EMBO reports

Specific *N-glycans* regulate an extracellular adhesion complex during somatosensory dendrite patterning

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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 **22nd Nov 2021**

Dear Dr. Bülow

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed.

Please note that we also ask from the editorial side to call out figures in a chronological order, to specify the number of experiments (biological, technical repeats) in the figure legends and to show all data (lect-2, sax-7, currently 'data not shown').

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) 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 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

- Please upload the Reagent or Resource table that is now part of the manuscript as "Reagents and Tools Table". This table can be typeset into the methods section. More information and a template for download is available at .

- Please combine the Results and Discussion section as your manuscript will be considered in our 'Reports' section. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures.

***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 is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

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.***

When submitting your revised manuscript, we will require:

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).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) 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.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines

()

6) 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:

- 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.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) Figure legends and data quantification:

The following points must be specified in each figure legend:

- 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 {less than or equal to} 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.
See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images.

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

10) Our journal 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 .

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You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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

Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

Specific N-glycans regulate an extracellular adhesion complex during somatosensory dendrite patterning

In this manuscript, Rahman and colleagues report that specific N-glycan structures can regulate dendrite patterning through modulating activity of Menorin adhesion complex. Through an enhancer screen, they isolated a loss-of-function allele of AMAN-2 with ability to enhance lec-2(weak allele) phenotypes. They further showed AMAN-2 regulated PVD dendrite patterning via modulating glycosylation of DMA-1/LRR-TM. Through carefully genetic analyses and biochemical studies, the authors concluded that alteration of glycosylation could change the interaction ability of DMA-1/LRR-TM with other members of Menorin complex in PVD dendrite patterning. Overall, it is an interesting and important work. In particularly, the data for DMA-1 glycosylation are very convincing, and the experiments were very designed and elegant. I only have some minor suggestions on the experiments and statements.

1. Does aman-2(lf) enhance dma-1(null) phenotypes?

2. In the last figure, by mutating the potential glycosylation sites in dma-1, the authors concluded "The results in an aman-2 mutant background suggest that having some type of N-glycan on site 4 of DMA-1/LRR-TM, even if abnormal, is better than having no N-glycan at all" (Page 13). However, in Figure 3 C D, mgat1(null) suppressed aman-2 phenotypes. Does this genetic data suggest that "without glycosylation" is better than "wrong glycosylation"?. Also, one missing piece of data is whether mgat1 will also suppress the abnormal N-glycans of dma-1 in aman-2(null) animals.

3. Do DMA-1 mutations examined in Figure 4 result in misfolding, expression, or localization changes of DMA-1?

4. For the final conclusion (Figure. 4E), the authors propose that the N-glycans on specific N-glycosylation sites in DMA-1/LRR-TM function permissively to maintain high-affinity binding of DMA-1/LRR-TM to other members of the Menorin complex. To reach this conclusion, some co-IP data is needed.

5. In previously published paper by the authors (Tang et al, 2019; also cited in this manuscript), the average number of 4º branches in control worms is about 25 (Figure. 1I & Figure. 2G); while they used the same marker (wdIs52) to visualize PVD neurons, in this manuscript the average number in control animals is about 30. This needs to be addressed or clarified because the average number of 4º branches in aman-2 mutant worms is about 26, similar to that in the control animals in their previous publication.

Referee #2:

In their paper Rahman et al. present compelling evidence for a novel function for Golgi alpha mannosidase II (aman2), in regulating the stereotypical dendritic patterning of the PVD C. elegans somatosensory neuron. The authors further narrow down to a major target of regulation by aman2 identifying DMA-1, a key cell adhesion protein involved in the menorin pathway. The data and the appropriate controls are presented in a concise and well-structured manner, using a clear language throughout the paper. The graphical representation of the different glycans, their glycosylation pathways and the cleavage patterns of the different drugs employed enhances the manuscripts accessibility. Their findings will be useful for scientist working in the field as an important addition to the growing list of factors shaping dendritic development. Furthermore, the data presented here emphasizes the specificity of posttranslational glycosylation and its relevance in developmental processes.
The scope as well as the quality of the data presented warrant consideration for publication in EMBO reports, provided the points below are answered.

Major comments/additional experiments
1. The overall phenotype of the aman-2 mutants should be described: is viability impaired, are there additional major visible phenotypes? The way the story is currently written, it seems as if the effect of aman-2 and of swainsonine are very specific only to the PVD dendrites. If this is correct and mutant defects are really aminly affecting PVD, are there expression data that could support this specificity?
2. The evidence provided against DMA1 transport or folding defects in Aman2 mutants is quite indirect and should therefore be down-toned a bit.
3. Some of the described differences in molecular weight shown in the western blots (Figure 3 & EV4) are quite minute. In the interest of improved visualization, can the authors try to achieve better band separation? The number of repetitions should be

indicated in the figure legend. Are there alternative methods to indicate the presence of N-glycan modifications of DMA-1 (not requiring MS on very large amounts of the protein)?

