preps and the PTM state should be quantified with western blots that are in the linear range. There seems to be an inverse relationship between alpha-tubulin acetylation and alpha-tubulin polyE (Figure 1E). Is this true across preps?

We have carefully repeated the western blots for three independent sets of tubulin purifications, and now shown this in the new Fig EV1. It is very clear from these experiments that the PTM patterns are highly reproducible in multiple tubulin preps.

There was only one variation of a PTM in response to another PTM: we observed an increase of acetylation on microtubules from *Ttll1*$^{-/-}$*Ttll7*$^{-/-}$ double-KO brains, in which tubulin polyglutamylated is very low. This is a very exciting observation, and should be followed up in the future. However, for the current manuscript, this potential co-regulation has no implications for the conclusions, as we show that tubulin acetylation has no impact on Tau binding or Katanin severing, and Kinesin-1 has previously been shown to be insensitive to tubulin acetylation.

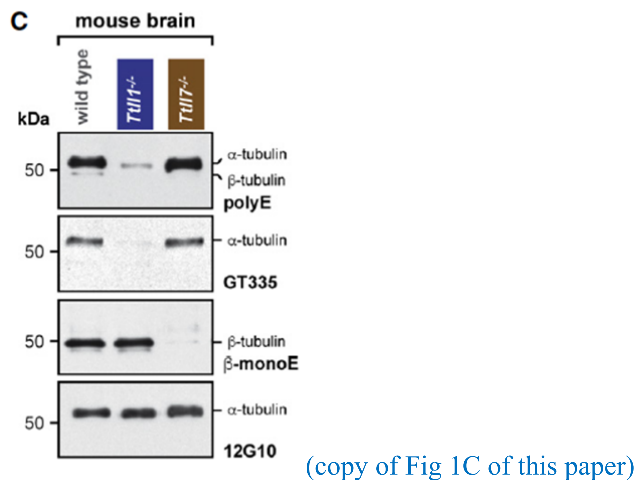
Concerning the suggestion to quantify our western blots: this was also suggested by referee 2, and we answered the following:

We would like to refrain from quantifying western blots for two reasons: First, our past experience shows that quantification of western blots has many caveats and can easily lead to incorrect data. Second, and most importantly, the message of our experiments is that very obvious, strong changes of tubulin PTMs (basically presence vs. absence of a PTM) have only gradual effects on the measured microtubule interactions. Quantifying the levels of the tubulin PTMs would suggest that we can correlate PTM precise levels the measured microtubule interactions, which is, given the above-discussed intrinsic variations of our experiments, not true. Therefore, quantifying the western blot signals would transmit a misleading message to the reader.

2. It would be nice to know the state of the polyglutamylation for each tubulin prep by mass spectrometry. For example, in the Ttll-/- mice, there is no alpha tubulin glutamylation by western blotting but is this just below the level of detection? And are there compensatory changes in the length of polyglutamylation on beta tubulin?

We agree with the referee that a precise mass spectrometry method to quantify tubulin polyglutamylation levels and patterns would be the best tool to employ. Unfortunately, while several laboratories are working intensely on solving this problem, there is yet no reliable method available.

Concerning the question about the residual glutamylation on α-tubulin: in our previous publication (Bodakuntla et al. 2021) we show that there is some polyE reactivity left in *Ttll1*$^{-/-}$ brains, but we also show that another glutamylation antibody, GT335, also detects the loss of glutamylation, indicating that not only long, but also short glutamate chains are lost in the absence of this enzyme:



(copy of Fig 1C of this paper)

Moreover, there is no visible compensation on β-tubulin. How do we know this? The specificity of the β-mono-E antibody to β-tubulin is related to the highly specific epitope of the antibody, --GEF--. This epitope does only exist on β-tubulin (E435). By contrast, GT335 has a much more common epitope, and would easily detect glutamylation on many of the potential sites on β-tubulin. As we do not see an increase of GT335 in the above blot from Bodakuntla et al. 2021, we conclude that there are no other sites than E435 modified β-tubulin in the *Ttll7*$^{-/-}$ brain. As a positive control, we have seen such over-glutamylation (detected with GT335) happening on β-tubulin in the absence of the deglutamylase Ccp1 (same paper).

