THE EMBO JOURNAL

A dual role of human tRNA methyltransferase hTrmt13 in regulating translation and transcription

Hao Li, Han Dong, Beisi Xu, Qing-Ping Xiong, Cai-Tao Li, Wen-Qing Yang, Jing Li, Zhi-Xuan Huang, Qi-Yu Zeng, En-Duo Wang and Ru-Juan Liu
DOI: 10.15252/embj.2021108544

Corresponding author(s): En-Duo Wang (edwang@sibcb.ac.cn) and Ru-Juan Liu (liurj@shanghaitech.edu.cn)

Review Timeline:

Submission Date:	22nd Apr 21
Editorial Decision:	28th May 21
Revision Received:	23rd Aug 21
Editorial Decision:	15th Sep 21
Revision Received:	19th Oct 21
Accepted:	29th Oct 21

Editor: Stefanie Boehm

Transaction Report:

(Note: 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.)

Re: EMBOJ-2021-108544
Human hTrmt13 regulates translation via tRNA modification and controls transcription independent of catalytic activity

Dear Prof. Wang,

Thank you for submitting your manuscript to The EMBO Journal. We have now received three referee reports on your study, which are included below for your information. Given the referees' comments, we would like to invite you to prepare and submit a revised manuscript.

As you will see, all reviewers appreciate the comprehensive analysis and the reported dual function of human Trmt13. However, they do raise several concerns and technical issues that must be addressed in the revised version. For the majority of these issues, it seems that adding further information on materials, methods, statistics or discussion of results, will likely be sufficient to address the points. Specifically it will be crucial to carefully discuss the findings in context of the current literature and potential mechanisms in more detail (ref#1- point 1, 2, 8; ref#2; ref#3- point 2, 5, 8) and to resolve the duplication issues in Fig. 4 and Fig. 5 (ref#1- point 4, 5; ref#2- [*] Figs. 4f and 4g..; ref#3- point 4, 7). Furthermore, the description of material and methods must be expanded and the required information added in this section, as well as in the figure legends. Please also remember that all datasets must be deposited in public repositories and made available (please see specifics on "Data Availability" below). In addition, the necessary and appropriate controls and statistical analyses have to be added, this includes the knockout and knockdown controls the referees request (ref#1- point 3; ref#2- [*]KO of hTrmt13; ref#3-point 1, 7, 11), as well as adding information on replicates and quantification of imaging experiments. Please also carefully consider and respond to all other referee comments and revise the manuscript accordingly.

Please note that it is our policy to allow only a single round of major revision. We realize that lab work worldwide is currently affected by the COVID-19/SARS-CoV-2 pandemic and that an experimental revision may be delayed. If you foresee any potential issues that may significantly delay a revision, please contact us to discuss this as soon as possible. In addition, I would encourage you to contact me to discuss a preliminary revision plan with a brief point-by-point response to ensure the critical issues will be resolved in the course of the revision.

Thank you again for the opportunity to consider your work for publication. Please contact me if you would like to discuss the revision plan or if you have any other questions.

Kind regards,

Stefanie Boehm

Stefanie Boehm
Editor
The EMBO Journal

-

Referee #1:

Posttranscriptional modification of individual ribonucleotides plays an important role in the biogenesis and functions of cellular RNAs. Mature tRNAs are heavily decorated by various types of chemical modifications that support their correct folding, promote efficient tRNA amino acylation and anticodon-codon interaction. However, the identification and mechanism of tRNA modifying enzymes remain largely unclear, which has progressively become a hot topic of 'tRNA epitranscriptomics' in recent year.
In this manuscript, the authors identified a novel human tRNA Nm4 methyltransferase (hTrmt13) CCDC76, which could modulate cellular protein translation via tRNA methylation and a specific tRF in the cytoplasm. On the other hand, hTrmt13 directly bound to DNA in the nucleus and played as a transcriptional co-factor to regulate the transcription of several genes involved in cell migration. The functions of hTrmt13 were then validated in cell lines and breast tumor metastatic mice models, and further supported by clinical data from cancer patients.
Overall this is a very inspiring discovery. The manuscript is well written and provides novel insights into the dual functions of tRNA modifying enzymes. I am happy to recommend its publication after some revision as follow.
Specific comments:

1. In Figure 1: the authors carried out a CLIP-seq to search the interacting RNA of hTrmt13. It is well known that several reverse transcriptase such as TGIRT reverse transcriptase can read through the transcript and eliminate the bias of chemical modifications. I wonder why the authors took a step of removing modifications during the experiment and if it could truly improve the data quality.
2. In Figure 1: according to the results of CLIP-seq and in vitro enzymatic assay, hTrmt13 selects specific tRNA substrate dependent on the species of tRNA and anti-codon, instead of the modified nucleotides and their nearby sequence. I wonder if the authors can explain or infer the cause of this interesting phenomenon.
3. In Fig. 1 and 2, the authors should provide experiments for verifying the knockout and knock down efficiency of hTrmt13 both in mRNA and protein level.
4. In Figure 4: the results of Figure 5 had repeatedly emerged in Figure 4 as 4f and 4g. I believe this is overlooked during data assembly.
5. In Figure 4: according to the legend of 4e, the significant regulated genes were colored red, while the interesting oncogenic genes were labeled in blue or red. This needs correction.
7. The authors should use at least two siRNAs for the knockdown of hTrmt13, for example fig 2b, d-h, fig 6f, fig 8d, Supplementary fig. 3d-e, etc.
8. Page 5 the author demonstrated the CHHC zinc-finger domain2 is crucial for hTrmt13 binding of tRNA in cytoplasm (founction of tRNA methylation), and page 8 indicated CHHC Zn-2 responsible for hTrmt13 directly interacts with specific DNA motifs in nucleus. The authors should explain how the same domain showed different function?

Minor comments:
1. In Fig. 2i and j, there weren't statistical analysis.
2. In fig. 1h, RNA-mass spectrometry in hTrmt13-KO and wild type cells should be verified by more methods, such as primer extension.
3. In fig.6c, the IgG could pulldown USF1, of which the bonding is stronger than hTrmt13 antibody. Therefore, the interaction between hTrmt13 and USF1 should be further confirmed.

Referee #2:

The manuscript "Human hTrmt13 regulates translation via tRNA modification and controls transcription independent of catalytic activity" by Hao Li et al. reveals the role of human Trmt13 as a novel tRNA methyl transferase and a moonlight function (or viceversa) in regulating the transcription of genes involved in EMT and cancer. The biochemical and functional characterizations of Trmt13 are extensive and, in general, well-performed.
In short, the authors use a combination of CLIP-seq, in vitro methylation assays and mass spectrometry approaches to identify tRNA substrates of hTrmt13 and confirm that it methylates the Nm4 position of certain tRNAs. Interestingly, this methylation down regulates the formation of a particular tRF, the 5'-Gly-CCC, which in turn negatively impacts protein synthesis. The authors report that hTrmt13 localises to both the nucleus and the cytoplasm. Fractionation experiments followed by co-IPs reveal that nuclear hTrmt13 is bound to DNA rather than RNA. They identify that the CHHC Zn-2 domain is crucial for binding to RNA or DNA. In the nucleus, hTrmt13 interacts with transcription factors and promotes the transcription of genes involved in EMT and cancer, independent of its catalytic activity. This enhances cell migration, which the authors also correlate to cancer progression and severity of disease. Lastly, the authors use a mouse model to demonstrate that hTrmt13 promotes cancel cell metastasis in

vivo.
The content is relevant in different aspects and most conclusions are supported by the data. Yet, while the paper is rich in experiments, the authors fall short in investigating or at least discussing potential mechanisms underlying their observations. In other words, the paper is rather descriptive and superficial in some passages. In particular, I find the Discussion irrelevant; it does not discuss anything but rather adds more references.
Besides fully re-writing the Discussion, I prompt the authors to refine the text - English must be upgraded - and go an extra mile with the legends, which could be more detailed.

Title:
The title is somehow general and tasteless. The term methyltransferase is missing, as well as a transcriptional link to EMT and cancer, which is a main topic of the paper.

Introduction:
The authors may want to add a reference - if different to ref. 22 - when claiming that 2'-O-methylation at position 4th is conserved and therefore important.

Underlined are the most important points to be addressed. (denoted here by [*]...[*])

Results:
CCDC76 is the human tRNA Nm4 methyltransferase, hTrmt13:

It is not clear how the authors figured out that CCDC76 is the human homolog of Trmt13p. Which bioinformatics tool/alignment did they use?

Intuitively, I would expect Fig. 1b to be a tRNA methylation assay rather than a CLIP experiment, since the authors new that Trmt13p is a tRNA-methylation enzyme.

What do the authors mean with HctRNA? The expanded form must be mentioned in the text.

[*] Targets of hTrmt13: From the data presented, it appears that the main tRNAs that hTrmt13 binds to are HctRNAAgr, HctRNALys, HctRNAThr. While the EMSAs for HctRNAAgr, HctRNALys convincingly show that hTrmt13 does indeed bind to them, it is puzzling that they do not undergo methylation in vitro. This observation remains to be discussed in the manuscript. What role could this binding play? Is there another methyl transferase that could be methylating those? Can these HctRNAs in question be affinity-purified from the hTrmt13 KO cells and their methylation status compared?[*]

[*] Residual methylation in the KO: Importantly, the authors do not discuss the specificity of the enzyme neither here nor in the Discussion. Which enzymes could contribute to the residual levels of methylation seen in the KO? And, which tRNA methyltransferase(s) modify those (Arg, Lys, Thr) that are not methylated by hTrmt13?[*]
Could the authors silence additional candidates with siRNAs to fully abolish methylation? It would be worth to know, but not essential for the paper.

[*] KO of hTrmt13: Aside from the first figure, there is no more information on the KO, including in the methods section. A western blot with absence of hTrmt13 in the KO is crucial. How does the KO compare with the Knock Downs?[*]

Fig. 1h: Please briefly explain in the legend how the purification of those tRNAs was done.

hTrmt13 modulates protein translation depending on its tRNA modification activity:

Fig. 2a and text: why do we need to know the correlation with Eif4A2?

Fig. 2b: Soucrose or sucrose? I would write: "This difference could be overcome/rescued by expression of the wild type allele.

Previously, in the KO experiment, the authors could still see methylation - I already asked about the remaining activity. Here, in the knockdown experiment, the authors claim a significant decrease in protein synthesis. [*] What is the decrease in protein synthesis in the KO? [*]

[*] The authors convincingly show that the 5'-Gly-CCC increases upon silencing of Trmt13, but neither raise any hypothesis nor compare its sequence or predicted folding with fragments that are not affected. Thus, how do the authors envision that modifying position 4 in Gly-CCC could impair fragmentation? [*]

Page 7, line 7: the period (.) after U2OS cells is not correct.

hTrmt13 binds tRNA in cytoplasm and localizes to chromatin via directly binding to promoter DNA in the nucleus:

As an example, towards the lack of mechanistic insights, the authors say that the lack of serum in the media results in Trmt13 disappearing from the cytoplasm due to "some signaling pathway". Has such a phenomenon been reported in the past? Any reference at least?

[*] In general, IFs and in situs are shown in rather small and dark panels. Please improve.[*]

[*] Specifically, Figure 6e; supplementary Figure 4d: The confocal images are not very clear and can be improved upon. Neither the figure, nor the legend contains information about which color represents what in the images.[*]

The function of Trmt13 is said to be exerted in the cytoplasm. Any reference to the yeast enzyme?

I encourage the authors to improve Fig. 3g when showing binding to high molecular weight DNA. The smear at the top is not very convincing. The labeling is also displaced one lane to the left.

hTrmt13 promotes cell migration independent of its tRNA modification activity:

Fig. 4a: silencing of Trmt13 does not affect cell proliferation; however, the authors also show that silencing impacts protein synthesis. A comment on this would be appreciated.

Page 10, line 1: does not read well, please check.