4. The quantification of the rescue experiment presented in figure 2C is not clear as presented. It would strongly profit from a table in the supplementary materials. This could help delineating the specific genotypes examined (first column, second column?) especially given that the figure legend refers to 25 animals per line.

5. The effect of swainsonine only on aman-2 mutants should be included in the experiment in figure 2F.

6. The pharmacological approach might help timing the requirement for aman-2 function. Can the compound be given at later time points during development? Can it be discontinued? Can it be given to mature animals to test whether the effect might be also on maintenance of branches?

Minor comments:

7. In the material and methods section, the parts referring to the heterologous rescue as well as the construct cloning are mostly identical in content. They could therefore be shortened to one section or refer to one another.

8. The fluorescence images as well as the traces of the PVD are lacking a scale bar throughout all figures, addition of which would increase the comparability of the figures.

9. In figure 3F the legend wrongly refers to a downward shift in the mutant while the corresponding figure and the accompanying text in the manuscript show/refer an upwards shift.

10. In figure 1 G&I the explicit genotype of the graphs is referring to fig 1 F where no genotype for the control labeled group can be found. Therefore, a specific mention of the exact control genotype would be beneficial.

11. In figure 4D, the schematic next to the DMA-1 wild type does not match the tracing and fluorescence image or vice versa. In contrast to the other two schemes they do not represent the same genotype.

12. Statistical analysis and power throughout the manuscript could potentially benefit from probing for normality of data distribution and using parametric statistics where possible. Furthermore, many of the significance statements are lacking reference to the performed post-hoc tests for comparison in between individual displayed groups.

13. There are also some typos in the text and figures or wrong reference to the Figure number. These need to be corrected, too.

Referee #3:

EMBOR-2021-54163

In this study, Rahman and colleagues combined genetic and biochemical assays to investigate whether/how N-glycosylation regulates neuronal morophogenesis in C. elegans. They show that N-glycosylation regulates the activity of the Menorin adhesion complex, and likely that of other proteins, through the acquisition of specific N-glycotypes. The study addresses an important and poorly understood question and reports interesting and novel results that will be of great interest for people working inside and outside the field.

Overall, I found that the experiments were well conducted and the results convincing and have only minor comments to improve the manuscript.

1) aman-2 mutants phenotype. I am afraid that non-specialist may find that the observed phenotype is not so obvious. The authors may want to add other measurements (e.g. length and spacing distance of Ts and quaternary dendrites) to better document the observed morphological defects.

2) Logical organization of the manuscript. I found that some parts of the manuscript were hard to follow. This is especially the case of the beginning of the Results section. The two first sections are somehow redundant and the narrative progression between these convoluted as it seems to go forth and back. Also, some of the mutants are described with dissimilar names in the two sections and in the figure legends, which also makes the flow harder to follow.
This may be a matter of personal taste but, along the same line, I would recommend calling the figures items in chronological orders where they are first described (as opposed to aggregated calls, ex to Fig2A and 3A, where they are first mentioned). This may require also changing the order of some figure panels in the supplemental figures.

3) Abstract page 2: "AMAN-2 functions cell-autonomously to ensure decoration of the neuronal transmembrane receptor DMA-

1/LRR-TM with high-mannose/hybrid N- glycans." Shouldn't this state « with complex/paucimannose N-glycans» ? Also, consider replacing "ensure" by "allow".

4) Introduction page 3: "(...) protein-protein interactions and provide information during development by way of their structural diversity (Holt & Dickson, 2005; Bülow & Hobert, 2006; Poulain & Yost, 2015; Masu, 2016). Whether the structural diversity of other classes of glycans such as N- glycans and O-glycans serve similar functions is unclear." Consider rephrasing: there are many examples of protein interactions dependent of N and O-glycan types... see for example lectins of the immune system etc.

5) Introduction page 4: «The question of whether and how specific classes of N-glycans modulate extracellular pathways or complexes during nervous system development has not been explored.» The authors may want to tone this statement down and specifically refer to the wealth of studies that have characterized the critical role of PSA-NCAM in the development and plasticity of the mammalian nervous system, and more recently on the importance of N-glycosylation in cerebellar development (Medina-Cano eLife 2018;7:e38309) and hippocampal dendrites growth and maintenance (Hanus et al., elife 2016).
The authors may also want to elaborate on the role of DSCAM in neuronal morphogenesis and on the role of N-glycans in regulating DSCAM homo-interactions (ex: Li et al. Sci. Adv. 2016; 2 : e1501118).

