EMBO reports

METTL14 protects METTL3 from STUB1-mediated degradation to maintain m^{6}A homeostasis

Zhan-Cheng Zeng, Qi Pan, Yu-Meng Sun, Heng-Jing Huang, Xiao-Tong Chen, Tian-Qi Chen, Bo He, Hua Ye, Shun-Xin Zhu, Ke-Jia Pu, Ke Fang, Wei Huang, Michelle YQ Chen, and Wen-Tao Wang
DOI: 10.15252/embr.202255762

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Review Timeline:

Submission Date:	10th Jul 22
Editorial Decision:	12th Aug 22
Revision Received:	9th Nov 22
Editorial Decision:	2nd Dec 22
Revision Received:	14th Dec 22
Accepted:	22nd Dec 22

Editor: Achim Breiling/Martina Rembold

Transaction Report:

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

Dear Prof. Wang

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

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

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

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 (November 12). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Your study contains 6 figures and will therefore be published as Article with a separate Results and Discussion section. For a normal article there are no length limitations. The entire materials and methods must be included in the main manuscript file.

*****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.*****

When submitting your revised manuscript, we will require:

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

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

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

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

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

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

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

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

7) Before submitting your revision, primary datasets (such as proteomics data) produced in this study need to be deposited in an appropriate public database (see < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).

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) that follows the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

8) 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

9) Figure legends and data quantification:
The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

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

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images.

10) 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 .

11) 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 .

12) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File 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.

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

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

I look forward to seeing a revised form of your manuscript when it is ready and please let me know if you have questions or comments regarding the revision.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

In this manuscript, Zeng et al revealed that the m6A methyltransferase catalytic subunit METTL3 directly binds to the ubiquitin domain of METTL14 and protects its ubiquitin sites from STUB1-mediated ubiquitination degradation, suggesting the coordination of the ubiquitination system controls METTL14 levels and m6A homeostasis. Futher functional studies showed that genetic upregulation or downregulation of STUB1 effectively regulates METTL14 protein levels, m6A modification, and tumorigenesis. The topic of this article is interesting and expands the dynamic regulation mechanisms of m6A. I have serveral concerns need to be addressed to further improve the quality of the manuscript.

1. METTL3 knockdown cells had significantly lower METTL14 protein levels and a small decrease in METTL14 gene expression compared with those in control cells, but figure1a showing the gene expression trend of METTL14 has diversity in different cell lines and shMETTL3s, especially in RBE cell line? Whether the METTL3 directly regulates METTL14 mRNA expression or not?

2. In order to distinguish expression difference, the gray value of objective band should be analyzed in western blot figures, for example, figure 3b, figure 3l, figure 4f. and figure 4g.

3. In figure 3j, the protein expression level of STUB1 should be detected in knockdown of STUB1 and control cells.

4. In figure 4b, the internal reference was H4, but the figure legend described that β-actin was used as the negative control, so which internal reference was used in the experiment? And the expression of internal reference proteins was not equal.

5. For figure 4h, the western blot of WT and METTL14-3KR should be run in a same gel and do gray value analysis.

6. In order to improve the reproducibility of experiments, more than two shSTUB1s should be applied in SK-ChA-1 cell transplantation experiment in mouse.

7. The protein expression of METTL3 and METTL14 also should be detected in CCA patients.

8. As we know thousands of transcripts contain m6A modification, the regulatory mechanism of METTL3 directly binds to the ubiquitin domain of METTL14 and protect its ubiquitin sites from STUB1-mediated ubiquitination degradation is a wide phenomenon or just exits in some specific tissue cells and pathways.

Minor points:
1. In figure 1d, the manuscript describes the METTL3 is knocked down by shMETTL3, but the figure is marked with siMETTL3.

2. Check the consistency of figure marker in the text and figures, such as Figure 5c, HA-METTL14 and METTL14-HA.

3. In the method part, the authors need to correct writing about μM or μl.

4. In figure 6j, the #4 and #5 are omissions, and the bands of STUB1-FLAG also miss in #4.

Referee #2:

In the manuscript, the authors revealed the mechanism of METTL14 protein stability and its role in m6A homeostasis, and characterized its implications in the context of cholangiocarcinoma. They uncovered the competitive interaction of METTL14 with METTL3 and STUB1 that can control METTL14 levels and m6A homeostasis. Mechanically, STUB1 directly interacts with METTL14 to mediate its ubiquitination at lysine residues K148, K156, and K162 for subsequent degradation. Functional studies showed that genetic upregulation or downregulation of STUB1 can effectively regulate METTL14 protein levels and tumorigenesis. Overall, the manuscript is well-written and easy to follow. The scientific questions are answered by multiple methods from different angles and the manuscript provides consistent results underpinning the proposed mechanism.
I have a few comments.

