EMBO reports

Nuclear m^{6}A Reader YTHDC1 suppresses proximal Alternative Polyadenylation sites by Interfering with the 3' Processing Machinery

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Dear Prof. Xu,

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

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also have several suggestions for how the study could be further improved. I think all points raised are reasonable and should be addressed. Please let me know if you have any questions or comments.

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

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (31st May 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

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

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

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

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

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

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

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

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

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public

database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please 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. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

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

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The following points must be specified in each figure legend:

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

- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

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

- Please also include scale bars in all microscopy images.

11) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests

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

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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

Yours sincerely,

Esther Schnapp, PhD

Senior Editor
EMBO reports

Referee #1:

In this study, Chen & Fu and colleagues seek to establish a role for the nuclear m6A-binding protein YTHDC1 in the regulation of alternative polyadenylation (APA) site selection. The rationale for this study is that m6A has potential links to APA site selection established by earlier studies, but the mechanism underpinning the relationship between m6A and APA is unclear. The authors use a variety of NGS and biochemical data in their effort to demonstrate that interactions between m6A, YTHDC1, and the polyadenylation machinery (via direct FIP1L1 contact) ultimately govern APA site selection for many genes.

Although the link between distal APA site selection and m6A has been established in multiple studies, the precise mechanism linking the two has remained elusive. Thus, this paper, based on its contribution to further understanding this well-established connection, should be seriously considered for publication in EMBO Reports. However, while the authors use a diverse array of methods to make their case for the connection between m6A, YTHDC1, and APA site choice, the manuscript is missing critical elements required for contextual clarity for both the results and the methods employed in the paper. Therefore, some significant textual improvements will be required. There are also minor experimental revisions that should be considered in order to improve the quality of the paper and strengthen the conclusions made by the authors.
All issues requiring attention from the authors, major and minor, are enumerated below:

Major concerns

1. Results, page 6, paragraph 2 --- The number of genes found to change APA site usage after the knockdown of YTHDC1 seems relatively small (only ~600 genes in HEK293T cells, and less than 300 genes in MCF-7 cells). Without context, it's impossible to determine how significant this result is. How does this compare with the extent of APA site changes in other types of datasets? For example, if one were to knockdown a CPSF protein, how many site changes would be observed? Or, if one were to simply compare different control datasets, how many false positive APA changes would be detected? Without that type of comparison, it could be argued that this small number of genes changing could simply be due to experimental noise, and not a physiological effect. The significance of this result should be contextualized by the authors.

2. Results, page 6-7 --- if the authors argue that these effects are mediated by YTHDC1 via interactions with m6A, then it would stand to reason that many of the genes with altered APA site usage should contain at least one m6A site. However, the authors do not provide this information. If the authors wish to claim these effects are mediated by m6A, they need to provide proof to the reader that the genes they are studying from their APA analysis have m6A. If the genes don't have m6A, a rationale must be provided to explain why they see a YTHDC1-m6A mediated effect. Importantly, the authors should also ask if this effect is more pronounced on genes that have greater amounts of m6A, or even better, in transcripts that contain m6A in the terminal exon, or stop codon area, which would plausibly be the best candidates for mediating APA site selection via YTHDC1 interactions. There is currently little justification for choosing these genes other than the basis that they gave a positive result. The authors need to justify these results more thoroughly and make a firm connection to m6A while doing so.

3. Results, page 11, ref to S Fig 4 --- The authors should include blots from the YTHDC1 coIP for the other writer proteins here such as VIRMA, ZC3H13, RBM15, and CBLL1/HAKAI. These may provide valuable additional context for which MTC components are potentially interacting with YTHDC1 besides WTAP.

4. Results, page 18, 2nd paragraph --- the quantification of the immunofluorescence results in this section falls quite short of the level of quantification performed earlier in the paper for Figure 4. At a minimum, the authors should perform a co-localization analysis using an overlap coefficient for Figure 6I for a substantial number of cells that will allow a statistical analysis to be performed. Figure 6D should be similarly analyzed to appreciate differences in the efficiency of YTHDC1 recruitment into condensates under different RNA and salt conditions.

5. Discussion, paragraph 1, line 5 --- the authors ambiguously make reference to Ke and Molinie et al without being clear about which paper supports which model (Ke supports m6A associated with distal APA, Molinie supports m6A associated with proximal APA) --- this should be corrected to be more clear. We now know that the Molinie study was flawed because the antibody they used for their LAIC-seq method also recognized m6Am, a cap-adjacent modification, and thus severely biased their results since it fails to account for this non-specific interaction. No other studies were able to replicate their findings, while other studies have also found the connection between m6A and distal APA, similar to Ke et al and the present authors. Thus, the authors presenting the Ke and Molinie result as having equal weight and the source of ongoing controversy does not accurately reflect the thinking of the field currently. The passage should be changed to focus on the association with distal APA sites that is currently accepted, so that this is not interpreted as an ongoing controversy by readers unfamiliar with the body of m6A literature on distal APA site selection that has come after the Molinie study.

Minor concerns

1. Results, page 5, line 3 --- could the authors remark more precisely on the similarities between AS and APA and provide citations that compare/contrast them more extensively?

2. Results, page 6, line 3 --- The authors should remark more extensively on IVT-SAPAS. This is a relatively obscure method and it should not be assumed that the reader knows or understands this method without a more detailed description of what it does and how it works.

3. Results, page 6, line 6-7 --- "shown in the data" needs to point to something specific in the data, i.e. a specific figure or panel

4. Results, page 10, ref to Fig. 2G --- how many of these genes have m6A? And how much m6A do they have?
Results, page 10, paragraph 2, line 9 --- provide a citation for the 'nlme' package

5. Figures, Fig 4. --- the paper would benefit from a re-ordering of the panel presentation so that panel 'C' logically follows 'B'. The current orientation of the panels is confusing.

6. Results, page 17, line 11 --- 'high scores' are referred to here, but the authors fail to explain what these high scores mean or how they are defined. The text needs more detail to help the reader, especially those unfamiliar with IDRs and associated terminology.

7. Results, page 17, line 18, 21 --- both mScarlet and DsRed are mentioned here as knock-ins for YTHDC1. Which is it? These names should not be used interchangeably.

8. Figures, Fig 6 --- some of the immunofluorescence images in this figure are low resolution and poor quality. The authors should use higher-quality vector images when creating this figure so that the immunofluorescence results and labels are clear and not pixelated.