[*] Figs. 4f and 4g need to be removed as they are duplicates of Fig 5d, 5e. [*]

hTrmt13 promotes transcription of key cell migration-related factors:

Page 11, line 1: remove "gene".

hTrmt13 directly interacts with transcription factors to exclude binding with tRNAs in the nucleus:

Here, the authors clearly show that hTrmt13 and USF1 interact, and partially revealed the mechanism by which Trmt13 binds DNA rather than tRNA in the nucleus. Two questions emerge from this result. a) Do FOSL1 and E2F1 show the same properties as USF1? Couldn't the authors find an alternative way to express those proteins (in baculovirus?) in recombinant form? b) What happens upon silencing of USF1? What is the transcriptional output of the targets of Trmt13?

hTrmt13 regulates in vivo cancer progression

Supplementary Figure 8c- labeling of the graph- intensity, instead of indensity

[*] Discussion:
There is a main problem with the current Discussion. It is trivial to say, that the authors should "discuss" their results beyond the data they provide in the Results section. In fact, when looking at the results, one hopes to find a new twist, limitations, perspectives, etc, in the Discussion. However, this is not the case. Reading it carefully, the Discussion is like a second Introduction, full of general knowledge and with few and short paragraphs related to their actual work. As an example, the authors solve the curious function of one single 5' tRNA fragment by saying it is "complicated". [*]

The authors should provide references to the first sentence about technology for tRNA biology. And, towards the end of the first paragraph: we report that tRNA...

I would reduce the size of the main figures by moving the following to the supplement:
1b and 1g; 2a; 3f and 3l, m and n; 4i and 4j (wrongly annotated); 6a and 7f.

Referee #3:

EMBOJ-2021-108544: Human hTrmt13 regulates translation via tRNA modification and controls transcription independent of catalytic activity by Hao Li, Han Dong, and Beisi Xu, et al.

Hao Li, Han Dong, and Beisi Xu, et al. present comprehensive evidence that human Trmt13 is a tRNA modifying enzyme that in addition can act as a transcriptional co-factor that regulates cancer-promoting genes. First, hTrmt13 is identified as the enzyme that modifies specific tRNAs with 2'-O-methylation at the acceptor stem loop. Knock down/out of hTrmt13 abolishes the modification, in vitro enzymatic activity could be shown, as well as sequence similarity to the bacterial version of the modifier. Apart from the expected tRNA modifying function, they discover that hTrmt13 also specifically binds genetic DNA motifs and show that it regulates gene expression by interaction with USF1 to regulated genes involved in EMT. Binding of hTrmt13 to a co-transcription factor is mutually exclusive with binding to tRNA. By studying available datasets from cancer patients, they deduce that hTrmt13 acts pro-tumorigenic by supporting tumor metastasis in cancer patients. Reduction of hTrmt13 attenuates metastatic spread in a mouse model of breast cancer. Together, this study is a highly admirable and thorough exercise in the characterization of what started as a tRNA modification enzyme and ends with the discovery of a potential therapeutic target in solid tumors.

The following points should be addressed to further increase the validity of the manuscript. It could be due to space limitations but occasionally it is not clear how the authors come to certain conclusions or why they decide to follow up on one pathway/interaction partner/consequence instead of another.

1. For CRISPR/Cas9 mediated knock out (referred to in Figure 1h), it is not clear if single clones were expanded or if bulk-transfected cells were used and no verification of the knock out is shown.

2. The effect on translation is not a strong one and there is no follow-up on the function of the tRNA fragment 5'Gly-CCC. Figure 2 i and j should be quantified. Given the later results, it seems surprising to assume that effects of hTrmt13 on tRNA modification are solely responsible for differences in translation. EMT can affect protein translation but the lack of rescue through the catalytically inactive E463A mutant and the subsequent data, which shows that transcriptional regulation by hTrmt13 is independent of its catalytic activity, suggest that they are unrelated, and that translational regulation is solely taking place through a tRNA fragment. Figure 2a: How was EIF4A2 picked - it does not show a strong correlation with hTrmt13, which other genes were tested and is there a general correlation between translation-associated genes and hTrmt13?

3. 3 m and n: At which concentration of hTrmt13 was the EMSA in 3 m run? The complete shift of the input DNA suggests that all DNA is hTrmt13 bound, however, if I understand the material and method section correctly, 3 n shows that at 400 nM hTrmt13 and 300 nM DNA, the majority of DNA is still free.

4. Figure 4 does not match the figure legend and the description in the text - for example, the legend for g should be for h, i and j are missing legends.

5. There are several GO terms associated with the regulation of cell cycle and cell cycle transition but no effect on cell proliferation was found?

6. I would like to see a paragraph on replicates, especially for the NGS-based data in the material and methods section. Additionally, the number of replicates should be clearly stated in each figure legend, except when bar graphs with replicates are shown. For example, how many replicates were run for RNAseq and how are biological replicates defined here?

7. 4f and g are the same as 5 d and e. The text mentions that Snail1 is reduced which does not seem obvious in the western blot shown in (now) Figure 4g and 5e? The blot should be quantified if this point is made. ZEB1 on the other hand seems clearly reduced but is not mentioned in the text.

8. Figure 6a: While it makes sense for me to look at whether FOSL1 and hTrmt13 co-regulate transcription based on the overlapping DNA recognition motif, it is less clear how the authors settled on E2F1 as an interactor. Was it purely based on gene overlap and if so, how many transcriptional regulators were tested? Hypergeometric testing would help to determine, whether the overlap is significant. Was there a significant overlap between JunB, BATF, or AP-1 controlled genes with hTrmt13 genes? How many genes are bound by both USF1 and hTrmt1? Do hTrmt13 and USF1 share a DNA motif that is mutually recognized? Were all peaks used for motif determination (Figure 3k) or only TSS-2kb peaks and if not, does the motif change, if only TSS-2kb peaks are used?

9. The hTrmt13 interactome is not described in material and methods. What does the first column in table S6 (Checked, False) mean? Supplementary Table legends would be highly appreciated.

10. Figure 6e should be quantified. A control should be added.

11. TGFB1, PKN2, and MEF2A levels could be measured in the breast cancer mouse model to support the statement that "hTrmt13 promotes breast cancer development, putatively by transcriptionally activating TGFB1, PKN2, and MEF2A".

12. Is the dataset used for Figure 8f BRCA and S8e the same? Were the same criteria used for dividing patients into low expression and high expression groups?

13. Overall, material and methods could be more detailed. If word limits are given by the journal, a Supplementary method section could be added. The variety of methods and the thoroughness of the study are greatly appreciated, which makes the lack of transparency as to how the data was obtained more frustrating.

14. The use of spaces between numbers and units is inconsistent (8M vs 100 mM, 2hr vs 30 min).

15. Was the anti-hTrmt13 antibody made in house? It is missing a commercial source. If so, verification of the antibody specificity should be added.

16. Figure S2: Is the motif found in Figure 3k also found in tRNA or does it show homology a to region within the modified tRNAs? What does Z1 bind to, if Z2 alone is necessary for recognition of both DNA and tRNA?

Response to comments from reviewers:

Referee #1:

Posttranscriptional modification of individual ribonucleotides plays an important role in the biogenesis and functions of cellular RNAs. Mature tRNAs are heavily decorated by various types of chemical modifications that support their correct folding, promote efficient tRNA amino acylation and anticodon-codon interaction. However, the identification and mechanism of tRNA modifying enzymes remain largely unclear, which has progressively become a hot topic of 'tRNA epitranscriptomics' in recent year.

In this manuscript, the authors identified a novel human tRNA Nm4 methyltransferase (hTrmt13) CCDC76, which could modulate cellular protein translation via tRNA methylation and a specific tRF in the cytoplasm. On the other hand, hTrmt13 directly bound to DNA in the nucleus and played as a transcriptional co-factor to regulate the transcription of several genes involved in cell migration. The functions of hTrmt13 were then validated in cell lines and breast tumor metastatic mice models, and further supported by clinical data from cancer patients.

Overall this is a very inspiring discovery. The manuscript is well written and provides novel insights into the dual functions of tRNA modifying enzymes. I am happy to recommend its publication after some revision as follow.

Response: We want to thank the reviewer for constructive comments. We have revised the manuscript carefully according to these valuable suggestions and added or updated the corresponding figures.

Specific comments:

Comment 1. In Figure 1: the authors carried out a CLIP-seq to search the interacting RNA of hTrmt13. It is well known that several reverse transcriptase such as TGIRT

reverse transcriptase can read through the transcript and eliminate the bias of chemical modifications. I wonder why the authors took a step of removing modifications during the experiment and if it could truly improve the data quality.

Response: Thanks! DM-TGIRT-seq (*Nat Methods* 12: 835-837 2015) and ARM-Seq (Nat *Methods* 12: 879-884 2015) are the two common methods for high-throughput sequencing of tRNA or tRNA fragments. In both methods, using AlkB to remove some modifications on tRNA is a key step and could efficiently improve the quality and integrity of the data. In DM-TGIRT-seq, in addition to the use of AlkB, TGIRT reverse transcriptase was also used to generate cDNA from highly structured tRNA (*Nat Methods* 12: 835-837 2015). However, due to the delivery time and other reasons, we could not obtain TGIRT conveniently, so we used Superscript IV as in the ARM-Seq method (*Nat Methods* 12: 879-884 2015). In our CLIP-seq experiment, we did find that the quality of the library (mainly refers to the concentration of the library here) with AlkB treatment was higher than that without. Specific modifications on tRNA (m^1A, m^1G, $m^{2,2}G$, and m^3C) could interfere reverse transcription during RNA-seq library preparation. And even using TGIRT as reverse transcriptase, adding AlkB treatment is an essential step (*Nat Methods* 12: 835-837 2015, and aslo through personal communication with Tao Pan's group in University of Chicago), so we think it is better to use AlkB for tRNA sequencing.

Comment 2. In Figure 1: according to the results of CLIP-seq and in vitro enzymatic assay, hTrmt13 selects specific tRNA substrate dependent on the species of tRNA and anti-codon, instead of the modified nucleotides and their nearby sequence. I wonder if the authors can explain or infer the cause of this interesting phenomenon.

Response: This is a very meaningful question. Indeed, it is not uncommon that some tRNA modifying enzymes can bind but not catalyze those tRNAs. For example, the tRNA modifying enzymes Nsun6 and PUS7 were reported to bind to non-substrate tRNAs (*RNA* 21:1532-1543 2015; *Cell* 173:1204-1216.e26 2018). In general, tRNA modifying enzymes fall into two groups that differ in their sensitivity to structural perturbations in tRNA. "Group I" tRNA modifying enzymes modify truncated tRNA fragments, whereas "group II" require well folded, full-length tRNA molecules as

substrates. We found hTrmt13 belongs to "group II" (**Response Figure 1A**) [Figures for referees not shown.]. Our results indicate that (1) hTrmt13 needs to recognize the complete tertiary structure of tRNA. (2) Besides the acceptor stem, hTrmt13 recognizes the D-stem of tRNA (**Response Figure 1B**)[Figures for referees not shown.]. We speculate that hTrmt13 first recognizes tRNA with tertiary structure, and then determines whether to catalyze the tRNA according to some specific discriminative elements. The detailed recognition and catalytic mechanism of hTrmt13 remains to be further investigated. Regarding the role of the binding with non-substrate tRNAs, we currently do not have any data to support this hypothesis, but one could speculate that they may have functions similarly to RNA binding proteins or as a molecular chaperone of tRNA. This phenomenon is indeed worth further investigation although out of scope of the current submission.

Comment 3. In Fig. 1 and 2, the authors should provide experiments for verifying the knockout and knock down efficiency of hTrmt13 both in mRNA and protein level.

Response: Thanks. We have completed the relevant experiments, and the data is shown in **Fig EV1A** (KO)**, Fig 2A** (sh-1, sh-2)**, Fig EV3D** (mRNA)**, Fig EV 4A, B** (si-1, si-2, protein and mRNA).

Comment 4. In Figure 4: the results of Figure 5 had repeatedly emerged in Figure 4 as 4f and 4g. I believe this is overlooked during data assembly.

