EMBO reports

The tRNA-like small noncoding RNA mascRNA promotes global protein translation

Xinping Lu, Jinliang Huang, Sipeng Wu, Qian Zheng, Peipei Liu, Huimin Feng, xiaoqing Su, Haipeng Fu, Qiaoran Xi, and Geng Wang
DOI: 10.15252/embr.201949684

Corresponding author(s): Geng Wang (wangengfuan@163.com)

Review Timeline:

Submission Date:	14th Nov 19
Editorial Decision:	29th Nov 19
Revision Received:	14th Jul 20
Editorial Decision:	5th Aug 20
Revision Received:	16th Sep 20
Accepted:	21st Sep 20

Editor: Esther Schnapp

Transaction Report:

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

1st Editorial Decision 29th Nov 2019

Dear Dr. Wang,

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is pasted below.

As you will see, all referees acknowledge that the findings are potentially interesting. However, they also point out that significant revisions will be required before the study can be considered for publication here. Most important, it needs to be clarified whether QARS has a causal role in the effect of mascRNA on protein synthesis. It also needs to be demonstrated that endogenous QARS interacts with mascRNA, and it would strengthen the study if further data could be provided on how mascRNA promotes QARS protein levels. The referees further ask for several controls and explanations that need to be provided.

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

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further.

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

Please note that the EMBO reports reference style is numbered, this must be corrected.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) Your manuscript contains statistics and error bars based on n=2 or on technical replicates. Please use scatter blots in these cases. No error bars can be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

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

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

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

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

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

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

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

8) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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

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

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

Lu and colleagues address the function of mascRNA, a tRNA-like RNA processed from the MALAT1 long noncoding RNA. Using biotinylated mascRNA probes, the authors show that this transcript interacts with aminoacyl tRNA synthetases, in particular QARS. This interaction appears to promote global protein synthesis, although some of the details are still unclear at this point. Regardless, this manuscript represents an important advance as no function for mascRNA has been known. I suggest a number of experiments below to clarify the data interpretation and provide important controls.

(1) Data quality is low in Figure 1. In Part A, the gel shift band is largely missing in the "Cyto 2x" lane for unclear reasons. In Part B, multiple bands are observed for mascRNA rather than a single species. All of these gels should be repeated to improve quality.

(2) For all Northern blots, please add size markers.

(3) Figure 2D: Based on the data in Figure 2A, I was very surprised that only QARS was able to IP mascRNA. Does this mean that over-expressed QARS does not associate with the MSC? I would have expected multiple AARSs to be able to pull down mascRNA (at least to some degree) based on the data in Fig 2A. This makes me question the biological relevance of the overexpression cell lines. To address this point, the authors should use an antibody against endogenous QARS and prove that the endogenous QARS protein (when expressed at endogenous levels) interacts with mascRNA.

(4) Figure 3: Are the identity elements that dictate tRNA-Gln aminoacylation known? If yes, they should be mentioned.

(5) Figure 4A: The authors need to show Northern blots to prove that their mascRNA

overexpression constructs produce fully processed mascRNA and no other intermediates, improper products, etc.

(6) For all the qPCR on mascRNA, the primers that are used are able to detect both mascRNA and long polyadenylated isoforms of MALAT1. The authors' current approach therefore can not adequately distinguish between these very different RNAs. I recommend all qPCR on mascRNA to be removed from the manuscript and only Northern blots be used.

(7) Figure 4C: When mascRNA is transfected into cells, what is the level of mascRNA overexpression obtained? Also, please clarify how the authors purified the in vitro transcribed RNA and if they ensured that a 5' monophosophate was present on the end rather than the triphosphate that would be present from in vitro transcription.

(8) Figure 6: What is the effect of QARS overexpression on protein synthesis in MALAT1 or mascRNA knockout cells? This would help clarify the underlying molecular mechanism.

(9) The details of how mascRNA promotes QARS protein levels on the molecular level are very unclear at the moment. I do not expect the authors to define all the molecular details in an initial paper, but some more mechanism would be very helpful here.

(10) Does the MEN b tRNA-like small RNA also interact with QARS?

Other suggestions:

(1) Page 3: "other regulatory ncRNAs such as long noncoding RNA (lncRNA) and Piwi-associated RNAs have not been well studied". I would argue that much is known about many of these RNAs, especially MALAT1 so I would not say that they have not been well studied.

(2) Page 4, Line 3: Please cite Sunwoo et al. 2009 Genome Research. This paper first identified the MEN b tRNA like small RNA.

(3) Page 5: Please mention Figure 1B in the main text.

(4) Figure EV2: Please denote the expected sizes of each AARS in the legend.

(5) As currently drawn, Figure 3 spans multiple pages so I suggest moving the acceptor stem data to the supplemental material so that all the relevant data can be displayed on a single page in the main text.

(6) Page 7: Please provide a citation for the "previous findings that mascRNA is not aminoacylated in HeLa cells even thought it also has CCA addition."

(7) Figure EV5: mascRNA overexpression is only 2 fold, unlike the 3 fold observed in Figure 4. Please explain why these data are not consistent with one another.

Referee #2:

Summary

mascRNA is a short ncRNA, generated as a processing product of MALAT1. In this manuscript, the

authors identified that mascRNA interacts with the aminacyl tRNA synthetase of tRNAGln (QARS). They further tested the resemblance in recognition by QARS to the recognition of tRNAGln, and suggest different interaction mode. Furthermore, the tested the possible role of the interaction between QARS and mascRNA, and found an interesting role in regulation of QARS protein levels and consequently total protein synthesis in the cell. Overall, the results of this work are of broad interest and novel: interactions between tRNA-like RNAs and proteins are emerging as abundant mode of cellular regulation. The majority of the manuscript characterize the molecular interactions between mascRNA and QARS and while needs some additional work (see below) is very interesting and coherent. The last Figure (figure 7) attempts to provide physiological relevance, yet in my opinion is very preliminary.

