EMBO reports

Expanded tRNA methyltransferase family member TRMT9B regulates synaptic growth and function

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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.)

Dear Dr. O'Connor-Giles,

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is pasted below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also have several suggestions for how the study should be strengthened. Especially all comments by referees 1 and 2 should be addressed. Please let me know in case you disagree with any of the suggestions so that we can discuss this further, also in a video chat, if you like.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (30th May 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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- the name of the statistical test used to generate error bars and P values,

- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,

- the nature of the bars and error bars (s.d., s.e.m.),

- If the data are obtained from n Program fragment delivered error ``Can't locate object method "less" via package "than" (perhaps you forgot to load "than"?) at //ejpvfs23/sites23b/embor_www/letters/embor_decision_revise_and_review.txt line 56.' 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

- Please also include scale bars in all microscopy images.

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I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Esther Schnapp, PhD
Senior Editor

Referee #1:

Hogan et al report the role of one of the drosophila homolog of the yeast Trmt9 tRNA methyltransferase in the axonal growth and function of motorneurons. The authors identified Trmt9b using a targeted RNAi screen and validated its function in axonal growth by producing CRISPR/Cas9 loss of function mutant alleles. They further found that the synaptic function of motorneurons was impaired. By doing tissue specific rescue experiments they could show that Trmt9b regulates these processes through a postsynaptic function. Lastly they demonstrate that the structure of the methyltransferase domain is conserved and that the mutation of some key residues impairs the ability to rescue the synaptic growth.

While TRMT9B was previously associated with cancer its physiological and molecular role was not yet investigated. Here the authors describe for the first time a function in axonal growth using Drosophila as model organism, yet the molecular function still remains to be elucidated. The manuscript is well written and easy to ready. It is surprising that Trm9b is enriched in the nervous system, yet its function appears to be important in muscles. Overall the work is well done and the results are clear. However some information are missing and a few additional experiments should be made to be complete.

Comments:

1. The authors identified Trmt9B in a targeted RNAi screen. They selected candidates based on their expression during synaptogenesis. Can the authors better describe this screen? How many genes were selected, how many candidates were knocked down and how many hits were obtained? Without these pieces of information, the choice of investigating Trmt9B sounds a bit like cherry picking.

2. What is the expression pattern of Trmt9b during development?

3. A defect was found in third instar larvae, yet in Fig 5C and D the level of different modifications was examined in fly heads. Could it be that the absence of effect of the loss of Trmt9b due to its importance during larval stages rather than in adult fly. To address this the mass spectrometry analysis should be repeated using larval samples.

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5. The authors show a clear defect in axonal growth and synaptic function of the motorneurons, yet they do not assess the impact on larval mobility. This assay is relatively straightforward and could be included in the manuscript.

6. The expression of WT and mutated Trmt9b should be validated by western blot to compare their level and to show that the full-length products are indeed produced.

7. In Fig2 only a few genotypes were tested. Does the homozygous Trmt9B KO and Trmt9b HA+IC also have altered synaptic function?

8. Does the mutated Trmt9b also fail to rescue synaptic function?

Referee #2:

Hogan and colleagues present compelling evidence that TRMT9B, a tRNA methyltransferase, functions as a negative regulator of synaptic development at the Drosophila NMJ. It is increasingly uncommon to find negative regulators of NMJ formation, making the study important for its novelty, but that novelty is extended even further as it begins to address neuronal roles of tRNA methyltransferases, which are incompletely understood. The authors present evidence that, in the absence of TRMT9B, there are more boutons present at NMJs and the synapse is reduced in function. Specifically, TRMT9B appears to function postsynaptically despite its broad expression in neurons, glia, and muscles. Beyond the basic characterization of the mutant, the authors also present evidence that TRMT9B is not involved in baseline tRNA methylation but does require its methyltransferase function to regulate synaptic growth. The evidence presented here is strong and is supportive of the authors' claims. Moreover, the use of state-of-the-art genetics and editing approaches to produce mutants and tagged lines is exceedingly helpful in lending credence to the conclusions. This is a thorough analysis that is suitable for publication following some minor revisions and the potential addition of new data.