Response: Thank you very much for pointing out this mistake, those in Figure 5 should be removed. We have now corrected them accordingly (Now **Fig 4** and **Fig 5**).

Comment 5. In Figure 4: according to the legend of 4e, the significant regulated genes were colored red, while the interesting oncogenic genes were labeled in blue or red. This needs correction.

Response: We apologized for the confusion. We amend the figure legend to "Volcano plot of differential expressed genes analysis results for RNA-seq data comparing hTrmt13 knockdown MDA-MB-231 cells (N=2) to control cells (N=2), the dots for significant regulated genes (FDR corrected p-value < 0.05) were colored red, the dots and labels of interesting genes with oncogenic implications have been colored in blue or red."

Comment 6. The authors should use at least two siRNAs for the knockdown of hTrmt13, for example fig 2b, d-h, fig 6f, fig 8d, Supplementary fig. 3d-e, etc.

Response: We thank the reviewer for this kind remind, we have now added the experimental results of two siRNAs (or two shRNAs) in the related figures (**Fig 2B-F, Fig EV3F, EV3G** and **EV3I, Fig 6F, Fig EV5C**).

Comment 7. Page 5 the author demonstrated the CHHC zinc-finger domain2 is crucial for hTrmt13 binding of tRNA in cytoplasm (function of tRNA methylation), and page 8 indicated CHHC Zn-2 responsible for hTrmt13 directly interacts with specific DNA motifs in nucleus. The authors should explain how the same domain showed different function?

Response: Thanks! The CHHC Zn-2 is very special. Up to now, this type of CHHC Zn-finger was only identified in the Trmt13 family, U11/U12 small nuclear ribonucleoprotein 48 kDa protein (SNRNP48), and gametocyte specific factors (GTSF1 and GTSF1L) (*Bioinformatics* 24: 2277-2280 2008). The CHHC Zn-finger mainly binds double strand RNA in SNRNP48 (*Structure* 17:294-302, 2009), while in GTSF1 it interacts with protein (*EMBO J* 12: e99325 2018). In our work, the CHHC

Zn-finger is essential for hTrmt13 to bind DNA and the double stranded region of tRNA. However, due to the lack of structural information, we currently still cannot accurately characterize the molecular mechanism of how hTrmt13 and the CHHC Zn-finger recognize DNA and tRNA. Interestingly, previous studies showed that the structural fold of the CHHC Zn-finger from SNRNP48 is generally similar to the classical C2H2 Zn-finger (*Bioinformatics* 24: 2277-2280 2008). Particularly, it is most similar to Zn-finger 5 of Transcription Factor IIIA (TFIIIA) (*Structure* 17:294-302 2009). This Zn-finger 5 was reported to bind DNA and RNA in different ways. Specifically, it interacts with the backbone of a double helix of RNAs, while binding to the major groove of its target DNA (*Nature* 426: 96-100 2003; *Proc Natl Acad Sci U S A* 95:2938-43 1998). We added this comment in the discussion part on **page 16**

Minor comments:

1. In Fig. 2i and j, there weren't statistical analysis.

Response: Thanks, we now added the statistical analysis to these figures (now **Fig. 2G and 2H**) in extended **Fig EV3K** and **EV3L**.

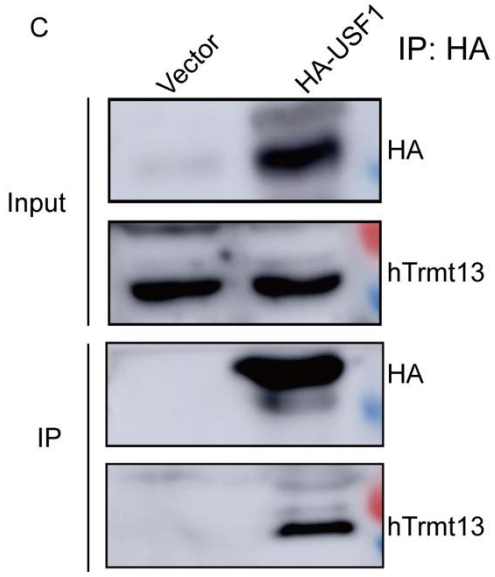
2. In fig. 1h, RNA-mass spectrometry in hTrmt13-KO and wild type cells should be verified by more methods, such as primer extension.

Response: Thank you for your advice. Exactly, RNA-mass spectrometry can only identify the modification type but not the modification positions. Thus, to confirm the modification site introduced by hTrmt13 is indeed at position 4, we mutated this site of $tRNA^{Gly}$-CCC into A, G, C, U respectively, and hTrmt13 could catalyze them into Am, Gm, Cm, Um respectively (extended figures: **Fig EV1E, F**), suggesting that hTrmt13 introduced 2'-*O* -methlyation at position 4 of tRNAs *in vitro*. Thanks for the reviewer's suggestion, we have now set up the primer extension for identification of Nm4 in $tRNA^{Gly}$-CCC and -GCC *in vivo*. These results showed that the Nm induced RT arrest products were observed in the low dNTPs groups of tRNAs purified from the WT MDA-MB-231 cells, but not in the hTrmt13-KO cells (CRISPR-Cas9 system) (New figures: **Fig EV1A, G**), suggesting that the modification introduced by hTrmt13

is most likely to be Nm modifications, which is consistent with our RNA-MS results.

3. In fig.6c, the IgG could pulldown USF1, of which the bonding is stronger than hTrmt13 antibody. Therefore, the interaction between hTrmt13 and USF1 should be further confirmed.

Response: The observed molecular weight of USF1 is ~ 40 kDa. In the WB membrane of IP samples, besides the band of USF1, we also observed a nonspecific band (we marked it with * in **Fig 6C**) larger than 40 kDa in both the control and experimental groups. While the ~40 KDa band of USF1 was only present in the htrmt13 experimental group but not in the IgG group. Thanks for the reviewer's advice, to further confirm the interaction between USF1 and hTrmt13, we performed additional new *in vivo* experiments, and found that USF1 could also pull-down endogenous hTrmt13 (**Response Figure 1C**). In addition, we have shown that USF1 and hTrmt13 could physically interact with each other *in vitro* without the interference from other factors (**Fig 7A and 7B**), thus we prefer to believe that the interaction between USF1 and hTrmt13 is reliable.



Response Figure 1C

1C. Interaction of HA-USF1 with endogenous hTrmt13. Whole-cell lysates from MDA-MB-231 cells stably expressing HA-USF1 were prepared and immunoprecipitation was performed with anti-HA followed by immunoblotting with

antibodies against hTrmt13.

Referee #2:

The manuscript "Human hTrmt13 regulates translation via tRNA modification and controls transcription independent of catalytic activity" by Hao Li et al. reveals the role of human Trmt13 as a novel tRNA methyl transferase and a moonlight function (or vice versa) in regulating the transcription of genes involved in EMT and cancer. The biochemical and functional characterizations of Trmt13 are extensive and, in general, well-performed.

In short, the authors use a combination of CLIP-seq, in vitro methylation assays and mass spectrometry approaches to identify tRNA substrates of hTrmt13 and confirm that it methylates the Nm4 position of certain tRNAs. Interestingly, this methylation down regulates the formation of a particular tRF, the 5'-Gly-CCC, which in turn negatively impacts protein synthesis. The authors report that hTrmt13 localises to both the nucleus and the cytoplasm. Fractionation experiments followed by co-IPs reveal that nuclear hTrmt13 is bound to DNA rather than RNA. They identify that the CHHC Zn-2 domain is crucial for binding to RNA or DNA. In the nucleus, hTrmt13 interacts with transcription factors and promotes the transcription of genes involved in EMT and cancer, independent of its catalytic activity. This enhances cell migration, which the authors also correlate to cancer progression and severity of disease. Lastly, the authors use a mouse model to demonstrate that hTrmt13 promotes cancel cell metastasis in vivo.

The content is relevant in different aspects and most conclusions are supported by the data. Yet, while the paper is rich in experiments, the authors fall short in investigating or at least discussing potential mechanisms underlying their observations. In other words, the paper is rather descriptive and superficial in some passages. In particular, I find the Discussion irrelevant; it does not discuss anything but rather adds more references.

Besides fully re-writing the Discussion, I prompt the authors to refine the text - English must be upgraded - and go an extra mile with the legends, which could be more detailed.

Response: We thank the reviewer for carefully reading our manuscript and providing constructive comments. We have revised the manuscript accordingly and added or updated the related figures.

Title:

Comment 1. The title is somehow general and tasteless. The term methyltransferase is missing, as well as a transcriptional link to EMT and cancer, which is a main topic of the paper.

Response: Thanks! In the current study, we focus on the dual functions of a tRNA modifying enzyme in both transcription and translation. The related physiological and pathological functions of hTrmt13 is further investigated in our lab with collaborators, hopefully we will get a more comprehensive understanding of hTrmt13. We now rewrite the title as "**Human RNA methyltransferase hTrmt13 regulates translation via tRNA modification and controls transcription independent of catalytic activity**"

Introduction:

Comment 2. The authors may want to add a reference - if different to ref. 22 - when claiming that 2'-O-methylation at position 4th is conserved and therefore important.

Response: Thanks. We've added all the related references, on **page 3**.

Comment 3. Underlined are the most important points to be addressed. (denoted here by [*]...[*])

Results:

CCDC76 is the human tRNA Nm4 methyltransferase, hTrmt13:

It is not clear how the authors figured out that CCDC76 is the human homolog of Trmt13p. Which bioinformatics tool/alignment did they use?

Response: Homologs of Trm13 were identified by BLAST search according to the sequence of yeast Trm13p which was first reported in *RNA* 13: 404-413, 2007. The human protein CCDC76 can be retrieved as the homologue of yeast Trm13p on the online tool UniProt (https://www.uniprot.org/uniprot/Q9NUP7). This have been further confirmed at EggNOG orthology database (http://eggnog5.embl.de/ search YOL125W).

Comment 4. Intuitively, I would expect Fig. 1b to be a tRNA methylation assay rather than a CLIP experiment, since the authors new that Trmt13p is a tRNA-methylation enzyme.

Response: During evolution, the substrates of RNA or DNA modifying enzymes usually change unexpectedly. For example, members of the NOL1/NOP2/SUN domain (NSUN) family have different RNA substrates when comparing lower species to higher eukaryotes (*RNA* 21:1532–1543 2015). In bacteria, only one NSun protein is present and a single rRNA is its substrate, while in human, seven NSun homologs are present, and their substrates include rRNA, tRNA, and mRNA. Similar substrate change is also observed in eukaryotic Pseudouridine synthase, such as PUS1 and PUS7 (*Nature* 515:143-6 2014). Another famous example is Dnmt2, which is a member of the animal DNA m^5C methyltransferase family and is a bona fide tRNA modifying enzyme *in vivo* (*Science* 311:395-8 2006; *Nat Rev Genet* 19:81-92 2018). Thus, we wanted to first identify whether hTrmt13 was mainly using tRNAs as a substrate *in vivo*.

Comment 5. What do the authors mean with HctRNA? The expanded form must be mentioned in the text.

Response: HctRNA means human cytoplasmic tRNA. Thank you for your advice. We've explained it in text on **page 4**.