9. Figures, Fig 6 --- the in vitro immunofluorescence images should be labeled with the protein that is being imaged. Right now, it's not clear from looking at the panels what they are supposed to represent (Fig 6B, D, and F)

10. Data availability --- the authors should ensure that their processed sequencing data used to generate all figures in the paper is available through GEO. Currently, only an SRA entry for raw sequencing data is provided and processed data cannot be verified.

11. Methods --- Authors should supply the manufacturer and lot number for the antibodies used in the IPs, western blots, and immunofluorescence experiments used in this study.

Referee #2:

This is overall an excellent and interesting paper with good quality data.
However, these issues need addressing before publication:

Figure 1: Although I don't doubt the conclusions, it appears a very arbitrary choice to only verify lengthened 3' UTRs in HEK293 and only shortened ones in MCF7. Is there a rationale for this? It would be good to have a somewhat more balanced verification, including lengthening and shortening 3' UTRs in both cell lines.

P7 "Both qRT-PCR (Supplemental Figure. S3A) and western blot (Supplemental Figure. S3B) showed that the expression levels of these related factors were not markedly altered after the knockdown of YTHDC1." In fact, the supplemental figure S3 only contains qPCR data for related factors, no western blots. Either this statement needs modifying (as protein level changes may still be happening) or the westerns need adding.

To what extent are the same genes affected in terms of APA in HEK293 and MCF7? Are the genes with lengthening and or shortening 3' UTRs functionally related?

P8 "the genes with a shortened 3' UTR due to the knockdown of YTHDC1, their proximal but not distal APA sites showed a higher density of YTHDC1 binding peaks compared to background or lengthened genes (Figure. 2C)" I don't think this is true for the comparison for the lengthened mRNAs and the shortened mRNAs, there is still a lower peak size at the distal sites for the lengthened mRNAs. I suggest "or lengthened" is deleted from this sentence. It is in fact interesting that the lengthening mRNAs have such low levels of YTHDC1 compared to control at both locations.

P9 The experiment with the dual luciferase reporter is complicated, considering all the potential roles of YTHDC1 in protein expression. A qPCR for the different open reading frames, or a classical Northern blot could have produced a clearer result.

Another confounding factor is that the authors don't determine YTHDC1 binding to the poly(A) sites used in the reporter. As it doesn't contribute a lot to the paper, it could be left out or moved to the supplement.

In contrast I think that the IP experiments described on p11 are interesting and Supplemental Figure 4 deserves to be in the main paper, provided sufficient replicates were done (if this is N=1 it should not be in the paper at all).

The data in this paper look very good, but there is a lack of key information on the western blots in this paper, and they are present in most of the figures. Which antibodies (manufacturer, catalogue number)? Are the sizes of proteins as expected? How often was each western blot repeated? It is a good idea to show replicates of western blots in the supplement, especially of the key data, given the known issues with data obtained by this method, especially when detecting endogenous proteins.

Minor problems
Throughout the paper, the authors refer to "lengthened genes" when in fact they mean "lengthened mRNAs". The genes are not changing in size!
P7 "in consistent with the proposed sequencing data (Supplemental Figure. S2)" - delete "in" or replace "consistent" with "agreement"
Supplemental figure S3C: Are the two Venn diagrams for the two cell lines? Please add labels to distinguish them
The line plot in Fig 2E is inappropriate, these are categorical values and should not be connected with a line. A categorical scatter plot showing all the replicate measurements is preferred. Even an old fashioned bar graph with error bars would be better.
For Fig 2G a categorical scatter plot would also work better.
P10 "(YTHDC1-Mut) in HKE293T cells (Figure. 2F)," Are HEK293T cells meant?
Size markers are required for Figure 5B.

Referee #3:

Manuscript: Nuclear m6A reader YTHDC1 regulates mRNA alternative polyadenylation by interfering with 3' processing machinery complex

By Chen et al.

This study by Chen et al. tackled the role of the m6Areader YTHDC1 in the regulation of alternative poly(A) (APA) site selection in human cells. Several prior studies have pointed to the potential of 3' UTR m6A modifications and the corresponding writers and readers in the regulation of 3' end mRNA processing. The regulation of 3' UTR length is one of the key factors that can affect gene expression. Here, authors show that YTHDC1 affects the APA selection in a subset of genes in HEK293T and MCF-7 cells. Furthermore, they reveal that YTHDC1 interacts with several 3' end processing factors in an RNA-dependent manner and FIP1L1 independently of RNA. By several additional experiments they tackle the mechanism of YTHDC1 - FIP1L1 APA regulation. By competitive IP experiments they suggest that it results from outcompeting FIP1L1 - CPSF4 interaction. This is a timely and important study. Most previous analyses focused on rather high-throughput analyses. This study brings more detailed and mechanistical insights. I believe that by addressing of several comments and additional experiments it is suitable to be published in EMBO Reports.

Comments and questions:
1. The first part that tackles the APA and 3' UTR length changes upon YTHDC1 downregulation in HEK293T and MCF-7 cells. I was surprised that the authors did not reveal the overlap of genes with shortened/lengthened 3' UTRs between the two siRNAs used in the same cell type, and between the two cell types. This analyses seems critical to strengthen the notion that the UTR phenotype observed is not stochastic. Why was the RT-qPCR validation focused only on genes with lengthened UTR in HEKs and only on shortened in MCF-7 cells (Figure 1D)? What led to the selection of the set of the genes in panel 1D?
2. Since the expression levels of YTHDC1 are loinked to the APA regulation by the authors, they should check the effect of WT and mutant YTHDC1 overexpression on the 3' UTR length in a subset of selected genes from their RNAseq analysis.
3. The second part deals with the regulation of APA in an m6A-dependent manner. Alike in point 1, I am missing an analysis of the overlap between the two siRNAs targeting YTHDC1.
The reporter system experiments reveal surprisingly clear indication for the regulatory hypothesis. The authors use a YTHDC1 mutant that doesn't bind m6A to prove the dependency of the regulatory mechanism on m6A. However, another they should also perform the same experiment in cells upon METTL3 knock down.
Minor question: why is there a double band observed in cells overexpressing YTHDC1?

4. Minor comment for the Figure 3. The panels B, C and D could be merged to one.

5. What exactly is the IP in Figure 5D, right panel showing? The signal for PAPOLA IPed with Myc-FIP1L1 seems not convincing due to the wage signal on the western blot.

6. Last part dealing with the nuclear condensates. The experiment in Figure 6I should be performed in cells upon METTL3 KD to again address the m6A dependency for YTHDC1 mediated droplet formation. Several times in the text, the FIP1L1 is misspelled as FIL1L1.

Dear Editor:

Our point-by-point responses are followed:

1. *A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.*

   Answer: We added a data availability section in our new version of manuscript.

