EMBO reports

MYC Promotes Cancer Progression by Modulating m^{6}A Modifications to Suppress Target Gene Translation

Ping Gao, Gongwei Wu, Caixia Suo, Ying Yang, Shengqi Shen, Linchong Sun, Shi-Ting Li, Yingli Zhou, Yongping Cai, Nana Wang, Huafeng Zhang, Yun-Gui Yang, Jie Cao, Dongdong Yang, and Yan Wang
DOI: 10.15252/embr.202051519

Corresponding author(s): Ping Gao (pgao2@ustc.edu.cn) , Yun-Gui Yang (ygyang@big.ac.cn), Gongwei Wu (gongwei_wu@dfci.harvard.edu), Jie Cao (eycaojie@scut.edu.cn)

Review Timeline:

Submission Date:	13th Aug 20
Editorial Decision:	3rd Sep 20
Revision Received:	16th Nov 20
Editorial Decision:	2nd Dec 20
Revision Received:	7th Dec 20
Accepted:	10th Dec 20

Editor: Achim Breiling

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.)

1st Editorial Decision 3rd Sep 2020

Dear Prof. Gao,

Thank you for the submission of your manuscript to EMBO reports. We have now received the enclosed referee reports on it.

As you will see, all referees acknowledge that the findings are potentially interesting. However, they also all have several suggestions for how the study should be further strengthened and improved. I think all referee comments are sensible and should therefore be addressed.

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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

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

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.

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."

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

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In this manuscript, Wu and co-authors showed that MYC suppresses several selected MRGs (eg. SPI1 and PHF12) via mechanisms involving inhibition of m6A of these genes by inducing the demethylase ALKBH5, thereby inhibiting the recruitment of the m6A-binding protein YTHDF3 to the MRGs and their translation. Functionally, they show that overexpression of ALKBH5 and knockdown of SPI1 or PHF12 promote cancer cell proliferation in vitro and in vivo. Also, Myc expression positively correlates with ALKBH5 expression whereas it reversely correlates with the expression of SPI1 or PHF12 in lymphoma patient samples. While this is an interesting finding delineating a novel mechanism for MYC-mediated gene suppression, there are a number of issues that should be addressed.

The main issue here is the specificity of the demonstrated MYC-ALKBH5-m6A-SPI1/PHF12 axis.

The data show that MYC regulates the global mRNA m6A and translation (polysome profiling assays). How the SPI1, PHF12 genes were specifically chosen for the study is not well presented. How about the well-known MYC target genes such as p21, p15? The MYC-induced genes should be used as controls to demonstrate the specificity. How does MYC specifically control mRNA m6A of a subset of genes? It is also somewhat confusing that while total M6A was increased by YTHDF3 KD, the total protein translation was not reduced (Fig. 3F, input). How YTHDF3 specifically increases the MRGs' translation should be further elaborated or experimentally tested.

Minor points:
• Fig. 4G-4I: Does it mean that unlike the endogenous SPI1 and PHF12, the overexpressed SPI1 and PHF12 are not subjected to the ALKBH5 regulation?
• Fig. S2E: It seems that SPI2 mRNA is increased. Please re-check the statistics.
• Fig. 3F is not convincing, what are the bands in IgG control?
• Figure 5: the model should be modified to reflect the increase of translation of MRGs in the context.
• The statement of the regulation as a transcription-independent mechanism is not accurate, as MYC drives the transcription of the upstream ALKNH5 gene.
• The role of FTO is not well consistent in Fig 2. There is no significant binding in PHF12 gene (Fig. 2E) and no rescue of MYC induced PHF12 expression (Fig. 2G).
• The discussion is not well written and should be more focused on the mechanism and implication and written cohesively.
• RIP protocol was not described in detail; RNA fragmentation is not described clearly.
• Some grammars and typos throughout: eg. Page 4 the last sentence should be "may overlap with the first,...". page 20 the second sentence "...and leads to cell proliferation and growth" should be "and leads to cell proliferation and growth inhibition".

Referee #2:

The manuscript by Wu et al. reveals MYC can suppress gene expression through tuning the m6A modification level on MRGs. The authors show that MYC knockdown leads to a global accumulation of m6A on mRNAs in B-cell lymphoma cells. MYC can promote the transcription of m6A demethylase ALKBH5 and FTO, therefore regulating the m6A methylation level. The authors study two specific MGRs, SPI1 and PHF12, which are effectively demethylated by ALKBH5 and bound by m6A reader YTHDF3. They show that YTHDF3 promotes the translation of SPI1 and PHF12 through m6A. Finally, they show that the MYC-ALKBH5- m6A-SPI1/PHF12 axis can be a potential clinical target for patients with aberrant MYC expression.

Figure 1: MYC is known to affect many genes through various mechanisms. The authors state that MYC knockdown leads to 2,542 differentially methylated. What percentage of these genes was previously known MRGs? What percentage of the MRGs was regulated by m6A? Are any of the genes previously known to be regulated by other mechanisms? From the GO analysis, some genes also regulate transcription; does MYC also employ m6A to regulate transcription of other genes?

Figure 2: The authors analyzed U2OS cells ChIP-seq data and found that MYC binds to the promoter regions of ALKBH5 and FTO to promote transcription; the same was confirmed in P493-6 cells. ALKBH5 is also overexpressed in many cancers, is this a general mechanism for cancer cells? The correlation from 78 lymphocyte cell lines may not seem as strong as the authors claimed (S3A).

Figure 2E - According to Figure 2A, Tet treated P493-6 cells express very little ALKBH5/FTO, how

do you ensure successful IP and how are the values normalized? Even though they have low protein expression level, successful IP should still show enrichment of their targets?

Figure 4 - More supportive tumor model would be comparing tumors with high and low MYC and overexpress ALKBH5 in these cells or treat with ALKBH5 inhibitor. As shown in S6C.

Minor questions:

Figure 1B: dot blot MB staining doesn't look right.

Figure 3H: in Raji, YTHDF3 and ALKHB5 double knockdown leads to even lower SPI1 and PHF12?

Referee #3:

In this study, the authors present nice pieces of evidence supporting a connection between the MYC proto-oncogene, the m6A mRNA demethylase ALKBH5, mRNA translation and cancer progression. First, they show that the MYC transcription factor activates the expression of the m6A mRNA demethylase ALKBH5 protein, thereby influencing the m6A status of mRNAs encoding for some MYC-repressed genes (notably SPI1 and PHF12). Indeed, the presence of m6A on these transcripts leads to the specific recruitment of the YTHDF3 reader, thereby enhancing their translation. On the contrary, upon MYC-induced ALKBH5 expression, these transcripts are demethylated by ALKBH5, which diminishes their translation and then reduces levels of SPI1 and PHF12. Interestingly, this control occurs at the level of translation and not mRNA decay as the levels of mRNA encoding for SPI1 and PHF12 are kept constant. This nice mechanistic study is complemented by in vivo experiments showing that low levels of either SPI1 or PHF12 proteins (due to silencing of these genes or over-expression of ALKBH5) favor cancer progression while high level of SPI1 or PHF12 reduces tumor size. Altogether, these are convincing data on an interesting topics and hence, it is my feeling that this study deserves publication in EMBO Reports. In addition, it is undoubtedly of general interest to the molecular biology community.

I have however comments that should be addressed in a revised version:
1) In Fig 2a, the author show that in the absence of Tet, the protein levels of MYC, ALKBH5 and FTO are high while in Fig 2e, in the absence of Tet, only the protein level of MYC is high although the same cell lines are used. I guess that this is because in Fig 2e, they use an anti-FLAG antibody to specifically detect the over-expression of either ALKBH5 or FTO but this is neither clear from the figure legend nor from the materials and methods section. This should be clarified.
2) Considering the functional redundancies described for YTHDF1, YTHDF2 and YTHDF3 (e.g. Zaccara and Jaffrey; Cell; 2020; PMID: 32492408), it is important to validate the model proposed by the authors as it involves only YTHDF3 protein. In Figure 2b and 2c, I would like to suggest to add WB showing that YTHDF1 and YTHDF2 protein levels are not affected by the shRNAs directed against YTHDF3.

