

The MTR4/hnRNPK complex surveils aberrant polyadenylated RNAs with multiple exons



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<http://creativecommons.org/licenses/by/4.0/>.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The function of RNA surveillance system is an interesting direction. This study determined aberrant RNA targeted by MTR4 by combining long-read direct RNA sequencing and 3' sequencing. Interestingly, intronic polyadenylated transcripts generated by transcriptional read-through over one or more exons are preferentially affected by MTR4. Mechanistically, the authors found that MTR4 destabilizes these aberrant RNAs together with hnRNPK. Furthermore, the authors found that the aberrant transcripts may lead to aberrant protein that forms aberrant condensate, which might link to Parkinson's and Alzheimer's diseases and in morphologically abnormal spermatozoa. The findings are potentially interestingly. However, the manuscript may not be suitable to be published in Nature Communications in the current shape. The main concerns are the conclusions largely built on qPCR analysis of very limited number of targets, the reliability of several piece of data, and the very weak link between dysregulation of MTR4/hnRNPK mediated RNA surveillance and the diseases mentioned. Please see the specific comments below.

Specific comments:

1. "We found 11,237 transcripts were increased in abundance and that 1,146 transcripts were stabilized by MTR4 depletion." 11,237 transcripts increased means most of the transcriptome increased which is very rare even for comparing different cell types. Can this analysis be better explained? How is the transcriptome analysis normalized?
2. In Extended Data Fig. 2a, DIS3 KD #2 works better than #1. However, the effect of DIS3 KD #2 on 3XTs is almost neglectable compared to #1. This makes me doubtful about the reliability of the data.
3. Line 158-160 says knockdown of hnRNPK but not PCBP2 caused upregulation of the multi-exon 3XTs. However, the data in Extended Data Fig. 3a,b is not as described. PCBP2 KD clearly affected SPRED2 and possibly KCTD13 as hnRNPK KD did. In addition, qPCR analysis of several limited number of targets may have bias. Therefore, transcriptome-wide analysis is needed for the KD samples to draw a solid conclusion for the analysis here, and also other similar analysis, such as Fig. 2a, b, and c.
4. The data in Extended Fig. 3f also makes me doubtful about the reliability of the data. The protein pulled down showed comparable (PABPN1) or higher (ZFC3H1) level than input, although the IP target showed only comparable intensity between Input and IP.
5. The KeXT body was observed in cells transfected with FLAG-tagged KCTF13 3XT. However, whether KeXT is observable in MTR4 KD cells is not analyzed. The KeXT body seen in transfected cells might be an artifact due to overexpression.
6. The potential contribution of defect of RNA surveillance by MTR4 and hnRNPK to Parkinson's and Alzheimer's diseases and abnormal spermatozoa is interesting. However, the evidence for this only relies on the moderately decreased in gene expression, which is far from convincing evidence for a direct link.

7. MTR4 is well-known to be in TRAMP complex. Is TRAMP complex involved in the function of MTR4 here?

8. Unlike MTR4, hnRNPK depletion had no effect on the expression of the mono-exon eXTs. This is an interesting difference, which shall be at least carefully discussed.

9. "Aberrant RNAs with intronic APA that generate aberrant proteins must be degraded by the RNA surveillance system." is not necessary. I'd suggest it to be removed.

Reviewer #2 (Remarks to the Author):

The nuclear exosome, MTR4 and PAXT components have previously been shown to prevent the accumulation of prematurely terminated RNAs with intronic polyadenylation. In this study using genomic analysis of cells depleted of MTR4, the authors show that longer extended species that they call multi-exon 3XTs are also destabilized by MTR4 and by the PAXT complex, but not by the NEXT complex. They identify hnRNPK as an additional protein involved in this pathway and show that hnRNPK interacts with MTR4 in an RNA-independent manner, and that it also binds the 3' extended region of multi-exon 3XTs. In the last part of the paper, the authors suggest that proteins generated by translation of multi-exon 3XT contain intrinsically disordered domain and they provide preliminary evidence that one of these may form liquid-liquid condensates.

Overall the study presents some new and interesting findings in a very concise manner. On one hand it seems like an extension of previous findings, as the nuclear exosome and MTR4 have previously been shown to target short transcripts prematurely polyadenylation. On the other hand the authors identify a new type of aberrant RNAs and a new factor involved in this pathway and suggest that these aberrant RNAs may form aggregates. So I think the paper has good potential for publication. That said there are a number of major and minor issues that need to be addressed.

Major

The authors perform mutational analysis of hnRNPK and show that amino acid 42 to 104 and 387 to 451 were required for the association of hnRNPK with β -globin-fused KCTD13 3XR. It is not clear to me that these mutants are stably expressed or localized properly in the cell, so western blot analysis and cellular localization analysis of these mutants should be performed.

The authors state that "hnRNPK depletion led to the cytoplasmic accumulation of KCTD13 3XTs in HeLa cells (Extended Data Fig. 4b)". The nuclear/cytoplasmic fractionation experiment lacks important controls – it is not clear that the cytoplasmic fraction is not contaminated by nuclear components, and the nuclear fraction is not shown. This experiment should be repeated with proper fractionation controls, and including both nuclear and cytoplasmic fractions.

