nature portfolio

Peer Review File

A cohort of mRNAs undergo high-stoichiometry NSUN6-mediated site-specific m5C modification

Corresponding Author: Professor Ru-Juan Liu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
RNA m5C methylation is a widespread post-transcriptional modification distributed in rRNA, tRNA, and mRNA, playing a crucial role in the regulation of gene expression. However, elucidating the regulatory roles of mRNA m5C methylation by manipulating the expression levels of m5C methyltransferases is very challenging, as these enzymes act on both tRNA and mRNA. In this study, Liu and colleagues developed a robust in vitro methyltransferase assay to identify the mRNA targets of NSUN6 and characterized the sequence and structural features of these targets. More importantly, through comprehensive biochemical analyses, they identified discrepancies in the mechanisms by which NSUN6 recognizes mRNA and tRNA targets. Using mutations that exclusively affected mRNA target recognition, the authors demonstrated that NSUN6-dependent mRNA targets contribute to breast cancer metastasis by stabilizing these transcripts. This manuscript is well-organized, and the data presented are solid and compelling. The findings are both intriguing and significant, offering a novel paradigm for investigating the functions of enzymes that act on both mRNA and tRNA. I have only a few minor comments.

Specifically,
1. The authors mentioned that the MARCKSL1 transcript could not be methylated by in vitro methyltransferase assay. Could the authors clarify why this site was not methylated in vitro? Is this a false positive identified by BS-seq, or does m5C methylation at this site require additional co-factors? A more detailed explanation or discussion of this observation is recommended.
2. The SHAPE assay is robust tool for identifying the structural characteristics of transcripts in a transcriptome-wide manner. The authors showed four examples in Figure 2b-e, demonstrating that the loop size in stem-loop varied from 10 to 13 nt. I recommend the authors to analyze the influences of the loop size in a transcriptome-wide manner. For example, the NSUN6 targets identified by BS-seq or UBS-seq can be divided into several groups according to their loop size, and a comparison of m5C methylation levels across these groups could provide insight into the global influence of loop size on m5C methylation.
3. The results in Figure 4a-b are striking and compelling, providing strong evidence for the regulatory roles of NSUN6-dependent mRNA m5C methylation in breast cancer metastasis. Since the primary focus of this study is on the function of NSUN6, I recommend that the authors analyze the changes in methylation level specially at NSUN6-dependent m5C sites, rather than all m5C sites as shown in Figure 4i.
4. In Supplementary Table 2, the authors provided detailed information for all m5C sites identified in MDA-MB-231, which will be very valuable for researchers following up on this study. Since this study focuses on NSUN6, I strongly recommend the authors add a column indicating whether this site is NSUN6-dependent.
5. Figure 4m shows that NSUN6-dependent m5C sites are mainly distributed in CDS and 3'-UTR. I wonder if the m5C sites in these different regions have distinct effects on mRNA stability. Could the authors compare the half-life of transcripts containing m5C sites in the CDS versus the 3' UTR, as shown in Figures 4p and 4r? Such a comparison could provide insights into the functional influences of m5C methylation in different regions.
6. This study characterizes YBX3 as a new m5C reader and demonstrates that its binding can stabilize the transcript. Since figure 6g shows that most of YBX3 targets can also be bound by YBX1, therefore I recommend the authors to discuss the potential relationship or selectivity between these two readers.

Reviewer #2

(Remarks to the Author)

In this manuscript, Zhang et al. investigated that NSUN6 employed different strategies to recognize tRNA and mRNA substrates by utilizing distinct combinations of RNA binding surfaces. By introducing mutations, they separated the catalytic capabilities of NSUN6 toward mRNA and tRNA, and found that NSUN6 promoted breast cancer cell migration depending on NSUN6-mediated site-specific m5C modification of mRNA. In addition, the introduction of high-level m5C can significantly improve the stability of therapeutic mRNA in cells. The key role of m5C-modified mRNAs in promoting breast cancer metastasis and its potential therapeutic application were emphasized. I love to recommend its publication after addressing my several questions.

For Figure 2h and i, in order to clarify the modification site characteristics of NSUN6, it is necessary to add a control group with C * UCCA motifs located in non-loop structures. Meanwhile, Figure 2h and i lack statistically significant difference analysis.
In Figure 4e, volcano diagram the color of upregulated and downregulated genes is red and blue, and the color scheme for genes of interest and migration related genes is also red and blue, which is very confused. It is recommended to change the color scheme for better differentiation.
In order to enhance the logic of the article, it is suggested to add the background introduction of Nsun6-mediated tumorigenesis, and why breast cancer is selected as the research object in the future.
In the manuscript, the authors described that 256 genes were significantly down regulated, but the number of genes indicated is 265 in the Figure.
It is unclear whether the 194 mRNA substrates of NSUN6 contain m5C modification in Figure 4o-r. Furthermore, compared to the two publicly available data, why did not all of NSUN6's target RNA appear in total mRNA. Therefore, it is recommended to increase the matching transcriptome data and conduct joint analysis to avoid errors in different laboratories.
Why do YBX1 and YBX3 need to jointly mediate the stability of the same target mRNA, and is it functional redundancy ?
In “A cohort of migration related mRNAs with high m5C levels, mediated by NSUN6, regulated breast cancer cell migration” partly increase the reasons for selecting MDA-MB-231 cell line or add several other breast cancer cell lines to exclude irrelevant factors.
In the “NSUN6 mediated m5C modification could increase the stability of clinical mRNAs” section, SARS-CoV-2 mRNA-1273 vaccine is used to verify the role of NSUN6-mediated m5C in improving vaccine efficacy. It is recommended to increase its development potential for the treatment of breast cancer metastasis and improve the logic coherence of the full text. Otherwise, it is felt that the results in this section are two independent parts from the previous verification.
For Figure 7, the aim of this experiment is to explore the stability of therapeutic mRNA, but the method is to use plasmid vectors instead of mRNA form for transfection, which is inconsistent with the title. It is recommended to further use mRNA molecules to directly transfect cells to study the half-life of mRNA molecules and protein expression.

Minor：
To better illustrate the distribution characteristics of m5C, it is recommended to label each sample’s specific cell line and sequencing method in Figure 1a.
In line 294, “twofold” should be modified to “two fold”.
In line 315-321, the article description is confusing and there are punctuation errors. It is recommended to reorganize the language.
Inconsistent writing of “m5C” and “m5C”. It is recommended to unify it with “m5C”.
Figure 4i lacks statistically significant difference analysis.

Reviewer #3

(Remarks to the Author)
Comments for the Authors

In this manuscript, the authors focus on dissecting the role of human NSUN6 at both molecular and pathological levels. A major challenge in the field of RNA modifications has been that most mRNA modifications are installed by enzymes that also install modifications on tRNA, which limits the ability to genetically dissect the functionality of mRNA modifications. Key contribution of the current study are (1) finding that activities on tRNA and on mRNA are mediated via different structural conformations, that hence allow such genetic decoupling, (2) identification of m5C readers, (3) establishing that m5C leads to increased mRNA half life. On the basis of such genetic decoupling the authors establish a model wherein methylation of a small set of targets in genes driving migrations leads to their binding via a set of readers, thereby to their stabilization, hence leading to increased tumor invasion in a breast cancer model.

Overall, we are enthusiastic about the contributions of this manuscript. Nonetheless, there are several points requiring clarifications prior to publication, as indicated below.

Major points that should be addressed:
• Figure 5b-c: Furin is among the major mRNA targets that were characterized in vitro as an NSUN6 substrates. Why are its levels not abolished – but merely minorly reduced - in a full NSUN6 KO? Moreover, given how mild the reduction is, the 3-fold drop in half-life appears all the more surprising. Additionally, the decay curves in 5c (and across other sections in the manuscript) are also not intuitive to me. Assuming the Y axis is plotting the abundance, as indicated, and not log-transformed abundance, the plots should show exponential decay.
• Figure 6c: The authors make strong claims about YBX1 acting as an m5C reader. The data does not appear to us to be

very compelling. The plot shows that Furin independent of the presence of m5C can be bound strongly by YBX1. This and other data pose the question whether the reader proteins bind to the specific NSUN6 structure and motif without requiring the m5C modification. Specially data shown for YBX3 knockdown (5i, 5j) show a minimal effect on RNA stability, partially within the error range. Additional experiments (ideally in-vitro, using controlled levels of enzymes and substrates) would be required to make a stronger case about these proteins serving as readers.
• Minor points
• Line 454: There is not substantial data for the claim that m5C modification increases the local luciferase reporter activity. The observed higher activity could simply result from C-A conversion at this specific site. This result could be strengthened by introuducing the reporter into a NSUN6 KO cell line, where the differences between C and A harboring reporters should be abolished.
• Figure 1E: For calculating a correct Km value, a saturated reaction/curve is required. However, especially for tRNAThr the curve is clearly not saturated. Therefore, care should be taken in stating absolute Km values.
• Line 144: The comment “towards certain mRNA substrates” might be toned down, because in Figure 1 this has only been proven for a single RNA substrate.
• Line 150: It is not very clear what is meant with “* is the catalytic site”, as this phrase is usually used for the catalytic site of an enzyme. For an RNA motif the better phrase would be “modified C”.
• In the current form some graphs have low quality.
• Figure 3G: Adding the positively charged patches to figure 3G would contribute to understanding the figure as the surface charge 3C and 3G look very different. Thus, in 3G the patch1 and 2 are not clearly identifiable
• Figure 3: How to rule out the possibility that the respective mutations especially in patch 2 are simply abrogating catalytic activity by disrupting the 3D fold of the complex. Potentially AlphaFold3 complexes with the mutants could be generated to see that there are not major changes for catalytically important residues upon mutating patch amino acids. Moreover, it would be great to see the activity of patch1 mutant on at least one other mRNA to show general validity of these in silico predicted mechanism for mRNA recognition. As the mutant is used in further experiments (Figure 4), such a validation would increase confidence in the differential tRNA and mRNA recognition (Figure 4).
• Line 286: While its true that NSUN6 KO seem to not impact tRNA aminoacylation and steady-state levels of tRNAThr and tRNACys, the tRNA substrate still could have been impacted in another way e.g. change of other tRNA modifications or mischarging. Therefore, toning down the statement that the “tRNA substrates were not impacted” would reflect the performed experiments in a better way.
• Line 308 ff: Do the identified sites align with previously reported sites? Is this overlap better for the highly modified sites. This info would add to the confidence of identified m5C sites.
• Line 411ff: Is there a specific reason why the model was built with PVRL2 mRNA, but the pulldown experiments done with Furin mRNA?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all my concerns. I strongly recommend the publication in Nature Communications.

Reviewer #2

(Remarks to the Author)
The authors have addressed most of my concerns.I recommend its publication.

Reviewer #3

(Remarks to the Author)
The authors have done an excellent job addressing my concerns. I congratulate them on an impressive and well conducted paper.