Comment 6 [*] Targets of hTrmt13: From the data presented, it appears that the main tRNAs that hTrmt13 binds to are HctRNAAgr, HctRNALys, HctRNAThr. While the

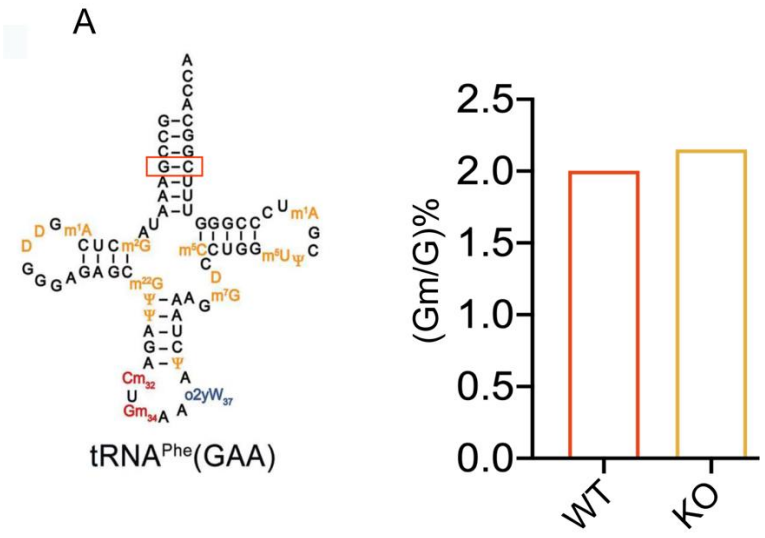
EMSAs for HctRNAAgr, HctRNALys convincingly show that hTrmt13 does indeed bind to them, it is puzzling that they do not undergo methylation *in vitro*. This observation remains to be discussed in the manuscript. What role could this binding play? Is there another methyl transferase that could be methylating those? Can these HctRNAs in question be affinity-purified from the hTrmt13 KO cells and their methylation status compared?[*]

Response: 1) "hTrmt13 binds to other tRNAs, but, they do not undergo methylation in vitro". What role could this binding play? As we described in our response to comment 2 from Reviewer 1, we think it is a very interesting phenomenon. Indeed, it is not uncommon that some tRNA modifying enzymes can bind but not catalyze those tRNAs. For example, the tRNA modifying enzymes Nsun6 and PUS7 were reported to bind to non-substrate tRNAs (*RNA* 21:1532-1543 2015; *Cell* 173:1204-1216.e26; 2018). In general, tRNA modifying enzymes fall into two groups that differ in their sensitivity to structural perturbations in tRNA. "Group I" tRNA modifying enzymes modify truncated tRNA fragments, whereas "group II" require well folded, full-length tRNA molecules as substrates. We found hTrmt13 belongs to "group II" (**Response Figure 1A**). Our results indicate that (1) hTrmt13 needs to recognize the complete tertiary structure of tRNA. (2) Besides the acceptor stem, hTrmt13 recognizes the D-stem of tRNA (**Response Figure 1B**). We speculate that hTrmt13 first recognizes tRNA with tertiary structure, and then determines whether to catalyze the tRNA according to some specific discriminative elements. The detailed recognition and catalytic mechanism of hTrmt13 remains to be further investigated. Regarding the role of the binding with non-substrate tRNAs, we currently do not have any data to support this hypothesis, but one could speculate that they may have functions similarly to RNA binding proteins or as a molecular chaperone of tRNA. This phenomenon is indeed worth further investigation although out of scope for this submission.

2) "Is there another methyl transferase that could be methylating those?" tRNA undergoes heavy chemical modification *in vivo*. Those tRNA modifying enzyme are usually type specific and site specific. For example, tRNAs have known 2'-*O*

-methylation modification at sites 4, 18, 32, 34, 44, and they are catalyzed by totally different modifying enzymes (Trmt13, Trmt3, FTSJ1/THADA, FTSJ1/WDR6, and TRMT44, respectively). These enzymes are evolutionarily irrelevant. Usually, modification at a certain position is only completed by a specific modifying enzyme. Since the tRNA substrate specificity of those tRNA 2'-*O* -methylation MTases in human remains largely unknown, we speculate that these tRNAs are likely to be modified by other enzymes. To our knowledge, hTrmt13 is the only MTase for 2'-*O*-methylation at position 4 of tRNA have been identified in human.

3) "Can these HctRNAs in question be affinity-purified from the hTrmt13 KO cells and their methylation status compared?". Up to now, only 17 human cytoplasmic tRNAs have been sequenced and modification at the rest of tRNAs remain unknown. From the sequenced database (https://iimcb.genesilico.pl/modomics/modifications; http://trna.bioinf.uni-leipzig.de/DataOutput/Result?ID=tdbR00000603), except for $tRNA^{Gly}$-GCC and -CCC, there is no 2'-*O*-methylation modification at the 4^{th} position of the remaining 15 human cytoplasmic tRNAs including *Hc*$tRNA^{Lys}$ and *Hc*$tRNA^{His}$. This is consistent with our *in vitro* methylation assays (**Fig 1F, EV1D**). In addition, as suggested by the reviewer, we purified $tRNA^{Phe}$(GAA) from WT and hTrmt13 KO cells and performed RNA-MS. The results showed that the proposed Nm4 (Gm for $tRNA^{Phe}$(GAA)) is not altered in this assayed tRNA in WT and KO cells (**Response Figure 2A)**. This result indicated that $tRNA^{Phe}$(GAA) was not the substrate of hTrmt13, and the Gm modification in this tRNA must be catalyzed by other modifying enzyme. Indeed, our previous report showed that the FTSJ1/WDR6 complex could introduce Gm at the 34^{th} position of $tRNA^{Phe}$(GAA) (*EMBO Rep* 21:e50095 2020).



Response Figure 2A

2A. 4th position of tRNA^{Phe}(GAA) is a G (Left, *EMBO Rep* :e50095 2020). The Gm modification level of tRNA^{Phe}(GAA) is not decreased in hTrmt13 KO cells when compared to WT (Right). The Gm modification is most likely coming from the 34th position which modified by FTSJ1/WDR6.

Comment 7. [*] Residual methylation in the KO: Importantly, the authors do not discuss the specificity of the enzyme neither here nor in the Discussion. Which enzymes could contribute to the residual levels of methylation seen in the KO? And, which tRNA methyltransferase(s) modify those (Arg, Lys, Thr) that are not methylated by hTrmt13?[*]

Could the authors silence additional candidates with siRNAs to fully abolish methylation? It would be worth to know, but not essential for the paper.

Response: Human tRNAs have known 2'-*O* -methylation modification at sites 4, 18, 32, 34, 44, and probably some unidentified sites. Different tRNAs have totally different modification patterns because those tRNA modifying enzymes are site specific and substrate selective. For example, although HctRNA^{Phe}(GAA) has no Nm modification at site 4, it does have Nm modification at sites 32 and 34 (*EMBO Rep* 21:e50095 2020). Similarly, HctRNA^{Gln} has no Nm modification at site 4, but has Nm modification at sites 18 and 32. Therefore, our data suggest that the remaining 2'-*O*-methylation modification (Nm) level found in KO cells is more likely to come

from Nm modification from other known or unknown positions on tRNA, rather than residual methylation activity of hTrmt13 in the KO cells. For tRNAs (Arg, Lys, Thr), combing our results and sequenced tRNA database, these tRNAs are not modified at site 4 by hTrmt13, but they may be modified with 2'-*O* -methylation by other enzymes including Trmt3, FTSJ1/WDR6, FTSJ1/THADA, and TRMT44.

Comment 8. [*] KO of hTrmt13: Aside from the first figure, there is no more information on the KO, including in the methods section. A western blot with absence of hTrmt13 in the KO is crucial. How does the KO compare with the Knock Downs?[*]

Response: Thanks! We 've now accomplished the related experiments, and added this results in **Fig EV1A** and in the methods section (**Page 26**). The details of KO have been descripted in method. In the western blot assays, the hTrmt13 band is disappear in the KO **(Fig EV1A right)**, and the hTrmt13 band is less than ~10% left in the Knockdown cells when compared with that WT **(Fig 2A, 4D, EV4A).**

Comment 9. Fig. 1h: Please briefly explain in the legend how the purification of those tRNAs was done.

Response: Thanks! The detailed procedure of purification of those tRNAs was in the method section (**Page 26**). We have now also added some essential information in the according figure legend (**Fig 1H**).

hTrmt13 modulates protein translation depending on its tRNA modification activity:

Comment 10. Fig. 2a and text: why do we need to know the correlation with Eif4A2?

Response: tRNA modification is often related to the translation process. Therefore, we first used bioinformatics methods to investigate whether there is any correlation between the expression of hTrmt13's gene and the expression of translation related genes using available database. We indeed found that the expression of eukaryotic

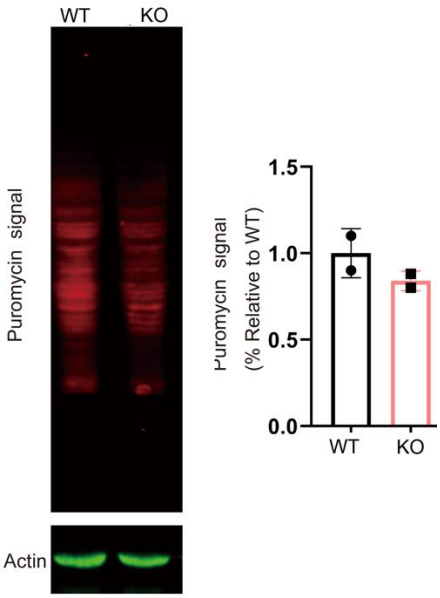
initiation factor 4A-II (EIF4A2) is correlated with that of hTrmt13. This result has implicated of hTrmt13's role in translation but is not strong evidence by itself. Thus, we move this result into the supplementary **Fig EV3A**.

Comment 11. Fig. 2b: Soucrose or sucrose? I would write: "This difference could be overcome/rescued by expression of the wild type allele.

Response: Thanks. We have revised it accordingly on **page 6**.

Comment 12. Previously, in the KO experiment, the authors could still see methylation - I already asked about the remaining activity. Here, in the knockdown experiment, the authors claim a significant decrease in protein synthesis. [*] What is the decrease in protein synthesis in the KO? [*]

Response: We have explained the reason why we still see some remaining Nm modification in KO cells in the responses to comment 6 and comment 7. We suggest that it mainly comes from Nm modifications in other positions of tRNA, but not from the 4th position by hTrmt13. Combining the result of the polysome profiling assay and *de novo* protein synthesis assay, we found that protein synthesis was mildly decreased in both hTrmt13 knock down (KD) cell lines, and the decrease was statistically significant. From WB assay, the protein level of hTrmt13 could barely be detected in KD cells (**Fig 2A, 4D** and **EV4A**), suggesting that the KD is quite potent and comparable to the effect of KO cell line. Taking the reviewer's advice, we performed new assays using the KO cell line, and the result from the *de novo* protein synthesis assay showed that protein synthesis was mildly inhibited in hTrmt13 KO cells than WT, the decrease level is ~18% (**Fig EV1A, Response figure 2B**), which is comparable to the ~13%-15% decrease in KD cells (**Fig 2A**).



Response Figure 2B

2B. *De novo* protein synthesis measured by puromycin incorporation in WT and hTrmt13 knockout cells (left), quantification was normalized to the protein level of actin (right, two duplicates experiment).

Comment 13. [*] The authors convincingly show that the 5'-Gly-CCC increases upon silencing of Trmt13, but neither raise any hypothesis nor compare its sequence or predicted folding with fragments that are not affected. Thus, how do the authors envision that modifying position 4 in Gly-CCC could impair fragmentation? [*]

Response: tRF is generated from tRNA, and its biogenesis is widely unknown. At present, Angiogenin (ANG) is thought to be the main enzyme for cutting tRNA into tRF (*J Biol Chem* 267:21982-21986 1992; *Trends Biochem Sci* 41:679-689 2016). However, ANG is not very sensitive to the sequence identity of tRNA, but indeed prefer to recognize the tertiary of tRNA (*RNA Biol* 2021 doi:10.1080/15476286.2021.1930758). Generally, all mature tRNA has a classical inverted L tertiary structure. Whether the subtle structural differences between different tRNA species could affect the recognition by ANG remains unknown. What's more, whether there are other enzymes that cleave tRNA to form tRF is also unknown (*Nat Rev Mol Cell Biol* 19:45-58 2018; *RNA Biol* 17:1149-1167 2020; *J Mol Med (Berl)* 96:1167-1176 2018). A couple of tRNA modifications have also been

reported to affect the formation of tRFs (*Nat Cell Biol* 2018 20:535-540 2018), however, the mechanism remains unknown. Further investigations are expected to comprehensively understand the relation between tRNA modification and tRF production. Here, the possible roles of Nm4 in regulating the level of tRF-CCC could be suspected as follows: it may work as a marker to recruit/exclude ANG or other RNA endonuclease directly, or via unknown reader proteins. In addition, the structures of tRNA are generally similar, whether modifications could introduce subtle structural differences and then affect the formation of tRF is also a question worthy of consideration. Furthermore, the modifications on tRNA could be delivered to tRF, whether they affect the structure or stability of tRF is also worth to be investigated. We added some of this discussion on **Page 15**.