The authors found that proteins generated from the Flag-tagged KCTD13 3XT construct

formed

granule-like structures, which they called KCTD. These granule-like structures are sensitive to treatment with 1% 1,6-hexandiol, which disrupts liquid-like assemblies. These experiments are presented in Figure 5c and extended data Fig.5a. The main issue with these experiments is that there is no evidence of how general and reproducible these observations are, as the authors only show one cell for each condition. The authors should quantitate the fraction of cells that exhibit granule like structures and the quantitative effect of 1,6-hexandiol treatment with statistical analyses to show evidence of reproducibility.

I did not find that the expression analysis presented in the last paragraph particularly important or impactful. It was very preliminary and I suggest that it should be removed from the manuscript, or alternatively supported by additional data.

Minor

The classification of the different RNA forms presented in Fig.1b is very difficult to understand and the labeling sometimes counter intuitive. I do not understand what the authors mean by 'intergenic'; also two of the species look almost identical but one is labeled 'included' and the other "known exon". I also do not understand the 5'/3' extended species. The authors should completely modify this figure to make it as explicit as possible.

The genome browser screenshots of Figure 1d are very difficult to read and analyze. I encourage the authors to use alternative ways to represent long-read sequencing data, such as the Nanoblot software, which can be used to generate a more compact visualization of long read sequencing data for multiple transcripts.

In general the paper would benefit from editing by a native English speaker. The following are non exhaustive examples of sentences/statements that could be edited.

Line 61 "the whole picture of the aberrant RNA is elusive"

Line 71 "The RNA helicase, MTR4" – remove comma

Line 108 "depletion"

Line 121 "isoform with an exon" – replace "an" with "one"

Reviewer #3 (Remarks to the Author):

Taniue et al. have used a long-read sequencing pipeline to identify several hundred aberrant transcripts bearing various APA signatures whose abundance is modulated by MTR4, PAXT and hnRNPK. They show that MTR4 and hnRNPK are associated with the 3' extended transcripts. Fusion of the 3' extension of KCTD13 to β -globin transcript induced its down-regulation, suggesting that the extension destabilizes the transcript. Further analysis led to the notion that the protein product of KCTD13-3XT may form phase-separated protein condensates that may lead to toxicity. Available microarray data showed that MTR4, Dis3 and hnRNPK are poorly expressed in brain in sperm and defects in these factors are known to be associated with neurological disease and male infertility. However, a direct link between the expression of 3XT transcripts and condensate formation in such tissues was not demonstrated. Overall, the findings are of interest to the field. However, many of the

claims made require further substantiation.

1. Fig 1f, the authors should show a schematic diagram of what is being amplified by PCR. The result should be normalized to a control region of the same transcript (not extended). It's not clear if normalization was applied.

2. Fig 3d, it appears that the hnRNPK RIP was performed in control cells, in which the 3XT transcripts should not be particularly abundant. It would be preferable to perform the RIP in control and MTR4-knockdown cells. A control region within the transcript should be amplified in addition to the 3XT region.

3. Fig 3e and extended data 3f, the IPs performed in the presence and in the absence of RNase need to be shown in order to conclude whether the interaction is dependent or not on RNA.

4. Lines 181 to 183, the authors conclude that hnRNPK associates with and downregulates 3XTs by interacting with MTR4. I don't think the authors have all the necessary information to support such a conclusion. Does MTR4 also interact with the 3XT transcript? Is it dependent on hnRNPK or not? The authors should either extend their analysis to address the recruitment mechanism or modify the conclusion.

5. Fig 4a, while the addition of 3XT appears to decrease the abundance of the fusion transcript, it is not clear whether that is dependent on MTR4 and/or hnRNPK. This should be addressed.

6. Fig 4b, why was over-expressed hnRNPK used in this RIP experiment? RIP experiment shown in Fig 3d used endogenous hnRNPK.

7. Extended data 4b, it is necessary to show the amount of 3XT in the nucleus, in addition to the cytoplasm to determine whether the transcript is better exported to the cytoplasm, or if simply more transcript is present in the cells.

8. Extended data 4c, it is necessary to show the implication of MTR4 or other exosome/PAXT subunits in the loss of 5BoxB transcript.

9. Fig 5c, what is the evidence that endogenous KCTD13 3XT forms condensates? Also, the images shown are not of very high quality. The condensates seem to be in both the cytoplasm and nucleus. The authors need to demonstrate the implication of MTR4 in formation of the condensates (endogenous or over-expressed 3XT)

10. Extended data Fig 6, this section is not compelling. Either the authors perform additional experiments showing 3XT in brain cells or sperm, or this section should be removed. The figures as shown are intriguing correlations but don't prove anything.

General comment:

The authors refer to 'stabilization' or 'degradation' of transcripts throughout the manuscript. While the transcripts are likely to be degraded, no experiment in the

manuscript formally demonstrates it. The DRB chase experiment might suggest transcript stabilization but it could be interpreted in other ways. Therefore, it is preferable if the authors use terms such as 'accumulate', or 'increase (or decrease) in abundance', which more correctly reflect the data shown.