Also page 4 : "Together, our experiments suggest that distinct classes of N-glycans serve specific functions beyond protein folding and localization". Here also, consider rewording: there are many examples of N-glycans regulating other things than protein folding and protein localization (see Moremen et al.,, and Scott et al., for reviews).

Page 4: "correct" N-glycosylation of DMA1 ... Can the authors really exclude that this protein doesn't have other forms of N-glycans in other neurons of C.elegans or other organisms)? Consider rewording.

6) Figure EV1B... I am not a geneticist but is a rescue in 3/5 lines really enough according to the authors' field standards? How many animals were scored?

7) Figure 2E: The authors may want to comment on the weird round fluorescent structures seen in worms treated with Sw... What are those? Debris from dead neurons or protrusions?

8) Figure3 and EV3: did I miss something or PCT mutants were indeed not investigated? It was not clear to me why, as this would have allowed the authors to investigate the entire glycan maturation pathway that is depicted in Figure 3A.

9) End of page 10. « These data indicate that one or more structurally abnormal N-glycans with a terminal N-acetylglucosamine are responsible for the observed defects in PVD patterning. » The authors may want to insist on the difference that was observed between Man5Gn2 and GnMan5Gn2, which I think is very interesting. The point could also be expanded in the discussion as this observation makes sense in regard to the fact that Man5Gn2 is particularly prevalent in the mammalian brain and neurons (ex : Hanus et al., elife 2016, Riley et al., Nat Commun 10, 1311 (2019). https://doi.org/10.1038/s41467-019-09222-w).

And also:
10) Figure 1A scale bar is missing.
11) Introduction : Congenital disorders of glycosylation -> congenital (?)
12) Page 4 spell out PVD
13) May be a few words on PVD neurons would be useful...
14) Page 5 : "complete loss of function (...) early stop codon." The authors may want to add « in the key enzymatic domain of the protein ».
15) Page 7 : why not show data for the lack of effect on lect2 sax 7 localization?
16) Figure2C: why two sets of numbers: 2 lines for each?
17) Page 9 last paragraph: Consider rewording into "therefore required for normal branching of PVD dendrites" as some branching still occurs without these glycans it seems..
18) Page 11: "The upward shift in DMA-1 size following the loss of aman-2 is consistent with our genetic data establishing that the presence of larger, abnormal GnMan5Gn2 N-glycans gives rise to the PVD mutant phenotype." The authors may want to be more convervative here (I am not convinced that the assay is resolutive enough to infer about size or the influence of specific sugar residues as GlcNAc which may also impact migration in other ways...).
19) Figure EV4: shift with PNGase for SAX7 and lect2 really not obvious (most especially that of lect2).. ... A better separation may help seeing this better.
Also, it would be nice to be reminded what are the expected molecular weight of the proteins.
20) Page12: "suggesting that they did not carry abnormal N-glycans in an aman-2 mutant background." This could also be due to the low resolution of the assay as well, could it not?
21) "Collectively, our data show that among the N-glycosylated proteins of the Menorin complex, only DMA-1/LRR-TM was significantly affected by the loss of aman- 2/Golgi alpha-mannosidase II and carried altered N-glycans in aman-2 null animals."
22) Pages 12-13 and figure 4: "Lastly, the mutant phenotype resulting from a presumptive loss of all N-glycans on DMA-1 (S1234) is enhanced in an aman-2 mutant background, suggesting that N-glycosylated proteins other than DMA-1 may serve additional functions during PVD morphogenesis or that abnormal N-glycans on cryptic N- glycosylation sites further compromise

function (Fig.4C)."
I would put this comment first, but that is a matter of personal taste.
23) Figure 4E: I find the term "stability" misleading: the stability of the protein complex has not been assessed in the present study: consider replacing with "branching defect" or a descriptor closer to the experimental assay.

Referee #1:

Specific N-glycans regulate an extracellular adhesion complex during somatosensory dendrite patterning

In this manuscript, Rahman and colleagues report that specific N-glycan structures can regulate dendrite patterning through modulating activity of Menorin adhesion complex. Through an enhancer screen, they isolated a loss-of-function allele of AMAN-2 with ability to enhance lec-2(weak allele) phenotypes. They further showed AMAN-2 regulated PVD dendrite patterning via modulating glycosylation of DMA-1/LRR-TM. Through carefully genetic analyses and biochemical studies, the authors concluded that alteration of glycosylation could change the interaction ability of DMA-1/LRR-TM with other members of Menorin complex in PVD dendrite patterning. Overall, it is an interesting and important work. In particularly, the data for DMA-1 glycosylation are very convincing, and the experiments were very designed and elegant. I only have some minor suggestions on the experiments and statements.

We thank the reviewer for these positive and constructive comments, to which we respond below in detail.