Comment 14. Page 7, line 7: the period (.) after U2OS cells is not correct.

Response: Thanks, we have revised it accordingly.

hTrmt13 binds tRNA in cytoplasm and localizes to chromatin via directly binding to promoter DNA in the nucleus:

Comment 15. As an example, towards the lack of mechanistic insights, the authors say that the lack of serum in the media results in Trmt13 disappearing from the cytoplasm due to "some signaling pathway". Has such a phenomenon been reported in the past? Any reference at least?

Response: The expression or localization of tRNA modifying enzymes have been found to be affected by stress stimuli (*RNA Biol* 14:156-163 2017). As reported, *Os*Trmt13 is helpful for rice to deal with saline alkali environment (*J Exp Bot* 68:1479-1491 2017), and yeast Trm13 is related to oxidative stress (*Plos One* 15: e0229103 2020). We want to explore whether hTrmt13 also responds to some stimuli in cells, and serum starvation affects many signal pathways (*Cell Metab* 30:157-173.e7 2019), which is conducive to our initial screening. Serum starvation not only cause nutrient deprivation, but also cut off many growth factors to cells. Further mechanistic insights to figure out what kinds of stress hTrmt13 response to and how will be performed in the future.

Comment 16. [*] In general, IFs and in situs are shown in rather small and dark panels. Please improve. [*]

Response: Thanks. We now have uploaded all the high-resolution IF and in situs figures **(Fig 3A, 3B** and **6E, Appendix Fig 1B-D).**

Comment 17. [*] Specifically, Figure 6e; supplementary Figure 4d: The confocal images are not very clear and can be improved upon. Neither the figure, nor the legend contains information about which color represents what in the images.[*]

Response: Thanks. We have improved them now and added the details in the figure legend (**Fig 6E, Appendix Fig S1D**).

Comment 18. The function of Trmt13 is said to be exerted in the cytoplasm. Any reference to the yeast enzyme?

Response: According to our data, hTrmt13 located in both cytoplasm and nucleus (**Fig 3A, B**). hTrmt13 in cytoplasm mainly binds to tRNA (**Fig 3G**). Therefore, we suggest that tRNA Nm4 modification is mainly completed in cytoplasm via hTrmt13. The localization of Trm13 in yeast remains unknown yet.

Comment 19. I encourage the authors to improve Fig. 3g when showing binding to high molecular weight DNA. The smear at the top is not very convincing. The labeling is also displaced one lane to the left.

Response: We agree with the reviewer that we could not deduced from Fig. 3g that hTrmt13 binds to DNA, it can only indicate that hTrmt13 in nucleus binds to some nucleic acids other than mature tRNA. We have thoroughly rewritten those sentences on **page 43**. And we have now clearly marked each lane of **Fig 3G**.

hTrmt13 promotes cell migration independent of its tRNA modification activity:

Comment 20. Fig. 4a: silencing of Trmt13 does not affect cell proliferation; however,

the authors also show that silencing impacts protein synthesis. A comment on this would be appreciated.

Response: Thanks! Actually, this is also a very interesting phenomenon. Indeed, except for few cases, knocking out of most known tRNA modifying enzyme didn't affect cell proliferation under normal culture condition. However, the combination of knocking out two or more modifying enzymes shows growth defects, as well as the knocking out of a single enzyme under stress conditions (high temperature, oxidative pressure, low sugar, etc.) (*DNA Cell Biol* 31:434-454 2012; *RNA* 24:410-422 2018; *J Exp Bot* 2017 68:1479-1491 2017; *Cell* 167:816-828.e16 2016). As we responded on comment 12, protein synthesis was mildly decreased in both hTrmt13 knock down (KD) cell lines, although the decrease was statistically significant. We speculate that, hTrmt13 KO is not enough to change cell proliferation under normal cell culturing supplemented with rich nutrients, but maybe change cell proliferation under stress condition.

Comment 21. Page 10, line 1: does not read well, please check.

Response: Thanks, we have rewritten this sentence on **Page 10, now Line 14**.

Comment 22. [*] Figs. 4f and 4g need to be removed as they are duplicates of Fig 5d, 5e. [*]

Response: Sorry that we overlooked during figures reorganization. Those in Figure 5 should be removed and we have now corrected them accordingly.

hTrmt13 promotes transcription of key cell migration-related factors:

Comment 23. Page 11, line 1: remove "gene".

Response: We have corrected it accordingly.

hTrmt13 directly interacts with transcription factors to exclude binding with tRNAs in the nucleus:

Comment 24. Here, the authors clearly show that hTrmt13 and USF1 interact, and partially revealed the mechanism by which Trmt13 binds DNA rather than tRNA in the nucleus. Two questions emerge from this result. a) Do FOSL1 and E2F1 show the same properties as USF1? Couldn't the authors find an alternative way to express those proteins (in baculovirus?) in recombinant form? b) What happens upon silencing of USF1? What is the transcriptional output of the targets of Trmt13?

Response: Thanks for the questions. Yes, we observed similar properties in FOSL1 and E2F1 to USF1, with different strength of interaction. Their binding at several tested gene promoters could be also validated and was shown to be significant changed upon hTrmt13 knockdown (**Fig 6**). We suspect that FOSL1 and E2F1 cannot be purified *in vitro* might because their interactions with hTrmt13 were not as strong as for USF1. As the reviewer suggested, we now have tried a new recombinant expression system. We co-expressed the *E2F1* or *FOSL1* with *hTrmt13* using the pet-Duet vector in bacteria, however, we found that neither *E2F1* nor *FOSL1* could be expressed. Unfortunately, due to the limitations of available conditions, we are not able to perform the proposed additional baculovirus experiments. Our goal here is to provide the proof of concept that a tRNA methyltransferase like hTrmt13 could also regulate target gene transcription. The detailed regulatory relationship and network between these factors remains for further investigation.

Comment 25. Supplementary Figure 8c- labeling of the graph- intensity, instead of indensity

Response: We have corrected it accordingly.

[*] Discussion:

Comment 26. There is a main problem with the current Discussion. It is trivial to say, that the authors should "discuss" their results beyond the data they provide in the Results section. In fact, when looking at the results, one hopes to find a new twist, limitations, perspectives, etc, in the Discussion. However, this is not the case. Reading

it carefully, the Discussion is like a second Introduction, full of general knowledge and with few and short paragraphs related to their actual work. As an example, the authors solve the curious function of one single 5' tRNA fragment by saying it is "complicated". [*]

Response: We thank the reviewer for this constructive suggestion, we have now removed all the irrelevant words and discussed more based on our results. We mainly added our comments on three aspects: 1) the non-substrate tRNA binding of hTrmt13, 2) the possible roles of Nm4 in regulating the level of tRF, 3) the role of CHHC-Zinc finger in RNA and DNA binding.

Comment 27. The authors should provide references to the first sentence about technology for tRNA biology. And, towards the end of the first paragraph: we report that tRNA...

Response: Thanks! We now have added all the relevant references on **Page 15**.

Comment 28. I would reduce the size of the main figures by moving the following to the supplement:

1b and 1g; 2a; 3f and 3l, m and n; 4i and 4j (wrongly annotated); 6a and 7f.

Response: Thanks! We have now move **Fig 2a** into extended **Fig EV3A**.

Referee #3:

EMBOJ-2021-108544: Human hTrmt13 regulates translation via tRNA modification and controls transcription independent of catalytic activity by Hao Li, Han Dong, and Beisi Xu, et al. present comprehensive evidence that human Trmt13 is a tRNA modifying enzyme that in addition can act as a transcriptional co-factor that regulates cancer-promoting genes. First, hTrmt13 is identified as the enzyme that modifies specific tRNAs with 2'-O-methylation at the acceptor stem loop. Knock down/out of hTrmt13 abolishes the modification, in vitro enzymatic activity could be shown, as well as sequence similarity to the bacterial version of the modifier. Apart from the

expected tRNA modifying function, they discover that hTrmt13 also specifically binds genetic DNA motifs and show that it regulates gene expression by interaction with USF1 to regulated genes involved in EMT. Binding of hTrmt13 to a co-transcription factor is mutually exclusive with binding to tRNA. By studying available datasets from cancer patients, they deduce that hTrmt13 acts pro-tumorigenic by supporting tumor metastasis in cancer patients. Reduction of hTrmt13 attenuates metastatic spread in a mouse model of breast cancer. Together, this study is a highly admirable and thorough exercise in the characterization of what started as a tRNA modification enzyme and ends with the discovery of a potential therapeutic target in solid tumors.

Response: We gratefully thank the reviewer for carefully reading our manuscript and providing constructive comments on RNA methylation and cancer biology. We have revised the manuscript based on these comments and added/updated the corresponding figures.

The following points should be addressed to further increase the validity of the manuscript. It could be due to space limitations but occasionally it is not clear how the authors come to certain conclusions or why they decide to follow up on one pathway/interaction partner/consequence instead of another.

Comment 1. For CRISPR/Cas9 mediated knock out (referred to in Figure 1h), it is not clear if single clones were expanded or if bulk-transfected cells were used and no verification of the knock out is shown.

Response: Thanks! Single clones were used in our experiments. We have now added the information about CRISPR/Cas9 mediated hTrmt13 knock out in the methods part (**Page 26**) and in **Fig EV1A**.

Comment 2. The effect on translation is not a strong one and there is no follow-up on the function of the tRNA fragment 5'Gly-CCC. Figure 2 i and j should be quantified. Given the later results, it seems surprising to assume that effects of hTrmt13 on tRNA modification are solely responsible for differences in translation. EMT can affect

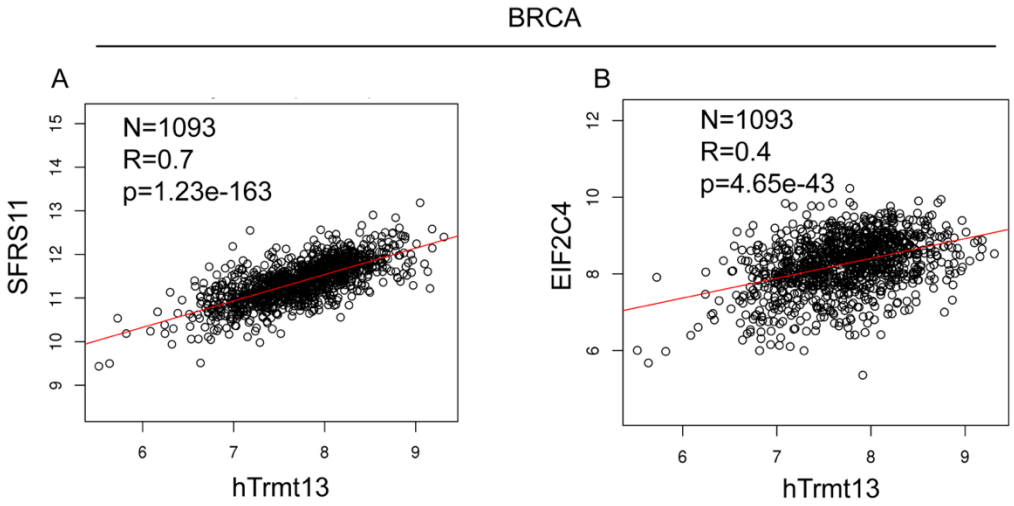
protein translation but the lack of rescue through the catalytically inactive E463A mutant and the subsequent data, which shows that transcriptional regulation by hTrmt13 is independent of its catalytic activity, suggest that they are unrelated, and that translational regulation is solely taking place through a tRNA fragment. Figure 2a: How was EIF4A2 picked - it does not show a strong correlation with hTrmt13, which other genes were tested and is there a general correlation between translation-associated genes and hTrmt13?