Specific Comments:

1) The allele notation makes the paper a little inaccessible to researchers unfamiliar with Drosophila methodology or design. A diagram in Figure 1 (or a supplemental figure) indicating the nature of the alleles, position of the tags, and design of the HA+IC concept would be very helpful.

2) Regarding the HA line - it seems to behave like a hypomorphic allele but this is not extensively discussed. It also has an unexpected mEJP phenotype. The paper would benefit from additional discussion of this and assessment / speculation on whether this influences the localization data (is the sfGFP line made the same way?).

3) The assertions of synaptic localization (esp. in Figure 3) are not optimally presented, given the high expression of TRMT9B throughout the muscle (and very difficult to discern in Figure S2C). Higher magnification images of the NMJ would be very helpful in both cases, as would additional marker staining (DLG, CSP, etc) to offer more evidence as to localization of the protein - at these magnifications, it looks all presynaptic, which is surprising given the postsynaptic rescues. If that does pan out, is the hypothesis that it is working elsewhere than the synapse to mediate its functions? Does it have a synaptic role versus a nuclear role?

4) Though the 24B / C155 rescue experiments are convincingly presented, since TRMT9B is amenable to RNAi (from the Honjo et al. 2016 study), could the authors present tissue-specific RNAi data that supports their assertion of postsynaptic function? Perhaps also tissue specific RNAi in the presence of one of their tagged versions to demonstrate knockdown? This last experiment would have the added bonus of presenting more data regarding the specificity of their endogenous epitope tagged versions.

5) The authors make an MTD (methyltransferase dead) transgene of TRMT9B based on homology to the yeast and human homologues. Is there any data to support that these mutations are doing the same thing in the fly TRMT9B homologue that they're doing in the yeast and human homologues? It would be helpful to see evidence that those residues function similarly. If that data does not exist, the language used to discuss these experiments should be tempered and the caveats thoroughly presented. Similarly to point 3, it's very difficult to tell if the MTD version localizes similarly to the wild-type 24B expression. Are there better / brighter images to assert this and perhaps higher magnification images of the NMJ to at least posit expression is not drastically impaired?

Referee #3:

In a screen for genes involved in synaptic growth regulation, Hogen et al identified TRMT9B, a methyltransferase involved in modifying the tRNA wobble position. They convincingly show with adequate genetics that TRMT9b activity is required post-synaptically in muscle 4 to restrict synapse formation. They also show that neurotransmitter release is reduced in muscle 6. They then find that the expected modifications did not change in TRMT9B mutants, but do so in ALKBH8 null mutants, but no characterization is given for ALBH8 mutants. Does an ALKBH8 KO also affect synaptic growth? Apparently, TRMT9b requires a co-factor , Trrm112. What is the phenotype of the knock-out of this gene? Given the lack of changes in methylation and a molecular target for TRMT9b, all attempts to make this to a coherent story rather raise more questions instead of providing mechanistic insights.

Other issues:
The methylation pathway shown in Fig 5B should be explained in detail, e.g. in the introduction. How does ALKBH8 fit in here? The amidation pathway should also be shown to visualize for the reader where ncm5U comes from.

A gene model with a depiction of the alleles in Fig 1 would help to understand the principally elegant genetics.
Depicting the expression profile in a supplemental Fig deduced from flybase would help for the 3 genes.

They only analysed muscle 4, how about muscle 6, where the mEJPs were done? Or muscle 12/13?

Does the modification landscape change with over expression of TRMT9b? It is not clear whether Trrm112 can substitute for TRMT9b. Does ALKBH8 act without Trrm112?

Response to reviews

Referee #1:

Hogan et al report the role of one of the drosophila homolog of the yeast Trmt9 tRNA methyltransferase in the axonal growth and function of motorneurons. The authors identified Trmt9b using a targeted RNAi screen and validated its function in axonal growth by producing CRISPR/Cas9 loss of function mutant alleles. They further found that the synaptic function of motorneurons was impaired. By doing tissue specific rescue experiments they could show that Trmt9b regulates these processes through a postsynaptic function. Lastly they demonstrate that the structure of the methyltransferase domain is conserved and that the mutation of some key residues impairs the ability to rescue the synaptic growth.

