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m6A is required for resolving progenitor identity during planarian stem cell differentiation

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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 **6th Dec 2021**

Dear Dr Wurtzel,

Thank you again for the submission of your manuscript (EMBOJ-2021-109895) to The EMBO Journal. Please accept my sincere apologies for the unusual delay with the peer-review of your manuscript. Your study has been sent to two reviewers for evaluation, and we have received reports from both of them, which I enclose below.

As you will see from their comments, the referees acknowledge the potential interest and value of your findings, although they also express major concerns. In more detail, referee #1 states
that a more extensive analysis of m6A dependent tissue alterations as well as complementary m6A pathway components is required to make the study conclusive (ref#1, pts 2,4,7,8). Referee #2 agrees in that a more rigorous characterisation of the RNAi scenario should be provided, including important validatory experiments (ref#2, pts 1-3).

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of the reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

Please feel free to contact me if you have any questions or need further input on the referee comments.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When submitting your revised manuscript, please carefully review the instructions below.

Thank you for the opportunity to consider your work for publication.
I look forward to your revision.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

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Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

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).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response 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 (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). 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.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).
In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.
*** Note - All links should resolve to a page where the data can be accessed. ***

7) 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 .

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can 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 or a single pdf per main figure 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 at .

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). 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 labelled 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.

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11) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

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See also guidelines for figure legends: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines
(https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
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Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 6th Mar 2022:

Link Not Available

Referee #1:

Dagan and colleagues aim to decipher the function of the m6A pathway in planarian flatworms, a model system for stem-cell driven regeneration. Following the discovery of all essential components of the m6A pathway in planarians, the authors study the role of individual m6A pathway genes on planarian homeostasis and regeneration. They find three components of the m6A pathway (mettl14, kiaa1429 and ythdc-1) to show severe, eventually lethal phenotypes, a finding they then follow up by mapping m6A-enriched regions in the planarian transcriptome and by uncovering a role of the m6A pathway in several important aspects of stem cell (neoblast) biology, such as the expression of polyadenylated histone variants, the coordinated expression of pseudogene clusters and the emergence of a novel cell cluster after m6A RNAi using single-cell sequencing.

Despite the primary weakness of the study at hand, which lies in an only indirect connection between the m6A mark and most of the described cellular disturbances, such as the changes in histone-mRNA polyadenylation, the expression of pseudogenes, and the connection to CHD4, I see the study by Dagan et al. as a major step forward in understanding the role of the epitranscriptomic mark m6A in planarian regeneration. Thus, I strongly recommend to invite a revision of the manuscript. Please see below the points that, in my opinion, need to be addressed during the revision process.

Major points:
• To aid comparability across the planarian community and to prevent contig-derived ambiguity, I urge the authors to include SMESGs in their study (e.g. in Suppl. Fig. 1).
• Line 119: The authors rightly mention that variable and inefficient knockdowns are an impediment to unequivocally uncovering essential genes in planarians. In light of this statement and the available preprint by Cui et al. (bioRxiv, 2021), which identifies wtap as an essential m6A pathway component in planarians, I urge the authors to experimentally revisit wtap in their experimental system and to discuss gained results in light of their own study.
• Fig. 2D (lines 162-163): I do not follow the authors line of reasoning regarding the observed broader peak distribution in their planarian m6A IP data. Why do the authors not discuss the obvious? - a worse resolution of their m6A enrichment data due to

multiple imaginable technical problems, e.g. a lack of antibody specificity coupled to the AT-rich planarian genome? Another explanation that comes to mind is that m6A peaks in planarians may more often occur in spatial proximity. Due to the coarse resolution of the presented data, I therefore suggest that the authors tune down their claims, in particular regarding a diverged m6A consensus motif in planarians.
• Fig. 4C: The authors show that gene expression changes correlate between mettl14 and ythdc1 knockdown conditions. How about the observed gene expression changes under kiaa1429 RNAi conditions? The authors need to address this open question, especially in light of the subsequent kiaa1429 RNAi experiments.
• Fig. 4E: Does a knockdown of mettl14 and ythdc1 show the same effects here? The authors need to show this experimentally here, especially given the knockdown variability in planarians and potential discrepancies to other studies (see Cui et al., bioRxiv 2021). Moreover, by showing similar effects after a mettl14, ythdc1 or kiaa1429 knockdown, the authors can substantiate their claim that intestinal cell depletion is an m6A-related phenotype and not related to other downstream effects of kiaa1429 RNAi.
• Fig. 5 (lines 343-361): Can the authors exclude effects of m6A on other polyA- transcripts (e.g. outside of the histone family)? A discussion of this possibility should be included at appropriate position in their study.
• Fig. 5A (lines 301-310): The authors connect effects of kiaa1429 RNAi (reduction of intestine cells) to observations after mettl14 RNAi (upregulation of histone encoding genes). This raises the following questions (as mentioned before regarding Figs. 4C+E): a) Do the gene expression changes under kiaa1429 RNAi conditions correlate with mettl14 and ythdc1 RNAi conditions. b) Is intestine cell depletion also found in mettl14 & ythdc1 RNAi animals? c) Fig. 5B shows that kiaa1429 RNAi leads to a dramatic overexpression of polyadenylated histone genes. Overexpression under mettl14 RNAi conditions can be mentioned, however, to aid the reader histone gene expression changes should be primarily connected to kiaa1429 RNAi conditions.
• Fig. 5F-G: As it stands, the authors need to characterize the overexpressed gene cluster further. Which pseudogenes are found in this cluster? What can be deduced by sequence analysis? Moreover, I dearly missed a connection to m6A-enriched peaks here. The authors need to connect the deregulated gene cluster to m6A marks, as they did in case of histone mRNAs (which the authors find, presumably to their disappointment, not to contain m6A marks).
• Fig. 7: Using a systematical analysis of planarian RNAseq datasets to identify the molecular function of m6A in planarians is an exciting idea. However, I miss any mentioning of signal/noise in this analysis. Was CHD4 the only candidate the authors found connected to the m6A pathway? In which way was CHD4 signal superior to all other possibilities? The authors need to include details regarding their choice for CHD4 in their manuscript.
• Fig. 7A: Regarding the IGV tracks for CHD4 RNAi conditions, only the bottom one seems to show an upregulation of the depicted gene cluster. The track on the top seems to display expression levels equal to control tracks. Having only one of two replicates show an effect is inconclusive and might be due to technical variation in library preparation. Thus, more convincing CHD4 RNAi data (triplicates) need to be presented in this figure, in order for the authors to be able to support their claims.

Minor points:
• Line 91: language: "expression of genes ... were expressed"
• Lines 158-160: The authors should better connect the fact that planarian 28S rRNA does not carry m6A marks to their findings concerning the presence of m6A in polyA-RNA vs. non-polyA-RNA (Fig. 1D). It would aid the instant understanding of their data if the reader knew about this planarian peculiarity before looking at Fig. 1D.
• Fig. 2B: The figure is supposed to show data for 740 high-confidence peaks. Does high-confidence mean 5-fold enrichment as mentioned in Suppl. Note 5? If yes, I suggest to indicate this in the figure description, e.g. "740 high-confidence peaks (>= 5-fold enriched)".
• Fig. 3D (line 204): What is the p-value associated with "significant upregulation" here?

Referee #2:

Summary:

In the manuscript 'm6A is required for resolving progenitor identity during planarian stem cell differentiation,' the authors present a series of very interesting experiments that support a model in which mRNA base-modification m6A is required for fate choice and cellular maturation in planarian stem cells. Perturbation of m6A pathway components resulted in progressive deterioration of tissues and animal death, increased expression of non-canonical histone variants, and accumulation of un-differentiated cells. A particular strength of the paper is the extensive sequencing analysis done on control vs. kiaa1429 RNAi treated animals, including single cell sequencing of ~20,000 cells. These sequencing studies provided additional support for the authors assertions that the stem cell population was not depleted, but stem cell differentiation and lineage specification was altered in the animals. In addition, single cell sequencing identified a previously un-described cell state that accumulated in RNAi treated animals and that expressed genes commonly expressed in neurons, glia, differentiated cells, as well as clusters of genes over-expressed after kiaa1429 RNAi treatment. Authors also provide evidence that depletion of NuRD component CHD4 results in a similar phenotype, but were unable to provide evidence that either pathway regulates the other.

Altogether, these results represent one of the first descriptions of mRNA modification in planaria and establish that mRNA modification is a critical regulator of stem cell differentiation and lineage specification in planarian stem cells. The manuscript is also the first to demonstrate m6A-seq2 in planaria, an important tool for the field. Thus, the paper will be of interest to both planarian researchers and the broader stem cell biology community. The discussion and analysis of the sequencing results is well done, but the paper's conclusions would be better supported with more rigorous characterization of the RNAi phenotypes

and additional quantitation and validation of predicted gene expression changes in vivo. In particular, the altered gut morphology observed after depletion of m6A pathway components is only minimally characterized and the novel cell state described by single cell sequencing is not adequately visualized or quantitated in the animal.

Major Criticisms:
1. Gut morphology phenotypes downstream of m6A pathway inhibition are insufficiently characterized given the correlation between intestinal branching and animal size/feeding. Would it be possible to quantitate branching or intestinal cell number per area/length of worm? Some attempt to control for animal size, quantify gene expression or cell abundance by FISH, or provide additional images (more individual animals, both max projections and z slices, etc.) would strengthen the claims in the text currently supported only by figure 4E.

2. Claims made by authors regarding stem cell proliferation/cell cycle following depletion of kiaa1429 are mostly based on FACS analysis (Figure 3E, F). In particular, the authors conclude that kiaa1429 RNAi results in an increase in immature post-mitotic progenitors based on the larger X2 cell abundance. This claim would be better supported by measuring progenitor abundance in vivo by FISH and/or quantitation of cell cycle indicators in vivo (EdU, phospho-Histone 3, PCNA, etc.). The authors could also test this model more explicitly during the discussion and analysis of the scRNAseq datasets.

3. Overall, claims made based on scRNAseq data should be validated more rigorously in vivo. These claims include those regarding stem cell differentiation, gut specification, and the identification of a kiaa1429 RNAi-specific cell state. Single cell sequencing data presented in Figure 6D should be validated in vivo by in situ hybridization and quantitation of cellular abundance. Abundance of cells expressing intestinal markers in Figure 6E could also be quantitated. Finally, co-expression of the repetitive gene clusters and smad6/7-2 and protocadherin-like gene dd_15376 in the kiaa1429 specific cluster is asserted in the text, but is not demonstrated in figure 6 or supplementary figure 5. Addition of neural and glial markers into Figure 6F and in vivo validation of co-localization by FISH would support this claim.

4. In my opinion, the results section regarding a connection between the m6A pathway and the NuRD complex is presented with a bias towards a model in which m6A regulates NuRD. While both RNAi depletions result in similar phenotypes, neither has been demonstrated to regulate the other and I think that the authors statement that 'the observation that CHD4 (RNAi) animals develop phenotypes more rapidly than following inhibition of m6A genes supports the possibility that inhibition of m6A progressively deteriorates NuRD activity' is too strong. There are many models that are consistent with this observation that are not discussed. In the absence of additional evidence, I would recommend revising the results section to more fully acknowledge the possibility that NuRD and m6A regulate similar processes independently. The discussion section does a much better job balancing these two models (Figure 8).
5. In the abstract, authors claim that m6A is critical for planarian stem cell homeostasis and gene regulation in regeneration, but most of their analysis of gene regulation was done at homeostasis and not during regeneration. This sentence could be changed to more accurately reflect the data in the manuscript.