Minor points:

line 108, depletion is misspelt

Fig 2 and extended data fig 2, please show WBs of the siRNA knock-downs, in addition to mRNA quantification.

Reviewer comments

Reviewer #1 (Remarks to the Author):

The function of RNA surveillance system is an interesting direction. This study determined aberrant RNA targeted by MTR4 by combining long-read direct RNA sequencing and 3'sequencing. Interestingly, intronic polyadenylated transcripts generated by transcriptional read-through over one or more exons are preferentially affected by MTR4. Mechanistically, the authors found that MTR4 destabilizes these aberrant RNAs together with hnRNPk. Furthermore, the authors found that the aberrant transcripts may lead to aberrant protein that forms aberrant condensate, which might link to Parkinson's and Alzheimer's diseases and in morphologically abnormal spermatozoa. The findings are potentially interestingly. However, the manuscript may not be suitable to be published in Nature Communications in the current shape. The main concerns are the conclusions largely built on qPCR analysis of very limited number of targets, the reliability of several piece of data, and the very weak link between dysregulation of MRT4/hnRNPk mediated RNA surveillance and the diseases mentioned. Please see the specific comments below.

Specific comments:

Comment:

1. "We found 11,237 transcripts were increased in abundance and that 1,146 transcripts were stabilized by MTR4 depletion." 11,237 transcripts increased means most of the transcriptome increased which is very rare even for comparing different cell types. Can this analysis be better explained? How is the transcriptome analysis normalized?

Response:

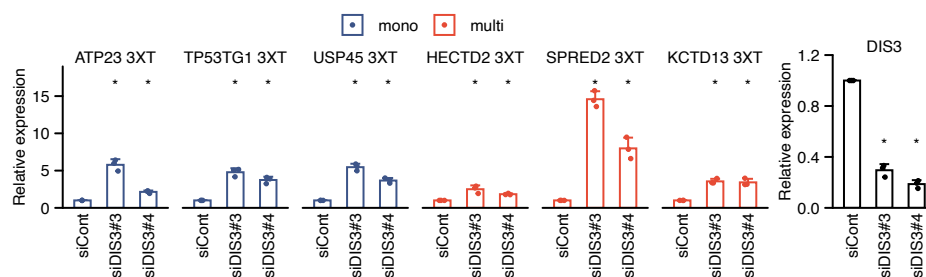
Thank you for carefully pointing out this issue. We indeed counted the number of transcripts that were increased in abundance based on raw counts, but noticed a potential bias due to different library sizes. We therefore normalized the counts (CPM, counts per million mapped reads), which resolved the issue. We applied a ≥ 1.5 -fold change cutoff to the normalized counts and re-evaluated the number of increased-abundance transcripts under MTR4 depletion and identified 8,043 transcripts. We have included a description of the normalization process in the Methods section and have revised the description of the number of increased-abundance transcripts in the main text on page 6, lines 111–114 of the revised manuscript. Importantly, re-analyzing the transcripts based on normalized counts did not impact the main results and conclusions of this article.

Comment:

2. In Extended Data Fig. 2a, *DIS3* KD #2 works better than #1. However, the effect of *DIS3* KD #2 on 3XTs is almost neglectable compared to #1. This makes me doubtful about the reliability of the data.

Response:

We designed an additional two siRNAs targeting *DIS3* and repeated the experiment. We found that *DIS3* knockdown upregulated both MTR4-target mono- and multi-exon 3XTs. These results are presented below.



Comment:

3. Line 158-160 says knockdown of *hnRNPK* but not *PCBP2* caused upregulation of the multi-exon 3XTs. However, the data in Extended Data Fig. 3a,b is not as described. *PCBP2* KD clearly affected *SPRED2* and possibly *KCTD13* as *hnRNPK* KD did. In addition, qPCR analysis of several limited number of targets may have bias. Therefore, transcriptome-wide analysis is needed for the KD samples to draw a solid conclusion for the analysis here, and also other similar analysis, such as Fig. 2a, b, and c.

Response:

We analyzed RNA-seq data from the cells depleted of *hnRNPK* or *PCBP2* and found that knockdown of *hnRNPK*, but not of *PCBP2*, led to the upregulation of multi-exon 3XTs. We also selected four more mono- and four more multi-exon 3XTs for qRT-PCR analysis and obtained similar results (Supplementary Fig. 3d,e), consistent with the RNA-seq analysis (Supplementary Fig. 3f). Unfortunately, we were unable to find RNA-seq data for *EXOSC5* knockdown cells; however, we analyzed RNA-seq data from the HeLa cells depleted of *PABPN1* or *RBM7*. We found that knockdown of *PABPN1*, but not of *RBM7*, led to the upregulation of both of mono- and multi-exon 3XTs (Supplementary Fig. 2h). In agreement with the RNA-seq results, qRT-PCR analysis revealed that knockdown of *EXOSC5* and *PABPN1*, but not of *RBM7* resulted in the upregulation of the other four mono- and four multi-exon 3XTs (Supplementary Fig. 2b–d).

Comment:

4. The data in Extended Fig. 3f also makes me doubtful about the reliability of the data. The protein pulled down showed comparable (PABPN1) or higher (ZFC3H1) level than input, although the IP target showed only comparable intensity between Input and IP.