Response to comments from reviewers:

Reviewer#1:

RNA m^5C methylation is a widespread post-transcriptional modification distributed in rRNA, tRNA, and mRNA, playing a crucial role in the regulation of gene expression. However, elucidating the regulatory roles of mRNA m^5C methylation by manipulating the expression levels of m^5C methyltransferases is very challenging, as these enzymes act on both tRNA and mRNA. In this study, Liu and colleagues developed a robust *in vitro* methyltransferase assay to identify the mRNA targets of NSUN6 and characterized the sequence and structural features of these targets. More importantly, through comprehensive biochemical analyses, they identified discrepancies in the mechanisms by which NSUN6 recognizes mRNA and tRNA targets. Using mutations that exclusively affected mRNA target recognition, the authors demonstrated that NSUN6-dependent mRNA targets contribute to breast cancer metastasis by stabilizing these transcripts. This manuscript is well-organized, and the data presented are solid and compelling. The findings are both intriguing and significant, offering a novel paradigm for investigating the functions of enzymes that act on both mRNA and tRNA. I have only a few minor comments.

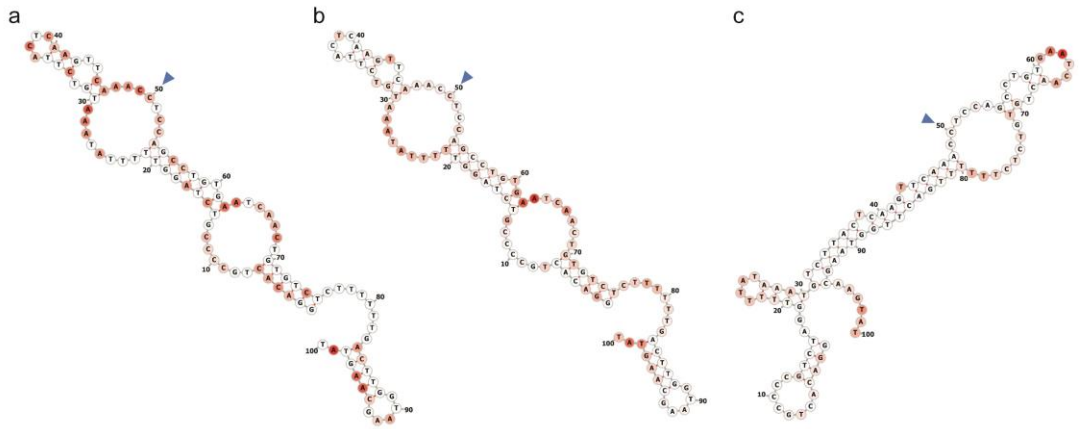
Response: We sincerely appreciate the reviewer for carefully reading our manuscript and providing constructive comments. We have revised the manuscript carefully according to these valuable suggestions and added or updated the corresponding figures and Supplementary Tables.

Comment 1. The authors mentioned that the *MARCKSL1* transcript could not be methylated by *in vitro* methyltransferase assay. Could the authors clarify why this site was not methylated *in vitro*? Is this a false positive identified by BS-seq, or does m^5C methylation at this site require additional co-factors? A more detailed explanation or discussion of this observation is recommended.

Response: Thanks for the questions! The *MARCKSL1* transcript has been reported as

an NSUN6 mRNA substrate in several m^{5}C-sequencing studies **(Fig. 1a)**. Based on the site information provided in these studies, we transcribed the reported 101-nt length RNA fragment surrounding the catalytic site (5'-GGACACUGCCCCGUCUAGGUUUUUAUAAAUGUCUUACUCAAGUUCAAA CC*UCCAGCCUGUGAAUCAACUGUGUCUCUUUUUUGACUUGGUAAGCAA GUAU-3') *in vitro*. However, we did not detect the activity of NSUN6 on this transcript in our *in vitro* methyltransferase activity assay. To further investigate, we examined the predicted secondary structure near the catalytic site of *MARCKSL1* using the RNA secondary structure database (rasp.zhanglab.net). Based on *in vivo* and *in vitro* results, we observed that the secondary structure of this region in *MARCKSL1* does not conform to the structural patterns we identified for NSUN6-methylated mRNA substrates **(Response Fig. 1a-c)**, which is consistent with the results of our *in vitro* activity assay.

Another study utilized a bisulfite-free method called m^{5}C-TAC-seq and did not detect m^{5}C modification at this site of *MARCKSL1* (*Mol Cell* 84: 2984-3000 2024). We think that the site on *MARCKSL1* is false positive, which may be attributed to incomplete bisulfite conversion caused by RNA secondary structure, antibody specificity, or the degradation of RNA fragments.



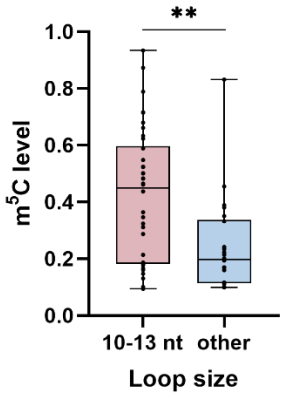
Response Figure 1

a-b, *In vivo* RNA secondary structure of *MARCKSL1* from *Cell* 169: 905-917 (2017) **(a)**, and *Cell* 165: 1267-1279 (2016) **(b)**. **c**, *In vitro* RNA secondary structure of *MARCKSL1* from *Cell* 165: 1267-1279 (2016). The predicted m^{5}C site is indicated by a blue triangle.

Comment 2. The SHAPE assay is robust tool for identifying the structural

characteristics of transcripts in a transcriptome-wide manner. The authors showed four examples in Figure 2b-e, demonstrating that the loop size in stem-loop varied from 10 to 13 nt. I recommend the authors to analyze the influences of the loop size in a transcriptome-wide manner. For example, the NSUN6 targets identified by BS-seq or UBS-seq can be divided into several groups according to their loop size, and a comparison of m^5C methylation levels across these groups could provide insight into the global influence of loop size on m^5C methylation.

Response: Your question is highly insightful! We integrated existing SHAPE results with the RNA secondary structure *in vivo* database of HEK293T cells (http://rasp.zhanglab.net/search/) to analyze the loop structures of NSUN6 mRNA substrates obtained from our UBS-seq. Based on loop size, we divided the substrates into two groups: one group with high activity defined as 10-13 nt (N=38), and the other group with the remaining loop sizes (N=22). We found that substrates with loop sizes of 10-13 nt exhibited significantly higher m^5C levels compared to those with other loop sizes **(Response Fig. 2)**. In addition to loop size, the base pairing of the first and second pairs on the stem also influences the m^5C level of mRNA **(Supplementary Fig. 4h-m)**. Therefore, we consider loop size could be one of the references for predicting NSUN6-mediated mRNA m^5C levels.



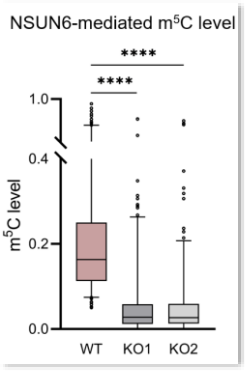
Response Figure 2

Boxplot showing the differences in m^5C levels between mRNA substrates with l0-13 nt loop size (N=38), and mRNAs with other loop sizes (N=22). Boxes represent the 25th-75th percentile (line at the median). Statistical significance was determined using a two-tailed *t*-test,

with P values indicated above the boxes. (**, P < 0.01)

Comment 3. The results in Figure 4a-b are striking and compelling, providing strong evidence for the regulatory roles of NSUN6-dependent mRNA m^5C methylation in breast cancer metastasis. Since the primary focus of this study is on the function of NSUN6, I recommend that the authors analyze the changes in methylation level specially at NSUN6-dependent m^5C sites, rather than all m^5C sites as shown in Figure 4i.

Response: Thanks for your suggestion! We analyzed the changes in NSUN6-dependent m^5C levels between WT and NSUN6 KO cells. The result showed that, for m^5C sites mediated by NSUN6, the m^5C level was significantly decreased in NSUN6 KO MDA-MB-231 cells compared to WT (Now **Fig. 4k**, also shown as below). We also described the results on current **Line 324-327**.



sites in MDA-MB-231 cells **(Fig. 4j)**. Remarkably, when NSUN6 was knocked out, the m^5C level of poly(A) RNA significantly decreased **(Fig. 4k)**, suggesting that NSUN6 is responsible for modifying the majority of high m^5C-level mRNA sites in MDA-MB-231 cells. Notably, our sequencing results indicated a strong sequence preference for

Comment 4. In Supplementary Table 2, the authors provided detailed information for all m^5C sites identified in MDA-MB-231, which will be very valuable for researchers following up on this study. Since this study focuses on NSUN6, I strongly recommend the authors add a column indicating whether this site is NSUN6-dependent.

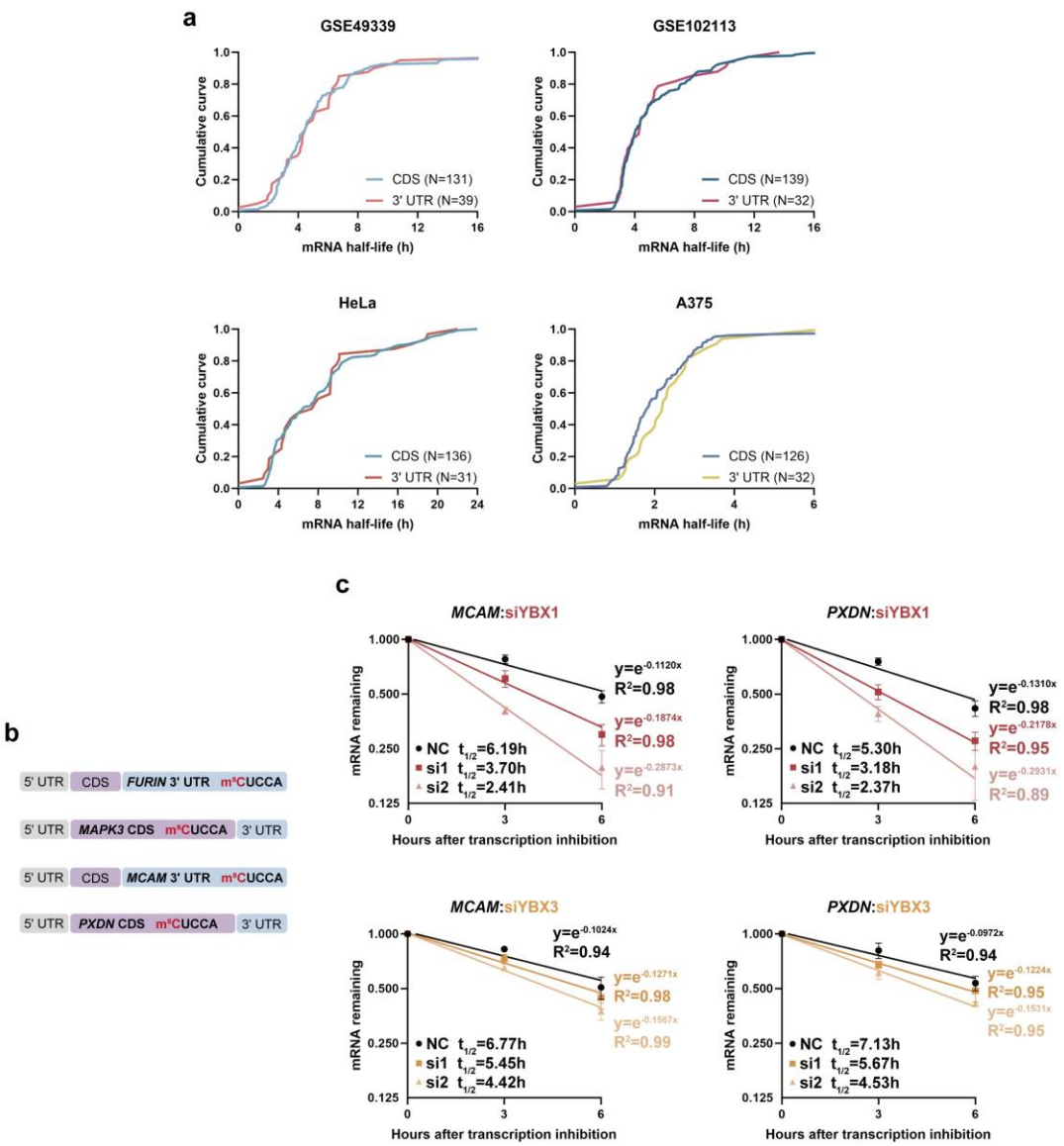
Response: Thank you for your valuable recommendation! We have added a column in

current **Supplementary Table 2** to label whether the substrates are NSUN6-dependent based on the conversion rate.