Finally, we would like to point out that for the current study, we did not aim for a precise quantitative measurement of tubulin polyglutamylation, but rather for a more coarse-grained test of the functions of polyglutamylation if it is added to α- vs. to β-tubulin as discussed about in point #1.

3. For the kinesin experiments, it looks like there is an increase in the number of motility events on the Ttll7-/- and Ttll1-/-Ttll7-/- tubulins. For microtubule associated proteins, tubulin PTMs are most likely to affect the affinity for the microtubule although this parameter is often ignored in the literature. This could be measured as either a landing rate (obtained for the existing motility data if equal amounts of motor were added under all conditions) or an affinity measurement via microtubule cosedimentation experiments.

This is a very important point and we would like to thank the referee for having raised it. We now determined the landing rates of three independent sets of experiments. While overall landing rates differed between different experimental sets, they are similar between different PTM variants of microtubules used in the kinesin experiments. This new figure is included in the new Fig EV4.

4. With respect to affinity, it is clear in Figure 2 that tau has a higher affinity for the wild type tubulin. What about katanin? The authors show changes in severing activity in Figure 3 but is this because katanin's binding has changed or its activity?

This is a very important question that we cannot answer with our current data. The binding and activation of Katanin are closely intertwined, in particular because the enzyme binds the C-terminal tail of tubulin as part of the severing process. We thus think answering this question is important, but beyond the scope of the current work. What is important here is that we show how polyglutamylation, but not acetylation, affects Katanin function.

5. Also for the kinesin data, what is the biological significance of the interaction time versus the run length? They seem very similar in terms of polyglutamylation impact. Probably one of them could be moved to the supplemental data and replaced by the more biologically-relevant landing rate or microtubule affinity data. I also do not understand the statistics tables.

Indeed, both data plots illustrate two different sides of the same process. However, both plots are equally important and we prefer to keep them in the main figure. As these plots look qualitatively similar (i.e. *Ttll1*$^{-/-}$ and wild-type traces colocalize and *Ttll7*$^{-/-}$ and *Ttll1*$^{-/-}$*Ttll7*$^{-/-}$ traces colocalize in both plots), their combination shows that kinesin molecules in our experiments don't pause much during their motility, a point raised by the Reviewer 1. As discussed in the reply to the Reviewer 1, if the motor would pause very often for example on the *Ttll1*$^{-/-}$*Ttll7*$^{-/-}$ microtubules, we would expect the dwell time to be identical to wild type, while its run length would decrease drastically. We now mentioned this explicitly in the manuscript, and kept both plots in the main figure.

To compare survival curves, hazard ratios and their (95%) confidence intervals are routinely used in the literature. The interpretation of this analysis is that if the confidence interval does not include 1, the difference between the survival curves is significant on the 5% significance level (marked by * in our table and mentioned in the figure caption).

6. For the Introduction, another way used in the literature to test the impact of polygutamylation is to compare kinesin activity on brain vs HeLa tubulin. Please add the Lessard et al 2019 reference which shows that polyglutamylation increases the landing rate of KIF1A (a kinesin-3 motor).

We apologize for this omission, which happened by mistake. We have now cited and discussed the paper in the introduction.

7. A most critical aspect of the entire manuscript lies in the relevance to the in vivo situation. The authors acknowledge this in the Discussion p. 13-15 where they provide several examples of where their in vitro data do not match the in vivo data including kinesin-1 sensitivity to polyglutamylation (Bodakuntla et al 2020 but also Maas 2009), katanin sensitivity to acetylation (Sudo and Baas 2010), and kinesin-1 sensitivity to acetylation (Reed et al 2006 but also Cai et al 2009, Guardia et al 2016, Tas et al 2017). The authors indicate the complexity of the cell as a reason for discrepancies between

their in-vitro and previous cell-based observations. An alternative explanation is just that the in vitro experiments cannot replicate what is happening inside a cell. The purification of tubulin and its reassembly in in vitro assays likely fails to replicate the microtubule architecture in cells where a microtubule is first assembled and then modified by PTM enzymes, MAPs, motors walking, plus tip proteins, etc. While both cellular and reconstitution experiments are important, the inability to replicate in vitro what is seen in a cellular environment should be considered a big red flag rather than a problem with the cellular experiments.