1. Does aman-2(lf) enhance dma-1(null) phenotypes?

We appreciate this comment — we have in fact conducted this experiment and found that the *dma-1; aman-2* double null mutant looked indistinguishable from the *dma-1(null)* phenotype of PVD, suggesting that the two genes may function in the same genetic pathway. Since we know that the dma-1 phenotype is genetically enhanceable (e.g. with clr-1, Liu et al., Dev Bio, 2016), this is important new data demonstrating that both genes function in the same pathway. This is now included in Fig. EV1F and on p.6

2. In the last figure, by mutating the potential glycosylation sites in dma-1, the authors concluded "The results in an aman-2 mutant background suggest that having some type of N-glycan on site 4 of DMA-1/LRR-TM, even if abnormal, is better than having no N-glycan at all" (Page 13). However, in Figure 3 C D, mgat1(null) suppressed aman-2 phenotypes. Does this genetic data suggest that "without glycosylation" is better than "wrong glycosylation"?. Also, one missing piece of data is whether mgat1 will also suppress the abnormal N-glycans of dma-1 in aman-2(null) animals.

In *mgat-1(null); aman-2(null) mnr-1(hyp)* animals (Figure 3D), a core *N*-glycan chain is likely still present on the sites of DMA-1, as upstream enzymes remain functional and thus wild type high-mannose *N*-glycans are still being formed (Figure 3A). Therefore, we are unable to classify this data as “without glycosylation” like we do in Figure 4C, where we block glycosylation of specific sites by mutation.
Thank you for the suggestion of testing whether loss of MGAT1 also suppresses the defects of *dma-1* point mutants in an *aman-2* mutant background. We created this quintuple mutant (+ reporter = 6 loci!) and now include the data in Figure 4 and page 13. We find that the loss of MGAT1 also suppresses the mutant phenotype of *dma-1(*S4*)* in *aman-2(null)* animals, likely because this prevents the abnormal *N*-glycans on site 123 of DMA-1 from forming. This reinforces the role of abnormal glycans in causing the mutant PVD phenotypes and the interplay between having occupied sites and mutant sites. Additionally, we now also include in Figure 3 and page 10, how *mgat-1(null)* also suppress the defects in quaternary dendrite formation in

aman-2(null) animals alone. Together, we believe that these two additional genetic experiments do strengthen our conclusions.

3. Do DMA-1 mutations examined in Figure 4 result in misfolding, expression, or localization changes of DMA-1?

The reviewer raises an important point, which is however not trivial to address experimentally, because endogenously tagged DMA-1 with GFP is rather weak (Dong et al, eLife, 2016). While we cannot formally exclude these possibilities, we consider them less likely, because if the effects of the point mutants were 'unspecific' (ie the result of misfolding, expression, or localization), we would expect the point mutants to be behave similarly in relation to each other independently of the genetic background. However, this is not the case (compare Fig.4B & C). We inserted a sentence to that effect on p.12 "We cannot formally exclude that these mutations affect stability, transport or folding of the mutant DMA-1/LRR proteins, but consider this less likely, because point mutants displayed different behaviors in different genetic backgrounds (see below)."

4. For the final conclusion (Figure. 4E), the authors propose that the N-glycans on specific N-glycosylation sites in DMA-1/LRR-TM function permissively to maintain high-affinity binding of DMA-1/LRR-TM to other members of the Menorin complex. To reach this conclusion, some co-IP data is needed.

We concur with the reviewer that ideally quantitative protein/protein interaction data would be desirable, although we are not sure whether Co-IP would be the best approach as Co-IPs are at best semi-quantitative. We believe that more involved, quantitative approaches such as single molecule pull downs or cell-cell interaction assays could address this question experimentally, but believe that this would go beyond the scope of the current paper. However, to prevent the impression that this is the only possible explanation of our genetic and biochemical data, we have included the following sentence: "Alternatively, the affinity of the proteins in the complex could be increased by different *N*-glycans, or modified in their activity by other means".

5. In previously published paper by the authors (Tang et al, 2019; also cited in this manuscript), the average number of 4º branches in control worms is about 25 (Figure. 1I & Figure. 2G); while they used the same marker (wdIs52) to visualize PVD neurons, in this manuscript the average number in control animals is about 30. This needs to be addressed or clarified because the average number of 4º branches in aman-2 mutant worms is about 26, similar to that in the control animals in their previous publication.

We appreciate the comment by the reviewer because this is an important clarification to make. In the current manuscript, the developmental stage of worms quantified was older than in Tang et al, 2019, causing this difference in number of 4º branches. Here, we observe young adult animals, while in the previous publication, younger L4 animals were quantified. This has been clarified in the Materials and Methods.