Minor Criticisms:
1. If stem cells are not depleted by inhibition of m6A pathway components, what is the cause of tissue degradation/lysis? While experiments to address this question may be beyond the scope of the manuscript, it would be interesting for the authors comment on this when discussing their models.
2. A size quantitation of kiaa1429 RNAi animals compared to controls should be added to Supplementary Figure 1F and J.
3. In Figure 2B, a visualization that allows for comparison of control and kiaa1429 RNAi on the same plot would be beneficial for data interpretation. Or perhaps bring supplementary figure 2b into main figure?
4. In Figure 5K, xbp-1 expression is shown in kiaa1429 RNAi-specific clusters, pharynx, and neoblasts. Why not neural or other differentiated tissues? Also, xbp-1 is not mentioned in the main text.
5. Statistical tests are missing in:
o Figure 3A, C, D
o Figure 7C, F
o Supp. Figure 6A, 6B

We are pleased to submit our revised manuscript EMBOJ-2021-109895 "m6A is required for resolving progenitor identity during planarian stem cell differentiation" to *The EMBO Journal*. We thank the reviewers for their comprehensive and thoughtful comments. We addressed the reviewer comments and suggestions, and further tested our findings by adding controls, in vivo analysis, and computational analyses. We added new data (Fig EV3, Fig EV4, Appendix Fig S1A-B, Appendix Fig S3, and Appendix Fig S4), additional supporting information, and formatted the paper according to *THE EMBO Journal* guidelines. We provide our replies to the reviewers comments below.

Summary of changes in manuscript item designation

Original item designation	Revised item designation
Supplementary Figure 1	Figure EV1
Supplementary Figure 2	Figure EV2
Supplementary Figure 3	Appendix Figure S1
Supplementary Figure 4	Appendix Figure S2
Supplementary Figure 5	Figure EV5
Supplementary Figure 6	Appendix Figure S5
Supplementary Table 1	Dataset EV1
Supplementary Table 2	Dataset EV2
Supplementary Table 3	Dataset EV3
Supplementary Table 4	Table EV1
Supplementary Table 5	Dataset EV4
Supplementary material item	Appendix Table S2
Supplementary material item	Appendix Table S3
Supplementary material item	Appendix Table S4
N/A (New EV figure)	Figure EV3
N/A (New EV figure)	Figure EV4
N/A (New Appendix figure)	Appendix Figure S3
N/A (New Appendix figure)	Appendix Figure S4
N/A (New Appendix Table)	Appendix Table S1

Reviewer #1:

Dagan and colleagues aim to decipher the function of the m6A pathway in planarian flatworms, a model system for stem-cell driven regeneration. Following the discovery of all essential components of the m6A pathway in planarians, the authors study the role of individual m6A pathway genes on planarian homeostasis and regeneration. They find three components of the m6A pathway (mettl14, kiaa1429 and ythdc-1) to show severe, eventually lethal phenotypes, a finding they then follow up by mapping m6A-enriched regions in the planarian transcriptome and by uncovering a role of the m6A pathway in several important aspects of stem cell (neoblast) biology, such as the expression of polyadenylated histone variants, the coordinated expression of pseudogene clusters and the emergence of a novel cell cluster after m6A RNAi using single-cell sequencing.

Despite the primary weakness of the study at hand, which lies in an only indirect connection between the m6A mark and most of the described cellular disturbances, such as the changes in histone-mRNA polyadenylation, the expression of pseudogenes, and the connection to CHD4, I see the study by Dagan et al. as a major step forward in understanding the role of the epitranscriptomic mark m6A in planarian regeneration. Thus, I strongly recommend to invite a revision of the manuscript. Please see below the points that, in my opinion, need to be addressed during the revision process.

Major points:
*** To aid comparability across the planarian community and to prevent contig-derived ambiguity, I urge the authors to include SMESGs in their study (e.g. in Suppl. Fig. 1).**

We added mapping to SMESG in all data figures, tables, and supplementary material based on the contig to SMESG association found in the planarian genome resource PlanMine (Rozanski et al, NAR, 2019).
The following text was added to Appendix Note 7 (lines 160-161): "Mapping of transcriptome assembly contig IDs to planarian gene models was fetched from PlanMine (Rozanski et al, 2019)". In addition, a new appendix table was added (Appendix Table S1), which includes the mapping of transcriptome IDs (i.e. dd) that are shown in the figures and their association with the current gene model.

• Line 119: The authors rightly mention that variable and inefficient knockdowns are an impediment to unequivocally uncovering essential genes in planarians. In light of this statement and the available preprint by Cui et al. (bioRxiv, 2021), which identifies wtap as an essential m6A pathway component in planarians, I urge the authors to experimentally revisit wtap in their experimental system and to discuss gained results in light of their own study.
We revisited experimentally the analysis of *wtap* function using two experimental protocols. First, we inhibited *wtap* expression by repeating the protocol used by Cui et al, which is reported to result in a highly penetrant regeneration failure phenotype. We used their reported RNAi protocol (3 RNAi feedings followed by cutting) but did not find any regeneration failure phenotype, or other effects compared to our control (see figure below). Importantly, the RNAi sequence that was used for inhibition of *wtap* by Cui et al is unavailable in their preprint, and therefore we used a different sequence. This may have contributed to the differences in RNAi outcome.

In addition, we performed the gene inhibition experiments of *wtap* several times using the RNAi protocol used to inhibit *mettl14* and *ythdc-1*. Briefly, animals were fed 9 times with high concentration of dsRNA (2 ug/ul) corresponding to *wtap, mettl14* (positive control), or *unc22* (negative control). The results from these experiments were variable, as reported in the manuscript. We did not detect lysis. Interestingly, in one of the biological replications of this experiment, we observed a significant size reduction in *wtap* (RNAi) animals (see figure for results from this experiment). We obtained RNA from this experiment for gene expression analysis: we examined by qPCR whether genes that were differentially expressed following *mettl14* (RNAi), *ythdc-1* (RNAi) and *kiaa1429* (RNAi) changed their expression similarly in the size-reduced *wtap* (RNAi) animals. We measured the expression of *h2b*, dd_3194 (intestine marker), and dd_11930, and did not find a significant difference in their expression following *wtap* (RNAi). Significant differential expression was observed in the positive control (*mettl14* (RNAi); see figure below). In conclusion, the use of a different RNAi sequence might have led to a different experimental outcome, including different morphological phenotype and gene expression changes, yet there are other potential explanations. We anticipate that once that the data from the preprint is released, including vector sequences, gene expression data, and m6A peak mapping, a systematic comparison could be made. However, since this data is unavailable, we prefer to maintain the current description of the results. Below is data from the experiments that we described, including the data from the single *wtap* (RNAi) experiment that resulted in significantly smaller animals without lysis:

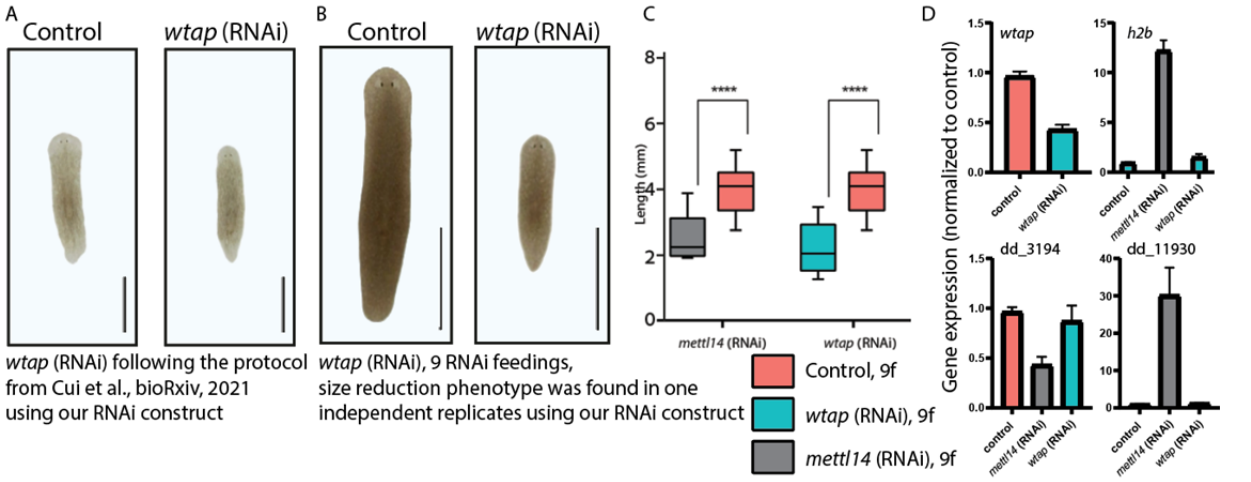


Figure. Inhibition of *wtap* using two dsRNA protocols. (A) Shown are regenerated fragments (10 days post amputation; n > 20 per group). We found no difference in the ability of the control and the *wtap* (RNAi) animals to regenerate. This might be a consequence of the different RNAi construct used. (B-C) Shown is the size reduction in *wtap* (RNAi) animals that was detected in only one of the biological replications of this experiment and a positive control *mettl14* (RNAi). (D) qPCR analysis showing the inhibition of *wtap* expression following RNAi (top-left). The genes *h2b*, dd_3194, and dd_11930 were not differentially expressed in the *wtap* (RNAi) samples, despite significant differential expression in the positive control (*mettl14* (RNAi)). Error bars represent the 95% confidence interval. Scale = 1 mm.

Fig. 2D (lines 162-163): I do not follow the authors line of reasoning regarding the observed broader peak distribution in their planarian m6A IP data. Why do the authors not discuss the obvious? - a

worse resolution of their m6A enrichment data due to multiple imaginable technical problems, e.g. a lack of antibody specificity coupled to the AT-rich planarian genome? Another explanation that comes to mind is that m6A peaks in planarians may more often occur in spatial proximity. Due to the coarse resolution of the presented data, I therefore suggest that the authors tune down their claims, in particular regarding a diverged m6A consensus motif in planarians.

We thank the reviewer for the comment. We agree with the reviewer that technical parameters or the high %AT planarian genome could result in the observed broad m6a peaks that we reported. We modified the text, added Figure EV3, and added data to Dataset EV1 to reflect this.

Following the reviewer comment, we evaluated the technical capacity and resolution of our m6A mapping by performing a mixed species m6A-seq2 experiment. RNA from yeast (*Saccharomyces cerevisiae* sk1) and from planarian was mixed together following RNA isolation to reduce biases that are associated with handling of RNA. We produced two pools of RNA: (1) Control planarian RNA mixed with RNA from *S. cerevisiae* lacking m6A, because of genetic mutation *Ime4*Δ/Δ/*Ndt80*Δ/Δ; and (2) planarian RNA from *kiaa1429* (RNAi) animals, which have a reduced m6A level, with RNA from *S. cerevisiae* having high level of m6A due to genetic background (*Ndt80*Δ/Δ). Biological replicates of the pools were generated. The pools were then processed according to the m6A-seq2 protocol by fragmenting the RNA, and molecularly tagging each pool using a 3'-end barcode. Then, the barcoded planarian-yeast RNA pools were combined, and immunoprecipitation of m6A was performed using an anti-m6A antibody. Library preparation and Illumina sequencing was performed according to the m6A-seq2 protocol. Then, sequencing reads were assigned to pools based on their molecular barcodes, and were assigned to planarian or yeast by mapping the sequences of each pool to a combined planarian-yeast genome or transcriptome (Rozanski et al., 2019).