Response:

For readers' convenience, we have mentioned the percentage of input because it differs between PABPN1 or ZFC3H1, and hnRNPK in Supplementary Fig. 3o of the revised manuscript. Similar findings have also been reported by Rolland in *Cell* 2014¹ and by Kwiatek in *RNA* 2023². These results are explained on page 9, lines 196–197 of the revised manuscript.

1. Rolland, T. *et al.* A proteome-scale map of the human interactome network. *Cell* **159**, 1212–1226 (2014).
2. Kwiatek, L., Landry-Voyer, A. M., Latour, M., Yague-Sanz, C. & Bachand, F. PABPN1 prevents the nuclear export of an unspliced RNA with a constitutive transport element and controls human gene expression via intron retention. *RNA* **29**, 644–662 (2023).

Comment:

5. The KeXT body was observed in cells transfected with FLAG-tagged KCTF13 3XT. However, whether KeXT is observable in MTR4 KD cells is not analyzed. The KeXT body seen in transfected cells might be an artifact due to overexpression.

Response:

We generated an anti-KCTD13 3XT protein antibody to detect endogenous KeXT bodies and then performed immunofluorescence analysis using HeLa cells depleted of MTR4 by siRNA. We found that endogenous KCTD13 3XT proteins formed KeXT bodies primarily in the cytoplasm of MTR4-depleted HeLa cells. These results are shown in Fig. 5c and explained on page 11, lines 252–254 of the revised manuscript.

Comment:

6. The potential contribution of defect of RNA surveillance by MTR4 and hnRNPK to Parkinson's and Alzheimer's diseases and abnormal spermatozoa is interesting. However, the evidence for this only relies on the moderately decreased in gene expression, which

is far from convincing evidence for a direct link.

Response:

Other reviewers suggested that we remove this section. We therefore decided to remove this section.

Comment:

7. MTR4 is well-known to be in TRAMP complex. Is TRAMP complex involved in the function of MTR4 here?

Response:

We performed qRT-PCR analysis of lysates from HeLa cells transfected with siRNA targeting *ZCCHC7* and *PAPD5*, components of the TRAMP complex, and confirmed that the TRAMP complex does not affect the expression of MTR4-target 3XTs. These results are shown in Fig. 2d and Supplementary Fig. 2g and are explained on page 7, lines 148–151 of the revised manuscript. Moreover, we added information concerning the TRAMP complex to the Introduction section on page 4, lines 69–80.

Comment:

8. Unlike MTR4, hnRNPk depletion had no effect on the expression of the mono-exon eXTs. This is an interesting difference, which shall be at least carefully discussed.

Response:

We have discussed the point indicated by the reviewer in the Discussion section on page 13, lines 298–302 of the revised manuscript.

Comment:

9. "Aberrant RNAs with intronic APA that generate aberrant proteins must be degraded by the RNA surveillance system." is not necessary. I'd suggest it to be removed.

Response:

At the suggestion of the reviewer, we have removed this sentence from the Introduction.

Reviewer #2 (Remarks to the Author):

The nuclear exosome, MTR4 and PAXT components have previously been shown to prevent the accumulation of prematurely terminated RNAs with intronic polyadenylation. In this study using genomic analysis of cells depleted of MTR4, the authors show that longer extended species that they call multi-exon 3XTs are also destabilized by MTR4 and by the PAXT complex, but not by the NEXT complex. They identify hnRNPK as an additional protein involved in this pathway and show that hnRNPK interacts with MTR4 in an RNA-independent manner, and that it also binds the 3' extended region of multi-exon 3XTs. In the last part of the paper, the authors suggest that proteins generated by translation of multi-exon 3XT contain intrinsically disordered domain and they provide preliminary evidence that one of these may form liquid-liquid condensates.

Overall the study presents some new and interesting findings in a very concise manner. On one hand it seems like an extension of previous findings, as the nuclear exosome and MTR4 have previously been shown to target short transcripts prematurely polyadenylation. On the other hand the authors identify a new type of aberrant RNAs and a new factor involved in this pathway and suggest that these aberrant RNAs may form aggregates. So I think the paper has good potential for publication. That said there are a number of major and minor issues that need to be addressed.

Major

Comment:

1. The authors perform mutational analysis of hnRNPK and show that amino acid 42 to 104 and 387 to 451 were required for the association of hnRNPK with β -globin-fused KCTD13 3XR. It is not clear to me that these mutants are stably expressed or localized properly in the cell, so western blot analysis and cellular localization analysis of these mutants should be performed.

Response:

We performed immunofluorescence and immunoblotting analysis using HeLa cells transfected with Flag-tagged wild-type hnRNPK and its deletion mutants and found that both wild-type hnRNP and its deletion mutants were localized in the nucleus and stably expressed. These results are shown in Supplementary Fig. 4c,d of the revised manuscript.

Comment:

2. The authors state that “hnRNPK depletion led to the cytoplasmic accumulation of

KCTD13 3XTs in HeLa cells (Extended Data Fig. 4b)”. The nuclear/cytoplasmic fractionation experiment lacks important controls – it is not clear that the cytoplasmic fraction is not contaminated by nuclear components, and the nuclear fraction is not shown. This experiment should be repeated with proper fractionation controls, and including both nuclear and cytoplasmic fractions.