Response: We thank the reviewer for this constructive comment. We agree with the reviewer's point that the evidence is not strong enough to support that the effect on translation is correspond to the production of tRNA fragment 5'Gly-CCC. We have checked the steady state level and aminoacylation level of tRNAs of hTrmt13, and found that they were unchanged upon hTrmt13 KD. However, we could not exclude the possibility that the modified tRNA and hypomodified one may perform differently on the ribosome during translation. Here, we did see that the level of tRNA fragment 5'Gly-CCC was modulated by hTrmt13. We speculate that that reason for this is that 5'Gly-CCC may affect translation based on former observation from Anderson' group, where they found that specific tRFs including 5'Gly-CCC could inhibit translation (*Mol Cell* 43:613-623 2011), and their follow-up studies showed that those tRFs binds directly to eIF4G, the major scaffolding protein in the translation initiation complex (*Nucleic Acids Res* 48 :6223-6233, 2020). Thus, we tuned down our conclusions in the manuscript. We planned follow-up studies on the function of 5'Gly-CCC, and figure out whether it has its main effect in regulating the translation by hTrmt13 in the future, although these were beyond the scope of this submission.

We have quantified the results of Figure 2 i and j (now **Fig 2G, H**) in **Fig EV3K, L**.

About Figure 2a: tRNA modification is often related to the translational process. Therefore, we first used bioinformatics methods to investigate whether there was any correlation between the expression of *hTrmt13* and the expression of translation related genes using available database. We indeed found that the expression of *eukaryotic initiation factor 4A-II* (*EIF4A2*) was correlated with that of *hTrmt13*. This

result has certain implications of hTrmt13's role in translation, but is not strong evidence by itself. Thus, we move this result into the supplementary **Fig EV3A**. Yes, we did see a general expression correlation between translation-associated genes (Serine/arginine-rich splicing factor 11 (*SFRS11*), Protein argonaute-4 (*EIF2C4*)) and *hTrmt13* (**Response figure 3A, B**).



Response figure 3 A, B

3A and 3B. Analysis of the TCGA dataset for the mRNA expression of hTrmt13 and SFRS11 (A) or EIF2C4 (B) in breast cancer patients. Spearman order correlation analysis was performed.

Comment 3. 3 m and n: At which concentration of hTrmt13 was the EMSA in 3 m run? The complete shift of the input DNA suggests that all DNA is hTrmt13 bound, however, if I understand the material and method section correctly, 3 n shows that at 400 nM hTrmt13 and 300 nM DNA, the majority of DNA is still free.

Response: Thanks! In figure 3m (now **Fig 3M**), we used 1μM hTrmt13. Sorry we didn't write them clearly, now we have marked them clearly in all the EMSA figure legends.

Comment 4. Figure 4 does not match the figure legend and the description in the text - for example, the legend for g should be for h, i and j are missing legends.

Response: Sorry that we mistakenly mixed them up during figures reorganization. We have now corrected them (now **Fig 4**) accordingly.

Comment 5. There are several GO terms associated with the regulation of cell cycle and cell cycle transition but no effect on cell proliferation was found?

Response: Thanks! It is indeed an interesting point. Actually, we were also interested by the result from the analysis. We have performed flow cell cytometry to check the effect of hTrmt13 KD on the cell cycle. However, no significant changes in the cell cycle were observed from the preliminary assays (**Response Figure 3C**) [Figures for referees not shown.]. Indeed, except for a few cases, knocking out of most known tRNA modifying enzyme didn't affect cell proliferation under normal culture condition. However, the combination of knocking out two or more modifying enzymes shows growth defects, as well as the knocking out a single enzyme under stress conditions (high temperature, oxidative pressure, low sugar, etc.) (*DNA Cell Biol* 31:434-454 2012; *RNA* 24:410-422 2018; *J Exp Bot* 68:1479-1491 2017; *Cell* 167:816-828.e16 2016). Thus, we speculate that the cell proliferation maybe affected under stress condition.

Comment 6. I would like to see a paragraph on replicates, especially for the NGS-based data in the material and methods section. Additionally, the number of

replicates should be clearly stated in each figure legend, except when bar graphs with replicates are shown. For example, how many replicates were run for RNAseq and how are biological replicates defined here?

Response: We thank the reviewer for pointing that out, for CLIP-seq, ChIP-seq and RNA-seq we all have two replicates, we now added this information in the method section on **Page 28, 32, 34** and also in the figure legends of **Fig 3-4**.

Comment 7. 4f and g are the same as 5 d and e. The text mentions that Snail1 is reduced which does not seem obvious in the western blot shown in (now) Figure 4g and 5e? The blot should be quantified if this point is made. ZEB1 on the other hand seems clearly reduced but is not mentioned in the text.

Response: Thanks for pointing that out, we have quantified the results of Western Blot (WB) in **Fig 4D** (quantification in **Fig EV4D**) and **Appendix Fig S4B**. The results of RNA-seq, RT-qPCR and WB all showed that the expression level of Snail1 decreased in hTrmt13 knockdown (KD) cell lines. Yes, ZEB1 is also decreased in RT-qPCR and WB in hTrmt13 KD cell lines, we have now added the description of ZEB1 on **Page 11**.

Comment 8. Figure 6a: While it makes sense for me to look at whether FOSL1 and hTrmt13 co-regulate transcription based on the overlapping DNA recognition motif, it is less clear how the authors settled on E2F1 as an interactor. Was it purely based on gene overlap and if so, how many transcriptional regulators were tested? Hypergeometric testing would help to determine, whether the overlap is significant. Was there a significant overlap between JunB, BATF, or AP-1 controlled genes with hTrmt13 genes? How many genes are bound by both USF1 and hTrmt1? Do hTrmt13 and USF1 share a DNA motif that is mutually recognized? Were all peaks used for motif determination (Figure 3k) or only TSS-2kb peaks and if not, does the motif change, if only TSS-2kb peaks are used?

Response: Thanks for the questions. For clarification, we went through all publicly available ChIP-seq data in MDA-MB-231 cells (FOXM1, MYC, E2F1, TP53, FOSL1,

EZH2) and found many of them enriched at hTrmt13 binding sites except EZH2 (**Fig EV5b**). In the mass spectrometry data (**Dataset EV5**) we found USF1 and E2F1. We next sought to check these factors' implication in EMT and decided to test E2F1, USF1 and FOSL1 because they could either interact or co-located with hTrmt13 and also have been reported to regulate EMT. Hypergeometric testing doesn't find any significant overlap between hTrmt13, FOSL1 and E2F1. Presumably because FOSL1 and E2F1 binding to many loci. Interestingly, if we only take the significant regulated genes, 157 out of 161 down-regulated genes promoters have E2F1 binding sites, 43 have FOSL1 binding sites (due to FOSL1 enriched for enhancers), 96 out of 103 up-regulates genes promoters have E2F1 binding sites, 33 have FOSL1 binding sites. Thus, we suspect hTrmt13 doesn't have to be significant overlapping those factors to practice its gene transcription regulation function.

We used all hTrmt13 reproducible peaks for motif analysis, we found that USF1 motif (CACGTG) could be slightly enriched (very weak, p = 0.01, 8.2% in peaks vs 5.9% in background) for hTrmt13 binding sites. Together, these indicated that it's more likely hTrmt13 recruit USF1 instead of vice versa. If we only use promoter hTrmt13 peaks, we found ELK1 (p = 1e-25, 22.1% in peaks vs 6.7% in background) and NFYA (p=1e-18, 9.9% in peaks vs 1.6% in background) motif instead. We think these were likely because FOSL1 were enriched for distal regions.

We thank the reviewer for sharing the interest, but unfortunately, we don't have ChIP-seq in MDA-MB-231 cells for USF1/JunB/BATF/AP-1 to answer the related questions. We agree there are many opportunities here, but we don't have enough resources to investigate all. Our main goal here is to provide the proof of concept that a tRNA methyltransferase like hTrmt13 could also regulates target genes' function by regulating their transcription. The detailed regulation relationship and network between these factors remain for further investigation.

Comment 9. The hTrmt13 interactome is not described in material and methods. What does the first column in table S6 (Checked, False) mean? Supplementary Table legends would be highly appreciated.

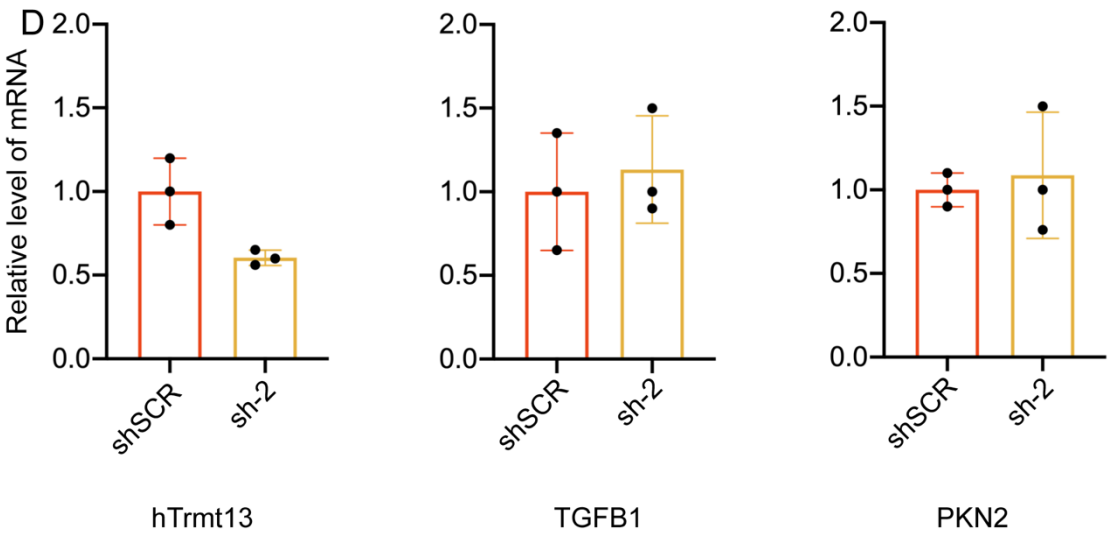
Response: Thanks! The hTrmt13 interactome has now been described in the methods section (**Page 34, 35**). “Checked, False” is a default column generated by the Masspec platform and they have no meaning in our case in this table, so we deleted this first column and other irrelevant columns and added legends in the current table (now **Dataset EV5**).

Comment 10. Figure 6e should be quantified. A control should be added.

Response: Thank the reviewer for pointing this out, we now have added the control in figure 6e (now **Fig 6E**) and added relevant legends.

Comment 11. TGFB1, PKN2, and MEF2A levels could be measured in the breast cancer mouse model to support the statement that "hTrmt13 promotes breast cancer development, putatively by transcriptionally activating TGFB1, PKN2, and MEF2A".

Response: Thanks for the suggestion! We checked the expression level of *hTrmt13*, *TGFB1* and *PKN2* levels in the lung metastatic breast tumors formed in the mice models. Surprisingly, we found that the expression level of *hTrmt13* in the KD group was largely restored in the tumor (**Response Figure 3D**), and the expression level of *hTrmt13* is almost 60% of the control group. We hypothesized that the reason may be because when the expression of *hTrmt13* was potently inhibited by shRNA in the MDA-MB-231 cells, those cells could barely move to the lung, while only those cells which escaped from strong shRNA and maintained a certain expression level of *hTrmt13* could move to the lung. When with the high expression level of hTrmt13 in the lung metastatic breast tumors, the expression level of *TGFB1* and *PKN2* were almost comparable in the control and KD groups (**Response Figure 3D**).



Response Figure 3D

3D. RT-qPCR analysis of hTrmt13, TGFB1, and PKN2 in lung metastatic breast tumors (shSCR, sh-2).

Comment 12. Is the dataset used for Figure 8f BRCA and S8e the same? Were the same criteria used for dividing patients into low expression and high expression groups?