The mapped m6A-pulldown and input reads were then used for detection of m6A-enriched regions, for each organism separately. The distribution of m6A peak length in the yeast RNA (median = 198 nt, SD = 156 nt) was similar to the distribution in the yeast m6A peaks in the REPIC database (Figure EV3). The distribution of planarian m6A peaks in the species mixing experiment (median = 298 nt, SD = 222) was significantly longer compared to the m6A peak length shown in the REPIC database (Fig 2D, Fig EV3; Student's t-test p-value < 2.2E-16). The most enriched sequence motif in the yeast m6A-regions, detected de novo using HOMER (Heinz et al, Mol Cell, 2010), was GGACA, which matches the consensus DRACH motif (Fig EV3D). Similar results were obtained using a separate analytic pipeline (Dierks et al., Nat Methods 2021). In the yeast negative control (*Ime4*Δ/Δ/*Ndt80*Δ/Δ), which lacks m6A, no such motifs were found. We next searched sequence motifs in all planarian m6A-enriched regions, yet did not find an enriched sequence motif using this approach. We reduced the computational complexity of motif finding by excluding all m6A-regions were longer than 250 nt, and retained the top 500 MeTPeak scoring peaks. Using this approach, CGACG was detected as the most enriched sequence motif (Fig EV3E). These results strongly suggest that the planarian MTC indeed recognizes a motif that is similar to the conserved consensus, at least in m6A-enriched peaks shorter than 250 nt. Moreover, the results of the species mixing experiment indicate that planarian m6A-enriched regions are indeed longer than m6A-enriched regions in other organisms, which, as pointed by the reviewer, could reflect co-occurrence (i.e., spatial proximity) of m6A in planarian RNAs.

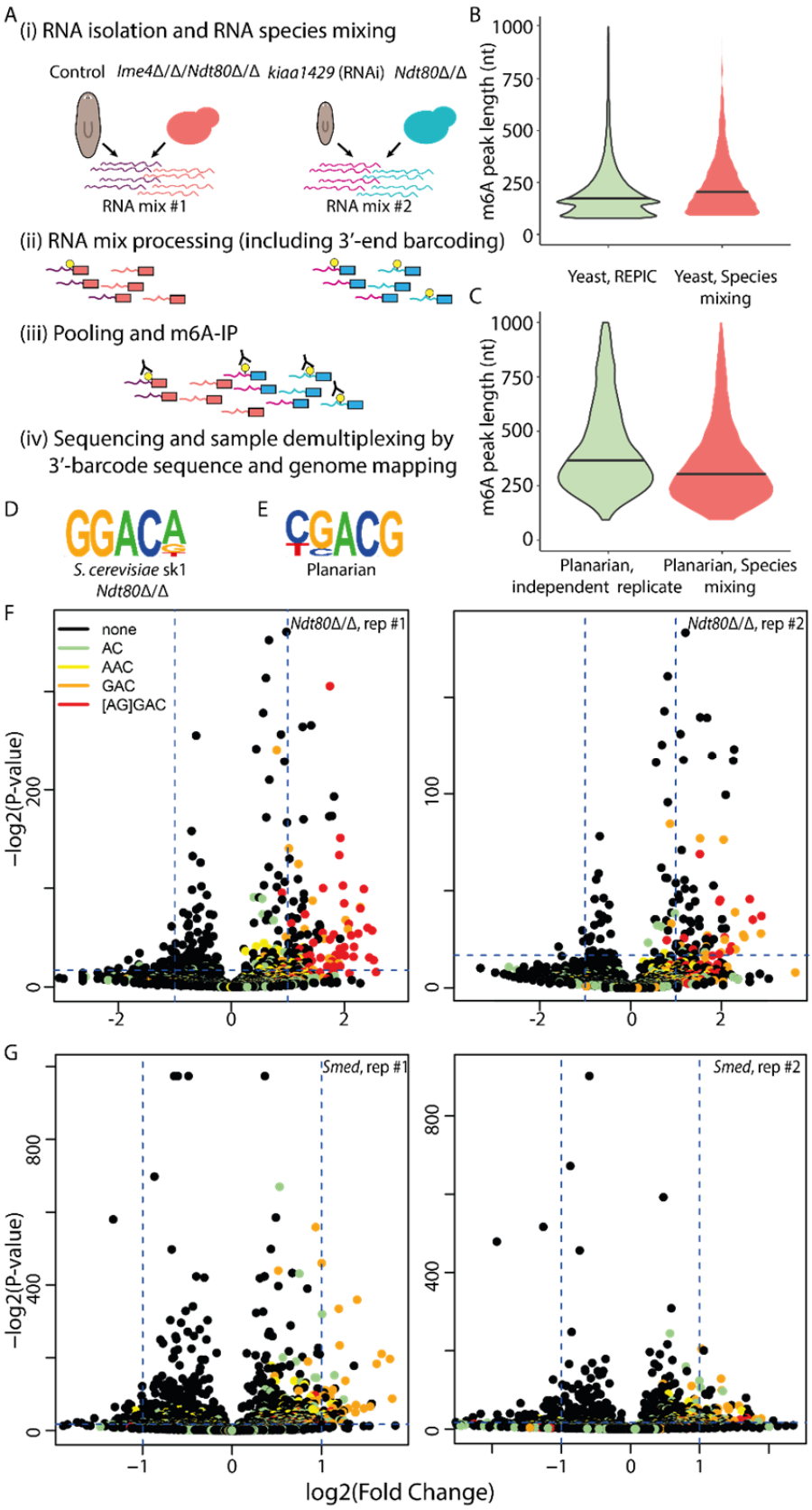
We made the following changes following this analysis:

- Result text revised with this data (152-172):

"We validated this result by mixing planarian and yeast (S. cerevisiae sk1) RNA and processing the mixed sample according to the m6A-seq2 protocol (Fig EV3, Dataset EV1; Appendix Note 5). The mixed RNA sample processing included RNA fragmentation, 3'-end barcoding, m6A enrichment, library preparation, and sequencing, therefore reducing many of the technical aspects that could contribute to the increased fragment length observed in planarian m6A peaks. We mapped the sequenced mixed libraries to the planarian and yeast genome and performed m6A peak detection (Fig EV3A-E; Appendix Note 5). Yeast m6A peak lengths (median = 198 nt, S.D. = 222) were similar to those previously published in the REPIC database (Liu et al, 2020c) (Fig EV3B), and were significantly shorter compared to planarian m6A peaks detected in this experiment (median 298 nt, S.D. = 222; Student's t-test = 2.2E-16). The distribution of planarian m6A peaks was similar to our initial mapping experiment (Fig EV3C), and longer than observed in other organisms".

The increased length of planarian m6A peaks rendered the detection of the m6A installment motif DRACH (D=A/G/U; R=A/G; H=A/C/U) (Linder *et al*, 2015) challenging. We therefore used high confidence m6A-enriched regions shorter than 250 bp (Fig EV3D-G; Appendix Note 5; top 500 enriched peaks). Using this approach, CGACG, which is similar to the DRACH motif, was found to be the most enriched sequence. These results were recapitulated on the basis of a separate analytic pipeline (Dierks *et al*, 2021; Schwartz *et al*, 2014), which we applied to both yeast and planarian data. This analysis also revealed an enrichment for 'GAC' harboring motifs in planarian, and the GGACA motif in yeast (Fig EV3F-G). Thus, m6A maps in planarians exhibit many of the classical hallmarks of m6A: they are enriched towards the ends of genes, depleted from highly expressed genes, are enriched in a 'GAC' motif, and are highly depleted upon inhibition of *kiaa1429*. The increased length of planarian m6A-enriched regions may suggest that planarian m6A sites are found in spatial proximity in RNA molecules, and higher resolution mapping of m6A sites could be used to further test this hypothesis".

- We added extended view figure, Figure EV3:


A
(i) RNA isolation and RNA species mixing
Control
Ime4Δ/Δ/Ndt80Δ/Δ
kiaa1429 (RNAi)
Ndt80Δ/Δ
RNA mix #1
RNA mix #2
(ii) RNA mix processing (including 3′-end barcoding)
(iii) Pooling and m6A-IP
(iv) Sequencing and sample demultiplexing by 3′-barcode sequence and genome mapping
B
m6A peak length (nt)
1000
750
500
250
0
Yeast, REPIC
Yeast, Species mixing
C
m6A peak length (nt)
Planarian, independent replicate
Planarian, Species mixing
D
S. cerevisiae sk1 Ndt80Δ/Δ
E
Planarian
F
none
AC
AAC
GAC
[AG]GAC
Ndt80Δ/Δ, rep #1
Ndt80Δ/Δ, rep #2
−log2(P-value)
G
Smed, rep #1
Smed, rep #2
log2(Fold Change)

- We added the following figure legend for Figure EV3 (Lines 1012-1031):

"Figure EV3. Analysis of m6A peak length by species mixing experiment. (A) RNA was extracted (1) from control or (2) *kiaa1429* (RNAi) animals in duplicates, with at least 10 animals in each replicate, (3) from meiosis-blocked *S. cerevisiae* strain with high levels of m6A (*Ndt80*Δ/Δ) (Dierks *et al*, 2021), and (4) from *S. cerevisiae* strain devoid of m6A, due to deletion of the methyltransferase (*Ime4*Δ/Δ/*Ndt80*Δ/Δ). RNA was combined into two pools (Appendix Note 5), and was processed according to the m6A-seq2 protocol (Dierks *et al*, 2021). Following sequencing, reads were associated with the original samples based on their 3'-end barcode (red and blue, representing mix #1 and mix #2, respectively), and then based on their mapping to either the planarian transcriptome or the yeast genome (Appendix Note 5). (B) Violin plot comparing the length of m6A peaks in yeast from either the REPIC database (Liu *et al*, 2020c), or from the data collected in this experiment; m6A regions larger than 1000 bp were excluded from the plot, horizontal line indicates the median length. (C) Comparison of planarian m6A peaks in our initial m6A-seq2 experiment, and in the m6a-seq2 profiling in the species RNA mixing experiment. Shown are peaks shorter than 1000 bp. To avoid comparison of lowly enriched peaks, shown are peaks with fold-enrichments that are larger than the median fold-enrichment. (D-E) Shown is the most frequent sequence motif detected in our mixed m6A-seq2 experiment (Appendix Note 5), as detected by HOMER (Heinz *et al*, 2010) for yeast (D) and planarian (E). (F-G) Shown are the fold change and associated p-values for each k-mer. DRACH-like k-mers are colored as in the figure legend. Data is shown for yeast (F, panels represent two replicates) and planarian (G, panels represent two replicates)".

- We added text the following description to Appendix Note 5 (lines 119-140):

"Mixed species m6A-seq2 experiment was performed by using RNA from yeast (*Saccharomyces cerevisiae* sk1) and from planarian (Fig EV3). RNA was mixed following RNA isolation and polyA-enrichment, prior to other processing to reduce biases that are associated with handling of RNA. Two pools of RNA were produced, in duplicates by mixing 80% of planarian RNA with 20% of yeast RNA: (1) Control planarian RNA mixed with RNA from *S. cerevisiae* lacking m6A, due to genetic mutation *Ime4*Δ/Δ/*Ndt80*Δ/Δ (Dierks et al. 2021); and (2) planarian RNA from *kiaa1429* (RNAi) animals, which have a reduced m6A level, with RNA from *S. cerevisiae* having high level of m6A because genetic background (*Ndt80*Δ/Δ) (Dierks et al. 2021). The pools were then processed according to the m6A-seq2. Briefly, the barcoded planarian-yeast pools were combined, and immunoprecipitation of m6A was performed using an anti-m6A antibody. Library preparation and Illumina sequencing were performed according to the m6A-seq2 protocol. Sequencing reads were assigned to respective planarian-yeast pool based on the sequence of their molecular 3'-end barcode (Appendix Table S3), and were then assigned to the planarian or yeast genome by mapping the sequences of each pool to a combined genome sequence of planarian (Rozanski et al., 2019), and *S. cerevisiae* (Dierks at al., 2021), or by mapping reads to a combined transcriptome sequence of planarian and the *S. cerevisiae* genome sequence. Enrichment in m6A sequence was performed by MeTPeak with default parameters (Cui et al, 2016), and motif finding was performed on regions enriched with m6A that are shorter than 250 bp using HOMER with parameters [-size given -len 5 using the 500 most enriched m6A regions] (Heinz et al. 2010), and using

an orthogonal m6A-associated k-mer enrichment detection strategy (Dierks et al. 2021; Schwartz et al. 2014)".