While TRMT9B was previously associated with cancer its physiological and molecular role was not yet investigated. Here the authors describe for the first time a function in axonal growth using Drosophila as model organism, yet the molecular function still remains to be elucidated. The manuscript is well written and easy to ready. It is surprising that Trm9b is enriched in the nervous system, yet its function appears to be important in muscles. Overall the work is well done and the results are clear. However some information are missing and a few additional experiments should be made to be complete.

Comments:

1. The authors identified Trmt9B in a targeted RNAi screen. They selected candidates based on their expression during synaptogenesis. Can the authors better describe this screen? How many genes were selected, how many candidates were knocked down and how many hits were obtained? Without these pieces of information, the choice of investigating Trmt9B sounds a bit like cherry picking.

We appreciate this point and should have described the identification of TRMT9B differently. We have updated the revised manuscript to better reflect our ultimate approach. Briefly, using ModENCODE data we found that a large number of Drosophila synaptic genes exhibit a unique spatio-temporal transcriptional profile during development. Expression is highly enriched in the nervous system, and transcription is generally low with peaks during each of the two periods of extensive synaptogenesis (late embryonic and mid-pupal). We reasoned that uncharacterized genes with this expression pattern might also encode proteins involved in synapse formation or function, so we compared the expression pattern of every gene in the genome with this profile and focused on those with the highest correlation coefficients that were both uncharacterized and conserved in mammals. We then conducted an RNAi screen of ~60 genes that met these criteria for altered synaptic growth at the NMJ. However, when we subsequently went to validate a small subset of our RNAi findings with classical alleles, we found that the results were not reliably recapitulated. There are a number of potential reasons for this, including off-target effects and incomplete knockdown – both well known drawbacks of RNAi approaches. As a result, we have been generating CRISPR alleles for our top candidate synaptic genes and screening through them over time.

We have changed the text in the introduction (lines 46-56) as follows and made corresponding changes to any other screen references:

> Previous: To uncover conserved, uncharacterized genes with roles in the formation of functional synapses, we conducted a genetic screen in Drosophila for regulators of synaptic growth at the well-characterized glutamatergic neuromuscular junction (NMJ). Computed Gene 42261 (CG42261) emerged from our screen as a negative regulator of synaptic growth.
>
> New: To uncover conserved, uncharacterized genes with roles in the formation of functional synapses, we first looked for genes expressed ‘in the right place at the right time.’ Using ModENCODE data, we observed that many *Drosophila* synaptic genes exhibit a unique spatio-temporal transcriptional profile during development. Expression is highly enriched in the nervous system, and transcription is generally low with peaks during each of the two periods of extensive synaptogenesis, late embryonic and mid-pupal (Fig. EV1). We reasoned that uncharacterized

genes with this expression pattern might be enriched for genes involved in synapse formation and/or function and compared the expression pattern of every gene in the genome with this profile. Focusing on genes with the highest correlation coefficients that were uncharacterized and conserved in mammals, we began generating CRISPR loss-of-function and endogenously tagged alleles to assess potential roles in synapse development at the well-characterized glutamatergic neuromuscular junction (NMJ).

2. What is the expression pattern of Trmt9b during development?

We have included ModENCODE developmental temporal expression data for TRMT9B in new Fig. EV1 as suggested by Reviewer 3. These data show that TRMT9B is expressed in the temporal pattern described above. Spatially, TRMT9B is largely nervous system specific with low expression in imaginal discs, fat body, and carcass (muscle and epidermis). This is noted on lines 415-418.

3. A defect was found in third instar larvae, yet in Fig 5C and D the level of different modifications was examined in fly heads. Could it be that the absence of effect of the loss of Trmt9b due to its importance during larval stages rather than in adult fly. To address this the mass spectrometry analysis should be repeated using larval samples.

This is an excellent suggestion. We repeated RNA mass spectrometry in larval ventral ganglia and body walls of control, *TRMT9B*^{KO}, and *ALKBH8*^{KO} animals and again observed a loss of wobble uridine methylation in the absence of ALKBH8 but not TRMT9B, suggesting that TRMT9B has a different role in both the larval and adult nervous systems. This data has been added to Fig. 5.

4. The authors mention that the number of synapses per bouton is reduced in Trmt9B mutants. Can they provide a magnification of the Brp staining and repeat this assay with other mutant alleles and rescue? It would be useful to have a table with the quantification?