Response:

We repeated the experiments and demonstrated changes in the amount of *KCTD13* 3XT in the nucleus and cytoplasm. As controls for fractionation, we used UHRF1 as a nuclear marker and α -Tubulin as a cytoplasmic marker. We found that hnRNPK knockdown resulted in the cytoplasmic accumulation of *KCTD13* 3XTs. These results are shown in Supplementary Fig. 4f and are explained on page 10, lines 218–220 of the revised manuscript.

Comment:

3. The authors found that proteins generated from the Flag-tagged KCTD13 3XT construct formed granule-like structures, which they called KCTD. These granule-like structures are sensitive to treatment with 1% 1,6-hexandiol, which disrupts liquid-like assemblies. These experiments are presented in Figure 5c and extended data Fig.5a. The main issue with these experiments is that there is no evidence of how general and reproducible these observations are, as the authors only show one cell for each condition. The authors should quantitate the fraction of cells that exhibit granule like structures and the quantitative effect of 1,6-hexandiol treatment with statistical analyses to show evidence of reproducibility.

Response:

We repeated the experiments and obtained images containing multiple cells under each set of conditions. Moreover, we calculated the proportion of cells containing KeXT bodies. These results are shown in Supplementary Fig. 5c–e and are explained on page 11, line 258 – page 12, line 263 of the revised manuscript.

Comment:

4. I did not find that the expression analysis presented in the last paragraph particularly important or impactful. It was very preliminary and I suggest that it should be removed from the manuscript, or alternatively supported by additional data.

Response:

At the suggestion of the reviewer, we have removed this section.

Minor**Comment:**

1. The classification of the different RNA forms presented in Fig.1b is very difficult to understand and the labeling sometimes counter intuitive. I do not understand what the authors mean by 'intergenic'; also two of the species look almost identical but one is labeled 'included' and the other "known exon". I also do not understand the 5'/3' extended species. The authors should completely modify this figure to make it as explicit as possible.

Response:

We generated a new classification diagram of APA features according to the status of the last exon with the addition of "last exon category". "Non-exonic" category contains "intergenic" and "intron-exonized" types. "Intergenic" type means that the last exon of the transcript is located between the last exon of the originally annotated mature mRNA and the downstream gene. "Intron-exonized" means the last exon is located within the intron. "Included" and "known exon" of original manuscript have been consolidated into a "known exon" type in "included in known exon" category. "Middle exon extended" category contains "5' extended", "3' extended", and "both extended", whose last exons have been extended into primarily intronic regions at the 5'-, 3'- or both ends. These results are shown in Supplementary Fig. 1 c and are explained on page 6, lines 114–116 of the revised manuscript.

Comment:

2. The genome browser screenshots of Figure 1d are very difficult to read and analyze. I encourage the authors to use alternative ways to represent long-read sequencing data, such as the Nanoblot software, which can be used to generate a more compact visualization of long read sequencing data for multiple transcripts.

Response:

At the suggestion of the reviewer, we performed NanoBlot analysis using direct RNA sequencing data. These results are shown in Fig. 1c and are explained on page 6, lines 129–132 of the revised manuscript.

Comment:

3. *In general the paper would benefit from editing by a native English speaker. The following are non exhaustive examples of sentences/statements that could be edited.*

Line 61 “the whole picture of the aberrant RNA is elusive”

Line 71 “The RNA helicase, MTR4” – remove comma

Line 108 “depletion”

Line 121 “isoform with an exon” – replace “an” with “one”

Response:

We corrected the errors highlighted by the reviewer and have once again had the manuscript proofread by a native English speaking editor from Edanz (<https://jp.edanz.com/ac>).

We have replaced “the whole picture of the aberrant RNA is elusive” with “the overall generation and turnover of aberrant RNAs remains unclear”.

We have replaced “The RNA helicase, MTR4” with “The RNA helicase MTR4”.

We have replaced “delpetion” with “depletion”.

We have replaced “isoform with an exon” with “isoform with one exon”.

Reviewer #3 (Remarks to the Author):

Taniue et al. have used a long-read sequencing pipeline to identify several hundred aberrant transcripts bearing various APA signatures whose abundance is modulated by MTR4, PAXT and hnRNPk. They show that MTR4 and hnRNPk are associated with the 3' extended transcripts. Fusion of the 3' extension of KCTD13 to β -globin transcript induced its down-regulation, suggesting that the extension destabilizes the transcript. Further analysis led to the notion that the protein product of KCTD13-3XT may form phase-separated protein condensates that may lead to toxicity. Available microarray data showed that MTR4, Dis3 and hnRNPk are poorly expressed in brain in sperm and defects in these factors are known to be associated with neurological disease and male infertility. However, a direct link between the expression of 3XT transcripts and condensate formation in such tissues was not demonstrated. Overall, the findings are of interest to the field. However, many of the claims made require further substantiation.

Comment:

1. Fig 1f, the authors should show a schematic diagram of what is being amplified by PCR. The result should be normalized to a control region of the same transcript (not extended). It's not clear if normalization was applied.