2. *Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.*

   Answer: We checked again and ensured none of our statistics and error bars based on n=2.

3. *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.*

   Answer: We have revised the manuscript as shown in text.

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

   Answer: We have replaced Supplementary Information with Expanded View (EV) Figures, and cited properly in the new version of our paper.

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

   Answer: We checked again and ensured that all have been properly set.

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

   Answer: We checked again and ensured that all have been properly set.

7. *a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part*

of the Review Process File (RPF), which will be published alongside your paper.

Answer: We agree with these policies.

8. *A complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>;. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.*

Answer: We updated the completed author information in the revised manuscripts.

9. *Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>;). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>;*

Answer: We linked ORCID ID to this journal account as you asked

10. *Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please 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. *If your study has not produced novel datasets, please mention this fact in the Data Availability Section.*

Answer: We submitted primary datasets in our new version of manuscript.

11. *We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>;.*

Answer: We submitted source data in the format of EMBO REPORT.

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

Answer: We revised data citations in the main test and reference list and ensured that all have been properly set.

13. *Regarding data quantification (see Figure Legends: https://www.embopress.org/page/journal/14693178/authorguide#figureformat)*

Answer: We revised data quantification part in our new version of paper.

14. *The following points must be specified in each figure legend:*

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

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- Please also include scale bars in all microscopy images.

Answer: We checked all figure legend and ensured that all have been properly set.

15. *The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests*

Answer: We made a statement in which all authors have declared no competing interests.

Referee #1:

In this study, Chen & Fu and colleagues seek to establish a role for the nuclear m6A-binding protein YTHDC1 in the regulation of alternative polyadenylation (APA) site selection. The rationale for this study is that m6A has potential links to APA site selection established by earlier studies, but the mechanism underpinning the relationship between m6A and APA is unclear. The authors use a variety of NGS and biochemical data in their effort to demonstrate that interactions between m6A, YTHDC1, and the polyadenylation machinery (via direct FIP1L1 contact) ultimately govern APA site selection for many genes.

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Answer: Thank you very much for your positive comment on our study, and we improved our manuscript based on your suggestions. A little tip: The order of the pictures in below response has changed as we added a new picture as Figure 3 in the main text of revised manuscript.

1. *Results, page 6, paragraph 2 --- The number of genes found to change APA site usage after the knockdown of YTHDC1 seems relatively small (only ~600 genes in HEK293T cells, and less than 300 genes in MCF-7 cells). Without context, it's impossible to determine how significant this result is. How does this compare with the extent of APA site changes in other types of datasets? For example, if one were to knockdown a CPSF protein, how many site changes would be observed? Or, if one were to simply compare different control datasets, how many false positive APA changes would be detected? Without that type of comparison, it could be argued that this small number of genes changing could simply be due to experimental noise, and not a physiological effect. The significance of this result should be contextualized by the authors.*

Answer: We are sorry for the confusion. Actually, the genes number with significant APA switching after knockdown of YTHDC1 is similar to knockdown of 3'end processing factors in previous study (Li et al. PLOS Genet, 2015; Yao et al. RNA, 2017). We added these references in the new version of manuscript, which provides the extent of APA site changes in other types of datasets.

2. *Results, page 6-7 --- if the authors argue that these effects are mediated by YTHDC1 via interactions with m6A, then it would stand to reason that many of the genes with altered APA site usage should contain at least one m6A site. However, the authors do not provide this information. If the authors wish to claim these effects are mediated by m6A, they need to provide proof to the reader that the genes they are studying from their APA analysis have m6A. If the genes don't have m6A, a rationale must be provided to explain why they see a YTHDC1-m6A mediated effect. Importantly, the authors should also ask if this effect is more pronounced on genes that have greater amounts of m6A, or even better, in transcripts that contain m6A in the terminal exon, or stop codon area, which would plausibly be the best candidates for mediating APA site selection via YTHDC1 interactions. There is currently little justification for choosing these genes other than the basis that they gave a positive result. The authors need to justify these results more thoroughly and make a firm connection to m6A while doing so.*

Answer: It is a good question. a) In the new version of manuscript, we compared the distribution of m^6A modification between genes with APA switching and background. (Figure. EV4). The results display that the genes with shortened 3' UTR show a higher m^6A modification near of proximal APA sites than the genes with lengthened 3' UTR or background (Figure. EV4). b) After interfering m^6A writer METTL3, the YTHDC1 target genes also show similar APA switching trends (Figure. EV5). Besides, we defined YTHDC1 target genes as those whose supporting reads of both MeRIP and YTHDC1 were greater than 50, and MeRIP reads were twice as numerous as the negative control with the MeRIP (m6A seq) and YTHDC1 iCLIP data (Meyer et al., 2012, Patil et al., 2016) in Figure. 2C, indicating the association of APA and m^6A to some extent. All of those results reveals that YTHDC1 can inhibit proximal APA sites in an m^6A-dependent manner.

3. *Results, page 11, ref to S Fig 4 --- The authors should include blots from the YTHDC1 coIP for the other writer proteins here such as VIRMA, ZC3H13, RBM15, and CBLL1/HAKAI. These may provide valuable additional context for which MTC components are potentially interacting with YTHDC1 besides WTAP.*

Answer: Your advices were well taken. We overexpressed the other writer proteins of CBLL1, RBM15, ZC3H13 and VIRMA, and co-IP results show that they barely pulldown YTHDC1 (Figure.3D). This result provides a solid evidence that YTHDC1 interacting with MTC complex via WTAP.

4. *Results, page 18, 2nd paragraph --- the quantification of the immunofluorescence results in this section falls quite short of the level of quantification performed earlier in the paper for Figure 4. At a minimum, the authors should perform a co-localization analysis using an overlap coefficient for Figure 6I for a substantial number of cells that will allow a statistical analysis to be performed.*

Answer: Your advices were well taken. We analyzed the overlap coefficient for DsRed-YTHDC1-WT/Mut and eGFP-FIP1L1, and the mutation of m^6A binding sites of YTHDC1 damages the co-localization of YTHDC1 and FIP1L1 (Figure. 7J).

5. *Figure 6D should be similarly analyzed to appreciate differences in the efficiency of YTHDC1 recruitment into condensates under different RNA and salt conditions.*

Answer: We tried to perform *in vitro* phase separation assay for YTHDC1, but failed to express and purify YTHDC1 protein in *E.coli*, and also could not get enough YTHDC1 proteins from mammalian expression systems to perform the *in vitro* assay. Besides, *in vivo* FRAP assay is more convincing in phase separation assay (Figure. 7H in the new version of manuscript), so we do not analyze it here.