We have conducted these analyses and found that similar to *TRMT9B*^{KO}, synapse number per bouton is reduced in *TRMT9B*$^{KO/HA+IC}$. Notably, this reduction offsets the increase in bouton number to yield a wild-type number of synapses per NMJ. These data indicate that a previously described homeostatic mechanism for maintaining synapse number is intact in the absence of TRMT9B and have been added to Fig. 4.

5. The authors show a clear defect in axonal growth and synaptic function of the motorneurons, yet they do not assess the impact on larval mobility. This assay is relatively straightforward and could be included in the manuscript.

We conducted larval crawling assays and observed a significant decrease in locomotion. However, we are unable to rescue the phenotype with restoration of endogenous expression. As noted below (Reviewer 2, comment 2), restoration of endogenously tagged TRMT9B only partially rescued synaptic growth, suggesting the tagged protein may have reduced function. We also attempted to rescue with Gal4-driven expression, however 24B Gal4 on its own increases locomotion complicating interpretation of the observed rescue. Because we can't be confident that the phenotype is fully attributable to loss of TRMT9B, we have not included it in the revised manuscript.

6. The expression of WT and mutated Trmt9b should be validated by western blot to compare their level and to show that the full-length products are indeed produced.

We have added this important control to Fig. EV5. Both transgenes express full-length proteins at similar levels.

7. In Fig2 only a few genotypes were tested. Does the homozygous Trmt9B KO and Trmt9b HA+IC also have altered synaptic function? 8. Does the mutated Trmt9b also fail to rescue synaptic function?

We appreciate this suggestion and have investigated the ability of methyltransferase-dead TRMT9B to rescue synaptic function and observe full rescue, suggesting that TRMT9B has evolved both methyltransferase-dependent and -independent functions. We have revised the text throughout to reflect this exciting finding. We have also added an analysis of homozygous *TRMT9B*^{KO}, which exhibits a similar deficit in neurotransmitter release. These data are presented in a new Fig. 8.

Referee #2:

Hogan and colleagues present compelling evidence that TRMT9B, a tRNA methyltransferase, functions as a negative regulator of synaptic development at the Drosophila NMJ. It is increasingly uncommon to find negative regulators of NMJ formation, making the study important for its novelty, but that novelty is extended even further as it begins to address neuronal roles of tRNA methyltransferases, which are incompletely understood. The authors present evidence that, in the absence of TRMT9B, there are more boutons present at NMJs and the synapse is reduced in function. Specifically, TRMT9B appears to function postsynaptically despite its broad expression in neurons, glia, and muscles. Beyond the basic characterization of the mutant, the authors also present evidence that TRMT9B is not involved in baseline tRNA methylation but does require its methyltransferase function to regulate synaptic growth. The evidence presented here is strong and is supportive of the authors' claims. Moreover, the use of state-of-the-art genetics and editing approaches to produce mutants and tagged lines is exceedingly helpful in lending credence to the conclusions. This is a thorough analysis that is suitable for publication following some minor revisions and the potential addition of new data.

Specific Comments:

1) The allele notation makes the paper a little inaccessible to researchers unfamiliar with Drosophila methodology or design. A diagram in Figure 1 (or a supplemental figure) indicating the nature of the alleles, position of the tags, and design of the HA+IC concept would be very helpful.

Thank you for this excellent suggestion. We have added a diagram, which was also suggested by Reviewer 3, to Figure 1.

2) Regarding the HA line - it seems to behave like a hypomorphic allele but this is not extensively discussed. It also has an unexpected mEJP phenotype. The paper would benefit from additional discussion of this and assessment / speculation on whether this influences the localization data (is the sfGFP line made the same way?).

We have increased our discussion of these points (lines 143, 154-156 and 206-7). Both tags were generated the same way and are tagged at the N terminus.

3) The assertions of synaptic localization (esp. in Figure 3) are not optimally presented, given the high expression of TRMT9B throughout the muscle (and very difficult to discern in Figure S2C). Higher magnification images of the NMJ would be very helpful in both cases, as would additional marker staining (DLG, CSP, etc) to offer more evidence as to localization of the protein - at these magnifications, it looks all presynaptic, which is surprising given the postsynaptic rescues. If that does pan out, is the hypothesis that it is working elsewhere than the synapse to mediate its functions? Does it have a synaptic role versus a nuclear role?