Response: Thanks for the questions. Yes, the same criteria were used for dividing the patients into groups. We got the results from http://kmplot.com/analysis/, the "high expression group" were those expression higher than 75 percentile and "low expression group" were those expression lower than 25 percentile, p-value were calculated by "survival" R package v2.38. More details could be found in their manuscript (https://doi.org/10.1038/s41598-021-84787-5) if further interested.

Comment 13. Overall, material and methods could be more detailed. If word limits are given by the journal, a Supplementary method section could be added. The variety of methods and the thoroughness of the study are greatly appreciated, which makes the lack of transparency as to how the data was obtained more frustrating.

Response: Thanks! We have improved the section of materials and methods with all the necessary details, so that the reader can know how we obtained the experimental data.

Comment 14. The use of spaces between numbers and units is inconsistent (8M vs 100 mM, 2hr vs 30 min).

Response: Thanks! We have now corrected them all.

Comment 15. Was the anti-hTrmt13 antibody made in house? It is missing a commercial source. If so, verification of the antibody specificity should be added.

Response: Thanks! The anti-hTrmt13 is customized from ABclonal (Wuhan, China). In general, rabbits were immunized with purified recombinant hTrmt13 proteins to obtain antibodies, and the anti-hTrmt13 were purified from the serum. We verified the specificity of the antibody, through western blot and Immunoprecipitation (**Fig EV1A**). One main band is observed using total cell lysate of MB-MDA-231 as input, and this band is disappeared in the *Trmt13*-KO cell line (**Fig EV1A**). We now added this information in the material and methods section.

Comment 16. Figure S2: Is the motif found in Figure 3k also found in tRNA or does it show homology a to region within the modified tRNAs? What does Z1 bind to, if Z2 alone is necessary for recognition of both DNA and tRNA?

Response: Thanks! This is a very interesting question. As we described in our response to comment 2 from Reviewer 1, our biochemistry assays showed that hTrmt13 needs to recognize the complete tertiary structure of tRNA (**Response Figure 1A)**. Besides the acceptor stem, hTrmt13 recognizes the D-stem of tRNA (**Response Figure 1B**), and two base pairs (G10:U25, U11:A24) are crucial for recognition. These results suggest that hTrmt13 mainly recognize double stranded RNA region. But the sequence in tRNA is not the same as what we found in DNA motif recognized by hTrmt13. Actually, this CHHC Zn-2 is very special. Up to now, this type of CHHC Zn-finger was only identified in the Trmt13 family, U11/U12 small nuclear ribonucleoprotein 48 kDa protein (SNRNP48), and gametocyte specific factors (GTSF1 and GTSF1L) (*Bioinformatics* 24: 2277-2280 2008). The CHHC Zn-finger mainly binds double strand RNA in SNRNP48 (*Structure* 17:294-302, 2009), while in GTSF1 it interacts with protein (*EMBO J* 12: e99325 2018). In our

work, the CHHC Zn-finger is essential for hTrmt13 to bind DNA and the double stranded region of tRNA. However, due to the lack of structural information, we currently still cannot accurately characterize the molecular mechanism of how hTrmt13 and the CHHC Zn-finger recognize DNA and tRNA. Interestingly, previous studies showed that the structural fold of the CHHC Zn-finger from SNRNP48 is generally similar to the classical C2H2 Zn-finger (*Bioinformatics* 24: 2277-2280 2008). Particularly, it is most similar to Zn-finger 5 of Transcription Factor IIIA (TFIIIA) (*Structure* 17:294-302, 2009). This Zn-finger 5 was reported to bind DNA and RNA in different ways. Specifically, it interacts with the backbone of a double helix of RNAs, while binding to the major groove of its target DNA (*Nature* 426: 96-100 2003; *Proc Natl Acad Sci U S A* 95:2938-43 1998). We added this comment in the discussion part on **page 16**.

Dear Prof. Wang,

Thank you for submitting your revised manuscript. We have now received reports from the three initial referees (see comments below) and I am pleased to say that they overall find that their main concerns have been satisfactorily addressed, and now overall support publication. Referees #2 and #3 have some remaining points, which you should carefully consider and revise the manuscript accordingly. In addition, I would now like to ask you to address a number of editorial issues listed in detail below. Please make any changes to the manuscript text in the data edited document only using the "track changes" option.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving your final revision. Please feel free to contact me if you have further questions regarding the revision or any of the specific points listed below.

Kind regards,

Stefanie Boehm

Stefanie Boehm
Editor
The EMBO Journal

Referee #1:

The authors have addressed most of my concerns. I am happy to recommend its publicaiton.

Referee #2:

In their manuscript, the authors Li et al. provide a comprehensive characterization and functional analysis of a novel tRNA modifying enzyme in humans, hTrmt13. They clearly demonstrate that hTrmt13 is a specific methyltransferase that methylates the Nm4 position on selected tRNAs, despite binding to others. The methyltransferase activity is negatively correlated with the formation of 5'-Gly-CCC tRFs, which appears to modulate protein synthesis levels. Furthermore, the analyses presented in the manuscript show that hTrmt13 plays a transcriptional role, independent of its catalytic activity, through the binding of chromatin in the nucleus. Lastly, the authors explore the physiological relevance of hTrmt13's activities in breast cancer metastasis using cell lines and a mouse model, demonstrating that hTrmt13 positively correlates with increased EMT and a poorer prognosis of the disease. Taken together, the manuscript entails an interesting and thorough exercise and presents a very comprehensive and cohesive picture of hTrmt13 and its roles. The manuscript has benefited greatly from the restructuring of certain sections and the substantial additions to the Discussion and Methods. The responses to the specific comments are also detailed and largely comprehensive.

Additional minor comments:

1."We first identified the tRNA substrate of hTrmt13 and deciphered its tRNA methylation mechanism." The underlined statement in the introduction should be rephrased as it may mislead the reader into assuming that mechanistic details of hTrmt13's catalytic activity and recognition of specific substrates have been worked out in the manuscript.

2. Figure EV1-G. A label should be added to indicate which panel corresponds to the Um4 or Cm4 detection.

Referee #3:

The manuscript is much improved, and the majority of issues have been addressed adequately. The text should be carefully proofread again though, spaces are missing occasionally, and capitalization is sometimes unusual, especially in the newly added sections. I would suggest adding line numbers to future manuscripts so that typos and missing spaces can be easily annotated during review. Overall, the writing has been much improved and it's much easier to follow the logical flow of the data.

The following points could be addressed to make the presented work clearer, and information could be added to figure legends:

Figure 1: Figure 1 overall looks much better and is clearer. It would help to add the chemical structure of the modified base(s) to scheme 1G. This would help readers later on to understand that hTrmt13 cannot methylate DNA as deoxyribose lacks the OH that is getting methylated by hTrmt13 in ribose/RNA (is that correct?) and makes the lack of catalytic activity needed for gene expression regulation much more intuitive.

Figure 2: Are both shRNAs against non-coding regions of endogenous hTrmt13? Otherwise, I would expect degradation of the recombinant hTrmt13 mRNA as well, although protein levels do look comparable.

EV3H: It is not clear to me how the authors discriminate between tRNA fragments and full-length tRNAs? The cited paper by Chen et al 2019 size selects using a gel, which should exclude full-length tRNAs but the selection over columns with a 200 bp cutoff as done here would not. Which libraries (poly A vs stem loop) was used for which subsequent figure? How were the 13 tRFs in EV3I selected?

Figure 2C: DA must be defined (deacylated?) in the figure legend. Are the tRNA fragments visible in the uncropped northern blots shown in 2B and C? If so, it would be worth showing that part of the blot.

While I understand that it is beyond the scope of revisions and likely technically difficult to do so after potentially a long time, I'd like to point out that more than 2 replicates are standard for RNAseq. If the program voom is specifically equipped to handle low n-number datasets, it should be noted. The read depth should be noted for all NGS methods to give an idea of how much coverage was achieved. Are inputs for Clip and ChIP deposited under GSE166278, I cannot see them among the samples?

I appreciate that the authors explain the connection to EIF4A2 better, but it still seems a bit random at the position in the text as it is now. Additionally, with the current structure it does not seem to add much as the data on tRNA modification, stability, fragment generation, and puromycin incorporation seems quite solid without it. The reply to comment 2 does help with understanding the rationale so I would suggest moving the correlation with EIF4A2 after the findings regarding 5'Gly CCC and introduce it correspondingly. Does EIF4A2 appear among the hTrmt13-bound or regulated genes?

Figure 2 G, H/EV3K, L: The quantification looks a lot more convincing than the western blot - the authors could consider swapping them.

Figure 5A: Is the enrichment of ChIP PCR over IgG and vector control or over input RNA? The figure legend could do with more details and an explanation of the conditions.

Figure 5: The color coding is confusing as the same colors are used for different conditions that should not show the same effect. For example, the pink color in A, B, and C correspond to wt, si-1, and E463A, respectively.

Response to comments from reviewers:

Referee #1:

The authors have addressed most of my concerns. I am happy to recommend its publication.
Response: We thank the reviewer for carefully reading our manuscript.

Referee #2:

In their manuscript, the authors Li et al. provide a comprehensive characterization and functional analysis of a novel tRNA modifying enzyme in humans, hTrmt13. They clearly demonstrate that hTrmt13 is a specific methyltransferase that methylates the Nm4 position on selected tRNAs, despite binding to others. The methyltransferase activity is negatively correlated with the formation of 5'-Gly-CCC tRFs, which appears to modulate protein synthesis levels. Furthermore, the analyses presented in the manuscript show that hTrmt13 plays a transcriptional role, independent of its catalytic activity, through the binding of chromatin in the nucleus. Lastly, the authors explore the physiological relevance of hTrmt13's activities in breast cancer metastasis using cell lines and a mouse model, demonstrating that hTrmt13 positively correlates with increased EMT and a poorer prognosis of the disease. Taken together, the manuscript entails an interesting and thorough exercise and presents a very comprehensive and cohesive picture of hTrmt13 and its roles. The manuscript has benefited greatly from the restructuring of certain sections and the substantial additions to the Discussion and Methods. The responses to the specific comments are also detailed and largely comprehensive.

Additional minor comments:

1."We first identified the tRNA substrate of hTrmt13 and deciphered its tRNA methylation mechanism." The underlined statement in the introduction should be rephrased as it may mislead the reader into assuming that mechanistic details of hTrmt13's catalytic activity and recognition of specific substrates have been worked out in the manuscript.
Response: Thanks! We removed the words “and deciphered its tRNA methylation mechanism” on Page 3, Line 11.

2. Figure EV1-G. A label should be added to indicate which panel corresponds to the Um4 or Cm4 detection.
Response: Thanks! We added a label to the bands that indicted the Um4 or Cm4 induced RT arrest products and updated the figure legends.

Referee #3:

The manuscript is much improved, and the majority of issues have been addressed adequately. The text should be carefully proofread again though, spaces are missing occasionally, and capitalization is sometimes unusual, especially in the newly added sections. I would suggest adding line numbers to future manuscripts so that typos and missing spaces can be easily annotated during review. Overall, the writing has been much improved and it's much easier to follow the logical flow of the data.

The following points could be addressed to make the presented work clearer, and information could be added to figure legends:

Figure 1: Figure 1 overall looks much better and is clearer. It would help to add the chemical structure of the modified base(s) to scheme 1G. This would help readers later on to understand that hTrmt13 cannot methylate DNA as deoxyribose lacks the OH that is getting methylated by hTrmt13 in ribose/RNA (is that correct?) and makes the lack of catalytic activity needed for gene expression regulation much more intuitive.
Response: Thanks! We added the chemical structure of Nm in Fig 1G.

Figure 2: Are both shRNAs against non-coding regions of endogenous hTrmt13? Otherwise, I would expect degradation of the recombinant hTrmt13 mRNA as well, although protein levels do look comparable.
Response: Thanks! When using recombinant hTrmt13 in the rescue experiments, the codon sequences of *htrmt13* have been modified in the gene by keeping the same amino acid readout, while these mutation on mRNA could avoid the targeting by these two shRNAs. We now added the details of this strategy in the method part (Page 20, line 7-10).