Below are detailed comments on the work:

Major points:

1. The physical interaction between mascRNA and QARS should be strengthen. In particular, the complex observed in EMSA need further explanation: 1. was the RNA denatured or structured? i.e. does the data support recognition of a structural element? 2. What is the protocol for cytosol and post nuclear membranes (PNM) preparation, and specifically does QARS maintained in the MSC or act independently under these conditions. 3. What is the signal on the PNM? Alternative MSC complex, that contains QARS, and resides in membranes? 4. Please do a supershift with antiQRS to show that it includes QARS. 5. Please do competition EMSA with tRNAGln, to support the differences in affinity claimed in the text. 6. Please do EMSA with representative mutants, to confirm loss of interaction. 1 to 3 should be clarified in text, while for 4 -6 experiments should be made.

2. Expression regulation of QARS: the authors claim that QARS levels are increased through reduced degradation. Both conclusions (i.e. the increase QARS levels and the decreased degradation) are derived from a biotinylation assay upon transfection/overexpression. I think that these conclusions need support by another experimental approach as they are very novel and one of the strongest points of the manuscript. Also, Fig. 5 indicates data regarding impact on QARS degradation, yet effect on protein synthesis (translation) is not excluded.

3. The authors claim that QARS increase leads to a general increase in translation (Fig.6). Yet, to pinpoint the involvement of mascRNA in this process the authors need to do the exp. in Fig 6 D-F also with mutants of mascRNA, that are not bound by QARS. These are expected not to interfere with increased general translation.

4. Physiological impact (Figure 7). The claimed physiological impact seems very preliminary (overexpression and a single assay). While I do not find it essential to expand this, it is necessary to show that the effect is mediated through QARS, or by other pathways that mascRNA is involved in. For example, by showing that the impact is lost when the antiMut are used.

Minor points

1. Figure 2: how do the authors explain the discrepancy in EPRS results: in C it is pulled down by the mascRNA while the reciprocal in E does not occur.

2. Figure 3: it will be helpful to include the structure of tRNAGln near the mascRNA structure (Fig. EV3) and also indicate important identity elements (for recognition by QARS).

Referee #3:

In the current manuscript, Xinping Lu and colleagues investigated the role of the MALAT1-derived

small cytoplasmic RNA (mascRNA). First, the authors show that mascRNA associates with proteins, especially subunits of the multi-tRNA synthetase complex. A detailed analysis revealed a direct interaction between mascRNA and QARS. This interaction seems to stabilize QARS protein which might be the cause for the observed increase in global protein synthesis rates seen after mascRNA overexpression. Finally, the author show that mascRNA levels dependent on FBS starvation and differentiation, i.e. cell proliferation, and mascRNA overexpression enhances colony formation in three different cell lines.
In general, this is a well-written and easy to follow study and most experiments are well controlled. The molecular function of mascRNA is largely unknown and this study suggests an interesting mode of action. However, I have some concerns that the authors should address:

Major

1) In general, an effect of mascRNA overexpression on global protein synthesis was identified and the authors claim that this is due to increased QARS expression. However, the experiments shown in Figures 5 and 6 do not fully support this model. The authors should reduce QARS expression while simultaneously overexpress mascRNA. Does the global protein synthesis rate normalize compared to mascRNA overexpression alone and untransfected cells?
Also, do the authors observe a dose-dependent effect of mascRNA on global translation, polysome profiles, and QARS levels? Are there higher levels of aminoacylated tRNAGlnUUG in mascRNA overexpressing cells?

2) The authors show that mascRNA overexpression increases cell proliferation and argue that this is due to increased protein synthesis mediated by increased QARS levels. Again, authors should overexpress mascRNA and simultaneously deplete QARS to show a causal connection. Furthermore, it would be highly interesting to analyse the proteome of mascRNA overexpressing cells to identify those proteins whose translation rates are most sensitive to mascRNA expression. These proteins could be involved in regulating cell proliferation.

3) In Figure 7, the authors show that serum starvation or cell differentiation reduce mascRNA levels, probably due to a reduced cell proliferation. However, the authors do not present evidence for a reduced proliferation rate, e.g. cell cycle profiles. Also, the mechanism of mascRNA downregulation remains unclear. Is this due to a reduced transcription of MALAT1? Or a reduced processing of MALAT1 by RNase P and Z? Or due to a reduced mascRNA stability?

4) The authors observed an increased global protein translation, yet also an increased turnover (Fig.4). So, the net amount is unchanged? Have the authors analysed total protein levels between mascRNA overexpressing cells and control cells? How do the authors explain the increased turnover? If total protein levels remain unchanged, how does this fit into the model of increased translation driving proliferation?

Minor

a) In Figure 1: Please add a size marker. Also, why is there no shift for 5S rRNA? Please introduce "Figure 1B" in the main text.

b) In Figure 3E: Please shift the labels to the right to match lanes better.

c) Figure 4: Please show a Northern Blot to confirm correct size of mascRNA after its

overexpression. Is it CCA-modified?

d) MascRNA can be overexpressed using an artificial precursor, e.g. GFP-mascRNA (see Gutschner et al., 2011; Wilusz et al., 2012). These constructs might be more suitable for mascRNA overexpression due to the correct processing steps.

e) The controls used in mascRNA overexpression experiments (scramble, or GFP fragment) are not ideal. Why did the authors refrain from using mascRNA antisense which they have used in the binding and interaction experiments and which seems to be a perfect control due to the lack of QARS binding?

f) MCF-7 cells have not been mentioned in the Experimental Procedures section.