Comment 1. In the manuscript, the author shows that the competitive interaction of METTL14 with METTL3 and STUB1, can control METTL14 levels and m6A homeostasis. The WATP is also a core member of MTase complex, does METTL14 protein stability also correlate with WATP? The author may also detect the METTL14 protein levels under the upregulation and/or downregulation WTAP.

Comment 2. Liao et al., has recently shown that Elvitegravir can suppress metastasis by directly targeting METTL3 and enhancing its STUB1-mediated proteasomal degradation in esophageal squamous cell carcinoma (ESCC) (Cancer Res. 2022 Jul 5;82(13):2444-2457.). In this study, the authors report a novel role of STUB1 on METTL14 metabolism in cholangiocarcinoma. The author might discuss the different regulatory functions in different context of cancers.

Comment 3. As shown in Fig.6, upregulation of STUB1 can significantly inhibit SK-Cha-1 cell growth. It is interesting to suggest ectopic STUB1 could thus be a promising therapeutic strategy against malignant cancers, such as CCA. The author may collect another CCA cell line, such as RBE, QBC939,..., to further verify the CCA cell growth suppression.

Comment 4. As shown in Fig. 6j, the labels of mouse number #4, #5 are missing, and the immunoblot of STUB1-FLAG in the mouse number #4 sample is over exposured.

Comment 5. Some words first emerge should be first defined by full name. Such as, MTase, STUB1, ...

Referee #3:

Zeng et all nicely show that that METTL14 protein stability is regulated by the competitive interaction of METTL3 with an E3 ligase, STUB1. This study provides a molecular explanation underlying how METTL3 controls METTL14 function and identifies a novel E3 ligase in M6A regulation. Overall the study is technically sound and data provided largely support their main conclusion. If with the following few concerns addressed, this reviewer will be happy to see its publication by EMBO report.
1. The interaction of endogenous STUB1 and METTL14 should be confirmed.
2. Since STUB1 targets many other proteins, its METTL14-dependent and independent tumorigenic functions should be studied.
3. The potential effect of STUB1 and METTL3 on METTL14 transcription should be analyzed as controls.

Point-by-point response to reviewers' comments and questions

We appreciated all the comments and suggestions.

Editors' comments:

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

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

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. 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 (November 12). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Your study contains 6 figures and will therefore be published as Article with a separate Results and Discussion section. For a normal article there are no length limitations. The entire materials and methods must be included in the main manuscript file.

Reply: We thank the reviewers' comments and suggestions. As suggested, we have performed additional experiments and revised the manuscript in accordance with the comments, including (1) Investigating the effect of STUB1 and METTL3 on METTL14 mRNA as suggested (new Fig EV 1A and 2F); (2) Redone the Figs 3J,

4B,and 4H following the comments and adding the gray value of objective bands; (3) Performing mouse transplantation experiments using another sh-STUB1 SK-Cha-1 cells (new Fig EV5F-H);（4）Performing additional experiments to demonstrate the relationship among METTL3, METTL14 and STUB1 in different cancer cell lines including Hela, HepG2 and K562(New Fig EV4); (5) Performing additional experiments to detect the METTL14 protein levels under the overexpression and/or knockdown of WTAP (New Fig EV 1C and D); (6) Conducting additional experiment to confirm the interaction of endogenous STUB1 and METTL14 in cell lines (New Fig EV 2D and E); (7) Performing a rescue experiment to investigate the function of STUB1 on CCA tumorigenesis is, at least partially METTL14-dependent (New Fig 6C and EV 5E). (8) As suggested, we have examined the statistics and error bars, and used scatter blots in the cases of n small. We have also addressed other issues raised by the three reviewers. Please find below our point-by-point responses and the revised text highlighted in the manuscript. According to your comments and suggestions, we have also revised the manuscript as Article format with a separate Results and Discussion section. As suggestion, the source data of blots or microscopy for figure panels have also been uploaded as “Figure Source Data File”.

All data to understand and assess the conclusions of this research are available in the main text and Expanded View (EV) Figures and Tables that are collapsible/expandable online. No primary datasets have been generated and deposited. The entire materials and methods have been included in the main manuscript file accordingly.

The input from you and the three colleagues has helped us greatly improve our manuscript. Thank you.