Comment 5. Figure 4m shows that NSUN6-dependent m^5C sites are mainly distributed in CDS and 3'-UTR. I wonder if the m^5C sites in these different regions have distinct effects on mRNA stability. Could the authors compare the half-life of transcripts containing m^5C sites in the CDS versus the 3' UTR, as shown in Figures 4p and 4r? Such a comparison could provide insights into the functional influences of m^5C methylation in different regions.

Response: Thank you for your insightful question! We have reanalyzed the half-lives sequencing data from multiple datasets, focusing on mRNAs with NSUN6-mediated m^5C sites located in the CDS and 3' UTR regions. The results showed that m^5C sites in both the CDS and 3' UTR had a similar impact on mRNA stability (**Response Fig. 3a**).

In the section "YBX1 and YBX3 preferentially recognize and stabilize mRNAs with NSUN6-mediated m^5C modifications", we examined the impact of readers on the stability of mRNA substrates—*FURIN* and *MAPK3*. In addition, we have now examined the effects of these two readers on the half-lives of other mRNA substrates of NSUN6. Specifically, the m^5C sites in *FURIN* and *PXDN* are located in the 3' UTR region, while the m^5C sites in *MAPK3* and *MCAM* are located in the CDS region **(Response Fig. 3b)**. The stabilities of these mRNAs were decreased upon knockdown of YBX1 or YBX3 (Now **Fig. 6j, k, Supplementary Fig. 7d, e, and Response Fig. 3c**). Therefore, we proposed that m^5C modifications in the CDS and 3' UTR regions of mRNA mediated by NSUN6 could enhance mRNA stability by recruiting the readers YBX1 and YBX3.



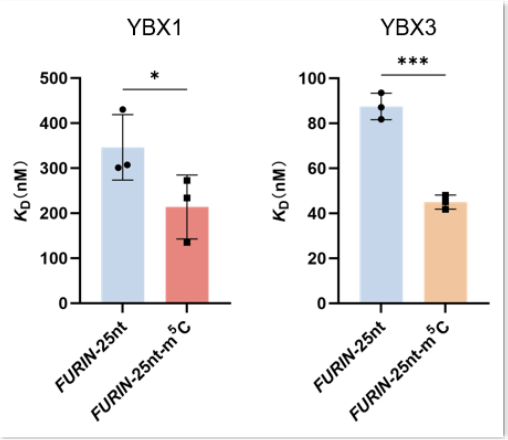
Response Figure 3

a, Comparison of mRNA half-lives between transcripts containing NSUN6-mediated m^{5}C sites in the CDS region and 3' UTR region. Data were reanalyzed from GEO: GSE49339, GEO: GSE102113, BRIC-seq data in HeLa cell line (*Genome Res* 22: 947-956 2012), and A375 cell line (*RNA Biol* 16: 1355-1363 2019). **b**, Schematic diagram indicating the location of NSUN6-mediated m^{5}C sites on mRNA. **c**, The RT-qRCR analysis of mRNA half-lives following YBX1 or YBX3 knockdown in MDA-MB-231 cells.

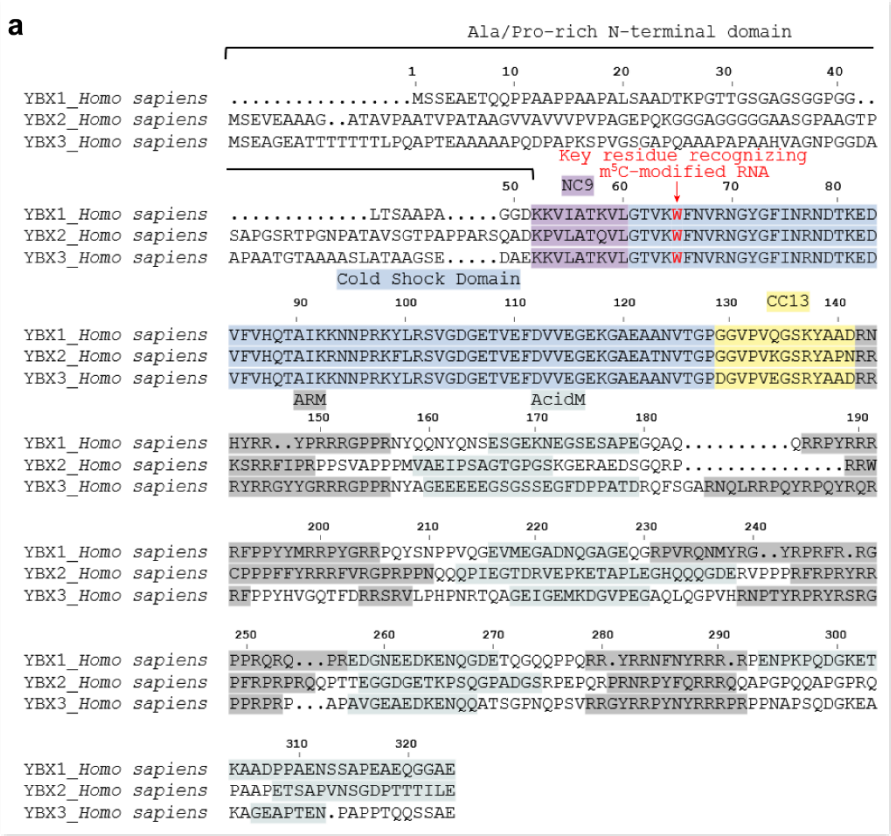
Comment 6. This study characterizes YBX3 as a new m^{5}C reader and demonstrates that its binding can stabilize the transcript. Since figure 6g shows that most of YBX3 targets can also be bound by YBX1, therefore I recommend the authors to discuss the

potential relationship or selectivity between these two readers.

Response: Thanks! YBX1 and YBX3 are members of the Y-box protein family, both containing a highly conserved CSD domain responsible for binding RNA. In our study, we found that the CSD domains of YBX1 and YBX3 exhibit stronger binding affinity to NSUN6-mediated m^5C-modified RNA than unmodified RNA. In terms of localization, both YBX1 and YBX3 are distributed in the nucleus and cytoplasm. Functionally, they are involved in stabilizing mRNA. However, there are differences between YBX1 and YBX3. In the UniProt database, sequence similarity inference indicates that YBX3 (UniProt: P16989) binds RNA sequences harboring the consensus motif 5'-UCCAUCA-3'. The sequence specificity of YBX3 exhibits a high degree of overlap with the sequence features of NSUN6 mRNA substrates. We have now added the *in vitro* RNA binding assays, and we observed that YBX3 has a stronger binding affinity to both m^5C-modified and unmodified RNA compared to YBX1 **(**Now **Fig. 6e, f**, also shown as below**)**. This result further indicates that YBX3 has a higher preference for NSUN6 mRNA substrates than YBX1.



Except the conserved CSD domain, YBX proteins possess variable acidic Ala/Pro-rich N-terminal domain, as well as C-terminal domain where motifs rich in arginine and aromatic amino acids (ARMs) alternate with motifs rich in acidic amino acids (AcidMs). These variable domains may interact with different proteins to stabilize mRNAs (Updated in **Supplementary Data Fig. 7a**, also shown as below).



Reviewer#2:

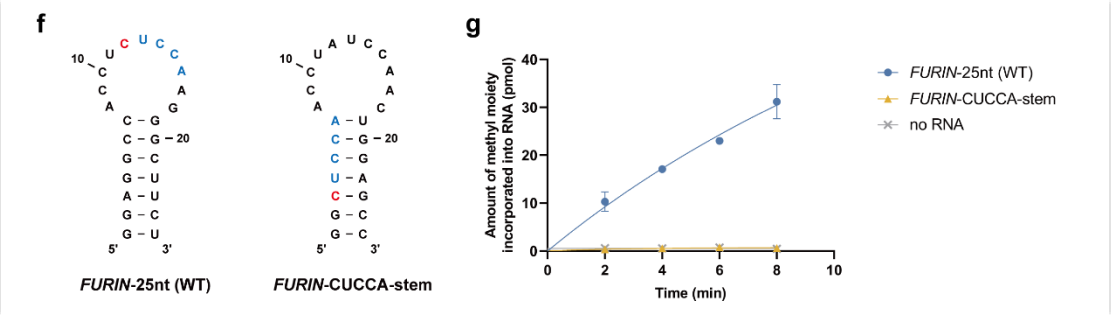
In this manuscript, Zhang et al. investigated that NSUN6 employed different strategies to recognize tRNA and mRNA substrates by utilizing distinct combinations of RNA binding surfaces. By introducing mutations, they separated the catalytic capabilities of NSUN6 toward mRNA and tRNA, and found that NSUN6 promoted breast cancer cell migration depending on NSUN6-mediated site-specific m^5C modification of mRNA. In addition, the introduction of high-level m^5C can significantly improve the stability of therapeutic mRNA in cells. The key role of m^5C-modified mRNAs in promoting breast cancer metastasis and its potential therapeutic application were emphasized. I love to recommend its publication after addressing my several questions.

Response: We sincerely appreciate the reviewer for constructive comments, your feedback has been pivotal in shaping our revisions. We have revised the manuscript accordingly and have supplemented or updated the relevant figures to better illustrate

our findings.