- The following discussion text was modified (lines 489-501):

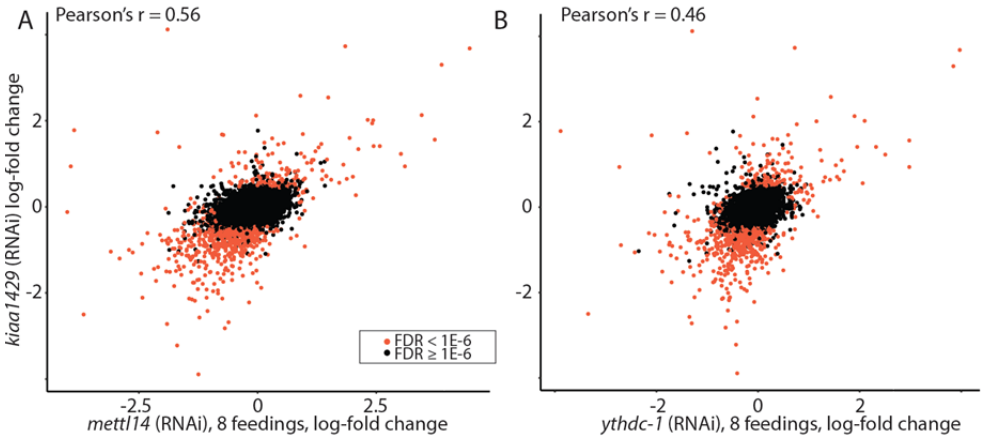
"We profiled the distribution of m6A in the planarian transcriptome, and identified 7,600 enriched regions in a diversity of cell types. Our profiling approach included mRNA enrichment, and was therefore focused on the detection of m6A enrichment in mRNAs. The m6A-enriched regions were found mostly near the 3'-end of the transcripts, as previously reported in other systems (Dominissini *et al*, 2012; Schwartz *et al*, 2014). This suggested that the role of m6A in planarians is similar to other systems. However, we identified a unique feature of planarian m6A-enriched regions: they are longer, on average, than reported m6A-enriched regions in other organisms (Liu *et al*, 2020c). This might be explained by lack of antibody specificity, yet we did not observe similar outcomes in our yeast samples. The detection of longer m6A-enriched regions might be a consequence of individual RNA molecules having multiple methylated adenosine sites in proximity, which could be therefore detected as a broad m6A-rich region. Single-nucleotide resolution based approaches for detection of m6A, such as miCLIP (Grozhik *et al*, 2017), could further elucidate these findings".

Fig. 4C: The authors show that gene expression changes correlate between mettl14 and ythdc1 knockdown conditions. How about the observed gene expression changes under kiaa1429 RNAi conditions? The authors need to address this open question, especially in light of the subsequent kiaa1429 RNAi experiments.

Following the reviewer's comment, we added plots showing the correlation in gene expression changes following the inhibition of *kiaa1429* and either *mettl14* or *ythdc-1* to Appendix Figure S1. There is a highly significant correlation (r = 0.56 and 0.46, for *mettl14* (RNAi) and *ythdc-1* (RNAi), respectively) with p-value < 2.2E-16 in changes to gene expression. The *kiaa1429* (RNAi) phenotype emerges more rapidly and therefore many genes change their expression more significantly.

The following changes were made:

- Panels were added to Appendix Figure S1



- Main text was changed to include these panels and now reads (lines 230-233):

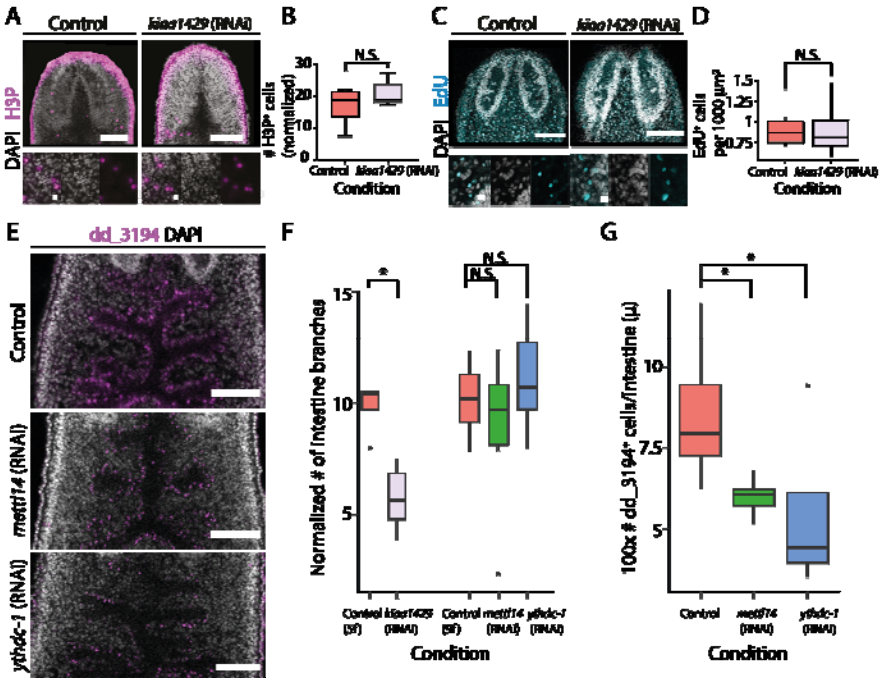
"The gene expression changes following *mettl14* (RNAi) and *ythdc-1* (RNAi) were remarkably similar with correlation > 0.7 between all time points (Fig 4A-C). Comparison of *mettl14* (RNAi) or *ythdc-1* (RNAi) with *kiaa1429* (RNAi) resulted in high correlation as well, 0.56 and 0.46, with *mettl14* (RNAi) or *ythdc-1* (RNAi), respectively (Appendix Fig S1A-B), despite the time point differences associated with the rapid development of the *kiaa1429* (RNAi)".

Fig. 4E: Does a knockdown of mettl14 and ythdc1 show the same effects here? The authors need to show this experimentally here, especially given the knockdown variability in planarians and potential discrepancies to other studies (see Cui et al., bioRxiv 2021). Moreover, by showing similar effects after a mettl14, ythdc1 or kiaa1429 knockdown, the authors can substantiate their claim that intestinal cell depletion is an m6A-related phenotype and not related to other downstream effects of kiaa1429 RNAi.

We thank the reviewer for the comment. We evaluated the effect of the RNAi on the intestinal cell population by using FISH with an intestine marker on *mettl14* (RNAi) and *ythdc-1* (RNAi) animals and observed a significant reduction of >40% of the intestine cells in both conditions. In agreement with the RNAseq results, this reduction was less severe than the reduction observed in the *kiaa1429* (RNAi) animals, and it did not involve a depletion of the intestine branching at the time point tested. These results further support the observation that the phenotypes observed following inhibition of these different m6A pathway genes are indeed similar, and that over the time points analyzed in our experiments the defects are detectable in specific tissues.

The following changes were made:

- We added Fig EV4E-F with these data. The panels include (a) FISH using an intestine-specific probe on *mettl14* (RNAi) and *ythdc-1* (RNAi) animals; (b) quantification of the number of intestine branches normalized by size; and (c) quantification of the number of intestine cells expressing the intestine marker following RNAi compared to controls.



- <u>The result section now includes these results and reads as follows (Lines 256-266)</u>:

"To test whether the reduction in intestinal gene expression represented downregulation of a gene expression program or a reduction in intestine cell number, we analyzed by FISH *kiaa1429* (RNAi), *mettl14* (RNAi), and *ythdc-1* (RNAi) and control animals using a riboprobe labeling the planarian intestine (Fig 4E, Fig EV4E-G). We found a severe defect in intestinal branching morphology following *kiaa1429* (RNAi) (Fig 4E, Fig EV4F, Methods), which suggested that the RNAi resulted in depletion of intestine cells. Indeed, following inhibition of *kiaa1429*, animals did not uptake food (Fig EV1F). Inhibition of *mettl14* (RNAi) and *ythdc-1* (RNAi) resulted in a significant decrease of 43% and 57% in the number intestinal cells, respectively, without loss of intestine morphology (Fig EV4E-G) at the time point tested (Bonferroni corrected Student's t-test $p < 0.05$ for both conditions). This result was consistent with the observation that inhibition of *kiaa1429* results in a more rapidly developing phenotype".

Fig. 5 (lines 343-361): Can the authors exclude effects of m6A on other polyA- transcripts (e.g. outside of the histone family)? A discussion of this possibility should be included at appropriate position in their study.

Current literature on m6A and its functions is mostly focused on the function of m6A on polyadenylated transcripts. Yet, it is correct that m6A is found on polyA- transcripts, for example, on circular RNAs (Yang et al., Biomarker research, 2020). The m6A mapping and RNAseq that we collected was highly enriched for polyadenylated RNAs, similar to the vast majority of datasets in the m6A literature. Following the reviewer's comment, we searched for representation of non-polyadenylated RNA species in our dataset, other than in the ribosomal RNA, which is shown in Figure EV2E. We found that abundant polyA- RNA species, like tRNA are not well represented in our dataset, and we could not find evidence of circRNA. We therefore have no evidence for the presence of m6A on polyA- RNAs. We cannot exclude the possibility that such transcripts are regulated by the m6A pathway or that they are affected by the

inhibition of m6A pathway genes, yet assessing this possibility requires experimental approaches (e.g., circRNA m6A-seq) that are not readily applicable here.

- We added the following text to the discussion (Lines 490-491):

"Our profiling approach included mRNA enrichment, and was therefore focused on the detection of m6A enrichment in mRNAs".

Fig. 5A (lines 301-310): The authors connect effects of kiaa1429 RNAi (reduction of intestine cells) to observations after mettl14 RNAi (upregulation of histone encoding genes). This raises the following questions (as mentioned before regarding Figs. 4C+E): a) Do the gene expression changes under kiaa1429 RNAi conditions correlate with mettl14 and ythdc1 RNAi conditions.
b) Is intestine cell depletion also found in mettl14 & ythdc1 RNAi animals?
c) Fig. 5B shows that kiaa1429 RNAi leads to a dramatic overexpression of polyadenylated histone genes. Overexpression under mettl14 RNAi conditions can be mentioned, however, to aid the reader histone gene expression changes should be primarily connected to kiaa1429 RNAi conditions.
We thank the reviewer for the comment. The consequences of *mettl14* (RNAi), *kiaa1429* (RNAi) and *ythdc-1* (RNAi) are very similar: (1) There is a high correlation in gene expression changes between the three RNAi conditions both on the resolution of the individual gene and the resolution of the cellular process or cell type; (2) the morphological and cellular phenotype is similar and includes depletion in intestinal cells, and (3) there is a similar reduction in m6A level following RNAi of the MTC components *metll14* and *kiaa1429* (but expectedly, not following the inhibition of the reader, *ythdc-1*). Data supporting our response was described in previous comment response text (Fig 4C, 4E).
Following the reviewer's comment, we emphasized the result on *kiaa1429* (RNAi):

- Revising the text to emphasize *kiaa1429* (RNAi) (lines 282-283):

"For example, seven and eight such genes were upregulated by over two fold in *kiaa1429* (RNAi) and *mettl14* (RNAi) animals, respectively (Fig 5A-B; Dataset EV2)".