6. *Discussion, paragraph 1, line 5 --- the authors ambiguously make reference to Ke and Molinie et al without being clear about which paper supports which model (Ke supports m6A associated with distal APA, Molinie supports m6A associated with proximal APA) --- this should be corrected to be more clear. We now know that the Molinie study was flawed because the antibody they used for their LAIC-seq method also recognized m6Am, a cap-adjacent modification, and thus severely biased their results since it fails to account for this non-specific interaction. No other studies were able to replicate their findings, while other studies have also found the connection between m6A and distal APA, similar to Ke et al and the present authors. Thus, the authors presenting the Ke and Molinie result as having equal weight and the source of ongoing controversy*

does not accurately reflect the thinking of the field currently. The passage should be changed to focus on the association with distal APA sites that is currently accepted, so that this is not interpreted as an ongoing controversy by readers unfamiliar with the body of m6A literature on distal APA site selection that has come after the Molinie study.

Answer: Thank you for your good advice. We did not realize that the Molinie study was flawed until you told us. Here, in the discussion part, we replaced "However, whether m^6A can promote or inhibit proximal APA sites (Ke et al., 2015, Molinie et al., 2016) continues to be debated, partly due to an unknown molecular mechanism. Knockdown/knockout of the m^6A "writers" or "erasers" still did not solve the contradiction (Bartosovic et al., 2017, Ke et al., 2015, Yue et al., 2018)." with "Consistent with previous studies (Bartosovic et al., 2017, Ke et al., 2015, Meyer et al., 2012), this study provides a detailed molecular regulatory mechanism (Figure. 7), which supports the model that m^6A inhibit proximal APA processing and associate with distal APA sites."

Minor concerns

1. *Results, page 5, line 3 --- could the authors remark more precisely on the similarities between AS and APA and provide citations that compare/contrast them more extensively?*

   Answer: We added new citations to provide the detailed mechanism of AS and APA in the new version of manuscript.

2. *Results, page 6, line 3 --- The authors should remark more extensively on IVT-SAPAS. This is a relatively obscure method and it should not be assumed that the reader knows or understands this method without a more detailed description of what it does and how it works.*

   Answer: We rewrote the details of IVT-SAPAS in materials and methods part.

3. *Results, page 6, line 6-7 --- "shown in the data" needs to point to something specific in the data, i.e. a specific figure or panel.*

   Answer: Your advice was well taken. We point to the number of annotated poly(A) sites and genes in our new version of paper, which are key summary of APA sequencing method. We rewrote the sentence as follows: "The numbers of annotated poly(A) sites and genes are similar with previous studies (Fu et al,

2018), and details of each sample of the sequencing data are shown in Table EV1."

4. *Results, page 10, ref to Fig. 2G --- how many of these genes have m6A? And how much m6A do they have?*

Answer: Actually, the genes in Fig.2 G all have m^6A modification, eight of those genes have greater than 50 support reads from MeRIP and YTHDC1 iCLIP data, and MeRIP reads were twice as numerous as the negative control.

5. *Results, page 10, paragraph 2, line 9 --- provide a citation for the 'nlme' package*

Answer: We added a citation for the 'nlme' package as your suggested.

6. *Figures, Fig 4. --- the paper would benefit from a re-ordering of the panel presentation so that panel 'C' logically follows 'B'. The current orientation of the panels is confusing.*

Answer: Your advice was well taken. We re-ordered the old Figure 4 in Figure 5 in the new version of manuscript.

7. *Results, page 17, line 11 --- 'high scores' are referred to here, but the authors fail to explain what these high scores mean or how they are defined. The text needs more detail to help the reader, especially those unfamiliar with IDRs and associated terminology.*

Answer: We are sorry for the simplified writing. We further explained IDRs and provided a citation of IDRs prediction tool to address this doubt. We rewrote the sentence as follows: "LLPS proteins usually contain intrinsically disordered regions (IDRs) with low hydrophobicity and high net charge polypeptide segments. With IDRs score prediction tool IUPred2A (Meszaros et al, 2018), we found that the key domain mediating the interaction between YTHDC1 and FIP1L1 (Figure. 7A black box) contained IDRs with high scores (score > 0.5)."

8. *Results, page 17, line 18, 21 --- both mScarlet and DsRed are mentioned here as knock-ins for YTHDC1. Which is it? These names should not be used interchangeably.*

Answer: We are sorry for the confusion. Actually, the endogenous

mScarlet-YTHDC1 protein is too weak to perform FRAP assay, and we overexpressed Dsred-YTHDC1 recombinant proteins instead. We clarified this as follows: “We also obtained homozygous mScarlet-YTHDC1 knock-in cell lines (Figure. 7E). However, the endogenous mScarlet-YTHDC1 proteins are too weak to perform FRAP assay. Thus, we overexpressed DsRed-YTHDC1 to perform FRAP assay, and found that DsRed-YTHDC1 also displayed dynamic and liquid-like properties *in vivo* (Figure. 7H).”

9. *Figures, Fig 6 --- some of the immunofluorescence images in this figure are low resolution and poor quality. The authors should use higher-quality vector images when creating this figure so that the immunofluorescence results and labels are clear and not pixelated.*

Answer: We updated them with new high-resolution pictures in the new version.

10. *Figures, Fig 6 --- the in vitro immunofluorescence images should be labeled with the protein that is being imaged. Right now, it's not clear from looking at the panels what they are supposed to represent (Fig 6B, D, and F)*

Answer: Your suggestion was well taken. We labeled the names of fluorescent proteins in our new version.

11. *Data availability --- the authors should ensure that their processed sequencing data used to generate all figures in the paper is available through GEO. Currently, only an SRA entry for raw sequencing data is provided and processed data cannot be verified.*

Answer: We submitted our sequencing data in GEO database and provided a GEO number (GSE198143) in data availability part.

12. *Methods --- Authors should supply the manufacturer and lot number for the antibodies used in the IPs, western blots, and immunofluorescence experiments used in this study.*

Answer: Your advice were well taken. We updated the detailed information of reagents and instruments in our new version.

Referee #2:

This is overall an excellent and interesting paper with good quality data. However, these issues need addressing before publication.

Answer: Thank you for your positive comment on our study. A little tip: The order of the pictures in below response has been changed as we added a new picture as Figure 3 in the main text of revised manuscript.

1. *Figure 1: Although I don't doubt the conclusions, it appears a very arbitrary choice to only verify lengthened 3' UTRs in HEK293 and only shortened ones in MCF7. Is there a rationale for this? It would be good to have a somewhat more balanced verification, including lengthening and shortening 3' UTRs in both cell lines.*

   Answer: We are sorry for the confusion. We verified genes lengthening and shortening 3' UTRs in both cell lines. We updated this result in our new Figure 1E.