Referee #2:

In their paper Rahman et al. present compelling evidence for a novel function for Golgi alpha

mannosidase II (aman2), in regulating the stereotypical dendritic patterning of the PVD C. elegans somatosensory neuron. The authors further narrow down to a major target of regulation by aman2 identifying DMA-1, a key cell adhesion protein involved in the menorin pathway. The data and the appropriate controls are presented in a concise and well-structured manner, using a clear language throughout the paper. The graphical representation of the different glycans, their glycosylation pathways and the cleavage patterns of the different drugs employed enhances the manuscripts accessibility. Their findings will be useful for scientist working in the field as an important addition to the growing list of factors shaping dendritic development. Furthermore, the data presented here emphasizes the specificity of posttranslational glycosylation and its relevance in developmental processes.
The scope as well as the quality of the data presented warrant consideration for publication in EMBO reports, provided the points below are answered.

We thank the reviewer for these positive and constructive comments, to which we respond below in detail.

Major comments/additional experiments
1. The overall phenotype of the aman-2 mutants should be described: is viability impaired, are there additional major visible phenotypes? The way the story is currently written, it seems as if the effect of aman-2 and of swainsonine are very specific only to the PVD dendrites. If this is correct and mutant defects are really aminly affecting PVD, are there expression data that could support this specificity?

Thank you for this suggestion. We observed no visible phenotypes in *aman-2* mutants in terms of uncoordinated movement (unc), small size (sma), or dumpy (dpy) worms. Moreover, the viability of aman-2 mutant animals is not obviously impaired, as they do not grow slowly or display an obvious difference in brood size. A quantification of brood size we have now included in Figure EV1 and page 5.
AMAN-2 has previously been reported to be expressed ubiquitously (Paschinger et al. JBC, 2006), and it is possible that other cells may be affected in aman-2 mutant animals. This possibility has been added to the discussion. Interestingly, expression data from the CeNGEN project (Taylor et al. Cell, 2021) reveals that AMAN-2 is most highly expressed in PVD neurons, consistent with an important function in this neuron. We have added a reference to this fact on page 14.

2. The evidence provided against DMA1 transport or folding defects in Aman2 mutants is quite indirect and should therefore be down-toned a bit.

We have toned-down these conclusions on p.7.

3. Some of the described differences in molecular weight shown in the western blots (Figure 3 & EV4) are quite minute. In the interest of improved visualization, can the authors try to achieve better band separation? The number of repetitions should be indicated in the figure legend. Are there alternative methods to indicate the presence of N-glycan modifications of DMA-1 (not requiring MS on very large amounts of the protein)?

We have made our best attempt to achieve the clearest separation possible by running the gels for long periods of time and by using different gradient gels. We eventually settled on a 4-12% polyacrylamide gels, which allowed reasonable separation. We now include the number of

replicates in the legends. An alternative method to determine *N*-glycans on proteins is mass spectrometry approaches. However, the amounts of DMA-1 in worm lysates are rather small (the protein is not visible in Western Blots and only following immunoprecipitation), and therefore likely below the detection limit.

4. The quantification of the rescue experiment presented in figure 2C is not clear as presented. It would strongly profit from a table in the supplementary materials. This could help delineating the specific genotypes examined (first column, second column?) especially given that the figure legend refers to 25 animals per line.

Thank you for this suggestion. Figure 2C and its legend have been amended for clarity.

5. The effect of swainsonine only on aman-2 mutants should be included in the experiment in figure 2F.

Thank you for this suggestion. This has been included in Figure 2F and 2G.

6. The pharmacological approach might help timing the requirement for aman-2 function. Can the compound be given at later time points during development? Can it be discontinued? Can it be given to mature animals to test whether the effect might be also on maintenance of branches?

The reviewer raises interesting points, i.e., the timing of *aman-2* function. One technical issue with the pharmacological approach is that we know little about the pharmacokinetics of this compound in worms, making it challenging at present to draw firm conclusions about timing. An alternative approach would be to flox the *aman-2* allele and use transgenic, heatshock-inducible Cre expression to knock out *aman-2* function at different developmental time points. We believe that pursuing either approach, would require substantial experimentation to establish the necessary bench marks, and therefore would be beyond the scope of the current paper.

Minor comments:

7. In the material and methods section, the parts referring to the heterologous rescue as well as the construct cloning are mostly identical in content. They could therefore be shortened to one section or refer to one another.
Thank you. We have shortened it to one section on page 31.

8. The fluorescence images as well as the traces of the PVD are lacking a scale bar throughout all figures, addition of which would increase the comparability of the figures.
We have now added scale bars to all the images and tracings.

9. In figure 3F the legend wrongly refers to a downward shift in the mutant while the corresponding figure and the accompanying text in the manuscript show/refer an upwards shift.
We have corrected this typo accordingly.

10. In figure 1 G&I the explicit genotype of the graphs is referring to fig 1 F where no genotype for the control labeled group can be found. Therefore, a specific mention of the exact control

genotype would be beneficial.
We have now clarified the genotype for the control in the figure legend.