- We changed the order of panels A and B in Figure 5.

Fig. 5F-G: As it stands, the authors need to characterize the overexpressed gene cluster further. Which pseudogenes are found in this cluster? What can be deduced by sequence analysis? Moreover, I dearly missed a connection to m6A-enriched peaks here. The authors need to connect the deregulated gene cluster to m6A marks, as they did in case of histone mRNAs (which the authors find, presumably to their disappointment, not to contain m6A marks).
We thank the author for comment. The genes found in the cluster are highly similar in sequence and most have an identifiable ubiquitin-like domain. The repeat cluster is found in multiple *S. mediterranea* genome assemblies, and genes that are highly similar to genes in the cluster are found in the available *S. polychroa* transcriptome, but not in transcriptomes of other species available in PlanMine (Rozanski et al, 2019). We identified m6A peaks in these regions, which were also detectable in the planarian-yeast species mixing experiment. Whether the change to m6A levels following RNAi is causing the overexpression is an open question that is challenging to answer with the available planarian methods.

In future studies, we plan to investigate the changes to the genomic region to further understand the basis for the silencing of this region in wildtype animals.

- We added the following text to the relevant section (lines 330-334):

"Several genes in these clusters had detectable m6A-enriched regions in our m6A-seq2 datasets (Dataset EV1), and encoded ubiquitin-like domain (Dataset EV2). We searched for similar sequences in other planarians transcriptomes (Rozanski et al, 2019) using BLAST and found similar sequences in the *Schmidtea polychroa* transcriptome, but not in other planarian species".

• Fig. 7: Using a systematical analysis of planarian RNAseq datasets to identify the molecular function of m6A in planarians is an exciting idea. However, I miss any mentioning of signal/noise in this analysis. Was CHD4 the only candidate the authors found connected to the m6A pathway? In which way was CHD4 signal superior to all other possibilities? The authors need to include details regarding their choice for CHD4 in their manuscript.

We expanded the description of this analysis in the supplementary material and added an Appendix Figure showing the ranking of the overlap of changes to gene expression across the available published datasets and our data. The success of the method in identifying potentially meaningful correlations is greatly dependent on the condition that is used for searching for association. For example, unsurprisingly, applying this analysis on dataset collected from irradiated animals easily detects similar conditions that are enriched for this population (e.g., Xins cell population sequencing or *h2b* (RNAi)). However, there are limitations to this approach, especially in analyzing conditions that have a small effect on the transcriptome.

Here, the analysis was performed using RNAseq libraries that had a large impact on the transcriptome, and moreover, having data collected in a time-course aided to manually examine the change in overlap of gene expression changes with other libraries as a function of time and of RNAi conditions. Multiple parameters affect the calling of differentially expressed genes, including the experimental design (e.g., number of replicates in each condition), and the thresholds used to determine whether a gene is differentially expressed. We therefore called the differentially expressed genes over a range of parameters (FDR range from 0.1 to 1E-10; minimal gene expression 1–20 transcript per million, TPM). The significance of overlap of differentially expressed genes was extremely robust to these thresholds, and we used conservative thresholds requiring corrected FDR < 1E-5 and TPM > 10 in order to include a gene in the list of differentially expressed genes for this analysis. The *CHD4* (RNAi) libraries at 9, 12, 15 days following RNAi had the most significant overlap of differentially expressed genes with the list of differentially expressed genes in our datasets with p-value, estimated by hypergeometric hypothesis testing of <1E-15 and often p-value <1E-60. For example, 31 out of the top 50 differentially expressed genes in following *mettl14* (RNAi) were differentially expressed in the *CHD4* (RNAi) 15 days, compared to a median overlap of 4 differentially expressed genes in all of the tested libraries. In addition to applying the hypergeometric test, we estimated the similarity between sets of genes that were the most significantly differentially expressed following RNAi. For this unbiased analysis, we selected up-to 200 genes with the lowest adjusted p-value from each condition and computed the Jaccard similarity coefficient (JI). The median JI in this analysis was < 0.02 compared to a maximum of JI > 0.14 at 9 and 15 days following *CHD4* (RNAi). The JI for the *CHD4* (RNAi) was significantly higher than JIs calculated for

the other groups as estimated by two-sided Wilcoxon rank sum (p = 0.0002). In summary, the use of this approach could be useful as a discovery tool for potential similarities of large datasets, yet it does not work equally well with every input dataset.

We made the following changes:

- The following description was added to Appendix note 11 (lines 215-232):

"We tested a range of parameters for calling differentially expressed genes in the published dataset (FDR range from 0.1 to 1E-10; minimal gene expression 1–20 transcript per million, TPM) and searched for overlap of gene expression with our dataset. The significance of overlap of differentially expressed genes was robust to these thresholds. To minimize spurious potential correlations, we used conservative thresholds requiring corrected FDR < 1E-5 and TPM > 10, in order to include a gene in the list of differentially expressed genes for this analysis.

The *CHD4* (RNAi) libraries at 9, 12, 15 days following RNAi had the most significant overlap of differentially expressed genes with the list of differentially expressed genes in our datasets with p-value, estimated by hypergeometric hypothesis test, of < 1E-15 and often p-value <1 E-60, with the highest ranking of overlap (Appendix Figure S4A) . For example, 31 out of the top 50 differentially expressed genes in following *mettl14* (RNAi) were differentially expressed in the *CHD4* (RNAi) 15 days condition, compared to a median overlap of 4 differentially expressed genes in all of the tested libraries. Furthermore, we selected up-to 200 genes with the lowest adjusted p-value, which meet the thresholds for differential expression, from each condition and computed the Jaccard similarity coefficient (JI). The median JI between our libraries and each of the libraries in this analysis was < 0.02, compared with JI > 0.14 at 9 and 15 days following *CHD4* (RNAi) with the *kiaa1429* (RNAi) library. The JI for the *CHD4* (RNAi) and our libraries was significantly larger than JIs calculated for the other groups as estimated by two-sided Wilcoxon rank sum (p = 0.0002)".

- Appendix Figure S2 was added (a higher resolution figure included in submission):

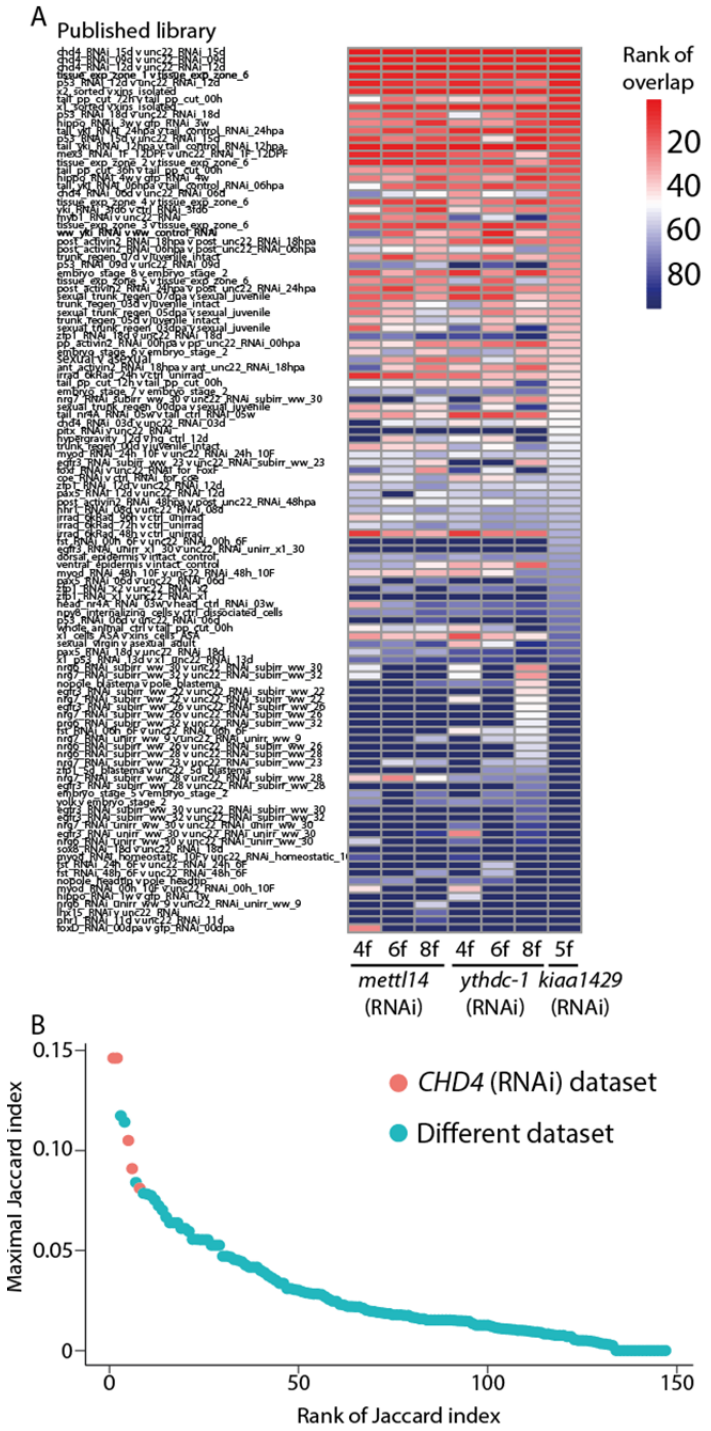
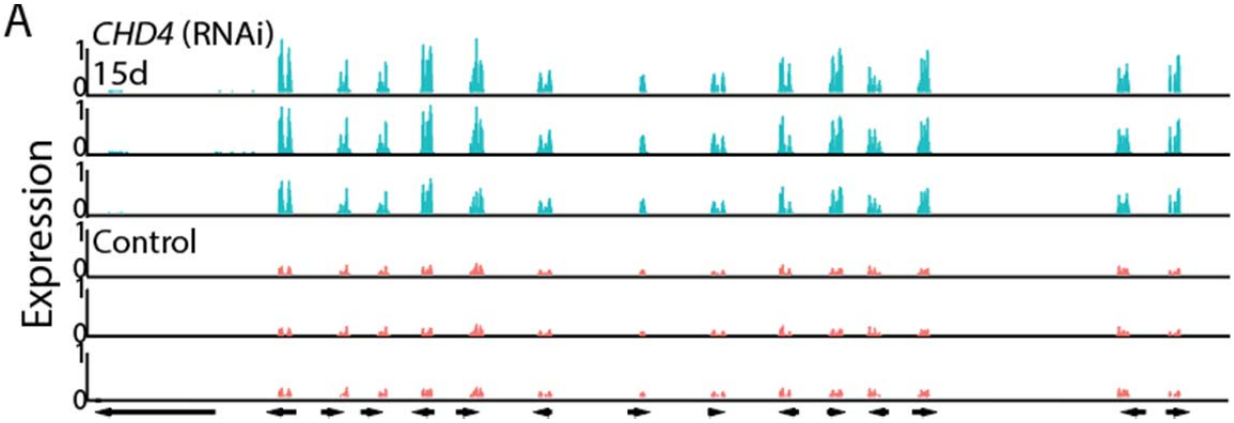


Fig. 7A: Regarding the IGV tracks for CHD4 RNAi conditions, only the bottom one seems to show an upregulation of the depicted gene cluster. The track on the top seems to display expression levels equal to control tracks. Having only one of two replicates show an effect is inconclusive and might be due to technical variation in library preparation. Thus, more convincing CHD4 RNAi data (triplicates) need to be presented in this figure, in order for the authors to be able to support their claims.