Referee #1:

In this manuscript, Zeng et al revealed that the m6A methyltransferase catalytic

subunit METTL3 directly binds to the ubiquitin domain of METTL14 and protects its ubiquitin sites from STUB1-mediated ubiquitination degradation, suggesting the coordination of the ubiquitination system controls METTL14 levels and m6A homeostasis. Futher functional studies showed that genetic upregulation or downregulation of STUB1 effectively regulates METTL14 protein levels, m6A modification, and tumorigenesis. The topic of this article is interesting and expands the dynamic regulation mechanisms of m6A. I have serveral concerns need to be addressed to further improve the quality of the manuscript.

Comment 1. METTL3 knockdown cells had significantly lower METTL14 protein levels and a small decrease in METTL14 gene expression compared with those in control cells, but figure1a showing the gene expression trend of METTL14 has diversity in different cell lines and shMETTL3s, especially in RBE cell line? Whether the METTL3 directly regulates METTL14 mRNA expression or not?

Reply: We thank the reviewer's comment and concern. We have carefully checked the qRT-PCR data in figure 1a, and found that METTL3 knockdown indeed slightly affected METTL14 mRNA levels. The slight change of METTL14 mRNA levels may be mainly due to the discrepancy within experiment error of qRT-PCR. To address the concern that the influence of METTL3 knockdown on METTL14 mRNA, we have redone this assay in RBE cell again and other several cell lines including Hela, HepG2 and three leukemia cell lines (K562, MV4-11 and MOLM-13). The results also showed that METTL14 mRNA levels in different cell lines presented a slight change when knocking down METTL3 (**New EV Fig 1A**). However, compared with mRNA levels, METTL14 protein showed a significantly decrease when knocking down METTL3 (Fig 1B), therefore, we mainly focused on the function of METTL3 upon METTL14 protein. According to the comment, we have rephrased the original sentence "…, despite showing only a small decrease in METTL14 gene expression" to "…., despite showing a slight change in METTL14 mRNA levels, ..." (pages 5,

lines 132-133) to make the description more accurately. The additional data have also included in the revised manuscript (pages 5, lines 132-133). Thank you!

Comment 2. In order to distinguish expression difference, the gray value of objective band should be analyzed in western blot figures, for example, figure 3b, figure 3l, figure 4f. and figure 4g.

Reply: We agree and have added the gray value of objective bands in almost of all western blot figures including Figs 1B-G, 1J, 2D, 2F, 3B,3E,3L, 4B,4F, 4H, 5A, 6K, 7B, and EV1B-D, 4A-C, 5C, 5E，5J in the revised manuscript. Thank you for the suggestions.

Comment 3. In figure 3j, the protein expression level of STUB1 should be detected in knockdown of STUB1 and control cells.

Reply: We agree. Following the suggestion, we have redone the IP and western blot and detected the protein expression level of STUB1 in knockdown of STUB1 and control cells. The new Fig 3J have been added into the revised manuscript.

Comment 4. In figure 4b, the internal reference was H4, but the figure legend described that β-actin was used as the negative control, so which internal reference was used in the experiment? And the expression of internal reference proteins was not equal.

Reply: We apologized for the typos and have corrected the typos. According to the comment, we have redone western blot and added the gray value of objective bands(New Fig 4B）. Thank you!

Comment 5. For figure 4h, the western blot of WT and METTL14-3KR should be run in a same gel and do gray value analysis.

Reply: We appreciate the comments. According to the suggestion, we have redone the experiment and run the western blot of WT and METTL14-3KR in the same gel and provided the gray value analysis. The new Figure 4H has been shown in the revised manuscript.

Comment 6. In order to improve the reproducibility of experiments, more than two shSTUB1s should be applied in SK-ChA-1 cell transplantation experiment in mouse.

Reply: According to the suggestion, we have performed additional transplantation experiment in mouse using another sh-STUB1-2 SK-Cha-1 cells (as shown in new **Fig EV5F-H**). Thank you!

Comment 7. The protein expression of METTL3 and METTL14 also should be detected in CCA patients.

Reply: Thanks for the comment. We have performed additional experiments and detected the protein expression of METTL3 and METTL14 in CCA patients as suggested. The data has been added in the revised manuscript (as shown in new **Fig EV5J**).

Comment 8. As we know thousands of transcripts contain m6A modification, the regulatory mechanism of METTL3 directly binds to the ubiquitin domain of METTL14 and protect its ubiquitin sites from STUB1-mediated ubiquitination degradation is a wide phenomenon or just exits in some specific tissue cells and pathways.