We have revised the text to make it clearer that we do not observe post-synaptic expression at the NMJ, but rather observe TRMT9B in a peri-nuclear pattern in muscle (lines 202-206). While we do not yet know the biochemical function of TRMT9B, we hypothesize that TRMT9B is functioning cytoplasmically to regulate the translation of key synaptic proteins. The presynaptic expression suggests that TRMT9B may also have additional functions in motor neurons that we have not yet identified.

4) Though the 24B / C155 rescue experiments are convincingly presented, since TRMT9B is amenable to RNAi (from the Honjo et al. 2016 study), could the authors present tissue-specific RNAi data that supports their assertion of postsynaptic function? Perhaps also tissue specific RNAi in the presence of one of their tagged versions to demonstrate knockdown? This last experiment would have the added bonus of presenting more data regarding the specificity of their endogenous epitope tagged versions.

Given our concerns about the RNAi lines (see response to Reviewer 1, Comment 1 above), which were not assessed for degree of knockdown or validated for specificity in the cited study, we believe that classical alleles and genetic rescue experiments are the stronger experiments. Notably, both synaptic growth and synaptic function are rescued by postsynaptic expression of TRMT9B. Similarly, since RNAi only results in partial knock down, we would expect TRMT9B-HA to continue to be expressed in an RNAi background making it difficult to make any interpretations about specificity.

5) The authors make an MTD (methyltransferase dead) transgene of TRMT9B based on homology to the yeast and human homologues. Is there any data to support that these mutations are doing the same thing in the fly TRMT9B homologue that they're doing in the yeast and human homologues? It would be helpful to see evidence that those residues function similarly. If that data does not exist, the language used to discuss these experiments should be tempered and the caveats thoroughly presented. Similarly to point 3, it's very difficult to tell if the MTD version localizes similarly to the wild-type 24B expression. Are there better / brighter images to assert this and perhaps higher magnification images of the NMJ to at least posit expression is not drastically impaired?

Unfortunately, without a known substrate we cannot directly test methyltransferase function, a point we make clearer in the revised results (lines 357-364) and discussion sections (lines 498-500). Instead, we used structural modeling to predict that these residues, which are conserved in Class I methyltransferases from yeast to humans and are critical in yeast Trm9, are also critical in flies. We've moved this figure from supplemental to Fig. 7 and tempered our language.

We have also added additional/better images of the expression patterns of the wild-type and methyltransferase-dead transgenes, including neuronal expression where TRMT9B expression is higher and easier to visualize, in Fig. EV5.

Referee #3:

In a screen for genes involved in synaptic growth regulation, Hogen et al identified TRMT9B, a methyltransferase involved in modifying the tRNA wobble position. They convincingly show with adequate genetics that TRMT9b activity is required post-synaptically in muscle 4 to restrict synapse formation. They also show that neurotransmitter release is reduced in muscle 6.
They then find that the expected modifications did not change in TRMT9B mutants, but do so in ALKBH8 null mutants, but no characterization is given for ALBH8 mutants. Does an ALKBH8 KO also affect synaptic growth? Apparently, TRMT9b requires a co-factor , Trrm112. What is the phenotype of the knock-out of this gene? Given the lack of changes in methylation and a molecular target for TRMT9b, all attempts to make this to a coherent story rather raise more questions instead of providing mechanistic insights.

Other issues:
The methylation pathway shown in Fig 5B should be explained in detail, e.g. in the introduction. How does ALKBH8 fit in here? The amidation pathway should also be shown to visualize for the reader where ncm5U comes from.

We have added the amide branch to the pathway in Fig. 5. ALKBH8 is not included because the figure depicts the yeast pathway, which has been well characterized.

A gene model with a depiction of the alleles in Fig 1 would help to understand the principally elegant genetics.
Depicting the expression profile in a supplemental Fig deduced from flybase would help for the 3 genes.

Thank you for these suggestions. We've added a model of the alleles to Figure 1 and ModENCODE spatial and temporal transcriptional expression profiles to new Fig. EV1.