EV3H: It is not clear to me how the authors discriminate between tRNA fragments and full-length tRNAs? The cited paper by Chen et al 2019 size selects using a gel, which should exclude full-length tRNAs but the selection over columns with a 200 bp cutoff as done here would not. Which libraries (poly A vs stem loop) was used for which subsequent figure? How were the 13 tRFs in EV3I selected?
Response: Thanks! Based on the different properties of tRNA and tRF, they could be distinguished based on the methods we improved of RT-PCR. Mature tRNAs from *in vivo* have three main characteristic properties: (1) compact tertiary structure, (2) heavily modified nucleosides, (3) almost fully aminoacylated. Our lab has been focused on the research of aminoacylation and modifications of tRNAs for about 30 years, we have tried many conditions and set up different RT-PCR procedures for distinguishing mature tRNAs and tRFs. For tRFs, normal conditions as for miRNAs could be used. While for full-length tRNA, most sequences are imbedded in the tertiary structure. When reverse transcription (RT) reaction was performed below 45 °C, no RT products were observed. Furthermore, since m^1A58 is present in almost all tRNAs, and if this modification is not removed, the RT will be halted at this

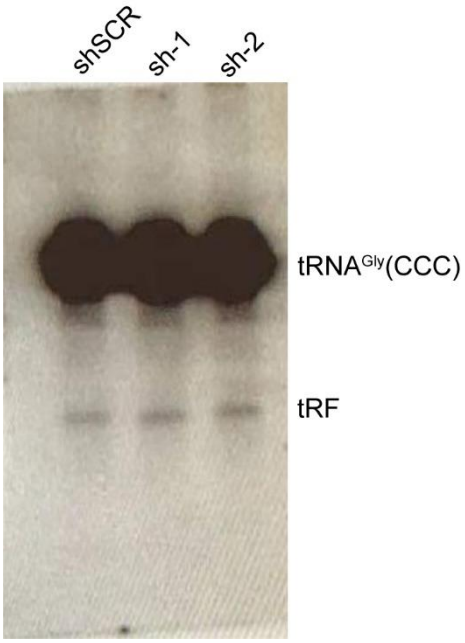
position. Even worse, without deacylation, the probes (poly A or etc.) could not be ligated to full-length tRNAs, thus no RT products of full-length tRNAs were observed.

In both stem loop or poly A methods of this manuscript, we performed RT at 37 ℃, and no denature process had been performed, nor any de-modification or deacylation treatments were performed. Thus, no RT products of full-length tRNAs could be observed. We now added the details of RT reaction in the method part (Page 21, line 7-8).

These 13 tRFs were selected because they could either be derived from hTrmt13 tRNA substrates or other tRFs that are known to affect protein synthesis (Anderson & Ivanov, 2014; Ivanov et al, 2011).

Figure 2C: DA must be defined (deacylated?) in the figure legend. Are the tRNA fragments visible in the uncropped northern blots shown in 2B and C? If so, it would be worth showing that part of the blot.

Response: Thanks! DA is deacylated, we now defined. The level of tRFs were very low when compared to the full-length tRNA under normal culture, thus they were hardly visible in the northern blots. Interestingly, the level of tRF-Gly-CCC could be detected at this culture condition as shown below: We could see that the level of tRF-Gly-CCC is higher in both of hTrmt13 knockdown cells when compared with WT. tRFs are mainly produced under stress conditions, we will investigate the regulation of hTrmt13 on tRF production under some stress in the future work.



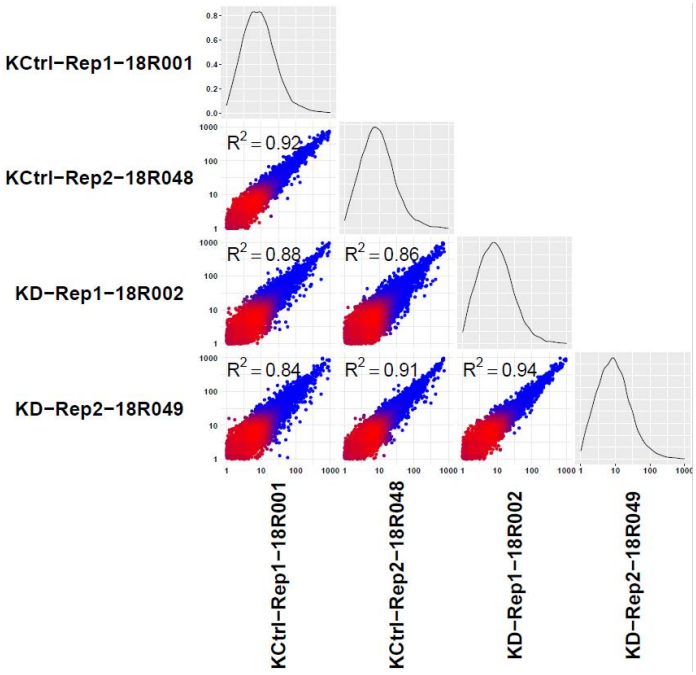
While I understand that it is beyond the scope of revisions and likely technically difficult to do so after potentially a long time, I'd like to point out that more than 2 replicates are standard for RNAseq. If the program voom is specifically equipped to

handle low n-number datasets, it should be noted. The read depth should be noted for all NGS methods to give an idea of how much coverage was achieved. Are inputs for Clip and ChIP deposited under GSE166278, I cannot see them among the samples?

Response: Thanks for the reviewer's remind. For RNA-seq, the program voom does not specifically designed for low n-number dataset, it helps the differential expressed gene detection by modeling the mean-variance trend thus the statical test won't strongly bias to highly expressed genes, this also account for unequal library size and achieved better false positive rate in many tests. For the proposed using 3 replicates, we agree that more replicates would give more reliable results. However, we followed the ENCODE guideline per quote: "In all cases, experiments should be performed with two or more biological replicates, unless there is a compelling reason why this is impractical or wasteful (e.g. overlapping time points with high temporal resolution)" (ENCODE Guidelines and Best Practices for RNA-Seq v2 at https://encodeproject.org). Because our RNA-seq data have good reproducibility (see below pairwise plot, the correlation between replicates were all > 0.9 as suggested by ENCODE guideline) and selected target genes have been validated thus we don't think it's necessary to add a third replicate. Sorry for not have mentioned the coverages. We usually followed ENCODE guideline that all our RNA-seq reached 40 million paired-end reads, we included these in method parts.

For ChIP-seq, we include INPUT for ChIP-seq to GSE166278. It also has more than 30 million reads as we mentioned for other ChIP-seq data.

For CLIP-seq, different from ChIP-seq, INPUT for CLIP-seq is not a common practice across the community yet, such as SRP030031 (Lovci et.al, *Nature Structural & Molecular Biology*, 2013), E-MTAB-2599 (Hauer et.al, *Nature Communication*, 2015), GSE109183 (Carter et.al, *Nucleic Acids Research*, 2019), GSE164238 (Sun et.al, Protein & Cell, 2021). The largest dataset implemented size-matched input (SMInput) have only been tested by ENCODE project recently but only for eCLIP, their results shown the main benefits by introducing SMI are improving signal-to-noise and reducing false positive rate for RNA-binding protein (RBP) (Nostrand et.al, *Nature Method*, 2016; Nostrand et.al, *Nature*, 2020). Thus, although we agree that including SMInput might improve the data quality, but we also concluded this is not essential for the investigations in current manuscript, because 1) there is no peak calling procedure involved in our analysis. 2) Unlike the RBPs from ENCODE, our target mainly binds to tRNA by CLIP-seq. At last, all our CLIP-seq samples got > 40 million (M) reads while ENCODE suggested > 1 million unique fragments for eCLIP. We also looked the data we tested from GSE109183 (Carter et.al, Nucleic Acids Research, 2019), for their 6 iCLIP-seq samples, they scatter from 3M, 7M, 7M, 14M, 16M, 48M. Thus, we conclude the coverage for our CLIP-seq were acceptable. We include these in method part and appendix material and methods part to help reader assess the coverage et.al.



I appreciate that the authors explain the connection to EIF4A2 better, but it still seems a bit random at the position in the text as it is now. Additionally, with the current structure it does not seem to add much as the data on tRNA modification, stability, fragment generation, and puromycin incorporation seems quite solid without it. The reply to comment 2 does help with understanding the rationale so I would suggest moving the correlation with EIF4A2 after the findings regarding 5'Gly CCC and introduce it correspondingly. Does EIF4A2 appear among the hTrmt13-bound or regulated genes?

Response: Thanks for the reviewer's suggestion! We now move this result into the supplementary Fig EV3A. EIF4A2 promoter does has implication of hTrmt13 binding but it was not as strong(below). We called low confidence peaks (FDR < 0.5) but not high confidence reproducible peaks for hTrmt13. EIF4A2 expression does not change in our RNA-seq. In the TCGA hTrmt13 low versus high analysis (Figure 8A), EIF4A2 were significant down-regulated but the fold change was not strong (log2 fold change = -0.41, FDR corrected p-value = 2.76E-16).

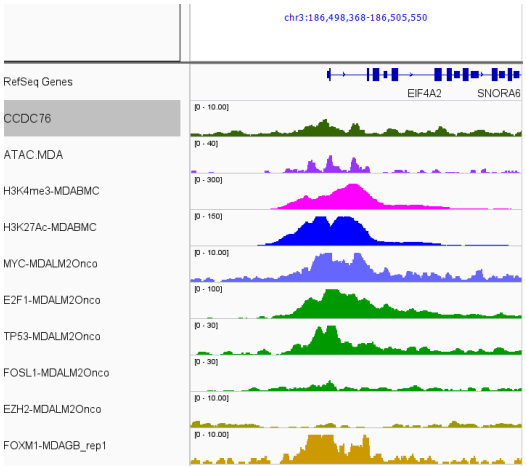


Figure 2 G, H/EV3K, L: The quantification looks a lot more convincing than the western blot - the authors could consider swapping them.
Response: Thanks! We took the reviewer's advice and revised them accordingly.

Figure 5A: Is the enrichment of ChIP PCR over IgG and vector control or over input RNA? The figure legend could do with more details and an explanation of the conditions.
Response: Thanks! The enrichment of ChIP PCR was over IgG and vector control. We added details in the legends accordingly.

Figure 5: The color coding is confusing as the same colors are used for different conditions that should not show the same effect. For example, the pink color in A, B, and C correspond to wt, si-1, and E463A, respectively.
Response: Thanks! We now recolor the panels to avoid confusion.

Thank you again for submitting the final revised version of your manuscript. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal. Please however ensure that the datasets are switched to public access as soon as possible. As of now this is not the case for GSE166278, GSE166274, GSE166276, and GSE166270.

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: En-Duo Wang, Ru-Juan Liu
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2021-108544

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 justified
- 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?	The effect size was not pre-specified. Sample size is indicated for all experiments in the corresponding figure legends.Each experiment was repeated at least three times
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size is indicated for all experiments in the corresponding figure and figure legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All results without exclusions.
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.	No specific randomization was used.
For animal studies, include a statement about randomization even if no randomization was used.	No specific randomization was performed.
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.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	No
5. For every figure, are statistical tests justified as appropriate?	Yes,all stasistical methods are discribed in corresponding figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, all methods were described in the manuscript.
Is there an estimate of variation within each group of data?	Yes, error bars were shown.

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
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

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).	Yes
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	MDA-MB-231 cell line is a gift from Dr Mofang Liu's Lab, other cell lines were purchsed from ATCC. They were tested for mycoplasma contamination.

* 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.	Reported in the methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Materials and Methods: The procedures for care and use of animals were approved by Institutional Animal Care and use Committee (China, SIBCB-S209-1705-007-c2) and all applicable institutional and governmental regulations (e.g., ARRIVE guidelines) concerning the ethical use of animals were followed.
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.	Materials and Methods: All applicable institutional and governmental regulations (e.g., ARRIVE guidelines) concerning the ethical use of animals were followed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
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 Our data are all from public dataset (e.g.,TCGA).

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	"Data Availability" section is provided at the end of the Materials and Methods.
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