Minor comments and typos :
-Figure 5: It is my opinion that this figure is difficult to read. I would like to suggest to split it in two scenarios. One where MYC levels are low and hence ALKBH5 also, leading to optimal mRNA translation of SPI1 and PHF12, thereby inhibiting cell proliferation and oncogenesis. Another one where MYC levels are high as ALKBH5 are, leading to reduced mRNA translation of SPI1 and PHF12

and thereby enhanced cell tumor size.
-Figure S1E. The IGV graph for HPRT1 would be useful as a comparison with the other mRNAs shown.
-Abstract : SPI should be SPI1.
-Page 15. In this section, the authors focus on two gene products SPI1 and PHF12, the two central MYC regulated genes (MRGs) studied in this manuscript, but give the feeling that their observations are applicable to all MRGs (i.e. « ...knocking down SPI1 or PHF12 significantly promoted cell proliferation, indicating that MRGs inhibit ... », « ...enhanced proliferation by ALKBH5, demonstrating that MRGs are involved in ALKBH5-regulated... »). They should be more cautious (as they are in the introduction and discussion sections) as they do not provide evidences that this is a general mechanism for all MRGs. At least, it is for SPI1 and PHF12, but we cannot exclude that other MRGs will behave differently.
-Page 15, « First, mice were xenografts with P493-6 cells that expressing shSPI1 or shPHF12 » should be « First, mice were xenografts with P493-6 cells expressing shSPI1 or shPHF12 ».
-Page 23, the plasmid used to over-express FTO should be described.
-Page 36, legend to figure 1E. This sentence is not correct. In my opinion, it should be « GO term analysis of transcripts containing unique (single ?) m6A peaks in P493-6 cells treated with Tea for 72h ». What unique means? mRNAs containing only one m6A site? This should be clarified.
-Page 36, legend to figure 1F. Should be « IGV graph showing location of m6A peaks on representative genes ». The same is true for legend to figure 2D and Fig. S1E.
-Legend to figure 1A. « The showing data is representative ... » should be « The shown data are representative ... ».

Point-by-point response to the comments of the reviewers

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Comments-1-1:

In this manuscript, Wu and co-authors showed that MYC suppresses several selected MRGs (eg. SPI1 and PHF12) via mechanisms involving inhibition of m^{6}A of these genes by inducing the demethylase ALKBH5, thereby inhibiting the recruitment of the m^{6}A-binding protein YTHDF3 to the MRGs and their translation. Functionally, they show that overexpression of ALKBH5 and knockdown of SPI1 or PHF12 promote cancer cell proliferation in vitro and in vivo. Also, Myc expression positively correlates with ALKBH5 expression whereas it reversely correlates with the expression of SPI1 or PHF12 in lymphoma patient samples. While this is an interesting finding delineating a novel mechanism for MYC-mediated gene suppression, there are a number of issues that should be addressed.

Response: We appreciate the Reviewer's summary of our work and thank you for the valuable comments and suggestions.

The main issue here is the specificity of the demonstrated MYC-ALKBH5-m^{6}A-SPI1/PHF12 axis. The data show that MYC regulates the global mRNA m^{6}A and translation (polysome profiling assays). How the SPI1, PHF12 genes were specifically chosen for the study is not well presented. How about the well-known MYC target genes such as p21, p15? The MYC-induced genes should be used as controls to demonstrate the specificity. How does MYC specifically control mRNA m^{6}A of a subset of genes?

Response: The Reviewer raises an interesting question about how we chose MYC target genes *SPI1* and *PHF12* in this study. First, the MYC-repressed genes were enriched top #1 by the GO analyses as we shown in Fig 1E. Compared to the other well-known MYC target genes, the MRGs are much less studied, which are more interesting to us; Secondly, considering the complexity of the genes that could be regulated by MYC at both mRNA level and m^{6}A modification level, such as *p21* (*CDKN1A*) (Fig R1A-R1D), we chose those genes that only

could be regulated by MYC at m^{6}A modification level; Thirdly, *SPI1* gene plays an important role in lymphomagenesis, which is well established, and we also included a not well studied gene *PHF12* and found it plays an important role in B-cell lymphomagenesis in this study. Inspired by the Reviewer's comments, we added a discussion to the revised text regarding how the SPI1, PHF12 genes were chosen for the study.

The Reviewer also makes a valuable point about controls. Following the suggestion of the Reviewer, we now looked at both the m^{6}A modification and gene expression of MYC target genes *p21* (*CDKN1A*) and *p15* (*CDKN1B*). From IGV analysis, we can see that the level of m^{6}A modification of *CDKN1A* is also increased when MYC is low (Fig R1A), suggesting the possibility that MYC regulates the gene expression of *CDKN1A* via m^{6}A modification. Of note, though there is a tiny peak at the 3'UTR region of *CDKN1B*, the m^{6}A modification level of *CDKN1B* is very low and there is no enriched m^{6}A peak/region for *CDKN1B* by our peak calling method, suggesting that *CDKN1B* is not an m^{6}A modification target in this content. Our RT-qPCR results showed that MYC represses the mRNA level of *CDKN1A* and *CDKN1B* (Fig R1B), indicating a transcriptional regulation by MYC. Western blot results showed that the protein level of CDKN1A and CDKN1B was increased in low MYC-expressing cells compared to the high MYC-expressing cells (Fig R1C and R1D). To further investigate whether ALKBH5 is involved in regulating CDKN1A and CDKN1B, we detected the protein level of CDKN1A and CDKN1B in the ALKBH5 knockdown P493-6 and Raji cell samples (same to Fig 2I and 2J), and found that the protein level of CDKN1A was increased when knocked down ALKBH5 but not as high as in low MYC-expressing cells (Fig R1C and R1D), and CDKN1B remaining the same (Fig R1C and R1D). These data suggest that, in addition to transcriptional regulation of CDKN1A, MYC might also regulate the mRNA m^{6}A modification of *CDKN1A* via ALKBH5, and then reduces its protein expression. As to *CDKN1B*, MYC might regulate it only by transcription.

Our data suggest that MYC may specifically regulate its target genes by regulating m^{6}A related enzymes, such as ALKBH5 in this study. Nevertheless, as we have discussed above,

the MYC-regulated genes, such as *CDKN1A* and *CDKN1B*, may not be good for controls since they are potentially regulated by additional different mechanisms.

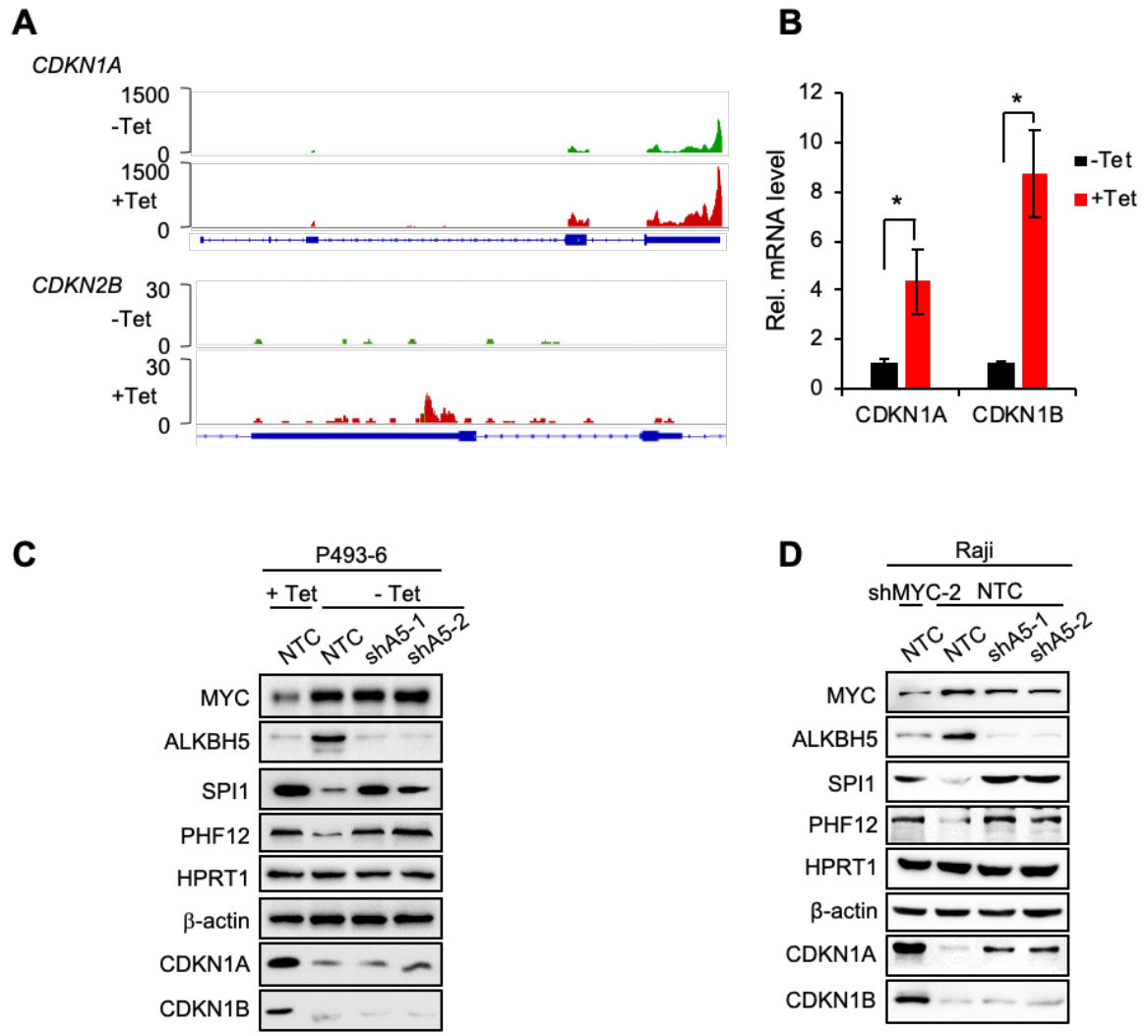

Fig R1. MYC down-regulates the level of mRNA m^{6}A modification and inhibits the expression of CDKN1A and CDKN1B. A: IGV showing the locations of m^{6}A peaks on *CDKN1A* and *CDKN1B*. **B**: RT-qPCR analysis of the mRNA levels of *CDKN1A* and *CDKN1B* in P493-6 cells treated with Tet for 0 hour or 24 hours, data were presented as mean (±SD), n = 3 biological replicates, * P<0.05 relative to corresponding -Tet group. **C, D**: Western blot analysis for protein levels in P493-6 cells that expressed NTC or ALKBH5 shRNAs and were then treated with or without Tet (**C**) or in Raji cells expressed NTC or ALKBH5 shRNAs with or without MYC knockdown (**D**). HPRT1 and β-actin serve as negative and loading controls, respectively.