July 14, 2020

Esther Schnapp, PhD
Senior Editor
EMBO reports

Re: Cover letter for revised manuscript EMBOR-2019-49684-T

Dear Dr. Schnapp:

Because of the Covid-19 situation and restriction on traveling, the revision took much longer than expected. We would like to apologize for the delay and thank you and the referees for your patience.

We were excited to obtain encouraging feedback from you and the three expert referees of our manuscript, entitled "The tRNA-like small noncoding RNA mascRNA promotes global protein translation". We have carefully and fully considered each referee's comment and provide definitive experiments to appropriately address the technical issues raised. We believe these insightful comments have led to a markedly strengthened manuscript, with the changes we have made detailed below.

Referee #1:

Comment 1: Lu and colleagues address the function of mascRNA, a tRNA-like RNA processed from the MALAT1 long noncoding RNA. Using biotinylated mascRNA probes, the authors show that this transcript interacts with aminoacyl tRNA synthetases, in particular QARS. This interaction appears to promote global protein synthesis, although some of the details are still unclear at this point. Regardless, this manuscript represents an important advance as no function for mascRNA has been known. I suggest a number of experiments below to clarify the data interpretation and provide important controls.
Response 1: We thank referee 1 for these and other summary comments.

Comment 2: (1) Data quality is low in Figure 1. In Part A, the gel shift band is largely missing in the "Cyto 2x" lane for unclear reasons. In Part B, multiple bands are observed for mascRNA rather than a single species. All of these gels should be repeated to improve quality.
Response 2: We have replaced Fig 1 with new data. The weak "Cyto 2x" bands in the old figure might be due to RNase activities in the cytosol. We have since added RNase inhibitors to the reactions and resolved the problem. Since mascRNA is a small RNA, a small shift or expansion of the gel during the transferring step makes the bands look like multiple bands, hence the low quality in old Fig 1B.

Comment 3: (2) For all Northern blots, please add size markers.
Response 3: Size markers have been added to all the Northern blots.

Comment 4: (3) Figure 2D: Based on the data in Figure 2A, I was very surprised that only QARS was able to IP mascRNA. Does this mean that over-expressed QARS does not associate with the MSC? I would have expected multiple AARSs to be able to pull down mascRNA (at least to some degree) based on the data in Fig 2A. This makes me question the biological relevance of the overexpression cell lines. To address this point, the authors should use an antibody against endogenous QARS and prove that the endogenous QARS protein (when expressed at endogenous levels) interacts with mascRNA.
Response 4: The level of overexpressed QARS is much higher than that of the endogenous QARS. Most over-expressed QARS does not associate with MSC, and only a small portion of MSC components can be co-immunoprecipitated with anti-FLAG, as shown by coomassie staining of FLAG pulldown samples (Fig EV2B). To examine whether endogenous QARS interacts with mascRNA, we performed RIP using antibodies against QARS/EPRS/GARS. The results show that both QARS and EPRS are able to pull down mascRNA, but not GARS (Fig 2F). EPRS and QARS are in the same complex, which explains why EPRS can pull down mascRNA. RIP using overexpressed proteins suggests a more direct interaction between QARS and mascRNA than the other MSC components.

Comment 5: (4) Figure 3: Are the identity elements that dictate tRNA-Gln aminoacylation known? If yes, they should be mentioned.
Response 5: Anticodon and acceptor stem nucleotides are major identity elements of *E. Coli* tRNA-Gln (Jahn and Roger, Nature, 1991). Reported tRNA-Gln identity elements in *E. coli* include U1:A72, G2:C71, G3:C70, G5, U6, A7, G10, C34, U35, G36, A37, and U38 (Perona, et al., Science, 1989; Ibba, et al., PNAS, 1996; Hayase, EMBO J, 1992). Identity elements of tRNA-Gln in human cells, however, have not been reported. Human tRNA-Gln sequences is quite different from those of *E. Coli* tRNA-Gln, and most tRNA-Gln identity nucleotides in *E. coli* are not conserved in human tRNAs.
Human tRNA sequences (obtained from GtRNAdb):