11. In figure 4D, the schematic next to the DMA-1 wild type does not match the tracing and fluorescence image or vice versa. In contrast to the other two schemes they do not represent the same genotype.
We have now changed to a separate panel for added clarity.

12. Statistical analysis and power throughout the manuscript could potentially benefit from probing for normality of data distribution and using parametric statistics where possible. Furthermore, many of the significance statements are lacking reference to the performed post-hoc tests for comparison in between individual displayed groups.
This has been clarified in the Material and Methods, where we indicate the post-hoc tests performed in GraphPad Prism. We have added statistical specifics to all legends.

13. There are also some typos in the text and figures or wrong reference to the Figure number. These need to be corrected, too.
Thank you. We have gone through corrected them to the best of our abilities.

Referee #3:

EMBOR-2021-54163

In this study, Rahman and colleagues combined genetic and biochemical assays to investigate whether/how N-glycosylation regulates neuronal morophogenesis in C. elegans. They show that N-glycosylation regulates the activity of the Menorin adhesion complex, and likely that of other proteins, through the acquisition of specific N-glycotypes. The study addresses an important and poorly understood question and reports interesting and novel results that will be of great interest for people working inside and outside the field.

Overall, I found that the experiments were well conducted and the results convincing and have only minor comments to improve the manuscript.

We thank the reviewer for these positive and constructive comments, to which we respond below in detail.

1) aman-2 mutants phenotype. I am afraid that non-specialist may find that the observed phenotype is not so obvious. The authors may want to add other measurements (e.g. length and spacing distance of Ts and quaternary dendrites) to better document the observed morphological defects.

We have now tried to clarify this phenotype by indicating the branches quantified and shown as full tertiaries, instead of using the more ambiguous “Ts”.

2) Logical organization of the manuscript. I found that some parts of the manuscript were hard to follow. This is especially the case of the beginning of the Results section. The two first sections are somehow redundant and the narrative progression between these convoluted as it seems to go forth and back. Also, some of the mutants are described with dissimilar names in the two sections and in the figure legends, which also makes the flow harder to follow.

This may be a matter of personal taste but, along the same line, I would recommend calling the figures items in chronological orders where they are first described (as opposed to aggregated calls, ex to Fig2A and 3A, where they are first mentioned). This may require also changing the order of some figure panels in the supplemental figures.

The consistency of allele names has been corrected, and we have fixed the chronological order of the figures in the text for hopefully a better flow. As for the order of the first paragraphs, we prefer to keep as is, because we want to make distinct points in both sections. 1. The identification of *aman-2* as a new gene important for dendrite morphogenesis and 2. its genetic placement in the Menorin pathway.

3) Abstract page 2: "AMAN-2 functions cell-autonomously to ensure decoration of the neuronal transmembrane receptor DMA-1/LRR-TM with high-mannose/hybrid N-glycans." Shouldn't this state « with complex/paucimannose N-glycans» ? Also, consider replacing "ensure" by "allow".

Thank you for this comment, because the way we had written the sentence was actually ambiguous and maybe non-intuitive, given that the normal function of *aman-2* is to synthesize complex and paucimannose type glycans. However, we show biochemically that DMA-1 has normally high-mannose/hybrid/paucimannose glycans, which change to high-mannose/hybrid only, in an *aman-2* mutant. Therefore, we have amended the sentence to now read “AMAN-2 functions cell-autonomously to allow decoration of the neuronal transmembrane receptor DMA-1/LRR-TM with the correct set of high-mannose/hybrid/paucimannose *N*-glycans.” “Ensure” has been replaced with “allow”.

4) Introduction page 3: "(...) protein-protein interactions and provide information during development by way of their structural diversity (Holt & Dickson, 2005; Bülow & Hobert, 2006; Poulain & Yost, 2015; Masu, 2016). Whether the structural diversity of other classes of glycans such as N- glycans and O-glycans serve similar functions is unclear." Consider rephrasing: there are many examples of protein interactions dependent of N and O-glycan types... see for example lectins of the immune system etc.

We have removed this sentence here and discuss the effects of *N*-glycosylation on protein-protein interactions in other places.

5) Introduction page 4: «The question of whether and how specific classes of N-glycans modulate extracellular pathways or complexes during nervous system development has not been explored.» The authors may want to tone this statement down and specifically refer to the wealth of studies that have characterized the critical role of PSA-NCAM in the development and plasticity of the mammalian nervous system, and more recently on the importance of N-glycosylation in cerebellar development (Medina-Cano eLife 2018;7:e38309) and hippocampal dendrites growth and maintenance (Hanus et al., elife 2016).
The authors may also want to elaborate on the role of DSCAM in neuronal morphogenesis and on the role of N-glycans in regulating DSCAM homo-interactions (ex: Li et al. Sci. Adv. 2016; 2 : e1501118).