Reply: We thank the reviewer for this thoughtful comment. Following the comments, we have performed additional experiments to demonstrate the relationship among METTL3, METTL14 and STUB1 in different cancer cell lines including Hela,

HepG2 and K562. As shown in new **Fig EV 4**, knockdown METTL3 could also decrease the METTL14 protein level, and the interaction between METTL14 and STUB1 was affected by overexpression of METTL3. These additional data might suggest a wide phenomenon that METTL3 directly binds to METTL14 and protect its ubiquitin sites from STUB1-mediated ubiquitination degradation. The new data has been added in the revised manuscript (new **Fig EV4**). Thank you again.

Minor points:

1. In figure 1d, the manuscript describes the METTL3 is knocked down by shMETTL3, but the figure is marked with siMETTL3.

Reply: We apologized for the typos and have carefully checked through the manuscript to correct the typos and to polish the manuscript. Thank you very much!

2. Check the consistency of figure marker in the text and figures, such as Figure 5c, HA-METTL14 and METTL14-HA.

Reply: We have carefully checked the consistency of figure marker in the text and figures to correct any typo. We appreciate the comment.

3. In the method part, the authors need to correct writing about μM or μl.

Reply: Thank you for pointing out the mistakes. We have carefully checked through the manuscript to correct the typos.

4. In figure 6j, the #4 and #5 are omissions, and the bands of STUB1-FLAG also miss in #4.

Reply: We thank the reviewer for this thoughtful comment. We have corrected it.

Referee #2:

In the manuscript, the authors revealed the mechanism of METTL14 protein stability and its role in m6A homeostasis, and characterized its implications in the context of cholangiocarcinoma. They uncovered the competitive interaction of METTL14 with METTL3 and STUB1 that can control METTL14 levels and m6A homeostasis. Mechanically, STUB1 directly interacts with METTL14 to mediate its ubiquitination at lysine residues K148, K156, and K162 for subsequent degradation. Functional studies showed that genetic upregulation or downregulation of STUB1 can effectively regulate METTL14 protein levels and tumorigenesis. Overall, the manuscript is well-written and easy to follow. The scientific questions are answered by multiple methods from different angles and the manuscript provides consistent results underpinning the proposed mechanism.

I have a few comments.

Comment 1. In the manuscript, the author shows that the competitive interaction of METTL14 with METTL3 and STUB1, can control METTL14 levels and m6A homeostasis. The WATP is also a core member of MTase complex, does METTL14 protein stability also correlate with WATP? The author may also detect the METTL14 protein levels under the upregulation and/or downregulation WTAP.

Reply: We appreciate the comments. According to the suggestion, we have performed additional experiments to detect the METTL14 protein levels under the overexpression and/or knockdown of WTAP. The results showed that upregulation and downregulation of WTAP did not show effects on the protein levels of METTL14. The data has been added in the revised manuscript (as shown in new **Fig EV 1C and D**). Thank you again.

Comment 2. Liao et al., has recently shown that Elvitegravir can suppress metastasis by directly targeting METTL3 and enhancing its STUB1-mediated proteasomal degradation in esophageal squamous cell carcinoma (ESCC) (Cancer Res. 2022 Jul 5;82(13):2444-2457.). In this study, the authors report a novel role of STUB1 on

METTL14 metabolism in cholangiocarcinoma. The author might discuss the different regulatory functions in different context of cancers.

Reply: We thank for the suggestion and have added discussion in the revised Discussion section. **(Page 16, line 458-461, marked in blue).**

Comment 3. As shown in Fig.6, upregulation of STUB1 can significantly inhibit SK-Cha-1 cell growth. It is interesting to suggest ectopic STUB1 could thus be a promising therapeutic strategy against malignant cancers, such as CCA. The author may collect another CCA cell line, such as RBE, QBC939,..., to further verify the CCA cell growth suppression.

Reply: We agree. According to the comment, we have performed upregulation of STUB1 in several other CCA cell line including RBE, QBC939, and MZ-Cha-1. As shown in new **Fig 6B** and **EV 5 C and D**, upregulation of STUB1 could indeed significantly inhibit RBE, QBC939, and MZ-Cha-1 cell growth. These additional data have been added into the revised manuscript (Page 11, line 317). Thank you very much!

Comment 4. As shown in Fig. 6j, the labels of mouse number #4, #5 are missing, and the immunoblot of STUB1-FLAG in the mouse number #4 sample is over exposured.

Reply: We thank the reviewer for this thoughtful comment. We have corrected it.

Comment 5. Some words first emerge should be first defined by full name. Such as, MTase, STUB1, ...