Gln tRNAs

```
>>>>>>>..>>>>..........<<<<.>>>>>.......<<<<<................>>>>>.......<<<<<<<<<<<<....
GGTTCCATGGTGTAAT.GGTgA.GCACTCTGGACTCTGAATCCAGCG...........A.TCCGAGTTCGAGTCTCGGTGGAACCT... tRNA-Gln-CTG-3-1 Sc: 71.9
GGTTCCATGGTGTAAT.GGTgA.GCACTCTGGACTCTGAATCCAGCG...........A.TCCGAGTTCGAGTCTCGGTGGAACCT... tRNA-Gln-CTG-3-2 Sc: 71.9
GGTTCCATGGTGTAAT.GGTaA.GCACTCTGGACTCTGAATCCAGCG...........A.TCCGAGTTCGAGTCTCGGTGGAACCT... tRNA-Gln-CTG-4-1 Sc: 71.9
GGTTCCATGGTGTAAT.GGTaA.GCACTCTGGACTCTGAATCCAGCG...........A.TCCGAGTTCGAGTCTCGGTGGAACCT... tRNA-Gln-CTG-4-2 Sc: 71.9
GGTTCCATGGTGTAAT.GGTtA.GCACTCTGGACTCTGAATCCAGCG...........A.TCCGAGTTCAAATCTCGGTGGAACCT... tRNA-Gln-CTG-1-1 Sc: 71.5
GGTTCCATGGTGTAAT.GGTtA.GCACTCTGGACTCTGAATCCAGCG...........A.TCCGAGTTCAAATCTCGGTGGAACCT... tRNA-Gln-CTG-1-2 Sc: 71.5
GGTTCCATGGTGTAAT.GGTtA.GCACTCTGGACTCTGAATCCAGCG...........A.TCCGAGTTCAAATCTCGGTGGAACCT... tRNA-Gln-CTG-1-3 Sc: 71.5
GGTTCCATGGTGTAAT.GGTtA.GCACTCTGGACTCTGAATCCAGCG...........A.TCCGAGTTCAAATCTCGGTGGAACCT... tRNA-Gln-CTG-1-4 Sc: 71.5
GGTTCCATGGTGTAAT.GGTtA.GCACTCTGGACTCTGAATCCAGCG...........A.TCCGAGTTCAAATCTCGGTGGAACCT... tRNA-Gln-CTG-1-5 Sc: 71.5
GGTTCCATGGTGTAAT.GGTtA.GCACTCTGGACTCTGAATCCAGCG...........A.TCCGAGTTCAAGTCTCGGTGGAACCT... tRNA-Gln-CTG-2-1 Sc: 70.9
GGTTCCATGGTGTAAT.GGTtA.GCACTCTGGACTCTGAATCCGGTA...........A.TCCGAGTTCAAATCTCGGTGGAACCT... tRNA-Gln-CTG-5-1 Sc: 66.2
>>.>>>>..>>>>..........<<<<.>>>>>.......<<<<<................>>>>>.......<<<<<<<<<.<<....
GGCCCCATGGTGTAAT.GGTcA.GCACTCTGGACTCTGAATCCAGCG...........A.TCCGAGTTCAAATCTCGGTGGGACCC... tRNA-Gln-CTG-6-1 Sc: 64.7
>>>>>>>..>>>>..........<<<<.>>>>>.......<<<<<................>.>>>.......<<<.<<<<<<<<....
GGTTCCATGGTGTAAT.GGTaA.GCACTCTGGACTCTGAATCCAGCC...........A.TCTGAGTTCGAGTCTCTGTGGAACCT... tRNA-Gln-CTG-7-1 Sc: 58.5
>>>>>>>..>>>>..........<<<<.>>>>>.......<<<<<................>>>>>.......<<<<<<<<<<<<....
GGTTCCATGGTGTAAT.GGTtA.GCACTCTGGACTCTGAATCCAGCG...........A.TCCGAGTTCAAATCTCGGTGGAACCT... DQ9990_CTG_HUMAN_CY
>>>>>>>..>>>>..........<<<<.>>>>>.......<<<<<................>>>>>.......<<<<<<<<<<<<....
GGTCCCATGGTGTAAT.GGTtA.GCACTCTGGACTTTGAATCCAGCG...........A.TCCGAGTTCAAATCTCGGTGGGACCT... tRNA-Gln-TTG-1-1 Sc: 72.6
GGTCCCATGGTGTAAT.GGTtA.GCACTCTGGACTTTGAATCCAGCA...........A.TCCGAGTTCGAATCTCGGTGGGACCT... tRNA-Gln-TTG-2-1 Sc: 70.0
>>.>>>>..>>>>..........<<<<.>>>>>.......<<<<<................>>>>>.......<<<<<<<<<.<<....
GGCCCCATGGTGTAAT.GGTtA.GCACTCTGGACTTTGAATCCAGCG...........A.TCCGAGTTCAAATCTCGGTGGGACCT... tRNA-Gln-TTG-3-1 Sc: 66.9
GGCCCCATGGTGTAAT.GGTtA.GCACTCTGGACTTTGAATCCAGCG...........A.TCCGAGTTCAAATCTCGGTGGGACCT... tRNA-Gln-TTG-3-2 Sc: 66.9
GGCCCCATGGTGTAAT.GGTtA.GCACTCTGGACTTTGAATCCAGCG...........A.TCCGAGTTCAAATCTCGGTGGGACCT... tRNA-Gln-TTG-3-3 Sc: 66.9
>>>>>>>..>>>>..........<<<<.>>>>>.......<<<<<................>>>>>.......<<<<<<<<<<<<....
GGTCCCATGGTGTAAT.GGTtA.GCACTCTGGCTTTGAATCCAGCA...........A.TCCGAGTTCGAATCTTGGTGGGACCT... tRNA-Gln-TTG-4-1 Sc: 58.1
GGTCCCATGGTGTAAT.GGTtA.GCACTCTGGACTTTGAATCCAGCG...........A.TCCGAGTTCAAATCTCGGTGGGACCT... DQ9992_TTG_HUMAN_CY
GGCCCCATGGTGTAAT.GGTtA.GCACTCTGGACTNTGAATCCAGCG...........A.TCCGAGTTCAAATCTCGGTGGGACCTcca RQ9990_NTG_HUMAN_CY
>>.>>>>..>>>>..........<<<<.>>>>>.......<<<<<................>>>>>.......<<<<<<<<<.<<.... RQ9990_NTG_HUMAN_CY
GGTCCCATGGTGTAAT.GGTtA.GCACTCTGGACTNTGAATCCAGCG...........A.TCCGAGTTCAAATCTCGGTGGGACCTcca RQ9991_NTG_HUMAN_CY
>>>>>>>..>>>>..........<<<<.>>>>>.......<<<<<................>>>>>.......<<<<<<<<<<<<.... RQ9991_NTG_HUMAN_CY
```

Comment 6: (5) Figure 4A: The authors need to show Northern blots to prove that their mascRNA overexpression constructs produce fully processed mascRNA and no other intermediates, improper products, etc.