It is also somewhat confusing that while total M^{6}A was increased by YTHDF3 KD, the total protein translation was not reduced (Fig. 3F, input).

Response: We apologize for not making this clear in the original figure. Our data didn't show that the total m^6A could be increased by YTHDF3 knockdown. As reported, YTHDF3 is a reader of m^6A modification instead of an enzyme responsible for regulating mRNA m^6A modification. Our m^6A-RIP data also showed that knockdown of YTHDF3 doesn't regulate the m^6A modification of *SPI1* and *PHF12* (as in Fig 3I of the original manuscript). In this study, our data showed that YTHDF3 facilitates the translation of the selected MRGs *SPI1* and *PHF12*. We believe that YTHDF3 could regulate many other genes' translation. As previous studies reported, the number of YTHDF3 target genes is about 1000 genes (Li, Chen et al., 2017, Shi, Wang et al., 2017), the percentage of YTHDF3 target genes among all genes would be low which is less than 10%. As a consequence, knockdown of YTHDF3 may not significantly impair the total protein translation, particularly in the ^{35}S pulse labeling experiment. We now performed the Click-iT AHA (L-Azidohomoalanine) assay and obtained the similar results (Fig R2C, also as Fig 3F in the revised manuscript).

How YTHDF3 specifically increases the MRGs' translation should be further elaborated or experimentally tested.

Response: We thank the Reviewer for the insightful suggestion. The Reviewer is correct, considering the functional redundancies of YTHDF1, YTHDF2 and YTHDF3, it is important to validate it is YTHDF3 that specifically promotes the MRGs' translation. We then added Western blot results showing that YTHDF1 and YTHDF2 protein levels are not affected by the shRNAs directed against YTHDF3 and knockdown of YTHDF3 significantly inhibits the protein level of MRGs SPI1 and PHF12 (Fig R2A and R2B, see also our response to the Reviewer #3 (Fig R11) as well as Fig 3B and 3C in the revised manuscript), suggesting that MRGs SPI1 and PHF12 protein levels are upregulated by YTHDF3. We also conducted a Click-IT AHA (L-Azidohomoalanine) assay to detect the nascent proteins. Our results showed that translation of SPI1 and PHF12 was significantly decreased in the absence of YTHDF3 (Fig R2C, also as Fig 3F in the revised manuscript). Taken together with our RIP assays, YTHDF3 knockdown experiments, polysome profiling analysis and ^{35}S pulse labeling experiment, we

thus conclude that YTHDF3 specifically facilitates the protein translation of MRGs SPI1 and PFH12.

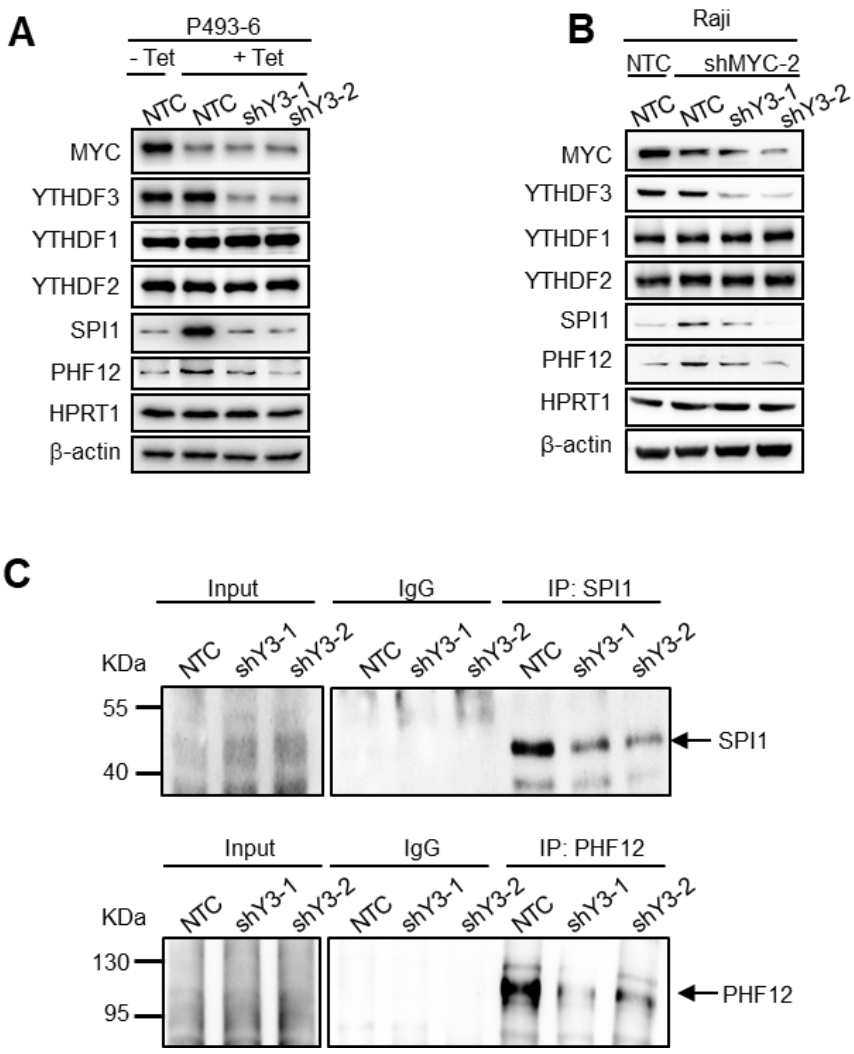

Fig R2. YTHDF3 promotes the translation of MRGs SPI1 and PHF12. A, B: Western blot analysis for protein levels in P493-6 cells that expressed NTC or YTHDF3 shRNAs in the presence or absence of Tet (**A**) or in Raji cells that expressed NTC or YTHDF3 shRNAs and knocked down MYC or not (**B**). HPRT1 and β-actin serve as negative and loading controls, respectively. **C**: Click-iT AHA (L-azidohomoalaine) experiments were performed using IgG, anti-SPI1 or anti-PHF12 antibody. P493-6 cells expressing NTC or YTHDF3 shRNAs were incubated for 1 hour in medium containing 100 ug/mL AHA. The translated proteins were detected by Western blot. Arrow indicates translated MRGs. See also Fig 3B, 3C and 3F in the revised manuscript.

Minor points:

• Fig. 4G-4I: Does it mean that unlike the endogenous SPI1 and PHF12, the overexpressed SPI1 and PHF12 are not subjected to the ALKBH5 regulation?

Response: We believe the overexpressed SPI1 and PHF12 are still subjected to the ALKBH5 regulation, at least partially. From Fig 1D, we can see that, apart from CDS region, the m^{6}A modification could be located at both 5' UTR and 3'UTR regions, which may also play regulatory roles. However, the overexpression plasmids only contain the CDS region. We actually compared the tumor size between 'EV-SPI1' group and 'A5-SPI1', or between 'EV-PHF12' group and 'A5-PHF12' group, respectively. As a result, we found the tumor size of 'A5-SPI1' group is slightly larger than 'EV-SPI1' group and that 'A5-PFH12' group is significantly larger than 'EV-PHF12' group (Fig R3), suggesting the overexpressed SPI1 and PHF12 are still subjected to the ALKBH5 regulation.

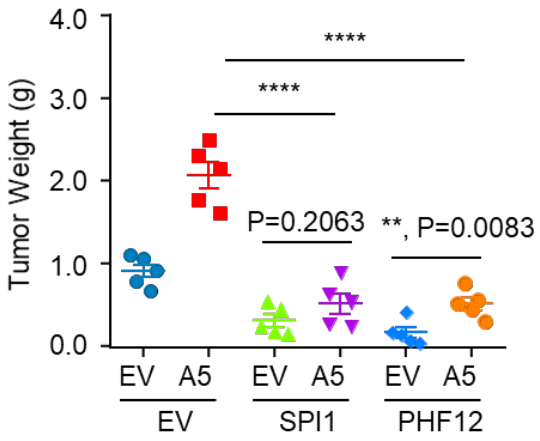

Fig R3. ALKBH5 promotes tumor growth even in the SPI1 and PFH12 overexpressed cells. Weight of tumors stably expressing EV or ALKBH5 and SPI1 or PHF12 at the end of the experiment in Fig 4I. Data are presented as mean (±SEM), n = 5 for each group. ** P<0.01 or **** P<0.0001 as compared between indicated groups.