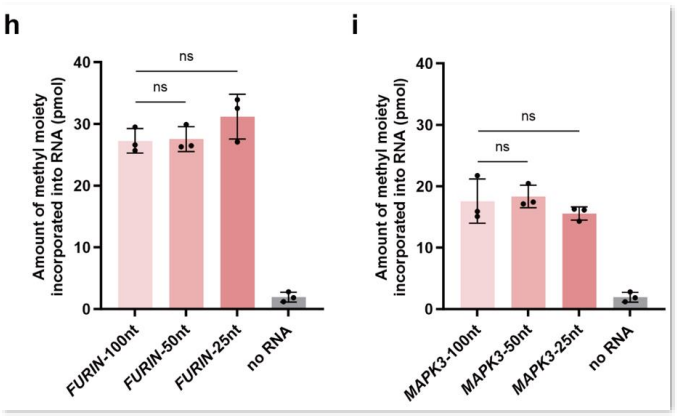
Comment 1. For Figure 2h and i, in order to clarify the modification site characteristics of NSUN6, it is necessary to add a control group with C*UCCA motifs located in non-loop structures. Meanwhile, Figure 2h and i lack statistically significant difference analysis.

Response: Thank you for your suggestion! We have added a control with C*UCCA located in the stem region of *FURIN*-25nt, and the results show that catalysis by NSUN6 only occurs when C*UCCA is positioned in the loop (Now **Supplementary Fig. 1f, g**, also shown as below). The results of this experiment are also described on current **Line 173-175**.



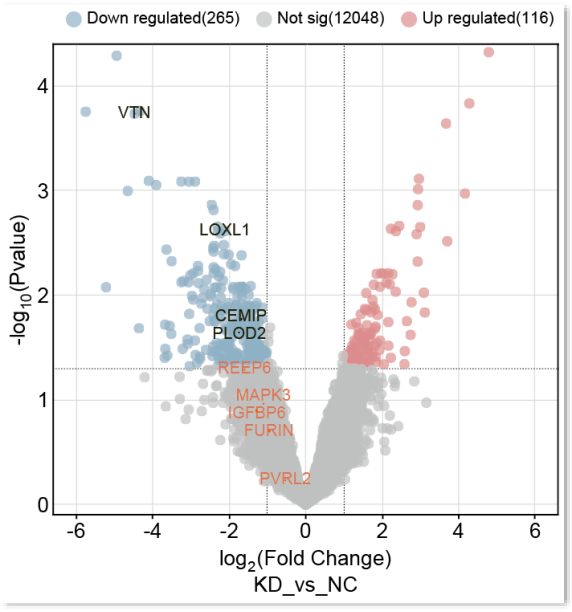
173 **1d, e)**. In addition, we relocated the C*UCCA motif to stem region while preserving
174 the stem-loop structure of *FURIN*-25nt, and we found that this mutant (*FURIN*-
175 CUCCA-stem) could not be methylated by NSUN6 (**Supplementary Fig. 1f, g**). The

Thanks for your carefully reading and we apologize for the mistake in data processing in Figure 2h and i, and we have now added the significance analysis (Updated in **Fig. 2h and i**, also shown as below).



Comment 2. In Figure 4e, volcano diagram the color of upregulated and downregulated genes is red and blue, and the color scheme for genes of interest and migration related genes is also red and blue, which is very confused. It is recommended to change the color scheme for better differentiation.

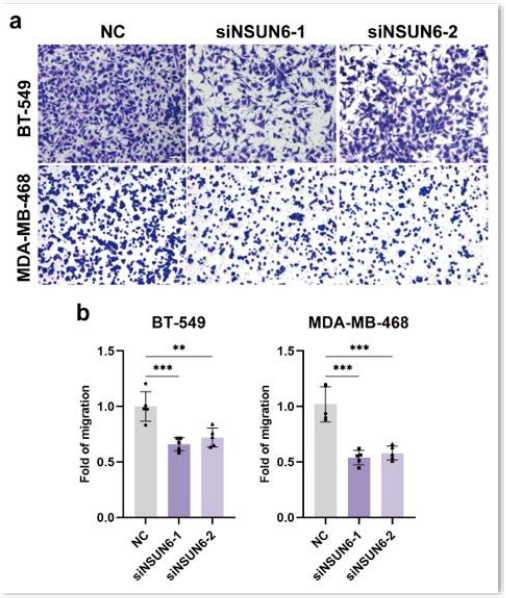
Response: Thanks for your suggestions! We have now changed the color of the interesting genes that were mRNA substrates of NSUN6 to orange, and the color of the genes associated with cell migration to black (Updated in **Fig 4e**, also shown as below).



Comment 3. In order to enhance the logic of the article, it is suggested to add the background introduction of Nsun6-mediated tumorigenesis, and why breast cancer is selected as the research object in the future.

Response: Thank you for your valuable suggestion! We have supplemented the background information on the high expression of NSUN6 in tumors (Now **Line 270-272**, also shown as below). We have added experiments showing that NSUN6 promotes the migration of other triple-negative breast cancer cells (BT-549 and MDA-MB-468) to enhance the logical coherence (Now **Supplementary Fig. 4a, b**, also shown as below).

NSUN6-mediated m^5C modification in tRNA and mRNA substrates. NSUN6 is highly expressed in various cancers, making it a potential indicator for predicting survival and progonosis[41]. A previous study has shown that knocking down NSUN6 expression inhibits the migration ability of MDA-MB-231 cells[35]. Similarly, we knocked down the expression of NSUN6 in the BT-549 and MDA-MB-468 cell lines and found that the migration ability of these cells were significantly restricted, indicating that NSUN6 facilitates cell migration in several triple-negative breast cancer cells **(Supplementary Fig. 4a, b)**. To elucidate the molecular mechanism of NSUN6 in breast cancer cell



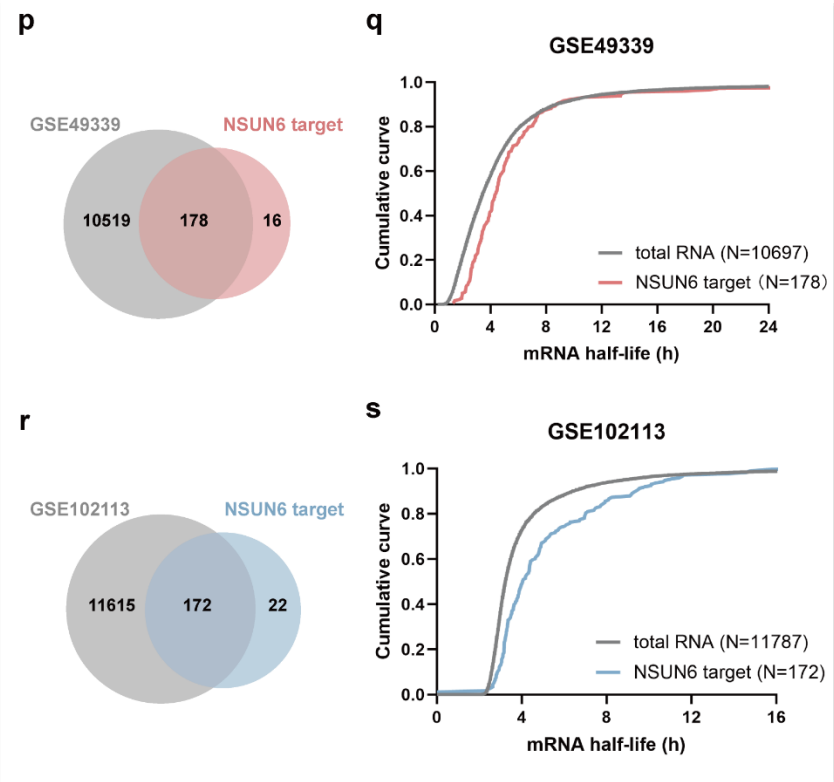
Comment 4. In the manuscript, the authors described that 256 genes were significantly down regulated, but the number of genes indicated is 265 in the Figure.

Response: Thank you very much for pointing out this mistake, we confirm the number and now correct 256 into 265 in the manuscript (Now **Line 302**, also shown as below).

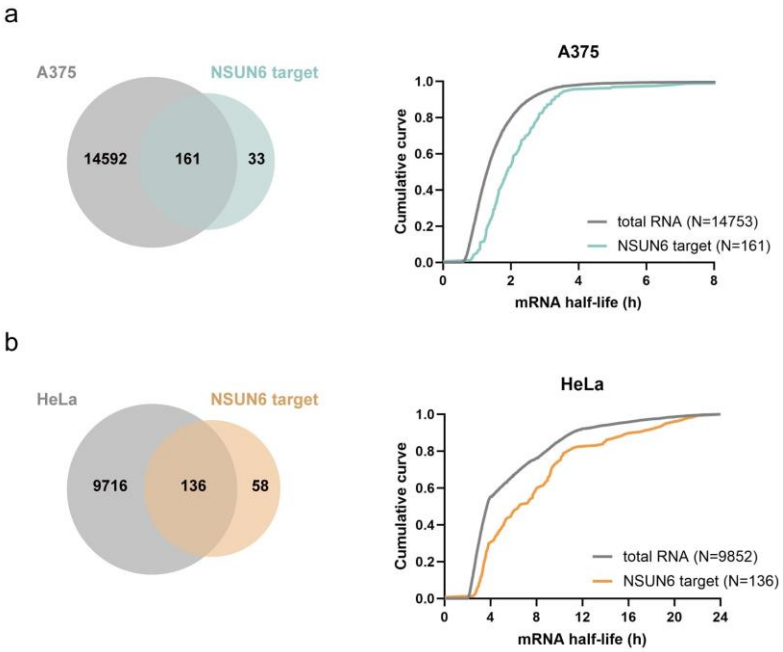
knockdown in MDA-MB-231 cells **(Supplementary Table 1)**. Compared to the control group, 265 genes were significantly downregulated, while 116 genes were significantly upregulated, with changes exceeding two fold **(Fig. 4e)**. Subsequently, these results

Comment 5. It is unclear whether the 194 mRNA substrates of NSUN6 contain m^5C modification in Figure 4o-r. Furthermore, compared to the two publicly available data, why did not all of NSUN6's target RNA appear in total mRNA. Therefore, it is recommended to increase the matching transcriptome data and conduct joint analysis to avoid errors in different laboratories.

Response: Thank you for your suggestions! These 194 mRNAs are the mRNA substrates of NSUN6 that we detected in the MDA-MB-231 cell line. When comparing half-lives, for the NSUN6 target samples, we only selected mRNAs that intersect with the publicly available data, rather than all 194 mRNAs. To avoid ambiguity, we have added labels indicating the sample size in the figures (Updated in **Fig. 4p-s**). We have reanalyzed the two GEO datasets, which now include a broader range of NSUN6-targeted mRNAs (Updated in **Fig. 4p-s**, also shown as below).



Additionally, we compared the 5'-bromouridine IP chase (BRIC)-Seq results in HeLa (*Genome Res* 22: 947-956 2012), and A375 cell lines (*RNA Biol* 16: 1355-1363 2019) (**Response Fig. 4**). The results from these datasets are quite consistent, indicating that the half-lives of mRNA substrates targeted by NSUN6 are higher than those of total transcripts.