2. *P7 "Both qRT-PCR (Supplemental Figure. S3A) and western blot (Supplemental Figure. S3B) showed that the expression levels of these related factors were not markedly altered after the knockdown of YTHDC1." In fact, the supplemental figure S3 only contains qPCR data for related factors, no western blots. Either this statement needs modifying (as protein level changes may still be happening) or the westerns need adding.*

   Answer: Thank you for your suggestion. We removed the result of western blot of METTL3 and METTL4, and rewrote the paragraph as: “The qRT-PCR (Figure. EV3A) showed that the mRNA expression levels of these related factors were not markedly altered after the knockdown of YTHDC1.”

3. *To what extent are the same genes affected in terms of APA in HEK293 and MCF7? Are the genes with lengthening and or shortening 3' UTRs functionally related?*

   Answer: A venn diagram in the new Figure. 1C shows that the APA genes between HEK293T and MCF7 are highly overlapped (12.5% for siRNA1 and 7.8%

for siRNA2) with statistical significance (p=2.068498e-24 for siRNA1 and p=4.875887e-17 for siRNA2 with hypergeometric test).

We performed gene function annotation with Metascape (http://metascape.org/gp/#/main/step1), and found that both genes with shortening and lengthening 3' UTRs are enriched in cell proliferation and metabolism related terms. The genes with shortening 3' UTRs are enriched in Rho GTPases signaling pathway, mitotic nuclear division and aspartate family amino acid biosynthetic process, and the genes with lengthening 3' UTRs are enriched in VEGFA-VEGFR2 signaling pathway, mRNA metabolic process and so on.

4. *P8 "the genes with a shortened 3' UTR due to the knockdown of YTHDC1, their proximal but not distal APA sites showed a higher density of YTHDC1 binding peaks compared to background or lengthened genes (Figure. 2C)" I don't think this is true for the comparison for the lengthened mRNAs and the shortened mRNAs, there is still a lower peak size at the distal sites for the lengthened mRNAs. I suggest "or lengthened" is deleted from this sentence. It is in fact interesting that the lengthening mRNAs have such low levels of YTHDC1 compared to control at both locations.*

Answer: Your advice was well taken. We deleted “or lengthened” in our new version of paper. Actually, the genes with lengthened 3’ UTRs have lower YTHDC1 binding even than background genes (Fig.2C), indicating that those genes are barely regulated by YTHDC1. Considering multiple roles of YTHDC1, the lengthened 3’ UTRs may be an indirect effect by some gene regulatory network caused by knockdown of YTHDC1.

5. *P9 The experiment with the dual luciferase reporter is complicated, considering all the potential roles of YTHDC1 in protein expression. A qPCR for the different open reading frames, or a classical Northern blot could have produced a clearer result. Another confounding factor is that the authors don't determine YTHDC1 binding to the poly(A) sites used in the reporter. As it doesn't contribute a lot to the paper, it could be left out or moved to the supplement.*

Answer: Thank you for your comment. Actually, we performed RT-qPCR for Firefly and Renilla luciferase open reading frames in our figure 2E (Left), and the

results show that λN-YTHDC1 but not YTHDC1 inhibits proximal APA sites （Figure 2E, left).

Bicistronic dual luciferase report assay is a common method to test a RBP role on APA sites processing (Deng, et al., 2018, Zhu, et al., 2018). λ bacteriophage anti-terminator protein N (λN-(1–22) or λN peptide) is used to tag the protein of interest, and it has been shown to have the high affinity binding to Boxb sequence (Baron-Benhamou et al., 2004), providing a reliable verification that YTHDC1 can inhibit the proximal APA sites by binding to upstream sites.

Base on above reasons, we think that this result is an orthogonal method to prove the YTHDC1 role on APA sites processing, and then we prefer to retain it in Figure 2.

6. In contrast I think that the IP experiments described on p11 are interesting and Supplemental Figure 4 deserves to be in the main paper, provided sufficient replicates were done (if this is N=1 it should not be in the paper at all).

Answer: Your advice was well taken. We put Supplemental Figure 4 in the main paper as Figure 3. We also explored the interaction between YTHDC1 and other writer proteins, such as VIRMA, ZC3H13, RBM15, and CBLL1/HAKAI (Figure. 3D). The results show that YTHDC1 are preloaded with the methyltransferase complex mainly via WTAP rather than other writer proteins (Figure. 3).

7. The data in this paper look very good, but there is a lack of key information on the western blots in this paper, and they are present in most of the figures. Which antibodies (manufacturer, catalogue number)? Are the sizes of proteins as expected? How often was each western blot repeated? It is a good idea to show replicates of western blots in the supplement, especially of the key data, given the known issues with data obtained by this method, especially when detecting endogenous proteins.

Answer: Thank you for your comment. We updated information of antibodies (including manufacturer, catalogue number) in the main text of revised manuscript, and other details of western blot (protein size, replicates and so on) along with results in the source data.

Minor problems:

1. *Throughout the paper, the authors refer to "lengthened genes" when in fact they mean "lengthened mRNAs". The genes are not changing in size!*

   Answer: We are sorry for the improper claims. We tuned down the tone in the new version of manuscript. We replaced "lengthen genes" with “lengthened transcripts” in the new version of paper.

2. P7 "in consistent with the proposed sequencing data (Supplemental Figure. S2)" - delete "in" or replace "consistent" with "agreement".

   Answer: Thank you very much for your comment! We delete “in” in our new version of paper.

3. Supplemental figure S3C: Are the two Venn diagrams for the two cell lines? Please add labels to distinguish them.

   Answer: Thank you for your common. The two venn diagrams are HEK293T (left) and MCF7 (right) cells, and we labeled the name of two cell lines in new Figure EV. 3B

4. *The line plot in Fig 2E is inappropriate, these are categorical values and should not be connected with a line. A categorical scatter plot showing all the replicate measurements is preferred. Even an old fashioned bar graph with error bars would be better.*

   Answer: Your advice was well taken. We replaced line plot with scatter plot with error bar in our new Figure of 2E.

5. *For Fig 2G a categorical scatter plot would also work better.*

   Answer: We updated Figure 2G as you suggested.

6. *P10 "(YTHDC1-Mut) in HKE293T cells (Figure. 2F)," Are HEK293T cells meant?*

Answer: We are sorry for our mistaken. We corrected it in the new version of paper.

7. *Size markers are required for Figure 5B.*

Answer: We provided size makers in the part of source data.