• Fig. S2E: It seems that SPI1 mRNA is increased. Please re-check the statistics.

Response: To confirm the result and conclusion, we re-prepared three biological replicates samples and re-detected the mRNA levels of *SPI1* and *PFH12*, which showed no significant increase (Fig R4A, also as Fig EV2E in the revised manuscript). Each biological replicate sample was detected with four technical replicates, all of them didn't show significant increase

of *SPI1* expression in low MYC-expression sample (Fig R4B). We replaced the old data with our new three biological replicates data (as Fig EV2E in the revised manuscript).

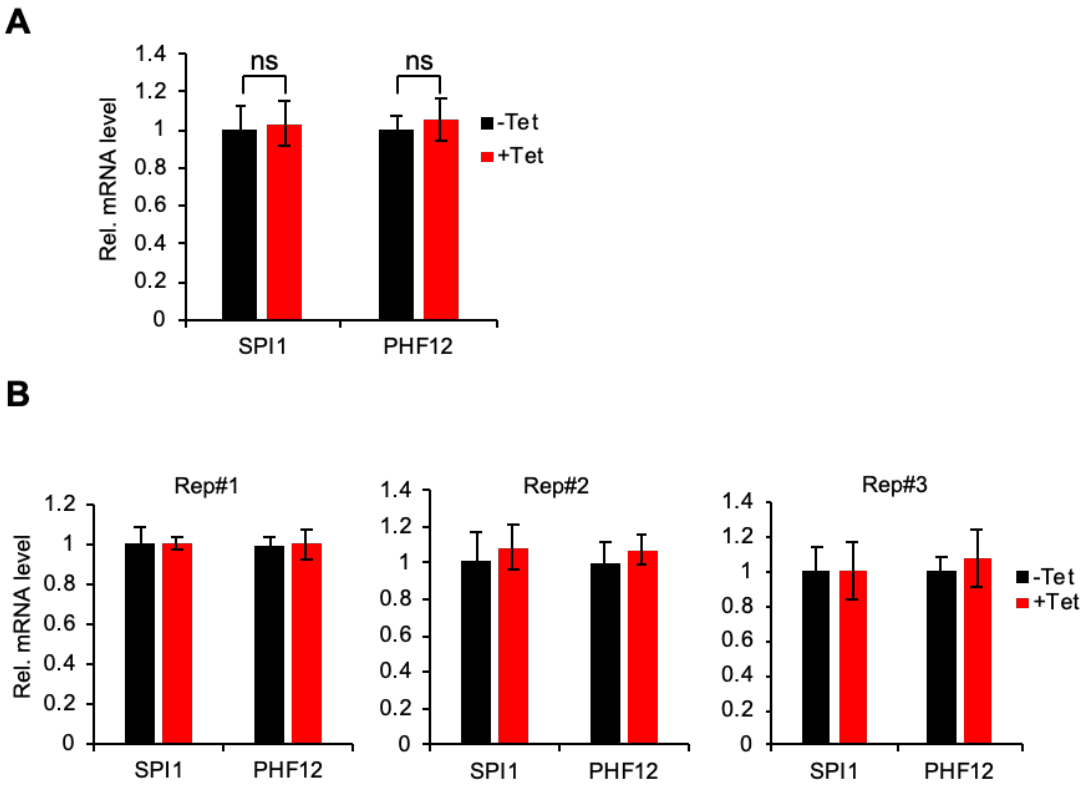

Fig R4. MYC doesn't regulate the mRNA level of SPI1 and PHF12. A, B: RT-qPCR analysis of the mRNA level of SPI1 and PHF12 in P493-6 cells treated with Tet for 0 hour or 24 hours. Data were presented as mean (±SD), n = 3 biological replicates (**A**). Each biological replicate was detected by four technical replicates (**B**). See also as Fig EV2E in the revised manuscript.

• Fig. 3F is not convincing, what are the bands in IgG control?

Response: The Reviewer is correct. The bands in IgG control group are non-specific bands. We tried to repeat this experiment to get a clean result, however, due to the limitation of ^{35}S labeling methionine and cysteine during this time of global pandemic, it's impossible for us to repeat the experiments using ^{35}S labeling materials which were not available. Instead, we conducted a Click-iT AHA (L-Azidohomoalanine) assay to detect the nascent proteins. Our Click-iT AHA assay showed that translation of SPI1 and PHF12 was significantly decreased in the absence of YTHDF3 (Fig R2C, also as Fig 3F in the revised manuscript). To avoid

confusion to the readers, we replaced the original ^{35}S pulse labeling results with our new Click-iT AHA assay data (as Fig 3F in the revised manuscript).

• Figure 5: the model should be modified to reflect the increase of translation of MRGs in the context.

Response: We appreciate the Reviewer's suggestion. We have revised the working model to reflect the changes of MRGs in the context (Fig R5, also as Fig 5 in the revised manuscript).

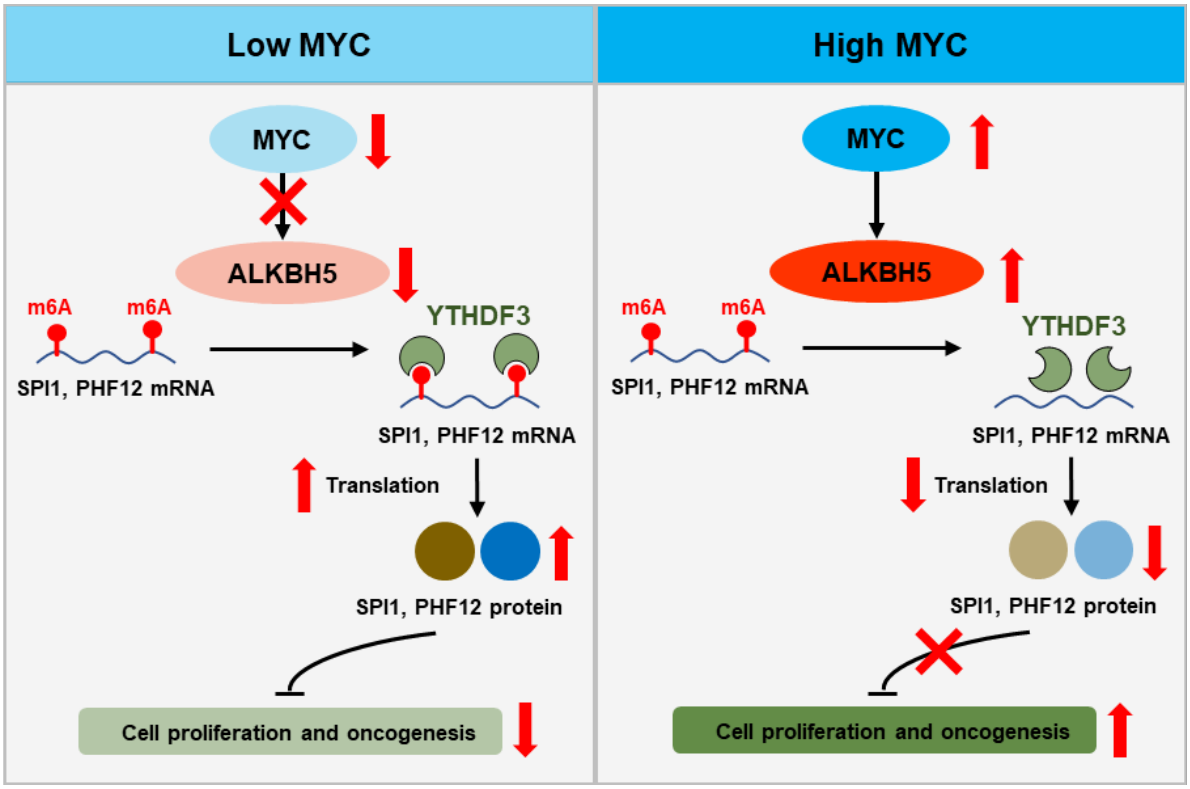

Fig R5. Working model: MYC suppression of gene expression via m^{6}A is critical for cancer progression. MYC down-regulates the m^{6}A modification preferentially in certain MRGs, by up-regulating the demethylase ALKBH5. The m^{6}A reader YTHDF3-mediated translation of MRGs SPI1 and PHF12 is attenuated as decreased m^{6}A modification, and thus releases the inhibitory effect of MRGs on cell proliferation, thereby promoting cancer progression. The up-direction red arrows indicate high and down-direction red arrows indicate low. See also as Fig 5 in the revised manuscript.