We have expanded the introduction to now include Hanus et al., which we had unintentionally omitted. We also mention polysialic acid modifications of *N*-glycans and its role in modulating adhesive properties of cell adhesion molecules. The corresponding section now reads: “Studies in vertebrates and invertebrates have shown that mutants that compromise *N*-glycan biosynthesis or *N*-glycan attachment result in defects in cell surface localization of cell adhesion

molecules and axon guidance cues (Medina-Cano et al., 2018, Sekine et al., 2013, Mire et al., 2018). Moreover, *N*-glycosylation *per se* has been shown to be important for dendrite development in dissociated rat neurons (Hanus et al., 2016), and the addition of polysialic acid chains to *N*-glycans can change the binding properties of cell adhesion molecules (reviewed in (Schnaar et al., 2014)). However, the question of whether and how specific classes and structures of *N*-glycans modulate extracellular pathways or complexes during nervous system development *in vivo* remains understudied."

The Li et al. study is interesting because it provides structural evidence for the involvement of *N*-glycans in dimerization of cell adhesion molecules. We now cite this paper in the context of two other papers (Fogel et al, 2010, Labasque et al, 2014), which show a role for *N*-glycans in protein-protein interactions. The corresponding sentence now reads "On the other hand, structural studies and *in vitro* experiments suggested that *N*-glycans can regulate protein-protein interactions of cell adhesion molecules (Fogel et al., 2010, Labasque et al., 2014, Li et al., 2016)."

Also page 4 : "Together, our experiments suggest that distinct classes of N-glycans serve specific functions beyond protein folding and localization". Here also, consider rewording: there are many examples of N-glycans regulating other things than protein folding and protein localization (see Moremen et al.,, and Scott et al., for reviews).

Thank you for this comment – we recognize the (inappropriately) limiting nature of the original sentence. This has been reworded to now read more generally "Together, our experiments suggest that distinct classes of *N*-glycans rather than *N*-glycosylation *per se* serve specific functions in dendrite branching, and contribute to developmental specificity during neuronal morphogenesis."

Page 4: "correct" N-glycosylation of DMA1 ... Can the authors really exclude that this protein doesn't have other forms of N-glycans in other neurons of C.elegans or other organisms)? Consider rewording.

This sentence has been updated to specify in PVD neurons.

6) Figure EV1B... I am not a geneticist but is a rescue in 3/5 lines really enough according to the authors' field standards? How many animals were scored?

We scored 20 animals per line, for a total of 5 lines. While 3/5 rescue is only partial rescue, the conclusion that the observed defects in dendrite patterning are due to loss of *aman-2* function is also supported by four additional and independently obtained alleles of *aman-2*, and cell specific rescue experiments. Together, these observations make a very strong argument that the dendritic defects we observe are a result of loss of *aman-2* function.

7) Figure 2E: The authors may want to comment on the weird round fluorescent structures seen in worms treated with Sw... What are those? Debris from dead neurons or protrusions?

Thank you for reminding us to point these out. These circles are gut autofluorescence visible in the GFP and RFP channels. This has been clarified and indicated in the figure legends.

8) Figure3 and EV3: did I miss something or PCT mutants were indeed not investigated? It was not clear to me why, as this would have allowed the authors to investigate the entire glycan maturation pathway that is depicted in Figure 3A.

We would have loved to test PCT mutant(s) as well. However, there are 30+ genes encoded in the worm with possible homology to PCT genes, and the "real" functional homologs in *C. elegans* have not been identified. Because of this uncertainty, we have to date not pursued this line of investigation.

9) End of page 10. « These data indicate that one or more structurally abnormal N-glycans with a terminal N-acetylglucosamine are responsible for the observed defects in PVD patterning. » The authors may want to insist on the difference that was observed between Man5Gn2 and GnMan5Gn2, which I think is very interesting. The point could also be expanded in the discussion as this observation makes sense in regard to the fact that Man5Gn2 is particularly prevalent in the mammalian brain and neurons (ex : Hanus et al., elife 2016, Riley et al., Nat Commun 10, 1311 (2019). https://doi.org/10.1038/s41467-019-09222-w).

Thank you for this suggestion. We now cite both papers in the manuscript.

And also:
10) Figure 1A scale bar is missing.
We have now added scale bars to all the images and tracings.

11) Introduction : Congenital disorders of glycosylation -> congenital (?)
Thank you. We have corrected this on page 3.

12) Page 4 spell out PVD
PVD stands for (Posterior cell body, Ventral cord process D) – now spelled out on first mention.

13) May be a few words on PVD neurons would be useful...
We agree and have included the functions of PVD neurons on page 4.

14) Page 5 : "complete loss of function (...) early stop codon." The authors may want to add « in the key enzymatic domain of the protein ».
We have incorporated this suggestion on page 5.