Response 6: Northern blots of mascRNA overexpressing and control samples have been added (Fig 4A, 4E, 4H and 6M). mascRNA overexpressing constructs produce fully processed mascRNA and no other intermediates.

Comment 7: (6) For all the qPCR on mascRNA, the primers that are used are able to detect both mascRNA and long polyadenylated isoforms of MALAT1. The authors' current approach therefore can not adequately distinguish between these very different RNAs. I recommend all qPCR on mascRNA to be removed from the manuscript and only Northern blots be used.

Response 7: *MALAT1* is retained in the nucleus while mascRNA is localized exclusively in the cytoplasm (Wilusz, et al., Cell, 2008). One study reported translocation of *MALAT1* from the nucleus to the cytoplasm in the G2/M cell cycle phase, which, however, requires further validation as *U6* snRNA was also found in the cytoplasm in their experiments (Yang, et al., FEBS Lett, 2013). RNA templates used for qPCR analysis of mascRNA were extracted from the cytoplasm.

We agree with the referee that Northern blotting is a better approach to look at mascRNA levels, and have added northern blots of mascRNA (Fig 4A, 4E, 4H and 6M).

Comment 8: (7) Figure 4C: When mascRNA is transfected into cells, what is the level of mascRNA overexpression obtained? Also, please clarify how the authors purified the in vitro transcribed RNA and if they ensured that a 5' monophosophate was present on the end rather than the triphosphate that would be present from in vitro transcription.

Response 8: The northern blots of mascRNA indicate that after mascRNA

transfection, mascRNA levels increased to about 3 folds of the endogenous levels (Fig 4A, 4H and 6M).

In vitro transcribed RNAs were treated with RNA 5′ Pyrophosphohydrolase (RppH) (NEB) to remove pyrophosphate from the RNAs, and then purified by ethanol precipitation. We have added the description in the Experimental Procedures section.

Comment 9: (8) Figure 6: What is the effect of QARS overexpression on protein synthesis in MALAT1 or mascRNA knockout cells? This would help clarify the underlying molecular mechanism.
Response 9: We have constructed *MALAT1* knockdown cell lines and found a reduction in both *MALAT1* and mascRNA levels (Fig 5E and 5F). QARS protein levels are also slightly decreased (Fig 5G and 5H). *MALAT1* knockdown leads to a decrease in global protein translation, and overexpression of QARS fully reverses the reduction (Fig 6J-L). These results indicate that mascRNA regulation of protein translation is dependent on QARS.

Comment 10: (9) The details of how mascRNA promotes QARS protein levels on the molecular level are very unclear at the moment. I do not expect the authors to define all the molecular details in an initial paper, but some more mechanism would be very helpful here.
Response 10: We have shown that mascRNA overexpression slows down QARS protein degradation (Fig 5J-L). To better understand the mechanism, we overexpressed mascRNA mutants and found that the physical interaction between mascRNA and QARS is essential for the effect on QARS levels. mascRNA mutants deficient in QARS binding lost their capacity to promote QARS stability and global protein translation, while the QARS binding mutants retain the activities (Fig 3G, 4H-J, 5C, and 5D). We have also obtained cell lines overexpressing mascRNA at different levels by colony selection. QARS protein levels increase in cells expressing higher levels of mascRNA (Fig EV4), indicating a dose-dependent effect of mascRNA on QARS protein levels.

Comment 11: (10) Does the MEN b tRNA-like small RNA also interact with QARS?
Response 11: We have performed RNA pulldown using biotinylated *MEN b* tRNA-like small RNA and found no evidence of menRNA interacting with QARS (Response Fig 1). We have shown that the anticodon stem-loop sequence of QARS is important for the interaction between mascRNA and QARS (Fig 3D-F). The anticodon stem-loop of menRNA is very different from that of mascRNA, which may explain its lack of interaction with QARS.

Comment 12: Other suggestions: (1) Page 3: "other regulatory ncRNAs such as long noncoding RNA (lncRNA) and Piwi-associated RNAs have not been well studied". I would argue that much is known about many of these RNAs, especially MALAT1 so I would not say that they have not been well studied.

Response 12: We thank the referee for the suggestion and have revised the sentence to "Only a small portion of other regulatory ncRNA have been well studied" (Page 3, Line 5-6).

Comment 13: (2) Page 4, Line 3: Please cite Sunwoo et al. 2009 Genome Research. This paper first identified the MEN b tRNA like small RNA.
Response 13: We thank the referee for the suggestion and have added the citation (Page 3, Line 28).

Comment 14: (3) Page 5: Please mention Figure 1B in the main text.
Response 14: We have added Fig 1B in the main text (Page 5, Line 12).

Comment 15: (4) Figure EV2: Please denote the expected sizes of each AARS in the legend.
Response 15: We have added the expected sizes of each AARS in the legend.

Comment 16: (5) As currently drawn, Figure 3 spans multiple pages so I suggest moving the acceptor stem data to the supplemental material so that all the relevant data can be displayed on a single page in the main text.
Response 16: Only five EV figures are allowed for each article. Moving the acceptor stem data to a new EV figure would make the number of EV figures exceed the limit.

Comment 17: (6) Page 7: Please provide a citation for the "previous findings that mascRNA is not aminoacylated in HeLa cells even though it also has CCA addition."
Response 17: The citation has been added (Page 8, Line 9).