We thank the reviewer for the comment. We added the genome snapshot of the same region showing triplicates. To enhance clarity, we used the *CHD4* (RNAi) 15 day samples, which, as expected, show a more exaggerated increase in expression. Importantly, the genes in this genomic neighborhood were significantly overexpressed at earlier time points including at the time point shown in the original figure.

- We included the following panel in Fig 7A:



- Figure legend was updated and now reads (lines 939-942):

"(A) Overexpressed gene neighborhood (contig: dd_Smes_g4_15:3.13-3.17 Mbp). Shown is the normalized and scaled gene expression in three *CHD4* (RNAi) samples, 15 days post feedings (top, blue) and in three corresponding control samples (bottom, red)".

Minor points:

Line 91: language: "expression of genes ... were expressed"

- We changed the text accordingly and now reads (lines 92-93):

"Genes that encode YTH-domain were expressed in largely non-overlapping cell types".

• Lines 158-160: The authors should better connect the fact that planarian 28S rRNA does not carry m6A marks to their findings concerning the presence of m6A in polyA-RNA vs. non-polyA-RNA (Fig. 1D). It would aid the instant understanding of their data if the reader knew about this planarian peculiarity before looking at Fig. 1D.

- The text was modified accordingly, it now reads (lines 146-149):

"Interestingly, m6A was not detected on rRNA (Fig EV2E), in contrast to mammalian systems where two sites are installed via a mechanism independent of METTL3, which could contribute to the lower m6A detection in non-polyA RNA (Fig 1D).

• Fig. 2B: The figure is supposed to show data for 740 high-confidence peaks. Does high-confidence mean 5-fold enrichment as mentioned in Suppl. Note 5? If yes, I suggest to indicate this in the figure description, e.g. "740 high-confidence peaks (>= 5-fold enriched)".

The 740 high-confidence peaks are enriched by at least 10 folds.

- We modified the figure legend accordingly and it now reads (line 853):

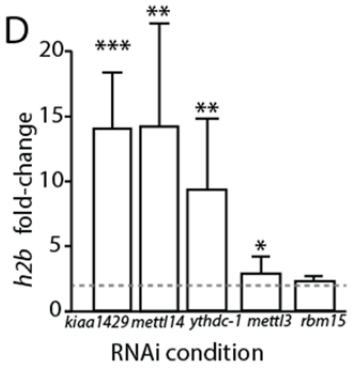
"... across 740 high-confidence peaks (≥ 10 fold-enriched)".

• Fig. 3D (line 204): What is the p-value associated with "significant upregulation" here?

The significance of upregulation was estimated by two-sided Student's t-test followed by Bonferonni's correction for multiple hypotheses and is summarized in the table below.

Condition	**Adjusted p-value**
kiaa1429 (RNAi)	6.92E-05
mettl14 (RNAi)	0.001036
ythdc-1 (RNAi)	0.001877
mettl3 (RNAi)	0.018423

- We added visual indicators to the Figure 3D with description in the figure legend (869-872):



"P-value was calculated by two-sided Student's t-test followed by Bonferroni correction for multiple hypotheses. Lack of asterisk indicated a non-significant change in gene expression. Error bars indicate the 95% confidence interval (*** - $p < 0.001$, ** - $p < 0.001$, * - $p < 0.05$)".

Reviewer #2:

In the manuscript 'm6A is required for resolving progenitor identity during planarian stem cell differentiation,' the authors present a series of very interesting experiments that support a model in which mRNA base-modification m6A is required for fate choice and cellular maturation in planarian stem cells. Perturbation of m6A pathway components resulted in progressive deterioration of tissues and animal death, increased expression of non-canonical histone variants, and accumulation of un-differentiated cells. A particular strength of the paper is the extensive sequencing analysis done on control vs. kiaa1429 RNAi treated animals, including single cell sequencing of ~20,000 cells. These sequencing studies provided additional support for the authors assertions that the stem cell population was not depleted, but stem cell differentiation and lineage specification was altered in the animals. In addition, single cell sequencing identified a previously un-described cell state that

accumulated in RNAi treated animals and that expressed genes commonly expressed in neurons, glia, differentiated cells, as well as clusters of genes over-expressed after kiaa1429 RNAi treatment. Authors also provide evidence that depletion of NuRD component CHD4 results in a similar phenotype, but were unable to provide evidence that either pathway regulates the other.

Altogether, these results represent one of the first descriptions of mRNA modification in planaria and establish that mRNA modification is a critical regulator of stem cell differentiation and lineage specification in planarian stem cells. The manuscript is also the first to demonstrate m6A-seq2 in planaria, an important tool for the field. Thus, the paper will be of interest to both planarian researchers and the broader stem cell biology community. The discussion and analysis of the sequencing results is well done, but the paper's conclusions would be better supported with more rigorous characterization of the RNAi phenotypes and additional quantitation and validation of predicted gene expression changes in vivo. In particular, the altered gut morphology observed after depletion of m6A pathway components is only minimally characterized and the novel cell state described by single cell sequencing is not adequately visualized or quantitated in the animal.

Major Criticisms:

1. Gut morphology phenotypes downstream of m6A pathway inhibition are insufficiently characterized given the correlation between intestinal branching and animal size/feeding. Would it be possible to quantitate branching or intestinal cell number per area/length of worm? Some attempt to control for animal size, quantify gene expression or cell abundance by FISH, or provide additional images (more individual animals, both max projections and z slices, etc.) would strengthen the claims in the text currently supported only by figure 4E.

We thank the reviewer for the comment. We added analyses of the intestinal cell population and intestinal morphology, and we expanded the analysis of intestinal morphology following inhibition of *kiaa1429*, *mettl14*, and *ythdc-1*. We used FISH with the intestinal phagocyte marker dd_3194 following RNAi and quantified the effect. We counted the number of intestinal branches and found that it was significantly reduced following inhibition of *kiaa1429* (Fig EV4), which reflected a broad defect in intestinal morphology. Consistent with RNAseq data, FISH following inhibition of *mettl14* and *ythdc-1* resulted in a large and highly significant reduction (>40%, $p < 0.05$) in the number of intestinal phagocytes in intestinal branches scaled to the length of the intestine. This reduction was observed without significant reduction in the number of intestinal branches (Fig EV4E-G). Indeed, *kiaa1429* (RNAi) phenotypes manifest more rapidly and dramatically, in agreement with these observations. In addition, we quantified several other intestine markers as detailed for comment #3 by the reviewer.
We made the following changes:

- The following text was added to the manuscript (Lines 256-266):

"To test whether the reduction in intestinal gene expression represented downregulation of a gene expression program or a reduction in intestine cell number, we analyzed by FISH *kiaa1429* (RNAi), *mettl14* (RNAi), and *ythdc-1* (RNAi) and control animals using a riboprobe labeling the planarian

intestine (Fig 4E, Fig EV4E-G). We found a severe defect in intestinal branching morphology following *kiaa1429* (RNAi) (Fig 4E, Fig EV4F, Methods), which suggested that the RNAi resulted in depletion of intestine cells. Indeed, following inhibition of *kiaa1429*, animals did not uptake food (Fig EV1F). Inhibition of *mettl14* (RNAi) and *ythdc-1* (RNAi) resulted in a significant decrease of 43% and 57% in the number intestinal cells, respectively, without loss of intestine morphology (Fig EV4E-G) at the time point tested (Bonferroni corrected Student's t-test $p < 0.05$ for both conditions). This result was consistent with the observation that inhibition of *kiaa1429* results in a more rapidly developing phenotype".

- We added a figure with this data (Fig EV4; included in this document).

- The following text was added to Appendix Note 13 (lines 252-260):

"Counting of intestinal phagocytes was performed on *mettl14* (RNAi) and *ythdc-1* (RNAi) animals. Intestinal phagocytes were labeled using a specific marker by FISH (*dd_3194*). Animals were imaged by confocal microscopy and the z-position showing the largest number of intestinal phagocytes anterior to the pharynx, as determined by two researchers, was selected for counting. Cells were counted using the Cell Counter module in ImageJ (Rueden *et al*, 2017; Schindelin *et al*, 2012). Then, the length of the intestinal branches was measured by tracing the intestine based on DAPI labeling next to the intestine lumen by using the segmented line tool in ImageJ. The normalized number of cells per micron of intestine branch was calculated based on these measurements".

2. Claims made by authors regarding stem cell proliferation/cell cycle following depletion of kiaa1429 are mostly based on FACS analysis (Figure 3E, F). In particular, the authors conclude that kiaa1429 RNAi results in an increase in immature post-mitotic progenitors based on the larger X2 cell abundance. This claim would be better supported by measuring progenitor abundance in vivo by FISH and/or quantitation of cell cycle indicators in vivo (EdU, phospho-Histone 3, PCNA, etc.). The authors could also test this model more explicitly during the discussion and analysis of the scRNAseq datasets.

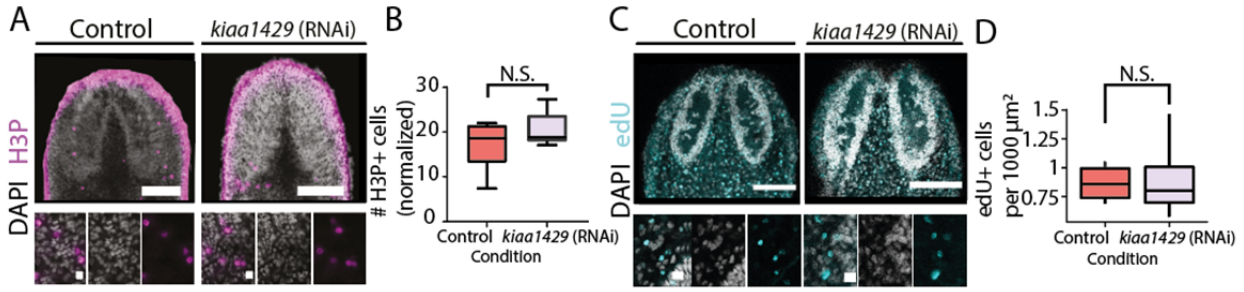
We addressed this comment by performing a set of experiments. First, we assessed whether there is a change in the number mitoses following inhibition of *kiaa1429* (RNAi) compared to control animals. We used an anti-phospho-histone 3 antibody (H3P) to label mitotic cells and counted H3P+ cells found in a defined region anterior to the pharynx. We found no significant increase ($p=0.59$) in the number of H3P+ cells. This result, together with the evidence that there is no increase in neoblasts (based on FISH, RNAseq, and scRNAseq) suggests that (a) the size of the neoblast population is similar between conditions, and that (b) this similarly-sized neoblast population produces a similar size of progeny. In addition, we evaluated the number of cells going through S-phase in *kiaa1429* (RNAi) and control animals by metabolic labeling using the thymidine analog F-ara-EdU. Animals were soaked in F-ara-EdU for 16 hours and were then fixed. The F-ara-EdU+ cells were counted in a ventral sub-epidermal layer that is distinguishable from the intestine. We found no significant difference in the number F-ara-EdU+ cells between in the *kiaa1429* (RNAi) and control animals, which further indicated that in the conditions tested here, there was no detectable defect in neoblast cell cycle progression. This data was added to figure EV4.