Response:

At the suggestion of the reviewer, we have indicated the positions of the primers used to detect MTR-target 3XTs and their corresponding originally annotated mature mRNAs in Supplementary Fig. 1e–j of the revised manuscript. We also normalized the values of target transcripts to the signal generated from *GAPDH* mRNA prior to fold-change calculation. We describe the normalization method in the “qRT-PCR analysis” section of the “Supplementary Methods”.

Comment:

2. Fig 3d, it appears that the hnRNPk RIP was performed in control cells, in which the 3XT transcripts should not be particularly abundant. It would be preferable to perform the RIP in control and MTR4-knockdown cells. A control region within the transcript should be amplified in addition to the 3XT region.

Response:

We performed RIP analysis with an anti-hnRNPk antibody using lysates from HeLa cells

transfected with siMTR4 and found that MTR4 did not affect the association between hnRNPk and multi-exon 3XTs. Unexpectedly, hnRNPk was also found to associate with normal *HECTD2*, *SPRED2*, and *KCTD13* mRNAs in an MTR4-independent manner. Importantly, depletion of hnRNPk or PABPN1 did not cause upregulation of normal *HECTD2*, *SPRED2*, and *KCTD13* mRNAs. These results are shown in Supplementary Fig. 3j–m and are explained on page 9, lines 187–191 of the revised manuscript.

Comment:

3. Fig 3e and extended data 3f, the Ips performed in the presence and in the absence of RNase need to be shown in order to conclude whether the interaction is dependent or not on RNA.

Response:

We conducted IP experiments in the presence of RNase A/T1 and presented the results in the original manuscript. To avoid misunderstanding, we have replaced “in an RNA-independent manner” with “in the absence of RNA” in the revised manuscript.

Comment:

4. Lines 181 to 183, the authors conclude that hnRNPk associates with and downregulates 3XTs by interacting with MTR4. I don't think the authors have all the necessary information to support such a conclusion. Does MTR4 also interact with the 3XT transcript? Is it dependent on hnRNPk or not? The authors should either extend their analysis to address the recruitment mechanism or modify the conclusion.

Response:

We performed RIP analysis using an anti-MTR4 antibody on lysates from HeLa cells. qRT-PCR analysis of the immunoprecipitates revealed that MTR4 associated with multi-exon 3XTs (Supplementary Fig. 1l). Moreover, hnRNPk knockdown reduced the association between MTR4 and multi-exon 3XTs (Supplementary Fig. 3n). These results indicate that hnRNPk may recruit MTR4 to multi-exon 3XTs.

Comment:

5. Fig 4a, while the addition of 3XT appears to decrease the abundance of the fusion transcript, it is not clear whether that is dependent on MTR4 and/or hnRNPk. This should be addressed.

Response:

We performed qRT-PCR analysis of β -globin-fused multi-exon 3XRs in HeLa cells transfected with MTR4 and/or hnRNPk and found that the overexpression of both hnRNPk and MTR4 led to the further downregulation of β -globin mRNA. These results support the idea that the multi-exon 3XR fusion-mediated down-regulation of β -globin mRNA is dependent on both MTR4 and hnRNPk. These results are shown in Supplementary Fig. 4a and are explained on page 9, lines 207–208 of the revised manuscript.

Comment:

6. Fig 4b, why was over-expressed hnRNPk used in this RIP experiment? RIP experiment shown in Fig 3d used endogenous hnRNPk.

Response:

To address the reviewer's concern, we performed RIP analysis with an anti-hnRNPk antibody using lysates from HeLa cells and found that endogenous hnRNPk associates with β -globin-fused multi-exon 3XRs. These results are shown in Supplementary Fig. 4b and are explained on page 9, line 208 – page 10, line 212 of the revised manuscript.

Comment:

7. Extended data 4b, it is necessary to shown the amount of 3XT in the nucleus, in addition to the cytoplasm to determine whether the transcript is better exported to the cytoplasm, or if simply more transcript is present in the cells.

Response:

At the suggestion of the reviewer, we show the amount of *KCTD13* 3XT in the nucleus and in the cytoplasm. These results indicate that hnRNPk knockdown led to the accumulation of *KCTD13* 3XT in the cytoplasm. These results are shown in Supplementary Fig. 4f and are explained on page 10, lines 218–220 of the revised manuscript.

Comment:

8. Extended data 4c, it is necessary to show the implication of MTR4 or other exosome/PAXT subunits in the loss of 5BoxB transcript.

Response:

We performed a tethering assay using Flag-tagged MTR4 as well as lambdaN-HA-tagged hnRNPk and β -globin RNA fused with 5BoxB. We found that MTR4 enhances the degradation of β -globin RNA fused with 5BoxB by lambdaN-HA-tagged hnRNPk. These results are shown in Supplementary Fig. 4h and are explained on page 10, lines 224–226 of the revised manuscript.