Comment 18: (7) Figure EV5: mascRNA overexpression is only 2 fold, unlike the 3 fold observed in Figure 4. Please explain why these data are not consistent with one another.
Response 18: In the old Figure EV5, mascRNA levels in control cells were set as 0.5, and the relative mascRNA levels in overexpression was ~1.7, roughly 3 folds of the levels in control cells. We apologize for the confusion, and have redrawn the graph, setting the control levels as 1 (New Fig EV3E).

Referee #2:

Comment 1: Summary
mascRNA is a short ncRNA, generated as a processing product of MALAT1. In this manuscript, the authors identified that mascRNA interacts with the aminacyl tRNA synthetase of tRNAGln (QARS). They further tested the resemblance in recognition by QARS to the recognition of tRNAGln, and suggest different

interaction mode. Furthermore, the tested the possible role of the interaction between QARS and mascRNA, and found an interesting role in regulation of QARS protein levels and consequently total protein synthesis in the cell. Overall, the results of this work are of broad interest and novel: interactions between tRNA-like RNAs and proteins are emerging as abundant mode of cellular regulation. The majority of the manuscript characterize the molecular interactions between mascRNA and QARS and while needs some additional work (see below) is very interesting and coherent. The last Figure (figure 7) attempts to provide physiological relevance, yet in my opinion is very preliminary.
Below are detailed comments on the work:
Response 1: We thank referee 2 for these and other summary comments.

Comment 2: Major points: 1. The physical interaction between mascRNA and QARS should be strengthen. In particular, the complex observed in EMSA need further explanation: 1. was the RNA denatured or structured? i.e. does the data support recognition of a structural element? 2. What is the protocol for cytosol and post nuclear membranes (PNM) preparation, and specifically does QARS maintained in the MSC or act independently under these conditions. 3. What is the signal on the PNM? Alternative MSC complex, that contains QARS, and resides in membranes? 4. Please do a supershift with antiQRS to show that it includes QARS. 5. Please do competitive EMSA with tRNAGln, to support the differences in affinity claimed in the text. 6. Please do EMSA with representative mutants, to confirm loss of interaction. 1 to 3 should be clarified in text, while for 4 -6 experiments should be made.
Response 2: 1. For EMSA studies, RNAs were first incubated at 65 ℃ for 10min, and then cooled down slowly to room temperature. Since mascRNA has a very stable tRNA-like structure, we expect it to assume a structured form.
2. The detailed protocol for preparing the cytosol and the post nuclear membranes (PNM) has been added to the Experimental Procedures section (Page 16, Line 30-Page 17, Line 11). We have shown that endogenous QARS interacts with mascRNA, and that EPRS also co-immunoprecipitates QARS and mascRNA (Fig 2F). In addition, overexpressed QARS that is not in the MSC complex also interacts with mascRNA (Fig 2D and 2E). These results suggest that both complex-forming QARS and free QARS are capable of interacting with mascRNA.
3. We have replaced Fig 1 with new data at the request of Referee 1. After optimizing our experiments, and with more thorough wash of PNM, the gel-shift band was no longer observed with the PNM lysates. The old signal might be due to non-specific binding. Since there seem to be some faint lower gel-shift bands with the cytosol, we agree with the referee that an alternative interaction with a preference for weak membrane binding might exist, which could be an interesting project for future studies.
4. We thank the referee for the suggestion and have performed EMSA with an anti-QARS antibody and observed a supershift band, indicating the existence of QARS in the shifts (Fig 2G).

5. We thank the referee for the suggestion and have performed competition EMSA with tRNA-Gln, and the shifts were not affected with 100 folds or 1000 folds of unlabeled tRNA-Gln (Fig EV3A).
6. We have performed EMSA with mascRNA mutants and have shown that anticodon stem-loop but not the acceptor stem is required for mascRNA-QARS interaction (Fig 3G).

Comment 3: 2. Expression regulation of QARS: the authors claim that QARS levels are increased through reduced degradation. Both conclusions (i.e. the increase QARS levels and the decreased degradation) are derived from a biotinylation assay upon transfection/overexpression. I think that these conclusions need support by another experimental approach as they are very novel and one of the strongest points of the manuscript. Also, Fig. 5 indicates data regarding impact on QARS degradation, yet effect on protein synthesis (translation) is not excluded.
Response 3: The increase of QARS protein levels in response to mascRNA overexpression is determined by western blotting of cytosolic proteins (Fig 5A-H), while reduced degradation is analyzed by in vivo protein degradation assay (Fig 5J-L). Biotinylation assays were used for analyzing global protein synthesis (Fig 4C, 4F, 4I, 6B, 6E, 6H, 6K and 6O) and degradation (Fig 4K). For QARS degradation assay, emetine and cycloheximide were used to block protein synthesis (Fig 5J-L).
We have also done more experiments to understand the mechanism of this effect. Our new data show that the effect of mascRNA on QARS protein levels relies on their interaction and is dose-dependent (Fig 3G, 4H-J, 5C, 6G-I, and EV4).

Comment 4: 3. The authors claim that QARS increase leads to a general increase in translation (Fig.6). Yet, to pinpoint the involvement of mascRNA in this process the authors need to do the exp. in Fig 6 D-F also with mutants of mascRNA, that are not bound by QARS. These are expected not to interfere with increased general translation.
Response 4: We thank the referee for the suggestion and have done the suggested experiments. We have overexpressed Anti-Mut1 that does not interact with QARS, which was confirmed by EMSA experiment (Fig 3G). Anti-Mut1 overexpression has no effect on global protein synthesis (Fig 4H-J), and overexpression of QARS on Anti-Mut1 overexpression background can increase translation, unlike on mascRNA overexpression background (Fig 6G-I). Therefore, mascRNA mutants without QARS-interacting activity do not affect QARS functions on global protein translation.