Referee #3:

This study by Chen et al. tackled the role of the m6A reader YTHDC1 in the regulation of alternative poly(A) (APA) site selection in human cells. Several prior studies have pointed to the potential of 3' UTR m6A modifications and the corresponding writers and readers in the regulation of 3' end mRNA processing. The regulation of 3' UTR length is one of the key factors that can affect gene expression. Here, authors show that YTHDC1 affects the APA selection in a subset of genes in HEK293T and MCF-7 cells. Furthermore, they reveal that YTHDC1 interacts with several 3' end processing factors in an RNA-dependent manner and FIP1L1 independently of RNA. By several additional experiments they tackle the mechanism of YTHDC1 - FIP1L1 APA regulation. By competitive IP experiments they suggest that it results from outcompeting FIP1L1 - CPSF4 interaction. This is a timely and important study. Most previous analyses focused on rather high-throughput analyses. This study brings more detailed and mechanistical insights. I believe that by addressing of several comments and additional experiments it is suitable to be published in EMBO Reports.

Answer: Thank you for your positive comments on our research. A little tip: The order of the pictures in below response has been changed as we added a new picture as Figure 3 in main text.

Comments and questions:

1. *The first part that tackles the APA and 3' UTR length changes upon YTHDC1 downregulation in HEK293T and MCF-7 cells. I was surprised that the authors did not reveal the overlap of genes with shortened/lengthened 3' UTRs between the two siRNAs used in the same cell type, and between the two cell types. This analyses seems critical to strengthen the notion that the UTR phenotype observed is not stochastic.*

Answer: This is a good advice. Because of the fewer genes with lengthened 3' UTRs, here we only provided a Venn diagram for the overlap of genes with

shortened 3' UTRs in Figure. 1C. High overlaps between two siRNAs used in the same cell type are found (42.9% for MCF7 and 26.1% for HEK293T), suggesting that the 3'UTR phenotype observed is reliable.

2. *Why was the RT-qPCR validation focused only on genes with lengthened UTR in HEKs and only on shortened in MCF-7 cells (Figure 1D)? What led to the selection of the set of the genes in panel 1D?*

Answer: We are sorry for the confusion. Actually, we intended to validate both genes with lengthening and shortening 3' UTRs. Considering number of genes with lengthening 3' UTRs is far less than genes with shortening 3' UTRs (Figure. 1B), we selected genes with lengthening 3' UTRs from HEK293T as more genes in this cell line (202 genes with lengthening 3' UTRs for HEK293T and 68 for MCF7). To test more cell line, we selected genes with shortening 3' UTRs in MCF7. To avoid this confusion, we now verified the genes with lengthening and shortening 3' UTRs in both cell lines. We updated this result in our new Figure 1E.

3. Since the expression levels of YTHDC1 are loinked to the APA regulation by the authors, they should check the effect of WT and mutant YTHDC1 overexpression on the 3' UTR length in a subset of selected genes from their RNAseq analysis.

Answer: Thank you very much for your comment! We did check the effect of YTHDC1-WT and YTHDC1-Mut overexpression on a subset of selected genes from RNA-seq analysis in Figure. 2G, demonstrating that YTHDC1-WT inhibits the proximal APA sites and produces shorter 3' UTRs transcripts but YTHDC1-Mut almost abolishes this inhibitory effect.

4. The second part deals with the regulation of APA in an m6A-dependent manner. Alike in point 1, I am missing an analysis of the overlap between the two siRNAs targeting YTHDC1.

Answer: Your advice was well taken. Consistent with result of two siRNAs in Figure.2A, the target genes tended to be markedly more common in the list of

genes with shortened 3'UTRs ($p=4.62\times10^{-3}$ for overlap of two siRNA with Fisher's exact tests) but not genes with lengthened 3'UTRs (p=0.92 for overlap of two siRNA with Fisher's exact tests).

5. *The reporter system experiments reveal surprisingly clear indication for the regulatory hypothesis. The authors use a YTHDC1 mutant that doesn't bind m6A to prove the dependency of the regulatory mechanism on m6A. However, another they should also perform the same experiment in cells upon METTL3 knock down.*

Answer: Your advice was well taken. To validate the m^6A dependent function of YTHDC1, we explored ten endogenous genes APA regulation with qRT-PCR after knock-down of METTL3. In new Figure EV5, eight of them show shorter 3' UTRs transcripts with knockdown of METTL3. These results reveal that YTHDC1 plays a suppressive role on APA sites in an m^6A-dependent manner.

Minor question:

1. *why is there a double band observed in cells overexpressing YTHDC1?*

Answer: Actually, the endogenous YTHDC1 protein also have double bands (Figure. 4C), which may be the result of the YTHDC1 protein modification.

2. *Minor comment for the Figure 3. The panels B, C and D could be merged to one.*

Answer: Your advice was well taken. We merged Figure. 3 panels B, C and D to B in our new Figure 4.

3. *What exactly is the IP in Figure 5D, right panel showing? The signal for PAPOLA IPed with Myc-FIP1L1 seems not convincing due to the wage signal on the western blot.*

Answer: The figure shows that IP of Myc-FIP1L1 cannot pulldown PAPOLA. The weak signal for PAPOLA may be due to the very weak interaction between Myc-FIP1L1 and FLAG-PAPOLA in our IP experiment. But the weak signal is not significantly affected when YTHDC1 expression was elevated (Figure. 6D), so this result does not affect our conclusions.

4. *Last part dealing with the nuclear condensates. The experiment in Figure 6I should be performed in cells upon METTL3 KD to again address the m6A dependency for YTHDC1 mediated droplet formation.*

Answer: This is a good advice. We found that DsRed-YTHDC1 condensates were significantly disrupted after METTL3 knockdown compared with control (Appendix Figure S2). This result strengthened the notion that YTHDC1 mediated droplet formation in a m^6A-dependent way.

5. *Several times in the text, the FIP1L1 is misspelled as FIL1L1.*

Answer: We are sorry for the mistaken. We corrected this in our new version of paper.

Dear Prof. Xu,

Thank you for the submission of your revised manuscript. We have now received the reports from the referees.

As you will see, all referees in principle support the publication of your revised study. However, referee 1 still has a few concerns that need to be addressed. Please co-submit a point-by-point response to these last concerns with your final submission.

A few editorial requests will also need to be addressed:

- Most figure callouts need correcting. They are named "Figure. 1A" etc but the full stop must be removed.

- Figure EV5 panel callouts are missing, please add.

- The table of content of the Appendix file needs to include page numbers.

- Some blots are over-contrasted compared with the source data. Figure 4A is an extreme example. Please improve the blots and avoid over-contrasting.