• The statement of the regulation as a transcription-independent mechanism is not accurate, as MYC drives the transcription of the upstream ALKNH5 gene.

Response: We completely agree with the Reviewer and we have changed the language for accuracy in the text.

• The role of FTO is not well consistent in Fig 2. There is no significant binding in PHF12 gene (Fig. 2E) and no rescue of MYC induced PHF12 expression (Fig. 2G).

Response: The Reviewer is correct and this is exactly what we observed and thus the reason why we focused on ALKBH5 instead of FTO: the regulation of ALKBH5 on SPI1 and PHF12 is very consistent, although we also believe some of MRGs might be regulated by FTO.

• The discussion is not well written and should be more focused on the mechanism and implication and written cohesively.

Response: Thank you and followed the suggestions by the Reviewer, we have revised our discussion on multiple occasions which are highlighted in the manuscript.

• RIP protocol was not described in detail; RNA fragmentation is not described clearly.

Response: We apologize and have added the details to the RIP protocol.

• Some grammars and typos throughout: eg. Page 4 the last sentence should be "may overlap with the first,...". page 20 the second sentence "...and leads to cell proliferation and growth" should be "and leads to cell proliferation and growth inhibition".

Response: We apologize for the errors and have corrected them throughout the revised manuscript.

Referee #2:

The manuscript by Wu et al. reveals MYC can suppress gene expression through tuning the m6A modification level on MRGs. The authors show that MYC knockdown leads to a global accumulation of m6A on mRNAs in B-cell lymphoma cells. MYC can promote the transcription

of m6A demethylase ALKBH5 and FTO, therefore regulating the m6A methylation level. The authors study two specific MGRs, SPI1 and PHF12, which are effectively demethylated by ALKBH5 and bound by m6A reader YTHDF3. They show that YTHDF3 promotes the translation of SPI1 and PHF12 through m6A. Finally, they show that the MYC-ALKBH5- m6A-SPI1/PHF12 axis can be a potential clinical target for patients with aberrant MYC expression.

Response: We appreciate the Reviewer's very nice summary and positive comments for our work.

Figure 1: MYC is known to affect many genes through various mechanisms. The authors state that MYC knockdown leads to 2,542 differentially methylated. What percentage of these genes was previously known MRGs? What percentage of the MRGs was regulated by m6A? Are any of the genes previously known to be regulated by other mechanisms? From the GO analysis, some genes also regulate transcription; does MYC also employ m6A to regulate transcription of other genes?

Response: To be honest, currently, we don't know for sure the percentage of m^{6}A modification regulated MRGs or the percentage of these genes that were previously known MRGs. As we know, MYC regulates many genes (up to 15% of all human genes) through various mechanisms and the MYC target genes are also various in different contexts. There's no universally recognized common list of MYC target genes available and thus it's difficult to calculate the percentage. What's more, though scientists have made many efforts, current knowledge about MRGs is still very limited, which warrants further studies. Trying to answer your questions, considering nearly one-third of the putative target genes are repressed by MYC and about 10,000 genes expressed in a cell, and there're 116 MRGs enriched by GO analysis, so the very rough percentage is 116/(15%*10000*1/3)=23.2%. Among those enriched MRGs, *p21* (*CDKN1A*), which is a well-known MRG, could be transcriptionally regulated by MYC (Fig R1B) and be partially regulated through m^{6}A modification (Fig R1A, R1C and R1D), supporting the Reviewer's comment that some of these MRGs could be regulated by other mechanisms instead of only by m^{6}A modification. Considering the complexity of the genes

that could be regulated by MYC at both mRNA level and m^{6}A modification level, we focused on those genes that only could be regulated by MYC at m^{6}A modification level in this study (see also our response to Reviewer #1 comment).

We completely agree with the Reviewer that MYC could employ m^{6}A modification to regulate transcription of other genes, which however is not addressed in this manuscript.

Figure 2: The authors analyzed U2OS cells ChIP-seq data and found that MYC binds to the promoter regions of ALKBH5 and FTO to promote transcription; the same was confirmed in P493-6 cells. ALKBH5 is also overexpressed in many cancers, is this a general mechanism for cancer cells? The correlation from 78 lymphocyte cell lines may not seem as strong as the authors claimed (S3A).

Response: We appreciate the Reviewer for the insightful points and believe that the Reviewer is correct. We analyzed the gene expression of ALKBH5 in all 1304 cell lines from CCLE datasets which showed that almost all of the cancer cell lines highly express ALKBH5 (Fig R6A). We also analyzed the co-expression of MYC and ALKBH5 in all 1304 cancer cell lines, which showed almost all of the cell lines highly express both MYC and ALKBH5 (Fig R6B). Next, we analyzed ENCODE Transcription Factor ChIP datasets and found that c-Myc (MYC) is a binding transcription factor at the promoter region of *ALKBH5* (Fig R6C), indicating a general regulation of MYC on ALKBH5. Finally, we analyzed published GEO ChIP-seq datasets and found that high level of MYC binds to the promoter regions of ALKBH5 in MCF-7 and MCF10A cell lines (Fig R6D). Together, our data and analyses suggest a strong correlation of MYC and ALKBH5 and the regulation of MYC on ALKBH5.

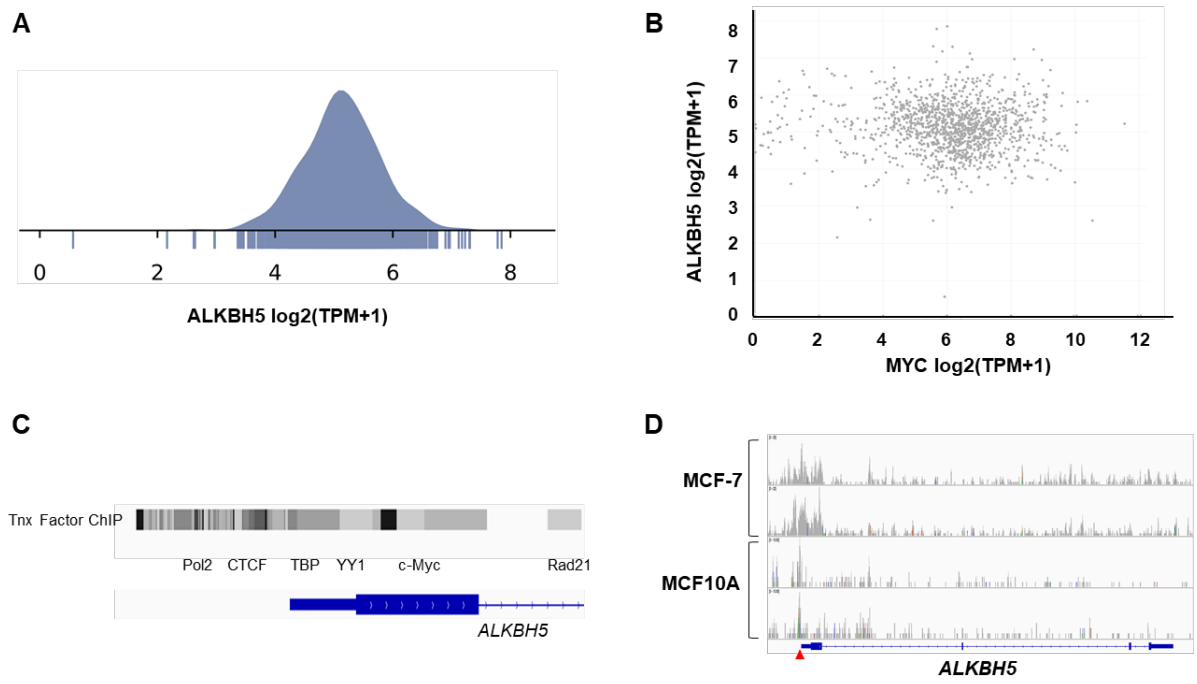

Fig R6. MYC and ALKBH5 are co-highly expressed in cancer cell lines and MYC binds to the promoter of ALKBH5. A: ALKBH5 expression in 1304 cell lines from CCLE datasets. **B**: Analyses of the co-expression of MYC and ALKBH5 in 1304 cell lines from CCLE. **C**: Analysis of ALKBH5 promotor in ENCODE Transcription Factor ChIP datasets (Data ref: Gene Expression Omnibus GSE33213 and GSE31477, 2011). **D**: IGV graph showing location of MYC binding peaks on ALKBH5 from published ChIP-seq datasets (Data ref: Gene Expression Omnibus GSE33213 and GSE31477, 2011). Red triangle indicates the binding peak.

Figure 2E - According to Figure 2A, Tet treated P493-6 cells express very little ALKBH5/FTO, how do you ensure successful IP and how are the values normalized? Even though they have low protein expression level, successful IP should still show enrichment of their targets?