Comment 5: 4. Physiological impact (Figure 7). The claimed physiological impact seems very preliminary (overexpression and a single assay). While I do not find it essential to expand this, it is necessary to show that the effect is mediated through QARS, or by other pathways that mascRNA is involved in. For example,

by showing that the impact is lost when the antiMut are used.

Response 5: We thank the referee for the suggestion and have done the suggested experiments. Unlike wild-type mascRNA and the mutants that still binds QARS, the mutants that do not interact with QARS do not promote cell proliferaction (Fig 7E and EV5B).

In addition, we have shown that QARS knockdown has a negative impact on cell proliferation and that overexpression of mascRNA does not fully rescue it (Fig 7F and EV5C). We have also constructed *MALAT1* knockdown cell lines (Fig 5E and 5F), and have shown that overexpression of QARS but not GARS partially reverses the negative effect on cell proliferation (Fig 7G and EV5D). Partial but not full rescue is probably due to mascRNA-unrelated *MALAT1* functions in cell proliferation.

Comment 6: Minor points 1. Figure 2: how do the authors explain the discrepancy in EPRS results: in C it is pulled down by the mascRNA while the reciprocal in E does not occur.

Response 6: The main reason is that majority of overexpressed proteins are not incorporated into MSC complex. Detailed explanation please refer to Response 4 to Referee 1.

Comment 1: 2. Figure 3: it will be helpful to include the structure of tRNAGln near the mascRNA structure (Fig. EV3) and also indicate important identity elements (for recognition by QARS).

Response 1: The structure has been added (Fig 3H). For explanation on identity elements please refer to Response 5 to Referee 1.

Referee #3:

Comment 1: In the current manuscript, Xinping Lu and colleagues investigated the role of the MALAT1-derived small cytoplasmic RNA (mascRNA). First, the authors show that mascRNA associates with proteins, especially subunits of the multi-tRNA synthetase complex. A detailed analysis revealed a direct interaction between mascRNA and QARS. This interaction seems to stabilize QARS protein which might be the cause for the observed increase in global protein synthesis rates seen after mascRNA overexpression. Finally, the author show that mascRNA levels dependent on FBS starvation and differentiation, i.e. cell proliferation, and mascRNA overexpression enhances colony formation in three different cell lines.

In general, this is a well-written and easy to follow study and most experiments are well controlled.

The molecular function of mascRNA is largely unknown and this study suggests an interesting mode of action. However, I have some concerns that the authors should address:

Response 1: We thank referee 3 for these and other summary comments.

Comment 2: Major 1) In general, an effect of mascRNA overexpression on global protein synthesis was identified and the authors claim that this is due to increased QARS expression. However, the experiments shown in Figures 5 and 6 do not fully support this model. The authors should reduce QARS expression while simultaneously overexpress mascRNA. Does the global protein synthesis rate normalize compared to mascRNA overexpression alone and untransfected cells?
Also, do the authors observe a dose-dependent effect of mascRNA on global translation, polysome profiles, and QARS levels? Are there higher levels of aminoacylated tRNAGlnUUG in mascRNA overexpressing cells?
Response 2: We thank the referees for the suggestions. We have constructed QARS and GARS knockdown cell lines. Knockdowns of QARS and GARS both significantly decrease global protein translation (Fig 6M-P). Simultaneous overexpression of mascRNA partially reverses the negative effect of QARS knockdown but not GARS knockdown. A partial but not full rescue is because mascRNA overexpression does not fully restore QARS protein levels (Fig 6N). In addition, we have also shown that QARS overexpression fully reverses the effect of *MALAT1* knockdown on protein synthesis (Fig 6J-L). Therefore, mascRNA's effects on global protein translation are dependent on its interaction with QARS.

We have also shown a dose-dependent effect of mascRNA on QARS protein levels (Fig EV4). Global protein synthesis also increases in response to the increase of mascRNA and QARS levels. However, the global protein synthesis levels do not continuously go up with the increase of mascRNA and QARS levels, but quickly reach a plateau, consistent with results in Fig 6D-F, suggesting a saturation point for QARS-regulated translation enhancement.

According to a high-throughput sequencing study, most cytosolic tRNAs are close to fully charged, and more than 90% of $tRNA^{Gln}_{UUG}$ is charged (Evans, et al., Nucleic Acids Research, 2017). So, it is technically challenging to detect the very small changes of $tRNA^{Gln}_{UUG}$ aminoacylation levels. As shown in Fig EV3D, our tRNA aminoacylation detection experiments failed to detect uncharged tRNAs under acidic conditions.

Comment 3: 2) The authors show that mascRNA overexpression increases cell proliferation and argue that this is due to increased protein synthesis mediated by increased QARS levels. Again, authors should overexpress mascRNA and simultaneously deplete QARS to show a causal connection. Furthermore, it would be highly interesting to analyse the proteome of mascRNA overexpressing cells to identify those proteins whose translation rates are most sensitive to mascRNA expression. These proteins could be involved in regulating cell proliferation.
Response 3: We thank the referee for the suggestion and have constructed QARS and GARS knockdown cell lines. Knockdowns of QARS and GARS both significantly decrease global protein translation (Fig 6M-P). Simultaneous overexpression of mascRNA partially reverses the negative effect of QARS

knockdown but not GARS knockdown. A partial but not full rescue is because mascRNA overexpression does not fully restore QARS protein levels (Fig 6N). In addition, we have also shown that QARS overexpression fully reverses the effect of *MALAT1* knockdown on protein synthesis (Fig 6J-L). Therefore, mascRNA's effects on global protein translation are dependent on its interaction with QARS.