Comment:

9. Fig 5c, what is the evidence that endogenous KCTD13 3XT forms condensates? Also, the images shown are not of very high quality. The condensates seem to be in both the cytoplasm and nucleus. The authors need to demonstrate the implication of MTR4 in formation of the condensates (endogenous or over-expressed 3XT)

Response:

We generated an anti-KCTD13 3XT protein antibody to detect endogenous KeXT bodies and then performed immunofluorescence analysis using HeLa cells transfected with siMTR4. We found that endogenous KCTD13 3XT proteins formed KeXT bodies primarily in the cytoplasm of MTR4-depleted HeLa cells. We also replaced the immunofluorescence images in Fig. 5c of the original manuscript with higher quality, high-resolution processed images. These results are shown in Figure 5c and Supplementary Fig. 5a and are explained on page 11, lines 252–258 of the revised manuscript.

Comment:

10. Extended data Fig 6, this section is not compelling. Either the authors perform additional experiments showing 3XT in brain cells or sperm, or this section should be removed. The figures as shown are intriguing correlations but don't prove anything.

Response:

At the suggestion of the reviewer, we have removed this section.

General comment:

The authors refer to 'stabilization' or 'degradation' of transcripts throughout the manuscript. While the transcripts are likely to be degraded, no experiment in the manuscript formally demonstrates it. The DRB chase experiment might suggest transcript stabilization but it could be interpreted in other ways. Therefore, it is preferable if the authors use terms such as 'accumulate', or 'increase (or decrease) in abundance', which

more correctly reflect the data shown.

Response:

At the suggestion of the reviewer, we have replaced “degradation” with “expression” in the third subtitle on page 7, line 158 of the revised manuscript. DRB chase experiments are presented in Fig. 1 and tethering assays in Fig. 4; therefore, we refer to ‘degradation’ or ‘stabilization’ in sections that include these results.

Minor points:

1. line 108, depletion is misspelt

Response:

We have replaced “delpetion” with “depletion”.

Minor points:

2. Fig 2 and extended data fig 2, please show WBs of the siRNA knock-downs, in addition to mRNA quantification.

Response:

At the suggestion of the reviewer, we have performed western blot analysis to check the knockdown efficiency. These results are shown in Fig. 2 and Supplementary Fig. 2a,e–g and are explained on page 7, lines 146–151 of the revised manuscript.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The revised version of the manuscript has been improvement. Nevertheless, several concerns remain:

1. In response to my previous Comment 1, It is still not clear how the 8043 transcripts identified. This is still a eronmously large number that is extremely rarely seen in transcriptome analysis. How many biological replicates analyzed? Has it been statistically evaluated? Additionally, there is a discrepancy in the numbers reported: 1164 transcripts are mentioned in line 112 of the current version, differing from the 1146 reported previously. Please clarify what has changed in your analysis approach.

In response to my previous Comment 2, although two additional siRNA-mediated knockdowns (KDs) have been incorporated, the old figures remain unchanged in the manuscript. This omission does not account for the new data for the revision. In addition, there is no explanation of the inconsistency between the two replicates for my previous comment.

Reviewer #2 (Remarks to the Author):

This is a revised version of a manuscript that I previously reviewed. The authors have done a very good job addressing the points I made in my previous review, as well as the issues raised by another reviewer. In particular they performed the control experiments which I thought were missing in the previous version of the manuscript (expression of mutants, fractionation controls etc).

One strength of the revised version is that the authors show that the endogenous KCTD13 resulting from misexpression with 3XT forms cellular bodies using antibodies recognizing the endogenous protein. This point was raised by reviewer1 as the previous version only used an exogenously expressed tagged protein.

There is however the same issue that I raised in the previous version of the manuscript on the Flag-tagged protein localization but this time with the data shown in Figure 5c for the endogenous protein. There is no evidence of reproducibility for formation of these punctates for the endogenous protein, and no quantification is shown to indicate the fraction of cells showing this pattern – only one cherry picked cell is shown. So the authors should provide a statistical analysis and indicate the numbers of cells analyzed and which fraction of cells show this pattern. The hexane-diol treatment should also be performed on the endogenous protein.

Similarly, the quantification of cells provided in Supplemental Figure S5 does not indicate how many cells were analyzed, only the % of cells showing this pattern. I could not find this number in the legend either so this should be provided.

A minor point is that I believe that the experiments shown in Figure S5 should be shown in the main Figure 5. The panel 5a in main figure is not essential and could be moved to supplemental data.

Finally in the abstract, the authors mention that “Our results demonstrate that RNA surveillance in human cells inhibits the formation of condensates of defective polyadenylated transcript-derived proteins.” – emphasis on the plural. I think this statement should be moderated because it has been demonstrated only for one protein, so the generality of this phenomenon is still not clear. So the abstract and rest of the paper should be moderated to clearly indicate that the ability of the aberrant proteins to aggregate in these bodies is shown for just one example.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily responded to the comments made.

Reviewer comments

Reviewer #1 (Remarks to the Author):

The revised version of the manuscript has been improvement. Nevertheless, several concerns remain:

Specific comments:

Comment:

1. In response to my previous Comment 1, It is still not clear how the 8043 transcripts identified. This is still a eronmously large number that is extremely rarely seen in transcriptome analysis. How many biological replicates analyzed? Has it been statistically evaluated? Additionally, there is a discrepancy in the numbers reported: 1164 transcripts are mentioned in line 112 of the current version, differing from the 1146 reported previously. Please clarify what has changed in your analysis approach.