Response: The Reviewer is correct, when P493-6 cells were treated with Tet, only very little ALKBH5/FTO were expressed. The same is true for MYC expression when treated with Tet. However, as we demonstrated in our previous ChIP-qPCR assays (Wu, Yuan et al., 2017), we were able to carry out the assays successfully even when the protein level is low. Basically, we used the same cell numbers and performed the RIP under the same conditions at the same time, which means we used the same amount of antibodies to pull down for each sample. We also normalized the enrichment of targets by cognate input. Actually, from the Tet treated group samples, some target RNAs were pulled down when compared to IgG control (Fig R6, as Fig

2E in the manuscript), but the amount is significantly lower than those from the no Tet treated group samples in which RNA enriched abundantly. Thus, the reviewer is correct, even though the Tet treated P493-6 cells have low protein expression level, successful IP still shows enrichment of their targets.

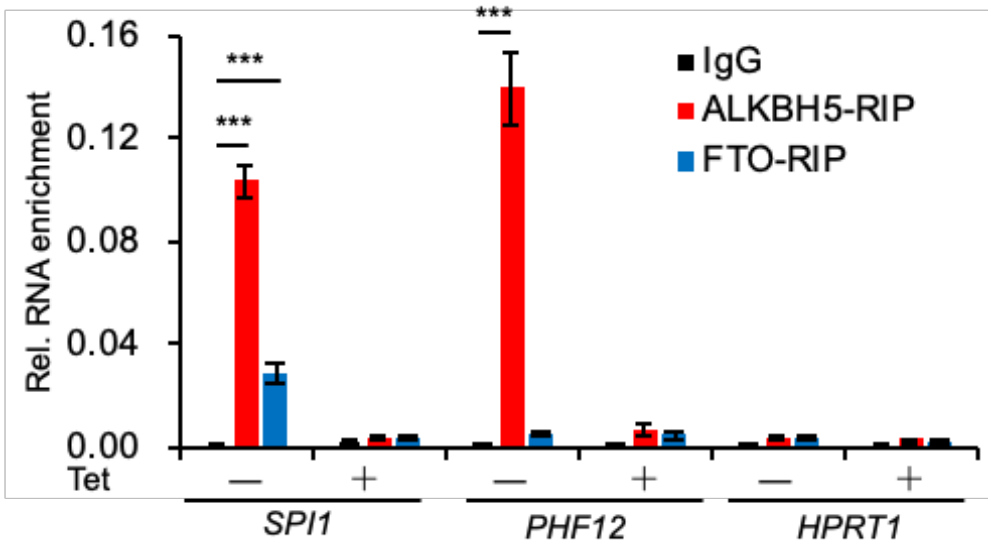

Fig R6. ALBKH5 bound to the selected MRGs *SPI1* and *PHF12* but notably less FTO. RIP assay, using ALKBH5, FTO or IgG antibody to detect the binding to MRGs (SPI1 and PHF12) in P493-6 cells treated with Tet or not. *HPRT1* serves as negative control. *** P<0.001 as compared to corresponding IgG group; Student's *t*-test. See also as Fig 2E in the manuscript.

Figure 4 - More supportive tumor model would be comparing tumors with high and low MYC and overexpress ALKBH5 in these cells or treat with ALKBH5 inhibitor. As shown in S6C.

Response: Following the Reviewer's suggestion, we performed a mouse xenograft experiment using the cells in Fig EV5C (also as Fig S6C in the original manuscript) that Raji cells stably expressing MYC and ALKBH5 shRNAs. Our results showed that knockdown of ALKBH5 alone significantly impaired tumor growth, and overexpression of MYC promoted tumor growth, which was eliminated by further knockdown of ALKBH5 (Fig R8, also as Fig 4D and 4E in the revised manuscript).

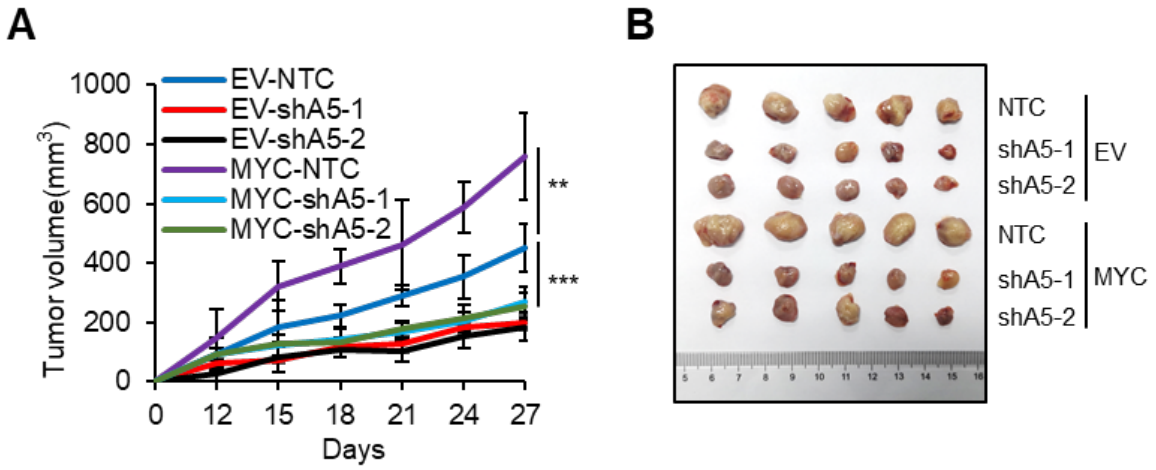

Fig R8. Raji cells stably expressing EV or MYC were infected with shALKBH5. Cells were injected subcutaneously into nude mice (n=5 for each group). Tumor growth curves were measured starting from 12 days post injection (**A**). Photo of tumors collected at the end of the experiment (day 27) (**B**). Data are presented as mean (±SEM). ** P<0.01 or *** P<0.001 as compared between indicated groups. See also as Fig 4D and 4E in the revised manuscript.

Minor questions:

Figure 1B: dot blot MB staining doesn't look right.

Response: Thank the Reviewer for the point. We have repeated this experiment. To avoid confusion, we ~~also~~ replaced the old data with our new results in the revised manuscript (Fig R9, also as Fig 1B in the revised manuscript).

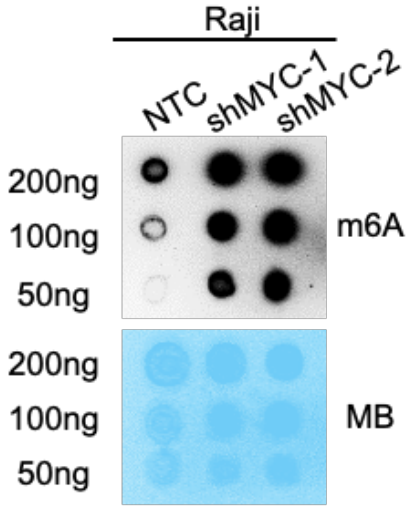

Fig R9. MYC down-regulates m^{6}A level. m^{6}A dot blot of Raji cells that expressed NTC or MYC shRNAs. Equal mRNA loading was verified by methylene blue staining. See also as Fig 1B in the revised manuscript

Figure 3H: in Raji, YTHDF3 and ALKHB5 double knockdown leads to even lower SPI1 and PHF12?

Response: Our data documented that ALKBH5 down-regulates the protein levels of SPI1 and PHF12, and YTHDF3 promotes the protein levels of SPI1 and PHF12. We have repeated this experiment multiple times (Fig R10A and R10B) and the results are consistent. To avoid confusion, we have replaced this result in the revised manuscript with Fig R10A (also as Fig 3H in the revised manuscript).

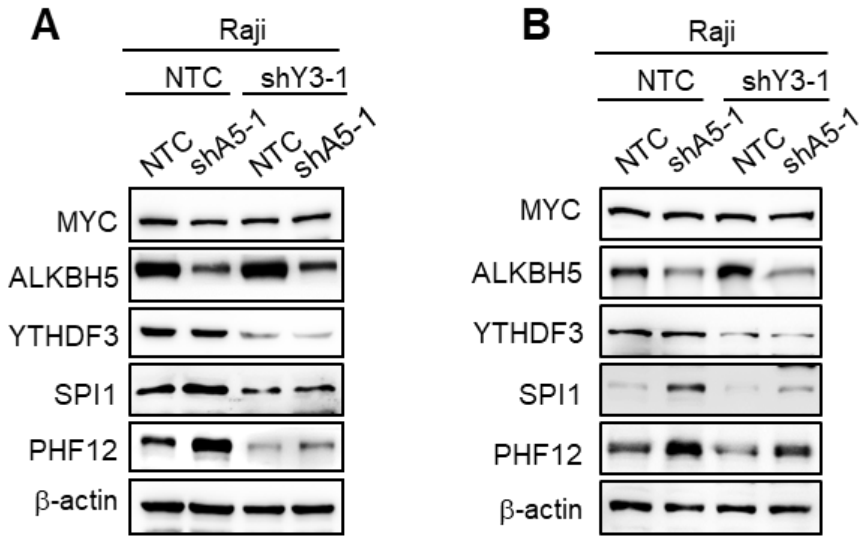

Fig R10. ALKBH5 down-regulates the protein levels of SPI1 and PHF12 and YTHDF3 promotes the protein levels of SPI1 and PHF12. Western blot analysis for protein levels in Raji cells that expressed NTC, or ALKBH5 shRNA, or YTHDF3 shRNA. β-actin serve as negative and loading controls, respectively. **A** and **B** are two Western blot representatives.