They only analysed muscle 4, how about muscle 6, where the mEJPs were done? Or muscle 12/13?

Because TRMT9B exhibits similar expression across all motor neurons, we conducted our synaptic growth assays at the representative NMJ4 to isolate a single type Ib motor neuron synapsing with a single postsynaptic muscle.

Does the modification landscape change with over expression of TRMT9b? It is not clear whether Trrm112 can substitute for TRMT9b. Does ALKBH8 act without Trrm112?

We appreciate these interesting questions. Experiments addressing them are the focus of ongoing, independent projects in the lab and beyond the scope of this manuscript.

Dear Kate,

Thank you for the submission of your revised manuscript files. We have now received the comments from all referees, as well as cross-comments, all pasted below.

As you will see, while referees 1 and 2 are satisfied with your revisions, referee 3 is not. However, in their cross-comments the other 2 referees agree that not much more experimentation is needed, and that - if possible - just the first 2 points by referee 3 could be addressed.

A few editorial requests will also need to be addressed:

- Please add up to 5 keywords to the ms file.

- Please add a "Disclosure and Competing Interest Statement".

- Please remove the authors credits from the ms file.

- Please correct the reference format to the EMBO reports (Harvard) style. The current format is not correct.

- Fig 1F is called out after 1G. Fig 8H-J are called out after 8D.
Fig 7B-F, EV3A-C, EV5A-H callouts are missing. Please correct.

- Please remove the author credits and ORCIDs from the cover page of the manuscript.

- Fig EV5 and perhaps also Fig 5 is too long. Please check again. All main and EV figures need to fit on one printed page. Here is the relevant info from our guide to authors for more information:
"The final size of figures will be between 87 mm and 180 mm wide on the printed page. Please bear this in mind when submitting your manuscript for review and allow for sufficient resolution at a suitable size. For help preparing figures, please download our figure guide PDF."

I would like to suggest some changes to the abstract that needs to be written in present tense. Please let me know whether you agree with the following:

Nervous system function rests on the formation of functional synapses between neurons. We here identify TRMT9B as a new regulator of synapse formation and function in Drosophila. TRMT9B has been studied for its role as a tumor suppressor and is one of two metazoan homologs of yeast tRNA methyltransferase9 (Trm9), which methylates tRNA wobble uridines. Whereas Trm9 homolog ALKBH8 is ubiquitously expressed, TRMT9B is enriched in the nervous system. However, in the absence of animal models, TRMT9B's role in the nervous system has remained unstudied. We generate null alleles of TRMT9B and show that it acts postsynaptically to attenuate synaptogenesis and promote neurotransmission. Through liquid chromatography-mass spectrometry, we find that ALKBH8 catalyzes canonical tRNA wobble uridine methylation, raising the question of whether TRMT9B is a methyltransferase. Our structural modeling studies suggest TRMT9B retains methyltransferase function. In vivo, disruption of key methyltransferase residues blocks the ability of TRMT9B to rescue synaptic overgrowth, but not neurotransmitter release. These findings reveal distinct roles for TRMT9B in the nervous system and highlight the significance of diversification of the tRNA methyltransferase family in metazoans.

I added Drosophila to the abstract, to be more specific. If you also have data on other model organisms, please modify (I am sorry I dont remember now). Thank you.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the final manuscript.

I look forward to seeing a final form of your manuscript when it is ready. Please use this link to submit your revision:
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Best regards,
Esther

Esther Schnapp, PhD

Senior Editor
EMBO reports

Referee #1:

The authors addressed most of my concerns. Even though there is still a lack of molecular understanding, the findings are nevertheless interesting and timely for publication.

Referee #2:

I thank the authors for their measured and serious consideration of both my and the other reviewers' comments. They have adequately answered all of my queries and I support publication.

Referee #3:

The authors have not addressed the main issues raised and leave the reader confused what Alkbh8 is doing and whether the loss of TRMT9b has a general effect on synaptic growth as inferred from the title or if its loss is specific to muscle 4. Since the molecular analysis showed no effect of TRMT9b on methylation of tRNA wobble uridines, TRMT9b likely only affects a subset of tRNAs. They need to quantify bouton numbers at least in muscle 6 where they did the NMJ recordings.