We made the following changes:

- We added a description of the experiment to the main text (lines 210-221):

"First, we used an anti-H3P antibody to label mitotic cells and counted H3P+ cells found in a defined region anterior to the pharynx (Fig EV4A-B; Methods). We found no significant increase (p=0.59) in the number of H3P+ cells following inhibition of *kiaa1429*, which indicated that the neoblast population mitotic rate is unperturbed. In addition, we estimated the number of cells going through S-phase in *kiaa1429* (RNAi) and control animals by metabolic labeling using the thymidine analog, F-ara-EdU. Animals were soaked in F-ara-EdU for 16 hours and were then immediately fixed for further processing. Following F-ara-EdU detection, the F-ara-EdU+ cells were counted in a ventral sub-epidermal layer that is distinguishable from the intestine (Fig EV4C-D; Methods). We found no significant difference in the number F-ara-EdU+ cells between the *kiaa1429* (RNAi) and control animals, which further indicated that in the conditions tested here, there was no detectable defect in neoblast cell cycle progression".

- We added figure panels (Fig EV4A-D):



- We added Methods text for H3P labeling (lines 618-624):

"H3P labeling was performed based on published protocols (Wenemoser & Reddien, 2010; LoCascio *et al*, 2017) on animals following fixation with NAC. Briefly, following fixation blocking was performed with 10% heat inactivated horse serum (HIHS). Anti-phospho-Histone H3 Antibody (sigma 04817) was added 1:100 overnight in 4°C. Samples were washed 7 times with PBSTx (0.1% triton) for 20 minutes, and were labeled using goat anti-rabbit-HRP secondary antibody (Abcam ab6721, 1:300) overnight at 4°C in block. Samples were developed using rhodamine tyramide diluted 1:1000 in PBSTi".

- We added Methods text for F-ara-EdU labeling (lines 610-616):

"Animals were soaked for 16 hours in 2.5 mg/ml F-ara-EdU (Sigma-Aldrich T511293) diluted in planarian water. Then, animals were fixed in NAC and bleached with formamide and H_2O_2 for two hours on a light table. Animals were treated with 0.2 µg/ml Proteinase K for 10 min, 4% FA for 10 min, and were then washed 3 times in 3% PBSB (PBS supplemented with 3% BSA). Click reaction was performed using baseclick kit (cat. back-edu488) and was followed by nuclear staining (DAPI, 1:5000) overnight in 4°C. Samples were washed 3 times in PBSTx (0.1%) for 10 minutes and then mounted for imaging".

3. Overall, claims made based on scRNAseq data should be validated more rigorously in vivo. These claims include those regarding stem cell differentiation, gut specification, and the identification of a kiaa1429 RNAi-specific cell state. Single cell sequencing data presented in Figure 6D should be

validated in vivo by in situ hybridization and quantitation of cellular abundance. **Finally, co-expression of the repetitive gene clusters and smad6/7-2 and protocadherin-like gene dd_15376 in the kiaa1429 specific cluster is asserted in the text, but is not demonstrated in figure 6 or supplementary figure 5. Addition of neural and glial markers into Figure 6F and in vivo validation of co-localization by FISH would support this claim.**

We thank the reviewer for the comment. We tested whether there is a decrease in the integration of new intestinal progenitors to the intestine. Post-mitotic progenitors express SMEDWI-1+ for roughly 48-72 hours following cell division. We detected recently integrated intestine cells by co-labeling *kiaa1429* (RNAi) and control animals with anti-SMEDWI-1 antibody and with a combination of FISH probes that detects intestine-specific transcription factors (i.e., *nkx-2.2*, *gata4/5/6-1*, and *hnf4*; intestine mix). SMEDWI-1+/intestine mix+ cells were counted in the intestine, anterior to the pharynx in z-stacks. We found a significant decrease of > 50% (two-way Student's t-test p = 0.02) in the number of recently integrated progenitors normalized to the size of the quantified region. This data was added to Appendix Fig S4. Therefore, despite no change in the number of mitoses, as indicated by H3P and edU labeling, there was a decrease in the number of newly integrated cells to the intestine. We further quantified the change in the number of intestine cells following *kiaa1429* (RNAi) by FISH with 3 additional intestine cell markers by FISH (shown in the figure) dd_72, dd_115 and dd_888. We found that the number of cells expressing either dd_72 or dd_115 was reduced by over two-folds (p=0.001 and 1.2E-7, for dd_72 (RNAi) and dd_115 (RNAi), respectively). The decrease in the number of dd_888 was not significant, yet the FISH signal following *kiaa1429* (RNAi) was qualitatively lower. These results support the scRNAseq analysis and demonstrate that there is indeed a reduction in the number of intestine cell following *kiaa1429* (RNAi), and that the integration of new intestine progenitors is reduced, which is consistent with the reduced number of intestine progenitors in the scRNAseq data. Finally, we tested several markers for FISH co-localization assay of the repetitive gene clusters, including smad6/7-2 and protocadherin-like gene dd_15376, but were not able to get sufficient technical labeling *in situ* with these genes as a second in situ probe; text was added.

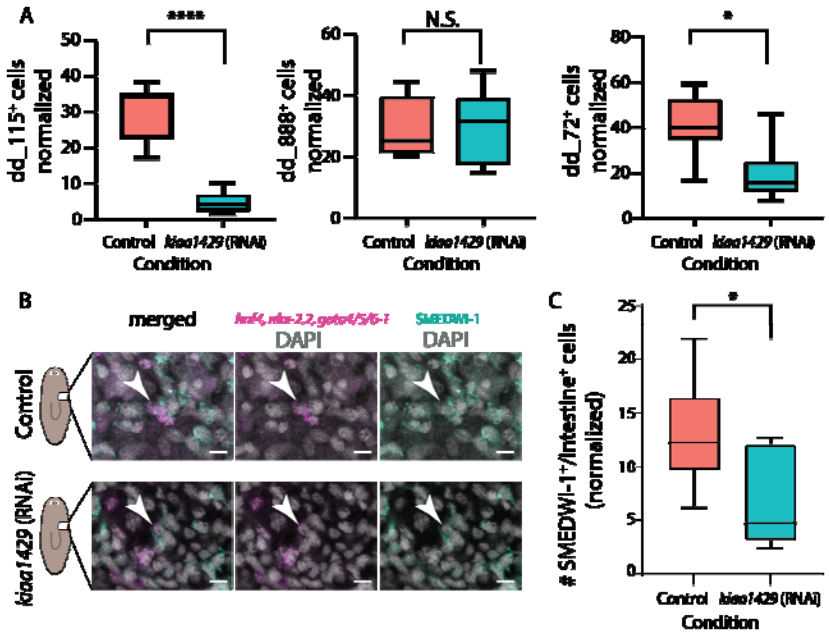
- The following text was added (lines 374-377):

"We quantified the reduction of the intestine markers dd_72, dd_115, and dd_888 (Fig 6E; Appendix Fig S3A) by FISH and found a highly significant reduction in the number of dd_72+ and dd_115+ cells (Appendix Fig S3A), with only qualitative difference in the expression of dd_888".

- The following text was added (lines 384-393):

"We tested whether there were indeed less recently produced progenitors integrated to the intestine. Recently integrated intestine cells were detected by co-labeling *kiaa1429* (RNAi) and control animals with anti-SMEDWI-1 antibody and with a combination of FISH probes that detects intestine-specific transcription factors (i.e., *nkx-2.2*, *gata4/5/6-1*, and *hnf4*; intestine mix). SMEDWI-1^{+}/intestine mix^{+} cells were counted in the intestine anterior to the pharynx. We found a significant decrease of > 50% (two-way Student's t-test p = 0.02) in the number of recently integrated progenitors normalized to the size of the animal (Appendix Fig S4B-C). Therefore, despite no detectable change in the number of mitoses, as indicated by H3P and F-ara-EdU labeling (Fig EV4A-D), there was a decrease in the number of newly integrated cells in the intestine".

- An appendix figure was added (Appendix Fig S3):



- Re: the *kiaa1429* (RNAi)-specific cluster we added the following text:

Results, (lines 400-401): "The scRNAseq analysis indicated that cells in the cluster highly expressed genes that are associated with neurons and glia…"

Discussion, (line 519): "...yet further in vivo evidence is required for determining their identity"

4. In my opinion, the results section regarding a connection between the m6A pathway and the NuRD complex is presented with a bias towards a model in which m6A regulates NuRD. While both RNAi depletions result in similar phenotypes, neither has been demonstrated to regulate the other and I think that the authors statement that 'the observation that CHD4 (RNAi) animals develop phenotypes more rapidly than following inhibition of m6A genes supports the possibility that inhibition of m6A progressively deteriorates NuRD activity' is too strong. There are many models that are consistent with this observation that are not discussed. In the absence of additional evidence, I would recommend revising the results section to more fully acknowledge the possibility that NuRD and m6A regulate similar processes independently. The discussion section does a much better job balancing these two models (Figure 8).

We thank the reviewer for the comment and agree with the reviewer's analysis. Following the reviewer's suggestion, we changed text used in the results for interpreting the outcomes of the experiments.

- The following text was *removed*:

"This observation supports the possibility that inhibition of the m6A pathway may progressively deteriorate NuRD activity, directly or indirectly, and indicates a potential link between m6A and chromatin regulation".

- The following text was added (lines 452-455):

"It is possible that inhibition of the m6A pathway progressively deteriorates NuRD activity, directly or indirectly. However, it is also possible that the m6A pathway and NuRD function independently and their inhibition results in similar phenotypes".

- Discussion text was changed and it now reads (lines 546-547):

Alternatively, m6A and NuRD regulate similar processes, such as chromatin accessibility or emergence of a new cell state (Benham-Pyle et al., 2021), independently.

5. In the abstract, authors claim that m6A is critical for planarian stem cell homeostasis and gene regulation in regeneration, but most of their analysis of gene regulation was done at homeostasis and not during regeneration. This sentence could be changed to more accurately reflect the data in the manuscript.

Following the reviewer's comment, we added the words "tissue homeostasis" to the abstract to better reflect the majority of the presented data.

Minor Criticisms:

1. If stem cells are not depleted by inhibition of m6A pathway components, what is the cause of tissue degradation/lysis? While experiments to address this question may be beyond the scope of the manuscript, it would be interesting for the authors comment on this when discussing their models.

We thank the reviewer for the comment. Our interpretation of the results is that the continuous decline in production of functional progeny (primarily intestine) leads to an increased damage to differentiated tissue and its integrity, which eventually results in lysis. Inhibition of genes that are required for the production of the intestine and its integrity are known to result in lysis phenotypes even without complete arrest of blastema production (Forsthoefel et al., *Dev Cell*, 2012). Similarly, Inhibition of *Smed-wwp*, a gene that is broadly expressed in the planarian intestine, results in dorsal lesions and incomplete regeneration (Henderson et al., *Developmental Biology*, 2015).

2. A size quantitation of kiaa1429 RNAi animals compared to controls should be added to Supplementary Figure 1F and J.

A size quantification was added to the figure.

3. In Figure 2B, a visualization that allows for comparison of control and kiaa1429 RNAi on the same plot would be beneficial for data interpretation. Or perhaps bring supplementary figure 2b into main figure?

Following the comment by the reviewer, we designated supplementary figure 2 as an expanded view figure (Fig EV2), which will enhance the visibility of this data.

4. In Figure 5K, xbp-1 expression is shown in kiaa1429 RNAi-specific clusters, pharynx, and neoblasts. Why not neural or other differentiated tissues? Also, xbp-1 is not mentioned in the main text.