This is a very important point; however, we would politely disagree with the last sentence of this referee's comment. Obviously, *in-vitro* experiments in general do not reproduce what happens in the cell. What *in-vitro* reconstitutions with purified compounds do is to determine which single molecular component is regulated by which single event. How these single components combine their effects in a living cell is an exciting question that should be answered next and apparent discrepancies between our *in-vitro* results and previous *in-vivo* findings provide direct starting points for this future work. We have carefully revised the text to avoid any notion that could insinuate that we reconstitute what is happening in the cell. Yet, what brings our investigation closer to the intracellular situation than any other *in-vitro* work before is that our tubulin represents native variants generated by the loss of endogenous modifying enzymes.

1st Revision - Editorial Decision | **5th Dec 2022**

Thank you for submitting a revised version of your manuscript. Your study has now been seen by all original referees, who find that their previous concerns have been addressed and now recommend publication of the manuscript. There remain only a few minor editorial points that have to be addressed before I can extend formal acceptance of the manuscript .

Please let me know if you have any further questions regarding any of these points. You can use the link below to upload the revised files.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

Referee #1:

The authors addressed all my concerns appropriately which improved the clarity of presentation. The conclusions are well supported by the data and the reported results are interesting. It's quite a nice EMBO paper in the view of this reviewer.

Referee #2:

The authors have addressed all of our major concerns in this revised manuscript. The changes to the text and additions to the EV Figures improve the clarity of the manuscript. This will be a valuable contribution to the field.

Referee #3:

I am satisfied with the revisions made to the manuscript

All editorial and formatting issues were resolved by the authors.

2nd Revision - Editorial Decision 22nd Dec 2022

Thank you for addressing the final editorial issues. I am now pleased to inform you that your manuscript has been accepted for publication.

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For all studies, sample size was chosen to be larger than 5 experiments per condition to be able to make statistical analyses. Often the sample size was much bigger.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Mice used in this studies were exclusively source of biological material.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	n/a
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No specific randomization procedures were performed.
For animal studies, include a statement about randomization even if no randomization was used.	n/a
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No specific blinding procedures were performed.
4.b. For animal studies, include a statement about blinding even if no blinding was done	n/a
5. For every figure, are statistical tests justified as appropriate?	We have chosen appropriate statistial tests for each figure. Specific details such as paired vs unpaired, parametric vs non-parametric test etc were mentioned in the legend of the respective figures, as well as in the materials and methods section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Data were analysed using Prism software or Mathlab, and appropriate tests were performed in the course of the analyses. Whenever the sample size is high, it was assumed to follow normal distribution.

Is there an estimate of variation within each group of data?	Data shown in Fig. 2,3,4,EV2,EV3,EV4,appendix Fig S2 are represented as scatter dot plots to highlight the variation in the parameters measured within each data set .
Is the variance similar between the groups that are being statistically compared?	The variance was similar between the groups that were tested for significance.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All manufacturers' details are described in the materials & methods section and in the supplementary tables in the appendix.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	There are no cell lines used in this study.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Ttll1 Ttll1tm1a(EUCOMM)Wtsi mice were generated at EUCOMM (http://www.mousephenotype.org/data/alleles/MGI:2443047/tm1a(EUCOMM)Wtsi). Ttll7
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal care and use for this study were performed in accordance with the recommendations of the European Community (2010/63/UE) for the care and use of laboratory animals. Experimental procedures were specifically approved by the ethics committee of the Institut Curie CEEA-IC #118 (authorization n°04395.03 given by National Authority) in compliance with the international guidelines.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Our animal studies are in line with all major animal study guidelines. Our study was approved by an ethics committee.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	n/a
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	n/a
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n/a
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	n/a
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n/a
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	n/a
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	n/a

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	There is no such data generated in our study.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	There is no such data generated in our study.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	n/a
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	n/a

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	n/a