- The Heading "Supporting Information" should be removed from the manuscript file.

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final ms file.

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

I would like to suggest some minor changes to the title and abstract. Please let me know whether you agree with the following:

Nuclear m6A Reader YTHDC1 Regulates mRNA Alternative Polyadenylation by Interfering with the 3' Processing Machinery

N6-methyladenosine (m6A) and alternative polyadenylation (APA) are important regulators of gene expression in eukaryotes. Recently, it was found that m6A is closely related to APA. However, the molecular mechanism of this new APA regulation remains elusive. Here we show that YTHDC1, a nuclear m6A reader, can suppress proximal APA sites and produce longer 3' UTR transcripts by binding to their upstream m6A sites. YTHDC1 can directly interact with the 3' end processing factor FIP1L1 and interfere with its ability to recruit CPSF4. Binding to the m6A sites can promote liquid-liquid phase separation of YTHDC1 and FIP1L1, which may play an important role in their interaction and APA regulation. Collectively, YTHDC1 as an m6A reader links m6A modification with pre-mRNA 3' end processing, providing a new mechanism for APA regulation.

I look forward to seeing a final version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

Overall, I think this manuscript is improved. However, there are still some central questions that remain that are important because they get to whether the mechanism described here is specific to m6A/YTHDC1, or potentially a nonspecific effect. The experiments described below are simply re-analysis or computational, and do not require bench work. I think they could however substantially improve this manuscript. I don't think they will take that much time or be a large burden. The specific comments are below:

1..I am fairly confident that there is an effect on 3' UTR length upon YTHDC1 depletion, I think the abstract and Discussion failed to convey that this effect occurs on a very small number of transcripts. The average reader would look at this manuscript and

conclude that the thousands of transcripts that are known to contain m6A undergo a substantial shortening of the 3' UTR upon YTHDC1 and/or METTL3 depletion. The abstract, the last paragraph in the Introduction, and the results section, along with the Discussion need to make it clear that the vast majority of m6A -containing transcripts are not affected by YTHDC1 depletion. The authors should report the percent of m6A-containing transcripts that are affected by YTHDC1 depletion. It is an interesting discussion point to discuss why so many transcripts are not affected by YTHDC1 depletion and what might make some transcripts affected. This is very important for the readers to understand - that this effect is not seen across all m6A transcripts and is not a general function of m6A.

2.. The authors should do a better job in conveying the fraction of transcripts that undergo shortening after YTHDC1 or METTL3 knockout. For instance, they mentioned that approximately 400 transcripts show shortening of the 3' UTR. However, some of these transcripts may have 90% of the isoforms with the long 3' UTR and 10% with the short 3' UTR, and this might change to 80%-20%. What is the magnitude of the changes? A histogram is the best way to show this across all 400 plus transcripts (and 200 plus in MCF7) cells. This is a relatively simple analysis using their existing software. A similar analysis for METTL3 was reported recently doi: https://doi.org/10.1101/2022.02.18.480977

3.. The authors were requested to compare the magnitude of the effect of YTHDC1 depletion with other known and validated regulators of alternative polyadenylation. However, they simply cited previous literature. The reason why this is not acceptable is that the results of an alternative polyadenylation analysis is very variable depending on the software and the settings that are used for the analysis. Therefore, the authors should have done side-by-side comparisons and presented the results graphically.

4.. The Kharas lab previously reported phase separation of YTHDC1 - see Cheng et al. As currently written, the manuscript gives the impression that it discovered this phenomenon. The correct paper should be cited https://doi.org/10.1016/j.ccell.2021.04.017

5..The authors were asked to report if m6A is found in the mRNAs that undergo shortening after YTHDC1 depletion. However the data in EV4 don't really show this. I think readers will not know the answer to this fundamental question. The authors can simply report the number of mapped sites in the mRNAs that undergo shortening, the number in the mRNAs that undergo lengthening, and overall distribution of the number of m6A site in the entire transcriptome. Hopefully we will see that mRNAs that undergo shortening have many more mapped m6A sites than the median in the transcriptome. They can also do this analysis for m6A sites near the stop codon, which are more likely to be associated with APA. The authors have not addressed this key question. If this analysis shows weak/no relationship to m6A, they should report this, and they can link their effect to YTHDC1 clip sites in the same groups of mRNAs.

Referee #2:

The authors have allayed all my concerns and I'm happy with the paper being published as it is.

Referee #3:

I have carefully read the revised version and how the authors addressed reviewers concerns and questions. I find the revised version suitable for publication in EMBO Reports.

Editor:

1. Most figure callouts need correcting. They are named "Figure. 1A" etc but the full stop must be removed.

Answer: We revised the figure callouts as shown in text.

2. Figure EV5 panel callouts are missing, please add.

Answer: We added EV5 panel callouts in our new manuscript.

3. The table of content of the Appendix file needs to include page numbers.

Answer: we added page numbers in our new version of Appendix file.

4. Some blots are over-contrasted compared with the source data. Figure 4A is an extreme example. Please improve the blots and avoid over-contrasting.

Answer: We updated Figure 3, Figure 4, Figure 5 and Figure 6 in new manuscript, to avoid over-contrasting.

5. The Heading "Supporting Information" should be removed from the manuscript file.

Answer: We deleted this part in new manuscripts.

6. I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final ms file.

Answer: We addressed all comments and ensured all have been properly set. The changes are highlighted in the new manuscript.

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

Answer: we have update this part along with the revised manuscript.

8. I would like to suggest some minor changes to the title and abstract. Please let me know whether you agree with the following:

Nuclear m6A Reader YTHDC1 Regulates mRNA Alternative Polyadenylation by Interfering with the 3' Processing Machinery

N6-methyladenosine (m6A) and alternative polyadenylation (APA) are important regulators of gene expression in eukaryotes. Recently, it was found that m6A is closely related to APA. However, the molecular mechanism of this new APA regulation remains elusive. Here we show that YTHDC1, a nuclear m6A reader, can suppress proximal APA sites and produce longer 3' UTR transcripts by binding to their upstream m6A sites. YTHDC1 can directly interact with the 3' end processing factor FIP1L1 and interfere with its ability to recruit CPSF4. Binding to the m6A sites can promote liquid-liquid phase separation of YTHDC1 and FIP1L1, which may play an important role in their interaction and APA regulation. Collectively, YTHDC1 as an m6A reader links m6A modification with pre-mRNA 3' end processing, providing a new mechanism for APA regulation.

Answer: We agreed with all changes and revised in new manuscript.