We thank the reviewer for the comment. Panels J and K in figure S5 (now designated Fig EV5) show the expression of *smedwi-1*, a pan-neoblast marker, and *xbp-1,* which is expressed specifically in differentiating or differentiated cells (Raz et al., 2021). Panel J shows that *smedwi-1* is expressed, but to a limited extent, in the *kiaa1429* (RNAi)-specific cluster compared to neoblast expression. However, the expression is higher than in fully differentiated cells, as found in the pharynx cluster. Panel K shows that *xbp-1* is broadly expressed in this cluster, consistent with the interpretation that cells of the *kiaa1429* (RNAi)-specific cluster are not neoblasts, despite their detectable *smedwi-1* expression. Pharynx cells are used as a visual indicator for expression (*xbp-1*) or lack of expression (*smedwi-1*) of these markers.

We now mention *xbp-1* in the main text (line: 398-399):

"… expression of genes associated with differentiation (e.g., *xbp-1*, Fig EV5K) (Raz *et al*, 2021)"

5. Statistical tests are missing in:

o Figure 3A, C, D

o Figure 7C, F

o Supp. Figure 6A, 6B

We added this information. Where the information did not contribute to the data presentation, it was added to the figure legend.

Dear Dr Wurtzel,

Thank you for submitting your revised manuscript (EMBOJ-2021-109895R) to The EMBO Journal, as well as for your patience with our feedback, which got protracted by delayed reviewer input. Your amended study was sent back to the referees for their re-evaluation, and we have received comments from both of them, which I enclose below. As you will see, the experts stated that the work has been substantially improved by the revisions and they are now broadly in favour of publication, pending minor revision.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

Please consider the remaining minor comments of the reviewers carefully and amend the text discussion and data presentation accordingly where appropriate.

Also, we now need you to take care of a number of minor issues related to formatting and data annotation as detailed below, which should be addressed at re-submission.

Please contact me at any time if you have additional questions related to below points.

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

Again, please contact me at any time if you need any help or have further questions.

with
Best regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Please provide maximally five keywords for the manuscript.

>> Data availability section: please add a hyperlink to the database entry and make sure privacy is released before online publication of your article.

>> Appendix file: move all technical 'Appendix notes' to the main manuscript text as Material and Methods. Adjust the Author Checklist accordingly.

>> Add a separate 'Statistical analysis' section to your manuscript, detailing the algorithms applied.

>> Please adjust the title of the 'Conflict of Interest' section to 'Disclosure and Competing Interests Statement'.

>> Figure callouts: recheck callouts for Figures 8A,B in the manuscript text.

>> Please remove the author contributions information from the manuscript text. Note that CRediT has replaced the traditional author contributions section as of now because it offers a systematic machine-readable author contributions format that allows for more effective research assessment. and use the free text boxes beneath each contributing author's name to add specific details on the author's contribution. More information is available in our guide to authors.
https://www.embopress.org/page/journal/14602075/authorguide

>> Please add origin boxes to Figure S2H; Cleary indicate image assembly in Figure 1B and clarify in Figure legend.

>> Please consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

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 (1st Sep 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

Referee #1:

In the revised version of their manuscript Dagan and colleagues have done a fantastic job in addressing all points I raised, major and minor. They have added substantial pieces of new data, such as a thorough analysis of a potential m6A consensus motif in planarians. Moreover, their point-by-point response is formatted in a very pleasant and comprehensive way.
I therefore enthusiastically recommend publication of the revised manuscript at this point. The only point I urge the authors to investigate further in the future is the very surprising lack of phenotype they observe after wtap (RNAi).

Referee #2:

The resubmitted manuscript by Omri Wurtzel's group is significantly improved over the prior version. They have added visualization and quantification of cell cycle indicators H3P and F-ara-EdU to demonstrate that neoblasts proceed through G1 and S phase at similar rates in kiaa1429 RNAi treated animals. They have also significantly improved their characterization and quantification of the intestinal morphology and intestinal differentiation phenotypes that they observe in kiaa1429 depleted animals. Finally, the authors have much improved their discussion to equally consider several models for how NuRD and m6a might be connected. Together, these data demonstrate the importance of m6a-modifications in cell fate choice and tissue homeostasis and mark an important advance for the field. The authors' efforts to complete these experiments, add additional data and methods to the manuscript, and revise their discussion should be appreciated. I have a few remaining minor criticisms that were not addressed, particularly regarding a more careful characterization of the kiaa1429-specific cluster identified by scRNAseq. However, I believe that these concerns could be easily addressed without additional experiments prior to publication and therefore believe that the revised manuscript is suitable for publication the EMBO journal with only minor changes.

Remaining Criticisms:

1. Lines 413 and regarding my major criticism number #3, since only a limited number of the kiaa1429-specific cluster markers could be visualized in vivo (dd_1837 and dd_585) and none of these markers have been co-localized in vivo, additional evidence should be presented that this cluster truly represents a 'molecularly defined progenitor population.' Compared to other clusters analyzed in Dataset EV3, the kiaa1429-specific cluster appears to have relatively few specific molecular markers and they have diverse cell type-specific gene expression in Fincher et al., so there is relatively little data to support a neural or glial cell identity. I strongly recommend that the authors expand Figure 6F to include smad6/7-2, protocadherin-like gene (dd_15376) and additional markers listed in Dataset EV3 to more rigorously depict the collection of genes that define this 'cell population.'
2. Similarly, mentioning 2 genes in lines 406-407 (smad6/7-2 and protocadherin-like gene) while only showing data for smad6/7-2 in figure EV5 seems odd if this is a cell population. For the best molecular markers of the kiaa1429-specific cluster identified in the requested heatmap, the authors should show feature plots of the whole dataset, instead of pooled violin plots from a selected lineages.
3. Please clarify what database the sequencing data produced for the manuscript has been deposited in. In fact, adding a link to the supplied datasets would be good.

We are pleased to submit our manuscript EMBOJ-2021-109895R2 “m6A is required for resolving progenitor identity during planarian stem cell differentiation”. We thank the reviewers for their supportive feedback on our revision. We made the requested changes, and they are detailed below.

>> Please provide maximally five keywords for the manuscript.

Planarian, m6A, Regeneration, Stem cells, Differentiation

>> Data availability section: please add a hyperlink to the database entry and make sure privacy is released before online publication of your article.

A hyperlink to the Sequence Read Archive database was added. The link will be activated upon publication.

>> Appendix file: move all technical 'Appendix notes' to the main manuscript text as Material and Methods. Adjust the Author Checklist accordingly.

We moved the appendix notes to the main manuscript text, adjusted the call outs accordingly, and modified the references section in the appendix file and in the main text accordingly.

>> Add a separate 'Statistical analysis' section to your manuscript, detailing the algorithms applied.

We added a statistical analysis section to the manuscript**.**

>> Please adjust the title of the 'Conflict of Interest' section to 'Disclosure and Competing Interests Statement'.

We changed the section title.

>> Figure callouts: recheck callouts for Figures 8A,B in the manuscript text.

Callouts were rechecked throughout the manuscript.

>> Please remove the author contributions information from the manuscript text. Note that CRediT has replaced the traditional author contributions section as of now because it offers a systematic machine-readable author contributions format that allows for more effective research assessment. and use the free text boxes beneath each contributing author's name to add specific details on the author's contribution. More information is available in our guide to authors.
https://www.embopress.org/page/journal/14602075/authorguide

Author contribution information was removed from the manuscript text. CRediT was added in the manuscript revision submission form.

>> Please add origin boxes to Figure S2H; Cleary indicate image assembly in Figure 1B and clarify in Figure legend.

The higher magnification image was taken from a different field-of-view. It is clarified in the text: “High magnification images (bottom) were acquired from different field-of-views from similar anatomical areas”.

>> Please consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

Referee #1:

In the revised version of their manuscript Dagan and colleagues have done a fantastic job in addressing all points I raised, major and minor. They have added substantial pieces of new data, such as a thorough analysis of a potential m6A consensus motif in planarians. Moreover, their point-by-point response is formatted in a very pleasant and comprehensive way.
I therefore enthusiastically recommend publication of the revised manuscript at this point. The only point I urge the authors to investigate further in the future is the very surprising lack of phenotype they observe after wtap (RNAi).

We thank the reviewer for the comments and the highly supportive feedback for our revised manuscript. We plan to revisit the *wtap* inhibition experiments when we have more information on the dsRNA vector.

Referee #2:

The resubmitted manuscript by Omri Wurtzel's group is significantly improved over the prior version. They have added visualization and quantification of cell cycle indicators H3P and F-ara-EdU to demonstrate that neoblasts proceed through G1 and S phase at similar rates in kiaa1429 RNAi treated animals. They have also significantly improved their characterization and quantification of the intestinal morphology and intestinal differentiation phenotypes that they observe in kiaa1429 depleted animals. Finally, the authors have much improved their discussion to equally consider several models for how NuRD and m6a might be connected. Together, these data demonstrate the importance of m6a-modifications in cell fate choice and tissue homeostasis and mark an important advance for the field. The authors' efforts to complete these experiments, add additional data and methods to the manuscript, and revise their discussion should be appreciated. I have a few remaining minor criticisms that were not addressed, particularly regarding a more careful characterization of the kiaa1429-specific cluster identified by scRNAseq. However, I believe that these concerns could be easily addressed without additional experiments prior to publication and therefore believe that the revised manuscript is suitable for publication the EMBO journal with only minor changes.

We thank the reviewer for the encouraging feedback and analysis of our manuscript.

Remaining Criticisms:

1. Lines 413 and regarding my major criticism number #3, since only a limited number of the kiaa1429-specific cluster markers could be visualized in vivo (dd_1837 and dd_585) and none of these markers have been co-localized in vivo, additional evidence should be presented that this cluster truly represents a 'molecularly defined progenitor population.' Compared to other clusters analyzed in Dataset EV3, the kiaa1429-specific cluster appears to have relatively few specific molecular markers and they have diverse cell type-specific gene expression in Fincher et al., so there is relatively little

data to support a neural or glial cell identity. I strongly recommend that the authors expand Figure 6F to include smad6/7-2, protocadherin-like gene (dd_15376) and additional markers listed in Dataset EV3 to more rigorously depict the collection of genes that define this 'cell population.'

We thank the reviewer for the comment. We added to Fig EV5 panel with feature plots (UMAP) showing *smad6/7-2* and *protocadherin-like* (dd_15376) and dd_1620. Four other genes that are highly enriched in the cluster are shown in Fig 6F in a single cell resolution heatmap.

2. Similarly, mentioning 2 genes in lines 406-407 (smad6/7-2 and protocadherin-like gene) while only showing data for smad6/7-2 in figure EV5 seems odd if this is a cell population. For the best molecular markers of the kiaa1429-specific cluster identified in the requested heatmap, the authors should show feature plots of the whole dataset, instead of pooled violin plots from a selected lineages.
We thank the reviewer for the comment. We added to Fig EV5 the requested feature plots (UMAP). Fig 6F is a single cell resolution heatmap of 4 genes that are expressed in the *kiaa1429* (RNAi)-specific cluster.

3. Please clarify what database the sequencing data produced for the manuscript has been deposited in. In fact, adding a link to the supplied datasets would be good.

We addressed the reviewer's comment, and added a link. The text now reads:
"High-throughput sequencing data produced in this project was deposited to the Sequence Read Archive (Leinonen et al. 2011). The data is available under BioProject accession PRJNA747686".

2nd Revision - Editorial Decision **12th Jul 2022**

Dear Dr Omri Wurtzel,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper.

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Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- 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?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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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Newly Created Materials	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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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	
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