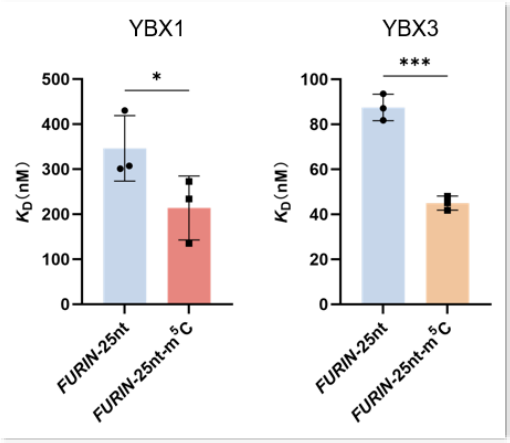
Response Figure 4

a-b, Comparison of mRNA half-lives between total transcripts and transcripts containing NSUN6-mediated m^5C sites. Left panels showed the Venn diagram of the overlap between NSUN6-targeted mRNAs in MDA-MB-231 cells and mRNAs detected in half-lives sequencing. Right panels showed the data reanalyzed from BRIC-seq in HeLa cell line (*Genome Res* 22: 947-956 2012) **(a)**, and A375 cell line (*RNA Biol* 16: 1355-1363 2019) **(b)**.

Comment 6. Why do YBX1 and YBX3 need to jointly mediate the stability of the same target mRNA, and is it functional redundancy?

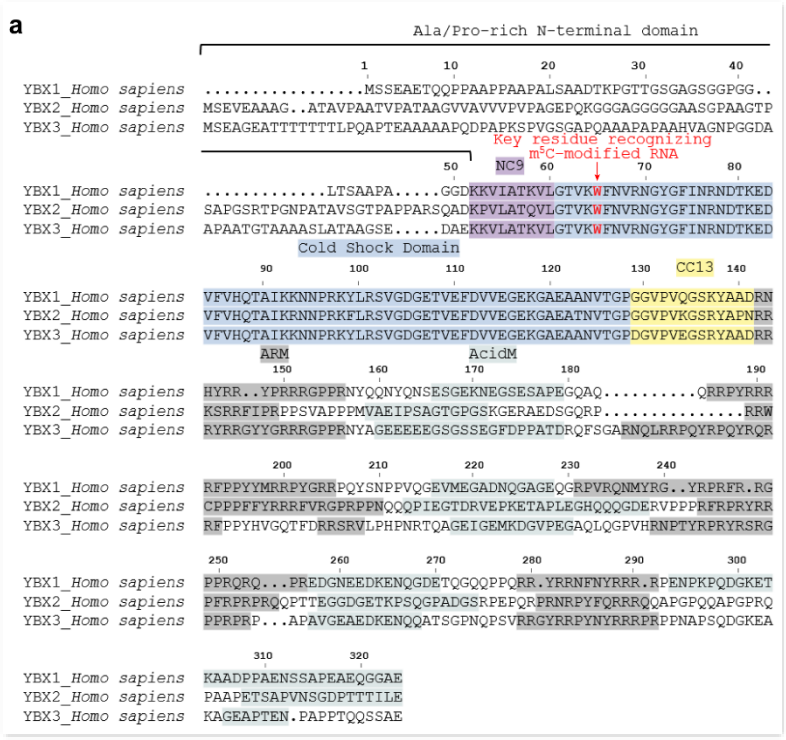
Response: Thanks for your insightful question! Both YBX1 and YBX3 are members of the Y-box protein family, sharing similarity in structure. Through validation in our study, we found that their conserved CSD domains are responsible for recognizing NSUN6-methylated RNA, thereby enhancing the stability of these RNAs. However, we observed in the UniProt database that YBX3 (UniProt: P16989) could bind full-

length mRNA and short RNA sequences containing the consensus motif 5'-UCCAUCA-3', which shows a high degree of overlap with the mRNA substrates of NSUN6. We have conducted Biolayer interferometry (BLI) experiments to assess the RNA binding affinity of purified YBX1 CSD and YBX3 CSD to mRNA substrates. The results demonstrated that YBX3 exhibited a higher binding affinity for both m^5C-modified and unmodified mRNA substrates compared to YBX1 (Now **Fig. 6e, f**, the results are described in current **Line 414-421**, also shown as below). This further indicates that YBX3 has a higher preference for mRNA substrates of NSUN6.



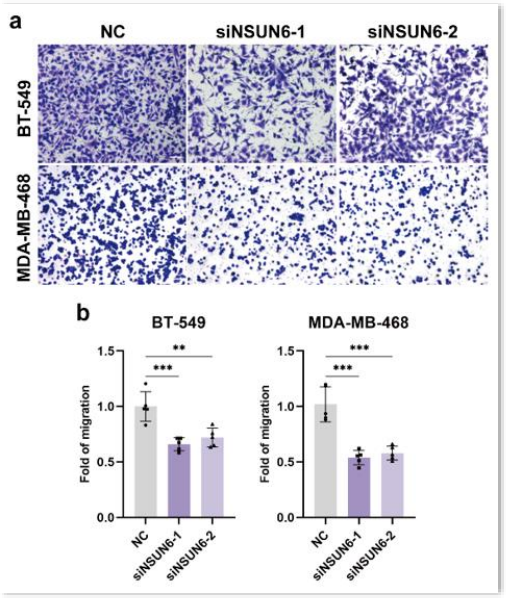
414 target RNA[61] **(Supplementary Fig. 7a)**. We examined the RNA-binding affinities of
415 the YBX1 CSD (residues 48-159) for unmodified and m^5C-modified *FURIN*-25nt using
416 Biolayer interferometry (BLI). The YBX1 CSD exhibited higher binding affinity for
417 m^5C-modified mRNA (K_D = 213.8 nM) compare to unmethylated mRNA (K_D = 346.1
418 nM) **(Fig. 6e)**. Similarly, the YBX3 CSD (residues 82-174) showed stronger binding
419 affinity to *FURIN*-25nt-m^5C (K_D = 45.0 nM) than *FURIN*-25nt (K_D = 87.5 nM) **(Fig.**
420 **6f)**. The results indicated that both YBX1 and YBX3 have a stronger preference for
421 NSUN6-methylated mRNAs than for unmodified ones. Previous structural studies

In addition, YBX proteins have variable N-terminal and C-terminal regions rich in acidic amino acids (Now **Supplementary Fig. 7a**, also shown as below), which are responsible for binding different proteins. In the NSUN6-regulated breast cancer migration progress, although YBX1 and YBX3 jointly mediate the stability of the same target mRNAs, the specific mechanisms by which they recruit different proteins to regulate downstream genes remain to be investigated.



Comment 7. In "A cohort of migration related mRNAs with high m^5C levels, mediated by NSUN6, regulated breast cancer cell migration" partly increase the reasons for selecting MDA-MB-231 cell line or add several other breast cancer cell lines to exclude irrelevant factors.

Response: Thank you for the great suggestion! We now performed additional tests in other triple-negative breast cancer cell lines, including BT-549, and MDA-MB-468. After knocking down NSUN6 in these cells, their migration abilities were significantly reduced compared to the negative control (NC) groups (Now **Supplementary Fig. 4a, b**, also shown as below). These results indicate that NSUN6 has a relatively consistent impact on the migration of triple-negative breast cancer cells, and the results were described in current **Line 273-277**.



273 inhibits the migration ability of MDA-MB-231 cells[35]. Similarly, we knocked down the
274 expression of NSUN6 in the BT-549 and MDA-MB-468 cell lines and found that the
275 migration ability of these cells were significantly restricted, indicating that NSUN6
276 facilitates cell migration in several triple-negative breast cancer cells **(Supplementary**
277 **Fig. 4a, b)**. To elucidate the molecular mechanism of NSUN6 in breast cancer cell

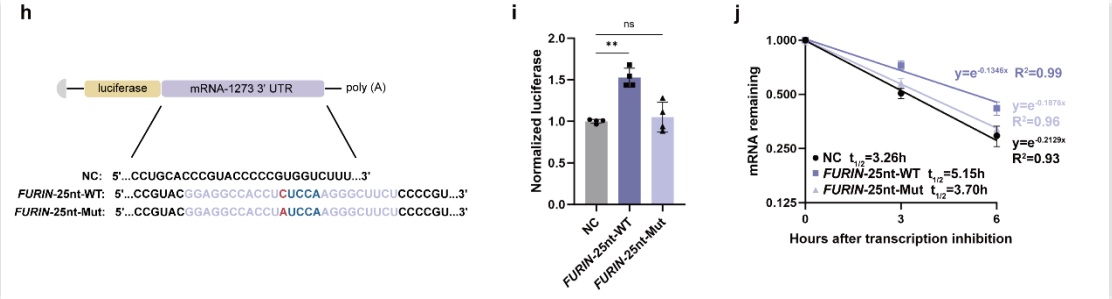
Comment 8. In the "NSUN6 mediated m^5C modification could increase the stability of clinical mRNAs" section, SARS-CoV-2 mRNA-1273 vaccine is used to verify the role of NSUN6-mediated m^5C in improving vaccine efficacy. It is recommended to increase its development potential for the treatment of breast cancer metastasis and improve the logic coherence of the full text. Otherwise, it is felt that the results in this section are two independent parts from the previous verification.

Response: Thank you for your suggestion! We have moved the summary of the molecular mechanisms by which NSUN6 promotes breast cancer metastasis to the previous section to ensure clear logic (Now **Line 453-458**). We have also added a description of the clinical significance of this mechanism in preventing breast cancer metastasis, thereby enhancing the logical coherence of the manuscript (Now **Line 458-462**, also shown as below).

Above, we demonstrated that NSUN6 catalyzes high-level m^{5}C modifications on specific mRNAs by recognizing certain stem-loops with the C*UCCA motif. In breast cancer MDA-MB-231 cells, a cohort of cell migration-related mRNAs, which possess these sequence and structure characteristics, are decorated by NSUN6 with high-level, site-specific m^{5}C modifications. These modifications subsequently enhance mRNA stability through recognition by m^{5}C readers, such as YBX1 and YBX3 **(Fig. 6n)**. The molecular mechanisms of NSUN6-mediated breast cancer metastasis that we have uncovered might provide a theoretical basis for the development of new prognostic biomarkers and therapeutic targets. Inhibiting the mRNA modification function of NSUN6 is expected to be an effective strategy for hindering breast cancer metastasis.

Comment 9. For Figure 7, the aim of this experiment is to explore the stability of therapeutic mRNA, but the method is to use plasmid vectors instead of mRNA form for transfection, which is inconsistent with the title. It is recommended to further use mRNA molecules to directly transfect cells to study the half-life of mRNA molecules and protein expression.