15) Page 7 : why not show data for the lack of effect on lect2 sax 7 localization?
We have now included these images in Figure EV2, panels F & G.

16) Figure2C: why two sets of numbers: 2 lines for each?
We agree that this is unclear and have clarified that the left column is data for the lect-2 aman-2 double mutant, while the right column is for the mnr-1 aman-2 double mutant.

17) Page 9 last paragraph: Consider rewording into "therefore required for normal branching of PVD dendrites" as some branching still occurs without these glycans it seems..

Thank you. We have incorporated this suggestion on page 9.

18) Page 11: "The upward shift in DMA-1 size following the loss of aman-2 is consistent with our genetic data establishing that the presence of larger, abnormal GnMan5Gn2 N-glycans gives rise to the PVD mutant phenotype." The authors may want to be more convervative here (I am not convinced that the assay is resolutive enough to infer about size or the influence of specific sugar residues as GlcNAc which may also impact migration in other ways...).

We have removed the reference to “larger” glycans. We believe it is safe to say that the change in mobility is consistent with abnormal glycans. The sentence now reads “The upward shift in DMA-1 size following the loss of *aman-2* is consistent with our genetic data establishing that the presence of abnormal GnMan5Gn2 *N*-glycans gives rise to the PVD mutant phenotype."

19) Figure EV4: shift with PNGase for SAX7 and lect2 really not obvious (most especially that of lect2).. ... A better separation may help seeing this better.
The size shifts are small (but clear in our opinion), and we tried our best to maximize the separation by running the gels for long periods of time and with different gradients. We included in the figure the most separation we were able to resolve. It should be noted that both proteins have also been shown by mass spec to be *N*-glycosylated, a fact we refer to on p.12 (Kaji et al., 2007).

Also, it would be nice to be reminded what are the expected molecular weight of the proteins.
We have included this is the figure legends for Figures 3 and EV4.

20) Page12: "suggesting that they did not carry abnormal N-glycans in an aman-2 mutant background." This could also be due to the low resolution of the assay as well, could it not?
We appreciate this caveat and have toned down this conclusion to now read “they may not carry abnormal *N*-glycans”.

21) "Collectively, our data show that among the N-glycosylated proteins of the Menorin complex, only DMA-1/LRR-TM was significantly affected by the loss of aman- 2/Golgi alpha-mannosidase II and carried altered N-glycans in aman-2 null animals."
We are not entirely sure what to make of this comment/reference.

22) Pages 12-13 and figure 4: "Lastly, the mutant phenotype resulting from a presumptive loss of all N-glycans on DMA-1 (S1234) is enhanced in an aman-2 mutant background, suggesting that N-glycosylated proteins other than DMA-1 may serve additional functions during PVD morphogenesis or that abnormal N-glycans on cryptic N- glycosylation sites further compromise function (Fig.4C)."
I would put this comment first, but that is a matter of personal taste.
We have changed the order of the observations as suggested.

23) Figure 4E: I find the term "stability" misleading: the stability of the protein complex has not been assessed in the present study: consider replacing with "branching defect" or a descriptor closer to the experimental assay.
We have toned down the conclusion and now say “function” of the complex in Figure 4E. We also provide alternative explanations in both the main text and figure legend to underscore the point that a decrease in complex stability may not be the only explanation.

Dear Dr. Bülow

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the reports from the referees who were asked to assess it (copied below).

As you will see, both referees are very positive about the revised study and recommend publication in EMBO Reports.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

- Thank you for submitting source data for all quantifications. Please combine all source data into one file per figure, e.g., by combining the .xls files into one file with several sheets. Please also double-check the naming of the files. In a spot check I noticed that you supplied source data for Fig. 1B, D, G but panel (D) shows no quantification while the data for (E) are missing.

- Please update the 'Conflict of interest 'paragraph to our new 'Disclosure and competing interests statement'. For more information see
https://www.embopress.org/page/journal/14693178/authorguide#conflictsofinterest

- References: The author names should not be in uppercase, the year needs to be in brackets and if there are more than 10 authors, please list only the first 10 followed by et al.

- Please add a callout to Fig. 4F.

- Please sort the following manuscript sections that follow the Results and Discussion part according to these guidelines and please remove the header 'Conclusions':
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We look forward to seeing a final version of your manuscript as soon as possible.

Kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

the authors have addressed all my questions. Congratulations on a beautiful study !

Referee #2:

I appreciate the revision work done by the authors and now support publication of the manuscript in the present revised version.

The authors have addressed all minor editorial requests.

Hannes Bülow
Albert Einstein College of Medicine
United States

Dear Dr. Bülow,

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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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.
- 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.

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?
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  - 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Materials & Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	no data points were excluded
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figures
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figures

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