Response:

We obtained long-read data from one sample each of the cells transfected with siCont, siMTR4#1, and siMTR4#2. In our previous revision, we normalized the long-read data by calculating CPM values. Since each sample size for each group is one, we did not apply statistical software such as EdgeR or EBseq for evaluation. We then applied a ≥ 1.5 -fold change cutoff to the normalized counts, comparing them to the control, and re-evaluated the number of transcripts with increased abundance under MTR4 depletion. This adjustment accounts for the potential small effects of the RNA decay system on expression levels. Next, we identified genes that overlap between different siRNAs targeting MTR4. The criteria were mentioned in the “Methods” section. The denominator in CPM calculation is the total number of reads mapped to all transcripts. To ensure consistency in the normalization of 3' seq data, we have re-normalized the 3' seq data. Specifically, we modified the CPM calculation by excluding reads that did not map to annotated transcripts from the total mapped reads in the denominator. This adjustment increased the CPM values calculated from the 3' seq data for all transcripts, as the number of filtered transcripts increased from 1146 to 1164. Importantly, this adjustment did not affect the main results and conclusions of this article.

Comment:

2. In response to my previous Comment 2, although two additional siRNA-mediated knockdowns (KDs) have been incorporated, the old figures remain unchanged in the manuscript. This omission does not account for the new data for the revision. In addition,

there is no explanation of the inconsistency between the two replicates for my previous comment.

Response:

Since the difference in the effect on siDIS3#1 and #2 could be due to the possible off-target effect of siRNA, we designed additional two siRNAs targeting DIS3 and repeated the experiment to validate the effect of DIS3 knockdown on the expression of MTR4-target 3XTs. Although the results of the four siRNAs targeting DIS3 were not completely consistent, a similar trend was observed with four siRNAs, supporting that DIS3 knockdown upregulates both MTR4-target mono- and multi-exon 3XTs. Moreover, we provided the additional figures with siDIS3#3 and #4 in Supplementary Figure 2a.

Reviewer #2 (Remarks to the Author):

This is a revised version of a manuscript that I previously reviewed. The authors have done a very good job addressing the points I made in my previous review, as well as the issues raised by another reviewer. In particular they performed the control experiments which I thought were missing in the previous version of the manuscript (expression of mutants, fractionation controls etc).

One strength of the revised version is that the authors show that the endogenous KCTD13 resulting from misexpression with 3XT forms cellular bodies using antibodies recognizing the endogenous protein. This point was raised by reviewer1 as the previous version only used an exogenously expressed tagged protein.

Specific comments:

Comment:

1. There is however the same issue that I raised in the previous version of the manuscript on the Flag-tagged protein localization but this time with the data shown in Figure 5c for the endogenous protein. There is no evidence of reproducibility for formation of these punctates for the endogenous protein, and no quantification is shown to indicate the fraction of cells showing this pattern – only one cherry picked cell is shown. So the authors should provide a statistical analysis and indicate the numbers of cells analyzed and which fraction of cells show this pattern. The hexane-diol treatment should also be performed on the endogenous protein.

Response:

We repeated the experiments and obtained images containing multiple cells under each set of conditions. Consistent with the results of Flag-tagged KCTD13-3XT protein, KeXT bodies which consisted of endogenous KCTD13 3XT under MTR4 depletion were also disrupted by treatment with 1,6-hexanediol. Moreover, we calculated the proportion of cells containing KeXT bodies. These results are shown in Fig. 5b,e and Supplementary Fig. 5b,c, and are explained on page 11, lines 255–258, and page 11, line 261 – page 12, line 272 of the revised manuscript.

Comment:

2. Similarly, the quantification of cells provided in Supplemental Figure S5 does not indicate how many cells were analyzed, only the % of cells showing this pattern. I could not find this number in the legend either so this should be provided.

Response:

We mentioned the number of cells that were analyzed in the legend.

Comment:

3. A minor point is that I believe that the experiments shown in Figure S5 should be shown in the main Figure 5. The panel 5a in main figure is not essential and could be moved to supplemental data.

Response:

At the suggestion of the reviewer, we changed the configuration of Figure 5 and S5.

Comment:

4. Finally in the abstract, the authors mention that “Our results demonstrate that RNA surveillance in human cells inhibits the formation of condensates of defective polyadenylated transcript-derived proteins.” – emphasis on the plural. I think this statement should be moderated because it has been demonstrated only for one protein, so the generality of this phenomenon is still not clear. So the abstract and rest of the paper should be moderated to clearly indicate that the ability of the aberrant proteins to aggregate in these bodies is shown for just one example.

Response:

At the suggestion of the reviewer, we have replaced “Our results demonstrate that RNA surveillance in human cells inhibits the formation of condensates of defective polyadenylated transcript-derived proteins” with “Our results suggest that RNA surveillance in human cells inhibits the formation of condensates of a defective polyadenylated transcript-derived protein”. We also have replaced “defective polyadenylated transcript-derived proteins” with “a defective polyadenylated transcript-derived protein” on page 5, line 100 of the revised manuscript.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily responded to the comments made.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The information in the responses is clear which has also been incorporated into the Methods in the revised manuscript. These responses and revisions have addressed all my remaining concerns.