Response: Thanks for your valuable suggestion! We have transcribed mRNAs containing the wild-type or mutant *FURIN*-25nt in the 3' UTR of mRNA-1273 *in vitro*, and subsequently transfected them into HEK293T cells (Now **Fig. 7h**). The results showed that the NSUN6-mediated m^{5}C site could enhance both the stability and translation efficiency of mRNA vaccines within cells (Now **Fig. 7i, j**, also shown as below). The results of mRNA transfection were similar to those of plasmid transfection, further demonstrating that the NSUN6-mediated m^{5}C site could efficiently enhance the stability and translation efficiency of the mRNA vaccine. The results were described on current **Line 493-497**.



translation efficiency and mRNA stability of mRNA-1273 **(Fig. 7f, g)**. Additionally, we confirmed this effect using *in vitro* transcribed mRNAs **(Fig. 7h)**. The results showed that adding the NSUN6-targeted *FURIN*-25nt sequence to the 3' UTR of the mRNA-1273 enhanced its stability and translation efficiency in the cells, whereas mutating the catalytic site of *FURIN*-25nt did not **(Fig. 7i, j)**. These results indicate that a single

Minor Comments:

1. To better illustrate the distribution characteristics of m^5C, it is recommended to label each sample's specific cell line and sequencing method in Figure 1a.

Response: Thank you very much for your suggestion! Due to space limitations in Figure 1a, we have added the cell lines and sequencing methods used in each study to the current **Supplementary Fig. 1a** to ensure a clear presentation.

	Sequencing Method	Cell Line
Fang *et al* (2020)	CIGAR-seq	HAP1
Liu *et al* (2020)	miCLIP	HEK293T
Selmi *et al* (2021)	miCLIP	HEK293T
Dai *et al* (2024)	UBS-seq	HeLa

2. In line 294, "twofold" should be modified to "two fold".

Response: Thanks for pointing out this mistake, we have now corrected it in the manuscript (Now **Line 303**, also shown as below).

knockdown in MDA-MB-231 cells **(Supplementary Table 1)**. Compared to the control group, 265 genes were significantly downregulated, while 116 genes were significantly upregulated, with changes exceeding two fold **(Fig. 4e)**. Subsequently, these results

3. In line 315-321, the article description is confusing and there are punctuation errors. It is recommended to reorganize the language.

Response: Thank you very much for pointing out our errors. We have corrected the punctuation in Line 317 and reorganized the language for better clarity in the manuscript (Now **Line 323-329**, also shown as below).

cells (**Fig.4i**). Further analysis showed that NSUN6 catalyzes 205 out of the 1,136 m^5C sites in MDA-MB-231 cells **(Fig. 4j)**. Remarkably, when NSUN6 was knocked out, the m^5C level of poly(A) RNA significantly decreased **(Fig. 4k)**, suggesting that NSUN6 is responsible for modifying the majority of high m^5C-level mRNA sites in MDA-MB-231 cells. Notably, our sequencing results indicated a strong sequence preference for C*UCYA at NSUN6-dependent sites compared to all intracellular m^5C sites **(Fig. 4l)**, consistent with our biochemical validation. Furthermore, we analyzed the distribution

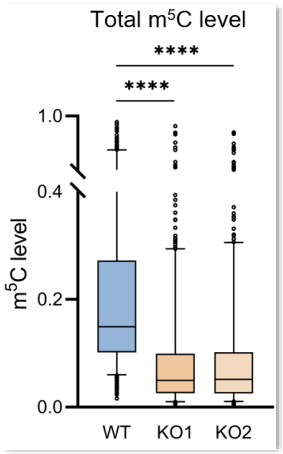
4. Inconsistent writing of "m5C" and "m^5C". It is recommended to unify it with "m^5C".

Response: We sincerely thank the reviewer for carefully reading! We have corrected the "m5C" into "m^5C" in Line 325 (Now **Line 333**, also shown as below) and rechecking all the pattern.

We also observed enrichment of NSUN6-mediated m^5C sites in the 3' UTR, comprising

5. Figure 4i lacks statistically significant difference analysis.

Response: We apologize for this mistake! We have now added the significant difference analysis (Updated in **Fig 4i**, also shown as below).



Reviewer#3:

Comments for the Authors

In this manuscript, the authors focus on dissecting the role of human NSUN6 at both molecular and pathological levels. A major challenge in the field of RNA modifications has been that most mRNA modifications are installed by enzymes that also install modifications on tRNA, which limits the ability to genetically dissect the functionality of mRNA modifications. Key contribution of the current study are (1) finding that activities on tRNA and on mRNA are mediated via different structural conformations, that hence allow such genetic decoupling, (2) identification of m^5C readers, (3) establishing that m^5C leads to increased mRNA half life. On the basis of such genetic decoupling the authors establish a model wherein methylation of a small set of targets in genes driving migrations leads to their binding via a set of readers, thereby to their stabilization, hence leading to increased tumor invasion in a breast cancer model.

Overall, we are enthusiastic about the contributions of this manuscript. Nonetheless, there are several points requiring clarifications prior to publication, as indicated below.

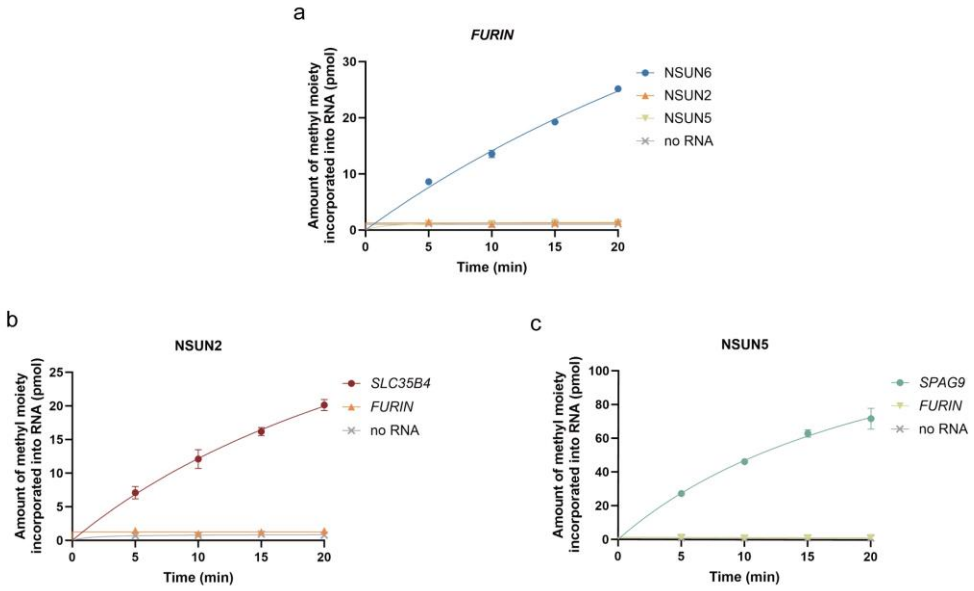
Response: We truly appreciate your insightful and constructive comments! These have guided us to refine the manuscript and enrich it with more comprehensive data and analyses. We have carefully revised the manuscript and figures, and we hope that these revisions could address the questions you raised.

Comment 1. Figure 5b-c: *Furin* is among the major mRNA targets that were characterized *in vitro* as an NSUN6 substrates. Why are its levels not abolished – but merely minorly reduced - in a full NSUN6 KO? Moreover, given how mild the reduction is, the 3-fold drop in half-life appears all the more surprising. Additionally, the decay curves in 5c (and across other sections in the manuscript) are also not intuitive to me. Assuming the Y axis is plotting the abundance, as indicated, and not log-transformed abundance, the plots should show exponential decay.

Response: We thank the reviewer for this question! When treating mRNA samples with the UBS-2 reagent, in order to minimize mRNA degradation as much as possible, we

opted for moderate treatment conditions (98° C for 9 minutes, which is recommended in *Nat Biotechnol* 42: 1559-1570 2024). However, this also led to partial C sites not being converted, resulting in the higher background. We think this maybe the reason why the m^{5}C level of *FURIN* in the NSUN6 KO samples did not decrease completely.

To verify that the m^{5}C modification at this site of *FURIN* is fully catalyzed by NSUN6, we used the *in vitro* methyltransferase activity assay to test the catalytic ability of two other reported mRNA m^{5}C modifying enzymes, NSUN2 and NSUN5 on *FURIN*. Our purified NSUN2 and NSUN5 could independently catalyze their known mRNA substrates, *SLC35B4* and *SPAG9*, respectively, but neither can catalyze *FURIN* **(Response Fig. 5a-c)**. This result may further indicate that the m^{5}C modification of *FURIN* is totally mediated by NSUN6.



Response Figure 5

a, The capacity of *FURIN* to be methylated by mRNA m^{5}C methyltransferase NSUN6, NSUN2, and NSUN5. **b**, The capacity of *SLC35B4*, and *FURIN* to be methylated by NSUN2. **c**, The capacity of *SPAG9*, and *FURIN* to be methylated by NSUN5.

We apologize for the unclear statement on Y-axis, which has caused confusion. We mainly referred to two publications (*Nat Cell Biol* 20: 285-295 2018; *Nature* 505: 117-120 2014). Both studies fitted the mRNA degradation curve using the **exponential decay model** $C = C_0 \times e^{-kt}$, where C$_0$ is the initial abundance of mRNA, k is the

decay constant, and t is time. Under a **log2 scale**,

$$log_2(C) = log_2(C_0 \times e^{-kt})$$

$$log_2(C) = log_2(C_0) + log_2(e^{-kt})$$

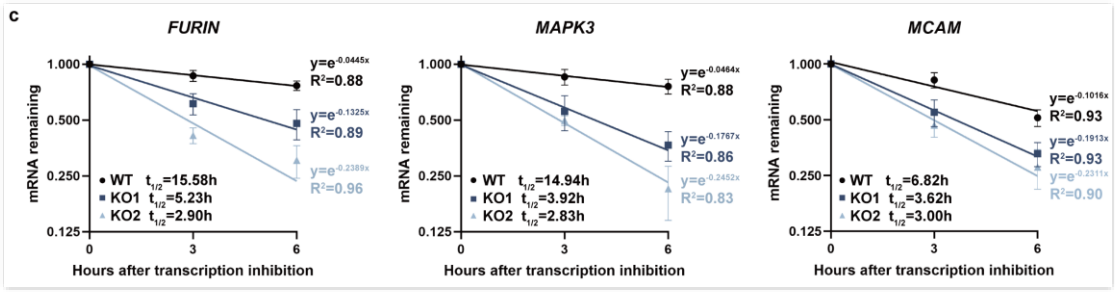
$$log_2(C) = log_2(C_0) - kt \cdot log_2(e)$$

since $log_2(e)$ is a constant,

$$log_2(C) = log_2(C_0) - \frac{k}{ln(2)}t$$

So, the degradation curve of mRNA will appear as **a straight line** with a slope of $-\frac{k}{ln(2)}$, which can intuitively reflect the degradation rate of mRNA. When the abundance of mRNA decreases to half of its initial value, substituting into the equation of the fitted straight line, the half-life of mRNA $t_{1/2} = \frac{ln(2)}{k}$. This result is the same as the calculation method without taking the logarithm, because the logarithmic transformation only changes the representation of the data, but does not alter the intrinsic kinetics of mRNA degradation.