Moreover, why did they remove the mcm5U data? This is confusing. Since Alkbh8 seems to act in the same step as the TRMT9b/Trrm112 heterodimer, why is this not shown in the diagram?

Also, they didn't bother about the role of Trrm112, despite an available transposon mutant in stock centres.
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The authors have addressed neuro-developmental and most genetic-related issues. Despite that all the resources are available, however, the characterization of the enzymatic pathways by genetic means remains fragmentary and is confusing to the reader. I would expect this to be resolved for publication in EMBO Reports as otherwise I recommend to reject the paper.

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> This is probably beyond the scope of this study.

Cross-comments from referee 2:

Reviewer #3 raises not unreasonable requests, but I think it's very clear that the requests are beyond the scope of one single manuscript. In my opinion, Hogan and colleagues have shown an interesting finding that should be published in EMBO Reports. It is absolutely true that they do not flesh out the entire mechanism - but that level of detail is not essential (again in my opinion) for the first story. A subsequent manuscript detailing the mechanism (that the authors themselves even say is part of an ongoing project in the lab) would be completely appropriate in this case.

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The appropriate characterization of a Trrm112 mutant would be beyond the scope of this study. The reviewer notes that an available transposon is available in a stock center, but whether that transposon is a mutant, what the nature of the mutant is, whether it is sufficiently viable to conduct third instar NMJ analyses, and what reagents would be needed to be made to confirm these facets is another paper in itself. The transposon is not guaranteed to be a sufficient mutant, and if it's not, then the authors would need to make a mutant, which would further vault this into another paper territory.

Finally, the reviewer requests an analysis of the ALKBH8 mutant since there is KO data provided in Figure 5 (regarding wobble uridine methylation). I can understand this request, but again, I see it as beyond the scope of this study. Speaking solely for myself here, I feel that the reviewers' classification of this study as a "fragmentary analysis" is incorrect and offensive to the very thorough characterization of TRMT9B that Hogan and colleagues have achieved.

The work does exactly what a good scientific paper should do - it offers a thorough characterization and then raises questions for the future. Science is built on raising questions for the future that new work by new trainees and new labs can answer. Reviewer #3 is asking for all of the questions to be answered in one shot, and while I respect that this is their right, I also feel that it is beyond the scope of a single scientific study. I, for one, would enjoy watching a tenure or promotion talk where all of those questions were answered over the first years of someone's career in multiple publications.

So my final assessment is that Reviewer #3 is asking reasonable questions in the larger scope of science, but that these specific asks are largely beyond the scope of the current study. I think it would be fair (though not essential) to ask the authors to quantify boutons at muscle 6 and demonstrate nearly identical phenotypes between two different muscles but I would not advocate for answering any of the further points in the current study.

Response to reviews

Referee #1:
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We thank Reviewer 1 for their constructive reviews and thoughtful assessment of our study.

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We have added analysis of bouton number at NMJ6/7, where we also observe synaptic overgrowth (Fig. EV2B).

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We have also added LC-MS data for mcm5U back to the text, along with the information that levels of this rare modification are close to our limit of detection (lines 200-203). As noted previously, ALKBH8 is not in Fig. 5B because it's the yeast pathway, which is presented as a reference for the LC-MS experiments before the results revealing ALKBH8's role in *Drosophila*.

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We believe we have conducted a thorough investigation of TRMT9B and significantly advanced understanding of this protein, whose role in the nervous system and tRNA methylation remained unknown despite it being studied over a decade.

Investigation of the remainder of the pathway is well beyond the scope of a single study. Given TRMT112's role as a cofactor for many enzymes, any loss-of-function findings would be highly complicated to interpret. As noted in the previous round, we have an entire study on ALKBH8 in progress.

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We are grateful to Reviewers 1 and 2 for their in-depth consideration of the merits of the study and thoughtful responses to the requests for additional experiments, all of which we agree with.

Dr. Kate O'Connor-Giles
Brown University
Department of Neuroscience
185 Meeting Street
Providence, RI 02912
United States

Dear Kate,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Esther

Esther Schnapp, PhD
Senior Editor
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Corresponding Author Name: Kate O'Connor-Giles
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Manuscript Number: EMBOR-2023-56808

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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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

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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.
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- 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.).
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- 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?
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  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
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