Referee #3:

In this study, the authors present nice pieces of evidence supporting a connection between the MYC proto-oncogene, the m^{6}A mRNA demethylase ALKBH5, mRNA translation and cancer

progression. First, they show that the MYC transcription factor activates the expression of the m^6A mRNA demethylase ALKBH5 protein, thereby influencing the m^6A status of mRNAs encoding for some MYC-repressed genes (notably SPI1 and PHF12). Indeed, the presence of m^6A on these transcripts leads to the specific recruitment of the YTHDF3 reader, thereby enhancing their translation. On the contrary, upon MYC-induced ALKBH5 expression, these transcripts are demethylated by ALKBH5, which diminishes their translation and then reduces levels of SPI1 and PHF12. Interestingly, this control occurs at the level of translation and not mRNA decay as the levels of mRNA encoding for SPI1 and PHF12 are kept constant. This nice mechanistic study is complemented by in vivo experiments showing that low levels of either SPI1 or PHF12 proteins (due to silencing of these genes or over-expression of ALKBH5) favor cancer progression while high level of SPI1 or PHF12 reduces tumor size. Altogether, these are convincing data on an interesting topics and hence, it is my feeling that this study deserves publication in EMBO Reports. In addition, it is undoubtedly of general interest to the molecular biology community.

Response: We thank you for the positive overall comments.

I have however comments that should be addressed in a revised version:

1) In Fig 2a, the author show that in the absence of Tet, the protein levels of MYC, ALKBH5 and FTO are high while in Fig 2e, in the absence of Tet, only the protein level of MYC is high although the same cell lines are used. I guess that this is because in Fig 2e, they use an anti-FLAG antibody to specifically detect the over-expression of either ALKBH5 or FTO but this is neither clear from the figure legend nor from the materials and methods section. This should be clarified.

Response: We thank the Reviewer for pointing this out. Now we have added the detailed information to the figure legend of Figure 2.

2) Considering the functional redundancies described for YTHDF1, YTHDF2 and YTHDF3 (e.g. Zaccara and Jaffrey; Cell; 2020; PMID: 32492408), it is important to validate the model

proposed by the authors as it involves only YTHDF3 protein. In Figure 2b and 2c, I would like to suggest to add WB showing that YTHDF1 and YTHDF2 protein levels are not affected by the shRNAs directed against YTHDF3.

Response: Following the Reviewer's suggestion, we added Western Blot results of YTHDF1 and YTHDF2 which were not changed by knocking down YTHDF3 (Fig R11, see also our response to the Reviewer #1 (Fig R2A and R2B) as well as Fig 3B and 3C in the revised manuscript).

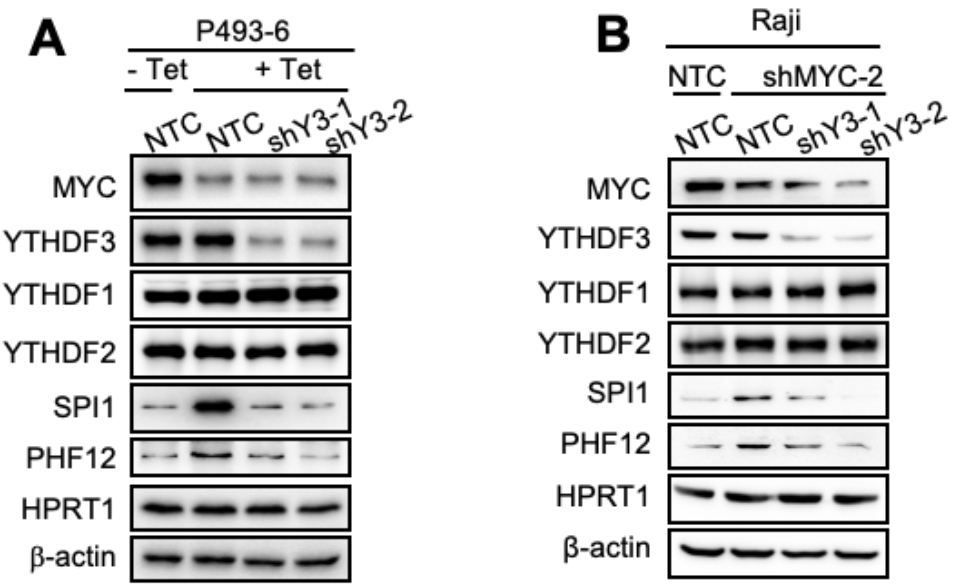

Fig R11. YTHDF3 shRNAs don't change the expression YTHDF1 or YTHDF2. A, B: Western blot analysis for protein levels in P493-6 cells that expressed NTC or YTHDF3 shRNAs in the presence or absence of Tet (**A**) or in Raji cells that expressed NTC or YTHDF3 shRNAs and knocked down MYC or not (**B**). HPRT1 and β-actin serve as negative and loading controls, respectively. See also Fig 3B and 3C in the revised manuscript.

Minor comments and typos :

-Figure 5: It is my opinion that this figure is difficult to read. I would like to suggest to split it in two scenarios. One where MYC levels are low and hence ALKBH5 also, leading to optimal mRNA translation of SPI1 and PHF12, thereby inhibiting cell proliferation and oncogenesis. Another one where MYC levels are high as ALKBH5 are, leading to reduced mRNA translation of SPI1 and PHF12 and thereby enhanced cell tumor size.

Response: We thank you for the suggestion and have revised the working model (Fig R12, also see our response to The Reviewer #1 (Fig R5) as well as Fig 5 in the revised manuscript).

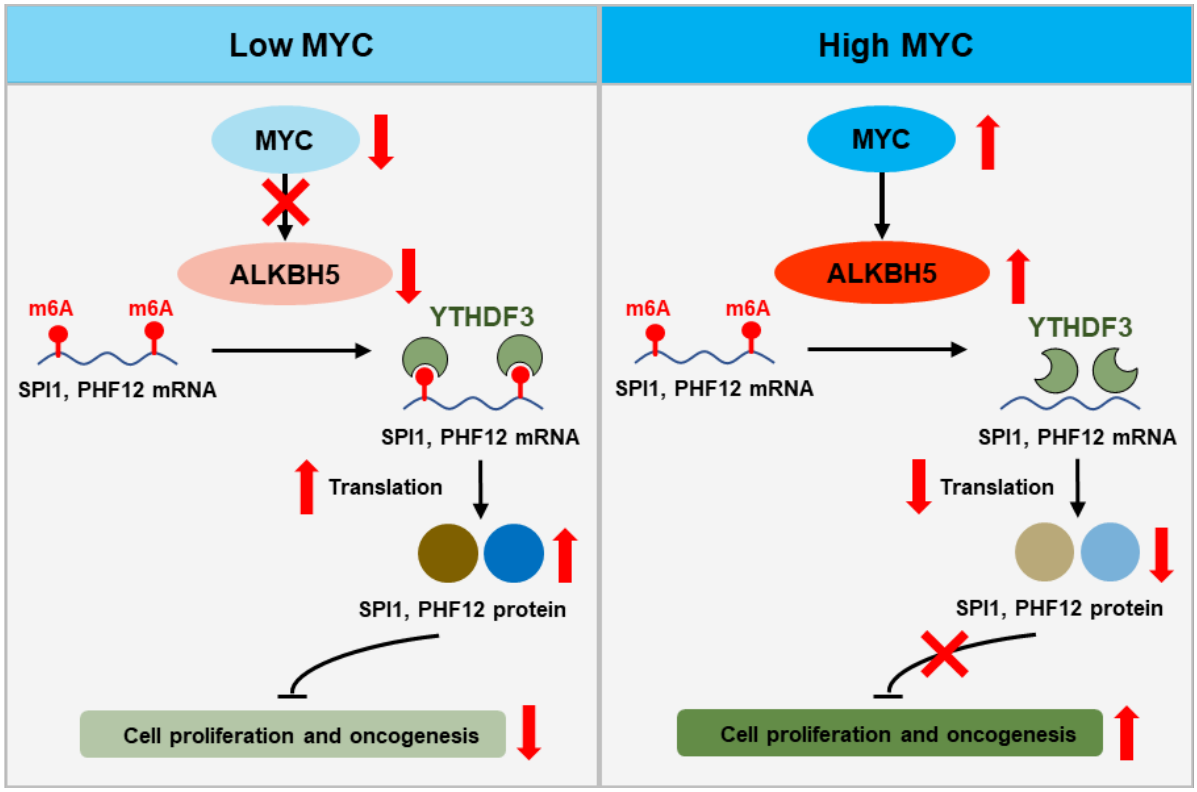

Fig R12. Working model: MYC suppression of gene expression via m^{6}A is critical for cancer progression. MYC down-regulates the m^{6}A modification preferentially in certain MRGs, by up-regulating the demethylase ALKBH5. The m^{6}A reader YTHDF3-mediated translation of MRGs SPI1 and PHF12 is attenuated as decreased m^{6}A modification, and thus releases the inhibitory effect of MRGs on cell proliferation, thereby promoting cancer progression. The up-direction red arrows indicate high and down-direction red arrows indicate low. See also our response to The Reviewer #1 (Fig R5) as well as Fig 5 in the revised manuscript.