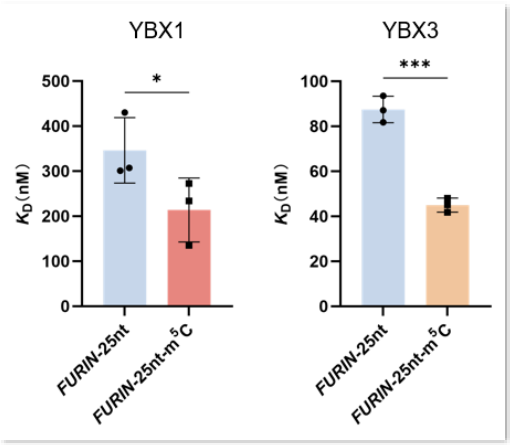
Thank you again for your questions! We have now modified the Y-axis of these graphs to represent the logarithmic form of the fold decrease in mRNA, namely 1, 0.5, 0.25, 0.125 (Updated in **Fig. 5c, 6j-m, 7c, 7d, 7g, 7j, and Supplementary Fig. 7d-g**, the example figure is also shown as below).



Comment 2. Figure 6c: The authors make strong claims about YBX1 acting as an m^5C reader. The data does not appear to us to be very compelling. The plot shows that Furin independent of the presence of m^5C can be bound strongly by YBX1. This and other data pose the question whether the reader proteins bind to the specific NSUN6 structure and motif without requiring the m^5C modification. Specially data shown for YBX3

knockdown (5i, 5j) show a minimal effect on RNA stability, partially within the error range. Additional experiments (ideally *in-vitro*, using controlled levels of enzymes and substrates) would be required to make a stronger case about these proteins serving as readers.

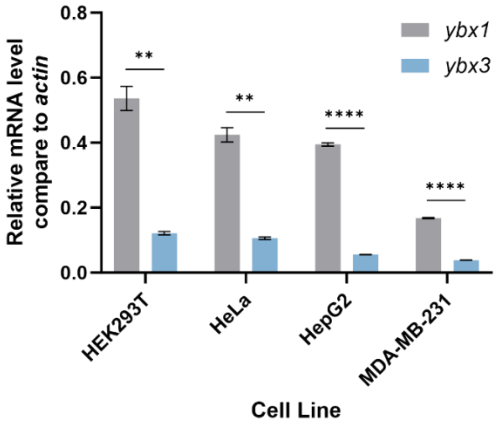
Response: Thanks for your insightful suggestion! We have used Biolayer interferometry (BLI) to measure the RNA-binding affinity of YBX1 CSD and YBX3 CSD to unmodified or *FURIN*-25nt-m^{5}C. The results showed that, compared to unmodified *FURIN*-25nt, YBX1 and YBX3 exhibited stronger binding affinity to mRNA exhibiting NSUN6-mediated m^{5}C modification (Now **Fig. 6e, f**, also shown as below). YBX1 and YBX3 could bind to unmodified RNAs, but they have binding preference to RNA with m^{5}C modification, which is consistent with previous study (*Nat Cell Biol* 21: 978-990 2019). These results were described on current **Line 414-421**.



414 target RNA[61] **(Supplementary Fig. 7a)**. We examined the RNA-binding affinities of
415 the YBX1 CSD (residues 48-159) for unmodified and m^{5}C-modified *FURIN*-25nt using
416 Biolayer interferometry (BLI). The YBX1 CSD exhibited higher binding affinity for
417 m^{5}C-modified mRNA (K_D = 213.8 nM) compare to unmethylated mRNA (K_D = 346.1
418 nM) **(Fig. 6e)**. Similarly, the YBX3 CSD (residues 82-174) showed stronger binding
419 affinity to *FURIN*-25nt-m^{5}C (K_D = 45.0 nM) than *FURIN*-25nt (K_D = 87.5 nM) **(Fig.**
420 **6f)**. The results indicated that both YBX1 and YBX3 have a stronger preference for
421 NSUN6-methylated mRNAs than for unmodified ones. Previous structural studies

Notably, based on sequence similarity, the UniProt database infers that YBX3 (UniProt: 16989) could bind mRNA containing the sequence 5'-UCCAUCA-3', which

is highly overlap with the characteristics of NSUN6 mRNA substrates. Also, *in vitro* experiments revealed that YBX3 has a stronger binding affinity to m^5C-modified mRNA (K_D = 45.0 nM) compared to YBX1 (K_D = 213.8 nM) **(**Now **Fig. 6e, f**, also shown as above**)**. These results indicate that YBX3 has a higher selectivity for the mRNA substrates of NSUN6. However, using RT-qPCR, we observed that the expression level of YBX1 is higher than that of YBX3 in multiple cell lines, including MDA-MB-231 **(Response Fig. 6)**. The limited expression of YBX3 restricts its function to stabilize mRNA substrates, which may be the reason why the knockdown of YBX3 in cells has a relatively minor effect on RNA stability.



Response Figure 6

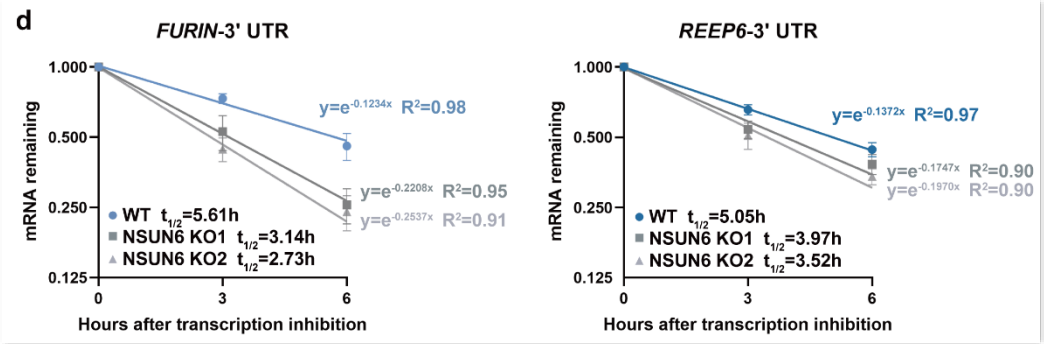
RT-qPCR analysis of the relative levels of YBX1 and YBX3 in HEK293T, HeLa, HepG2, and MDA-MB-231 cell lines. Error bars represent mean ± SD for three independent experiments. Data was analyzed using two-tailed Student's *t*-tests. **** P<0.0001, ** P<0.01.

Minor Comments:

1. Line 454: There is not substantial data for the claim that m^5C modification increases the local luciferase reporter activity. The observed higher activity could simply result from C-A conversion at this specific site. This result could be strengthened by introducing the reporter into a NSUN6 KO cell line, where the differences between C and A harboring reporters should be abolished.

Response: Thanks for giving our suggestions! We have now added the experiment results of introducing the reporter into the NSUN6 KO HEK293T cell line. The results

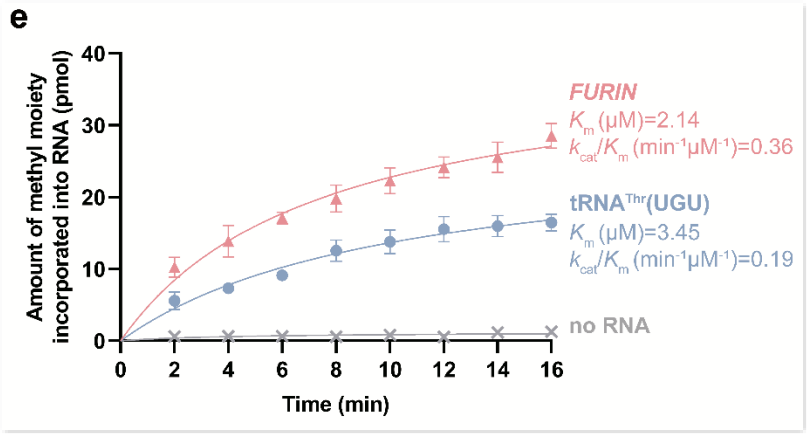
showed that introducing the 3' UTR of *FURIN* or *REEP6* did not enhance the stability of the mRNA in NSUN6 KO cells (Now **Fig 7d**, also shown as below). The results were described on current **Line 483-485**.



483 mRNA successfully increased mRNA stability **(Fig. 7c)**. However, introducing the 3'
484 UTR containing *FURIN* or *REEP6* did not enhance the stability of the mRNA in
485 NSUN6 KO cells **(Fig. 7d)**. These results suggest that introducing site-specific m^5C

2. Figure 1E: For calculating a correct K_m value, a saturated reaction/curve is required. However, especially for $tRNA^{Thr}$ the curve is clearly not saturated. Therefore, care should be taken in stating absolute K_m values.

Response: Thank you for pointing out our mistake! We have now repeated the determination of K_m to ensure that the reaction reached saturation (Updated in **Fig 1e**, also shown as below), and the results were described on current **Line 135-139**.



(Fig. 1d and Supplementary Fig. 1c). Subsequently, we measured the steady-state kinetic parameters of NSUN6 for *FURIN* under the same condition. The results showed that the K_m value for *FURIN* was 2.14 μM, which was lower than that for $tRNA^{Thr}$(UGU) (3.45 μM). Additionally, the k_{cat}/K_m value for *FURIN* was 0.36 $min^{-1}μM^{-1}$, which was approximately two folds for $tRNA^{Thr}$(UGU) (0.19 $min^{-1}μM^{-1}$) **(Fig. 1e)**. These results

3. Line 144: The comment "towards certain mRNA substrates" might be toned down, because in Figure 1 this has only been proven for a single RNA substrate.

Response: Thank you for your suggestion! In Figure 1, we demonstrated through *in vitro* methyltransferase activity assay that the catalytic activity of NSUN6 towards several mRNA substrates, including *FURIN*, *MAPK3*, and *PVRL2*, is comparable to that of tRNA **(Fig. 1d)**. We have now revised the term "certain" to "particular" in the manuscript, referring to the specific high-activity mRNA substrates (Now **Line 144**, also shown as below).

on examined mRNA substrates is indeed m^5C **(Fig. 1f)**. Collectively, we found that NSUN6 independently catalyzes high levels of m^5C on specific mRNAs *in vitro*, and emphasized that the catalytic activity of NSUN6 toward particular mRNA substrates is comparable to that for tRNA substrates.

4. Line 150: It is not very clear what is meant with "* is the catalytic site", as this phrase is usually used for the catalytic site of an enzyme. For an RNA motif the better phrase would be "modified C".

Response: Thank you for pointing out the error in our expression. We have made the corrections in the manuscript (Now **Line 150**, also shown as below).