Reply: We agree and have carefully checked through the manuscript and provided full names when first coming out. Thank you !

Referee #3:

Zeng et all nicely show that that METTL14 protein stability is regulated by the competitive interaction of METTL3 with an E3 ligase, STUB1. This study provides a molecular explanation underlying how METTL3 controls METTL14 function and identifies a novel E3 ligase in M6A regulation. Overall the study is technically sound and data provided largely support their main conclusion. If with the following few concerns addressed, this reviewer will be happy to see its publication by EMBO report.

Comment 1. The interaction of endogenous STUB1 and METTL14 should be confirmed.

Reply: We appreciate the comments. According to the suggestion, we have performed additional experiment to confirm the interaction of endogenous STUB1 and METTL14 in cell lines (please see the new **Fig EV 2D and E**). In addition, the immunofluorescence experiment in the Fig. 3d also showed the endogenous METTL14 (green) colocalized with that of STUB1 (red) in HEK293T cells. These additional data suggest the interaction of endogenous STUB1 and METTL14 protein. Thank you for the suggestion.

Comment 2. Since STUB1 targets many other proteins, its METTL14-dependent and independent tumorigenic functions should be studied.

Reply: We thank the reviewer for this thoughtful comment. To address this concern and investigate the function of STUB1 on tumorigenesis is METTL14-dependent or independent, we have performed the rescue experiments. We knocked down METTL14 in the STUB1 knockdown cells and found that both METTL14 protein level and cell proliferation increases caused by transfection of STUB1 siRNAs were reversed (as shown in the new **Fig 6C** and **EV 5E**), suggesting that the function of

STUB1 on CCA tumorigenesis is, at least partially METTL14-dependent. We have added this data into the revised manuscript. Thank you!

Comment 3. The potential effect of STUB1 and METTL3 on METTL14 transcription should be analyzed as controls.

Reply: We appreciate the comments. According to the suggestion, we have detected the METTL14 mRNA under knockdown of STUB1 and/or METTL3. The results showed that METTL14 mRNA levels in different cell lines presented a slight change when knocking down METTL3 or STUB1 (**New EV Fig 1A and 2F**), while METTL14 protein showed a significantly decrease when knocking down METTL3 or overexpressing STUB1(Fig 1B and 3E), therefore, we mainly focused on the function of METTL3 and STUB1 upon METTL14 protein. These additional data suggested that STUB1 and METTL3 might not regulate the METTL14 transcription, mainly regulate its protein stability. The data has been added in the revised manuscript (page 5, lines 132-133; page 8, lines 219-221). Thank you again!

Dear Prof. Wang,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that I asked to re-evaluate your study, you will find below. As you will see, the referees now fully support publication of your study in EMBO reports.

Before we can proceed with formal acceptance, Moreover, I have these editorial requests I ask you to address in a final revised manuscript:

- Please provide a more comprehensive and grammatically correct title without the comma (bodyguard cannot be used as verb).

- Please provide the abstract written in present tense throughout.

- Please have your final manuscript file carefully proofread by a native speaker.

- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. Thus, please remove the author contributions section from the manuscript text file. See also guide to authors: https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main, EV and Appendix figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams (main, EV and Appendix figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. If n<5, please show single datapoints for diagrams.

- Please shorten the data availability section. This should only refer to large scale datasets deposited at am external repository.

- Please make sure that all figure panels are called out separately and sequentially (main and EV figures). Presently, there seem to be no separate callouts for panels EV2B,D,E; EV 3D; EV5A-H and EV5K-M. Please check.

- Please add scale bars of similar style and thickness to the microscopic images (main and EV), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

- EV Table 1 is a dataset. Please upload this as original excel file as dataset using the name 'Dataset EV1'. Please add a title and a legend to the first TAB of the excel file. Finally, please change the callouts to this item using 'Dataset EV1'.

- I would suggest presenting the information shown in EV Tables 2 and 3 in an Appendix (as I do not think that this needs to be shown directly in the online version of the article - it is sufficient that interested readers can download the information as an Appendix). Please provide both tables with legend and title in an Appendix file with page numbers. Please add a title page with a title (Appendix for ...) and a table of contents (TOC) with page numbers to the Appendix file and upload this as single pdf file. Please use the nomenclature Appendix Table Sx and also use this for the callouts. Please make sure the respective callouts have been changed in the main manuscript text file.

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Sun Yat-sen University
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Guangzhou, Guangdong 510275
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1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:

  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
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  - definition of 'center values' as median or average;
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