Referee #1:

Overall, I think this manuscript is improved. However, there are still some central questions that remain that are important because they get to whether the mechanism described here is specific to m6A/YTHDC1, or potentially a nonspecific effect. The experiments described below are simply re-analysis or computational, and do not require bench work. I think they could however substantially improve this manuscript. I don't think they will take that much time or be a large burden. The specific comments are below:

Answer: Thank you for your positive comment on our revision, and we improved our manuscript based on your suggestions.

1. I am fairly confident that there is an effect on 3' UTR length upon YTHDC1 depletion, I think the abstract and Discussion failed to convey that this effect occurs on a very small number of transcripts. The average reader would look at this manuscript and conclude that the thousands of transcripts that are known to contain m6A undergo a substantial shortening of the 3' UTR upon YTHDC1 and/or METTL3 depletion. The abstract, the last paragraph in the Introduction, and the results section, along with the Discussion need to make it clear that the vast majority of m6A -containing transcripts are not affected by YTHDC1 depletion. The authors should report the percent of m6A-containing transcripts that are affected by YTHDC1 depletion. It is an interesting discussion point to discuss why so many transcripts are not affected by YTHDC1 depletion and what might make some transcripts affected. This is very important for the readers to understand - that this effect is not seen across all m6A transcripts and is not a general function of m6A.

Answer: This is a good question. To avoid this confuse, we tuned down the tone and point out number of significantly switching APA genes in results section, and discussed why so many m^6A transcripts are not affected by YTHDC1 depletion in Discussion section. We added the sentence as follows:

"Interestingly, our APA analysis show that only a subset of YTHDC1 target gene transcripts (about 11%) undergo a substantial shortening of the 3' UTR and most of m6A transcripts are not significantly affected upon YTHDC1 deletion. The regulation effect of YTHDC1 on APA needs its binding to m6A sites nearby the proximal APA sites (Figure 2C and Figure EV5A). Then it may only affect a subset of mRNAs with higher m6A level nearby the proximal APA sites but not all mRNAs with m6A modification. YTHDC1 inhibits proximal APA sites by interacting with FIP1L1, which preferentially binds to U-rich sequences of transcripts (Kaufmann et al., 2004). This also limits a subset of genes to be regulated. Furthermore, most genes exhibit less than 50% methylation levels (Molinie et al., 2016), which can decrease the power of detection of significant APA switching too. All of these can explain that only a subset of genes with m6A switched to proximal APA sites by knockdown of YTHDC1, and the regulation effects of YTHDC1 is cell context dependent."

2. The authors should do a better job in conveying the fraction of transcripts that undergo shortening after YTHDC1 or METTL3 knockout. For instance, they mentioned that approximately 400 transcripts show shortening of the 3' UTR. However, some of these transcripts may have 90% of the isoforms with the long 3' UTR and 10% with the short 3' UTR, and this might change to 80%-20%. What is the magnitude of the changes? A histogram is the best way to show this across all 400 plus transcripts (and 200 plus in MCF7) cells. This is a

relatively simple analysis using their existing software. A similar analysis for METTL3 was reported recently doi: https://doi.org/10.1101/2022.02.18.480977.

Answer: This is a good suggestion. a) To better display the magnitude of APA sites changes, we compared 3' UTR length between the control and YTHDC1 knockdown samples by calculated the standardized weighted average of the 3' UTR length for genes with shortening 3' UTR. We show this result in new Figure EV2, suggesting that the genes with the shortening 3' UTR undergo a significant APA sites switching. (Figure. EV2).

3. The authors were requested to compare the magnitude of the effect of YTHDC1 depletion with other known and validated regulators of alternative polyadenylation. However, they simply cited previous literature. The reason why this is not acceptable is that the results of an alternative polyadenylation analysis is very variable depending on the software and the settings that are used for the analysis. Therefore, the authors should have done side-by-side comparisons and presented the results graphically.

Answer: Thank you for your comments. We admit that different alternative polyadenylation analyses may be very variable. But we've done the comparison of the literature method (independence testing for the APA site switching) with our method (linear trend testing for the APA site switching) (Li *et al*, 2019, https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0124324), and the results is similar.

To further address this doubt, we have done the side-by-side comparisons of YTHDC1 and U1 snRNP complex proteins (Hu *et al*. 2022, In Press), which are validated regulators of alternative polyadenylation. The number of genes with significant APA switch is as follows [Figures for referees not shown.] :

4. The Kharas lab previously reported phase separation of YTHDC1 - see Cheng et al. As currently written, the manuscript gives the impression that it discovered this phenomenon. The correct paper should be cited https://doi.org/10.1016/j.ccell.2021.04.017

Answer: Thank you for your comment! We rewrote the related sentence as follow: “Recently, the m^6A reader YTHDF1-3 was reported to undergo liquid-liquid phase

separation (LLPS) by binding to modified m^6A RNA to regulate the stability and translation of transcripts (Fu & Zhuang, 2020; Ries *et al*, 2019), and YTHDC1 binds to m^6A and forms nuclear condensates mediated by LLPS (Cheng *et al*, 2021)".

5. The authors were asked to report if m6A is found in the mRNAs that undergo shortening after YTHDC1 depletion. However the data in EV4 don't really show this. I think readers will not know the answer to this fundamental question. The authors can simply report the number of mapped sites in the mRNAs that undergo shortening, the number in the mRNAs that undergo lengthening, and overall distribution of the number of m6A site in the entire transcriptome. Hopefully we will see that mRNAs that undergo shortening have many more mapped m6A sites than the median in the transcriptome. They can also do this analysis for m6A sites near the stop codon, which are more likely to be associated with APA. The authors have not addressed this key question. If this analysis shows weak/no relationship to m6A, they should report this, and they can link their effect to YTHDC1 clip sites in the same groups of mRNAs.

Answer: Actually, the analysis of m^6A peak density near APA sites is a common method, which was previously used to report the relationship of m^6A modification with APA sites (Ke *et al*, 2015) (http://genesdev.cshlp.org/content/29/19/2037.full.pdf+html). Our results of EV5A show that the mRNAs which undergo shortening after YTHDC1 depletion have a higher m^6A modification near of the proximal APA sites than the genes with lengthened 3' UTR or background.

We also analyzed m^6A peak density near the stop codon as your suggested. The new Figure EV5B shows m^6A enrichment near the stop codon in all mRNA transcripts, but that the mRNAs with shortening 3' UTR does not show higher m^6A level compared with lengthening or background mRNA transcripts near stop codon. All these results show that YTHDC1 regulate APA by binding to the nearby of the proximal APA sites via m^6A and inhibiting the generation of a shortened 3' UTR.

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