Previous studies showed that NSUN6 mRNA substrates contain the C*UCCA motifs[23, 35] (* represents the modified C). However, with approximately two hundred

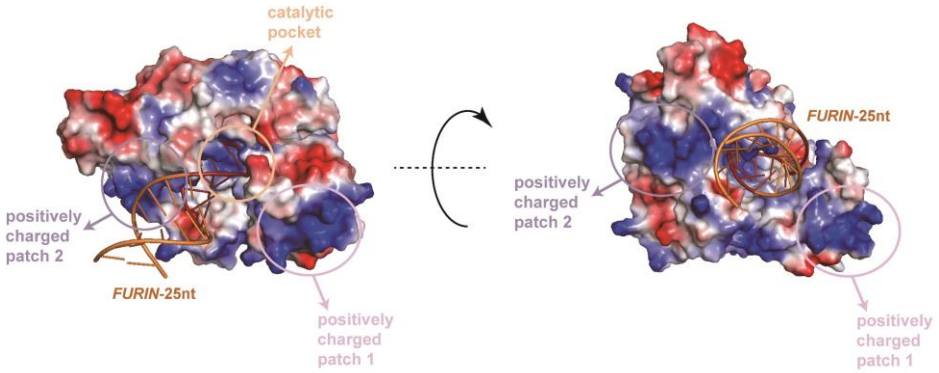
5. In the current form some graphs have low quality.

Response: We apologize for the low-quality graphs in the initial submission, which

were caused by the conversion from Word to PDF. We have now carefully reviewed and revised all the graphs to ensure they meet the required quality standards.

6. Figure 3G: Adding the positively charged patches to figure 3G would contribute to understanding the figure as the surface charge 3C and 3G look very different. Thus, in 3G the patch1 and 2 are not clearly identifiable.

Response: Thanks for your advice! In Figure 3G, we have labeled the catalytic pocket, positively charged patch 1, and positively charged patch 2 on NSUN6 to clearly illustrate the binding mode between NSUN6 and mRNA. We attempted to rotate NSUN6 to a position similar to that in Figure 3C but found that the mRNA would stack together **(Response Figure 7)**, making it difficult to distinguish the loop and stem regions of the mRNA.



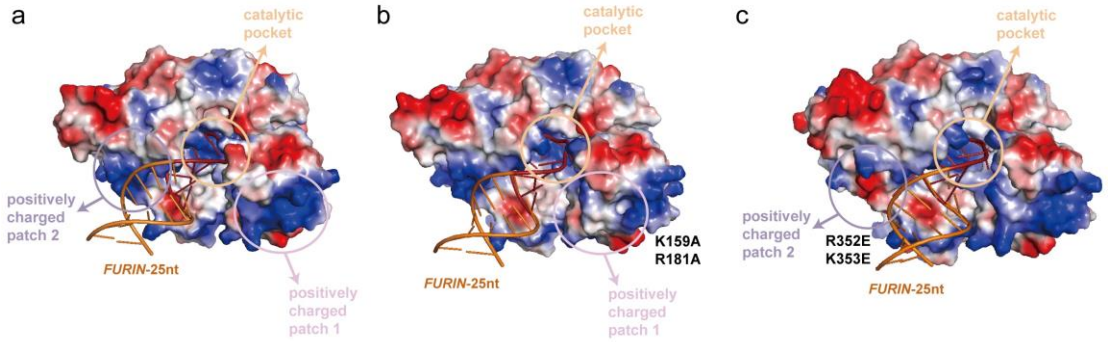
Response Figure 7

The structural model of NSUN6 and *FURIN*-25nt complex was generated using AlphaFold3. NSUN6 was shown as the electrostatics surface representation, the loop region of *FURIN*-25nt (dark red) was combined with and the catalytic pocket of NSUN6 and the stem region of *FURIN*-25nt (gold) was close to the positively charged patch 2 on NSUN6.

7. Figure 3: How to rule out the possibility that the respective mutations especially in patch 2 are simply abrogating catalytic activity by disrupting the 3D fold of the complex. Potentially AlphaFold3 complexes with the mutants could be generated to see that there are not major changes for catalytically important residues upon mutating patch amino acids. Moreover, it would be great to see the activity of patch1 mutant on at least one

other mRNA to show general validity of these in silico predicted mechanism for mRNA recognition. As the mutant is used in further experiments (Figure 4), such a validation would increase confidence in the differential tRNA and mRNA recognition (Figure 4).

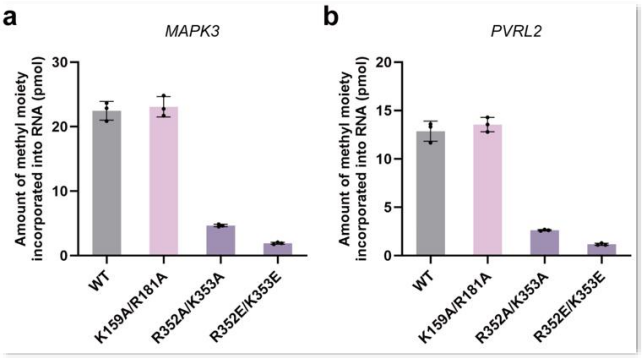
Response: Thanks for your suggestion! We utilized AlphaFold3 to generate the structures of NSUN6 mutants in complex with mRNA substrates. Since the mRNA substrate does not bind to patch 1, charge changes resulting from mutations on patch 1 (K159A/R181A) do not affect the binding affinity of NSUN6 to mRNA (**Response Fig. 8b**). The mutations on patch 2 (R353E/K353E) does not impact the 3D fold of the complex but instead hinder the binding of mRNA substrates to patch 2 by altering the protein surface charge (**Response Fig. 8c**).



Response Figure 8

a-c, The structural model of NSUN6 WT (**a**), K159/R181A (**b**), and R352E/K353E (**c**), in complex with *FURIN*-25nt were generated using AlphaFold3. NSUN6 was shown as the electrostatics surface representation, with the loop region of *FURIN*-25nt was in dark red and the stem region in gold.

We have also supplemented the *in vitro* methyltransferase activity assays of different NSUN6 enzymatic mutants on other mRNAs. The results are consistent with those observed for *FURIN*: mutations of key amino acids in patch 1 (K159/R181) do not affect NSUN6's catalytic activity on mRNA, while mutations of key catalytic amino acids in patch 2 (R352/K353) almost completely lost its activity on mRNA **(**Now **Supplementary Fig. 3**, also shown as below). These results demonstrate the general validity of the NSUN6 mRNA recognition mechanism.



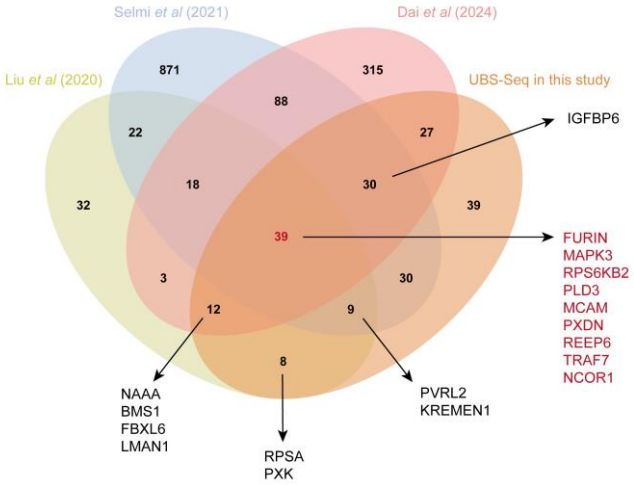
8. Line 286: While it's true that NSUN6 KO seem to not impact tRNA aminoacylation and steady-state levels of $tRNA^{Thr}$ and $tRNA^{Cys}$, the tRNA substrate still could have been impacted in another way e.g. change of other tRNA modifications or mischarging. Therefore, toning down the statement that the "tRNA substrates were not impacted" would reflect the performed experiments in a better way.

Response: Thanks for your advice! We have revised the statement "tRNA substrates were not impacted" to the more objective "the stability and aminoacylation levels of tRNA substrates" in the manuscript (Now **Line 295-297**, also shown as below).

295 **Supplementary Fig. 4i, j)**. This suggests that, under normal culturing conditions,
296 NSUN6-mediated m^5C modification did not significantly affect the stability and
297 aminoacylation levels of tRNA substrates in MDA-MB-231 cells.

9. Line 308 ff: Do the identified sites align with previously reported sites? Is this overlap better for the highly modified sites. This info would add to the confidence of identified m^5C sites.

Response: Thanks for your suggestion! We compared the results of our UBS-seq in MDA-MB-231 cells with other reported m^5C sites and found that the sites with m^5C levels > 0.5 demonstrated good concordance with other sequencing results **(Response Fig. 9)**, supporting the reliability of our data.

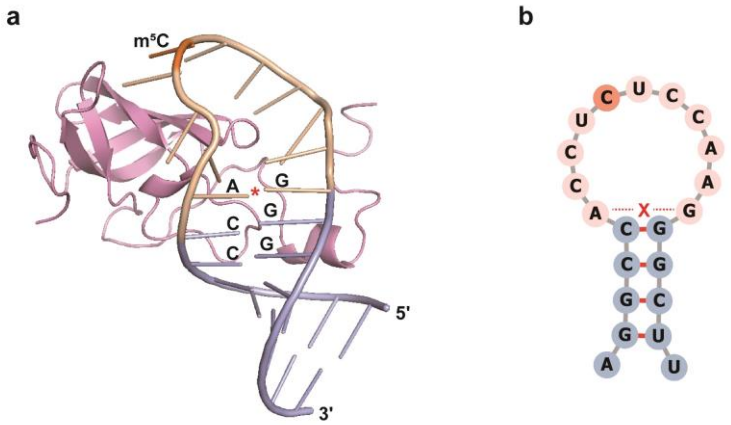


Response Figure 9

The Venn diagram shows the overlap of NSUN6-mediated mRNA m^5C sites in MDA-MB-231 cells with different datasets (*Natl Sci Rev* 31: nwaa273 2020; *Nucleic Acids Res* 49: 1006-1022 2021; *Nat Biotechnol* 42: 1559-1570 2024).

10. Line 411ff: Is there a specific reason why the model was built with *PVRL2* mRNA, but the pulldown experiments done with *FURIN* mRNA?

Response: Thank you for your question! In the *in vitro* methyltransferase activity assay, we identified NSUN6 exhibited high catalytic activity toward *FURIN*. Therefore, in subsequent functional validation experiments, including the pulldown assay you mentioned, we selected *FURIN* as a representative NSUN6 mRNA substrate. However, when using AlphaFold3 to generate models of the YBX1 and YBX3 complexes with mRNA, we observed significant discrepancies between the predicted secondary structure of *FURIN* and the experimental results from SHAPE probing. Specifically, there were errors in base pairing within the stem region and incorrect loop size **(Response Fig 10)**. To obtain a more accurate complex model, we attempted to fit other mRNA substrates with YBX proteins and ultimately selected *PVRL2*, which not only exhibited the correct base-pairing and secondary structure but also could be efficiently catalyzed by NSUN6 **(Fig. 1d)**.



Response Figure 10

a, Predicted structure of the YBX1-*FURIN*-25nt complex generated by AlphaFold3. YBX1 is shown in pink, the m^5C site of *FURIN*-25nt is highlighted in orange, wheat color represents loop regions of *FURIN*-25nt based on SHAPE experimental data, and blue indicates stem regions. The asterisk (*) denotes a wrong A-G pairing in the predicted structure, which results in a reduced loop size in *FURIN*-25nt. **b,** Secondary structure of *FURIN*-25nt derived from SHAPE experiments.