-Figure S1E. The IGV graph for HPRT1 would be useful as a comparison with the other mRNAs shown.

Response: The reason we use HPRT1 is because HPRT1 is a non-m^{6}A target and has been widely used as a negative control by many groups, such as Chuan He's group (Wang, Lu et al., 2014), Richard Gregory's group (Lin, Choe et al., 2016), Stacy Horner's group (Gokhale, McIntyre et al., 2016). Since HPRT1 is so widely used as a negative control in almost all of

the m^{6}A published papers that we think the IGV graph for HPRT1 might not be very informative to the readers, we thus did not compare HPRT1 with the other mRNAs shown.

-Abstract : SPI should be SPI1.

Response: Thank you for pointing this out.

-Page 15. In this section, the authors focus on two gene products SPI1 and PHF12, the two central MYC regulated genes (MRGs) studied in this manuscript, but give the feeling that their observations are applicable to all MRGs (i.e. « ...knocking down SPI1 or PHF12 significantly promoted cell proliferation, indicating that MRGs inhibit ... », « ...enhanced proliferation by ALKBH5, demonstrating that MRGs are involved in ALKBH5-regulated... »). They should be more cautious (as they are in the introduction and discussion sections) as they do not provide evidences that this is a general mechanism for all MRGs. At least, it is for SPI1 and PHF12, but we cannot exclude that other MRGs will behave differently.

Response: The Reviewer is correct. Following the suggestion, we have modified the languages.

-Page 15, « First, mice were xenografts with P493-6 cells that expressing shSPI1 or shPHF12 » should be « First, mice were xenografts with P493-6 cells expressing shSPI1 or shPHF12 ».

-Page 23, the plasmid used to over-express FTO should be described.

-Page 36, legend to figure 1E. This sentence is not correct. In my opinion, it should be « GO term analysis of transcripts containing unique (single ?) m^{6}A peaks in P493-6 cells treated with Tea for 72h ». What unique means? mRNAs containing only one m^{6}A site? This should be clarified.

-Page 36, legend to figure 1F. Should be « IGV graph showing location of m^{6}A peaks on representative genes ». The same is true for legend to figure 2D and Fig. S1E.

-Legend to figure 1A. « The showing data is representative ... » should be « The shown data are representative ... ».

Response: We apologize for the errors and have corrected them in the revised manuscript.

We thank you all for the constructive comments and valuable suggestions that help us improve the manuscript substantially.

References

Gokhale NS, McIntyre ABR, McFadden MJ, Roder AE, Kennedy EM, Gandara JA, Hopcraft SE, Quicke KM, Vazquez C, Willer J, Ilkayeva OR, Law BA, Holley CL, Garcia-Blanco MA, Evans MJ, Suthar MS, Bradrick SS, Mason CE, Horner SM (2016) N6-Methyladenosine in Flaviviridae Viral RNA Genomes Regulates Infection. *Cell Host Microbe* 20: 654-665

Li A, Chen YS, Ping XL, Yang X, Xiao W, Yang Y, Sun HY, Zhu Q, Baidya P, Wang X, Bhattarai DP, Zhao YL, Sun BF, Yang YG (2017) Cytoplasmic m(6)A reader YTHDF3 promotes mRNA translation. *Cell Res* 27: 444-447

Lin S, Choe J, Du P, Triboulet R, Gregory RI (2016) The m(6)A Methyltransferase METTL3 Promotes Translation in Human Cancer Cells. *Mol Cell* 62: 335-345

Shi H, Wang X, Lu Z, Zhao BS, Ma H, Hsu PJ, Liu C, He C (2017) YTHDF3 facilitates translation and decay of N(6)-methyladenosine-modified RNA. *Cell Res* 27: 315-328

Wang X, Lu Z, Gomez A, Hon GC, Yue Y, Han D, Fu Y, Parisien M, Dai Q, Jia G, Ren B, Pan T, He C (2014) N6-methyladenosine-dependent regulation of messenger RNA stability. *Nature* 505: 117-20

Wu G, Yuan M, Shen S, Ma X, Fang J, Zhu L, Sun L, Liu Z, He X, Huang, Li T, Li C, Wu J, Hu X, Li Z, Song L, Qu K, Zhang H, Gao P (2017) Menin enhances c-Myc-mediated transcription to promote cancer progression. *Nat Commun* 8: 15278

Data citations

Furey T, Zhang Z, Song L, Crawford G, Giresi P, Lieb J, Liu Z, McDaniell R, Lee B, Iyer V, Flicek P, Keefe D, Birney E, Graf S (2011) Gene Expression Omnibus GSE33213 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33213). [DATASET]

Snyder M, Gerstein M, Weissman S, Farnham P, Struhl K (2011) Gene Expression Omnibus GSE31477 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31477). [DATASET]

1st Revision - Editorial Decision 2nd Dec 2020

Dear Prof. Gao,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. Unfortunately, original referee #2 was not responsive to my invitations to re-assess the manuscript. However, going through your point-by-point response, I consider his/her concerns as adequately addressed. As you will see, the other two referees now fully support the publication of your paper in EMBO reports.

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

- I would suggest these modified titles:
MYC reduces target gene translation by modulating m6A modifications to promote cancer progression

or

MYC promotes cancer progression by modulating m6A modifications to reduce target gene translation

- As also indicated by referee #1, please add the additional data shown only in the point-by-point response to the main or EV figures of the final revised manuscript. We can accommodate up to 8 main figures. Please also provide the source data for the Western blots among these figures.

- We have recently changed our reference format. Please change this in the final manuscript text: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- Please remove the titles of the EV tables from the main manuscript text file. Please put these on the first TAB of their excel files.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. It seems, you already addressed most of these. Please check. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Kind regards,

Achim Breiling

Editor
EMBO Reports

Referee #1:

The authors have properly addressed my comments. I have no further comments but suggest the authors to add the additional data in their rebuttal /point-to-point response to the main or EV figures.

Referee #3:

The authors have addressed satisfactorily all my comments.

Authors made the requested editorial changes.

2nd Revision - Editorial Decision 10th Dec 2020

Prof. Ping Gao
University of Science and Technology of China
School of Life Sciences
University of Science and Technology of China, No.96, JinZhai Road Baohe District,Hefei,Anhui, 230027,P.R.China
Hefei, Anhui 230026
China

Dear Prof. Gao,

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

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, 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.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: 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."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

****

THINGS TO DO NOW:

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2020-51519V3 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ping Gao
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-51519V3

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs 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 and any statistical test employed should be iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship 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 $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No Pre-determination of sample sizes was done. Instead, at least three independent experiments were performed to support the statements.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For Figure 4D and 4E, equal numbers of the established Raji stable cells were injected subcutaneously into 5 mice for each group. For Figure 4F-4I, equal numbers of the established P493-6 stable cells were injected subcutaneously into 5 mice for each group. The mice were followed for a fixed amount of time and sacrificed.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded from the anlysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	For xenograft experiments (Figure 4D, 4E and 4F-4I), mice were randomly assigned to experimental groups.
For animal studies, include a statement about randomization even if no randomization was used.	For xenograft experiments (Figure 4D, 4E and 4F-4I), mice were randomly assigned to experimental groups.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The investigators were blinded to allocation during experiments and outcome assessment.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The investigators were blinded to allocation during experiments and outcome assessment.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All the antibody information were described in materials and methods section. (Manuscript PageS 26 and 27)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T cell line is from ATCC, Raji cell line is from the Type Culture Collection of Chinese Academy of Sciences, and P493-6 cell line is a gift from Dr. Chi V. Dang at Ludwig Institute for Cancer Research. All cell lines have been authenticated. All the cells have been tested and are free of mycoplasma contamination. （Manuscript Page 24）

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Male BALb/c nude mice were purchased from SJA Laboratory Animal Company of China and housed in a temperature controlled sepcific pathogen free environment with a 12 hours dark/light cycle.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal studies were conducted with approval from the Animal Research Ethics Committee of the South China University of Technology.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirmed compliance.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	For using these clinical materials for research purposes,approval from the Institutional Research Ethics Committee of Anhui provincial hospital was obtained.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	For using these clinical materials for research purposes, prior written informed consents from the patients was obtained. The studies were conducted in accordance with ethical guidelines of the Declaration of Helsinki.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A