We agree with the referee that it would be interesting to analyze the proteome of mascRNA overexpressing cells. It would be of great help to finding other mechanisms of mascRNA functions on cell proliferation and understanding other biological functions of mascRNA, which will be projects of future studies.

Comment 4: 3) In Figure 7, the authors show that serum starvation or cell differentiation reduce mascRNA levels, probably due to a reduced cell proliferation. However, the authors do not present evidence for a reduced proliferation rate, e.g. cell cycle profiles. Also, the mechanism of mascRNA downregulation remains unclear. Is this due to a reduced transcription of MALAT1? Or a reduced processing of MALAT1 by RNase P and Z? Or due to a reduced mascRNA stability?

Response 4: Serum starvation is widely used to synchronize cells and arrest cultured cells in the G0/G1 phase of the cell cycle (Khammanit, et al, Theriogenology, 2008; Rudkin, et al, EMBO J, 1989); Embryonic stem cells (ESCs) are supposed to have a short G1 phase and high proliferation rate. When ESCs undergo differentiation, G1 phase is expanded and cell cycle length increases (Filipczyk, et al, Stem Cell Res, 2007; Victor, et al., PNAS, 2012; Liu, et al., Nat Cell Biol, 2019). In hESCs, Nanog binds CDK6 and CDC25 genes and upregulate their expression, thereby promoting cell proliferation (Zhang et al., J Cell Biol, 2009).

We have measured *MALAT1* RNA levels and found the levels decrease in response to starvation, which could be the reason of mascRNA downregulation (Fig EV5A).

Comment 5: 4) The authors observed an increased global protein translation, yet also an increased turnover (Fig.4). So, the net amount is unchanged? Have the authors analysed total protein levels between mascRNA overexpressing cells and control cells? How do the authors explain the increased turnover? If total protein levels remain unchanged, how does this fit into the model of increased translation driving proliferation?

Response 5: The global protein translation and degradation are like inflow and outflow of water into a reservoir. An increase of inflow first increases the volume of the water in the reservoir, but the volume cannot go up continuously, and the outflow has to increase eventually for water to reach a stable level. For most cells, the total protein levels do not go up without the size of the cell going up or the cell going into division. Cell proliferation exerts higher demand on synthesis of new proteins, and protein synthesis is positively correlated with cell proliferation rate (Larsson, et al., J Cell Sci, 1985; Pardee, et al., Science, 1989). In our case, the total protein levels are not significantly different between mascRNA

overexpressing cells and control cells when the same number of cells were analyzed (Response Fig 2). If the same number of cells were cultured for 3 hours, and then all the cells were collected and total protein levels were compared, mascRNA overexpressing cells have higher levels of total proteins (Response Fig 2).

Comment 6: Minor
a) In Figure 1: Please add a size marker. Also, why is there no shift for 5S rRNA? Please introduce "Figure 1B" in the main text.
Response 6: Electrophoresis of EMSA samples are performed under native conditions and size markers are not reliable here.

We thank the referee for catching the mistake and have introduced Fig 1B in the main text (Page 5, Line 12).

There are shift signals for *5S* rRNA although not very strong. Since rRNAs are the most abundant RNAs in the cell, the amount of biotinylated *5S* RNA used for EMSA is likely not enough to compete with the endogenous RNA for protein binding.

Comment 7: b) In Figure 3E: Please shift the labels to the right to match lanes better.
Response 7: We have adjusted the labels.

Comment 8: c) Figure 4: Please show a Northern Blot to confirm correct size of mascRNA after its overexpression. Is it CCA-modified?
Response 8: Northern blots of mascRNA have been added (Fig 4A). The overexpressed mascRNA is CCA-modified as it has the exact same size of the endogenous RNA.

Comment 9: d) MascRNA can be overexpressed using an artificial precursor, e.g. GFP-mascRNA (see Gutschner et al., 2011; Wilusz et al., 2012). These constructs might be more suitable for mascRNA overexpression due to the correct processing steps.
Response 9: According to Gast et al., 2016, a precursor sequence is not required for mascRNA overexpression. Our constructs produced correct mascRNA, as shown by northern blots (Fig 4A, 4E, 4H and 6M).

Comment 10: e) The controls used in mascRNA overexpression experiments (scramble, or GFP fragment) are not ideal. Why did the authors refrain from using mascRNA antisense which they have used in the binding and interaction experiments and which seems to be a perfect control due to the lack of QARS binding?
Response 10: We stopped using antisense mascRNA as a control to avoid potential effects of the antisense RNA binding to mascRNA or *MALAT1*.

Comment 11: f) MCF-7 cells have not been mentioned in the Experimental Procedures section.

Response 11: We thank the referee for catching the mistake and have added MCF-7 cells in the Experimental Procedures (Page 16, Line 12).

Response references

Evans, M. E., Clark, W. C., Zheng, G., & Pan, T. (2017). Determination of tRNA aminoacylation levels by high-throughput sequencing. *Nucleic acids research*, 45(14), e133

Filipczyk, A. A., Laslett, A. L., Mummery, C., & Pera, M. F. (2007). Differentiation is coupled to changes in the cell cycle regulatory apparatus of human embryonic stem cells. *Stem cell research*, 1(1), 45–60

Hayase, Y., Jahn, M., Rogers, M. J., Sylvers, L. A., Koizumi, M., Inoue, H., Ohtsuka, E., & Söll, D. (1992). Recognition of bases in Escherichia coli tRNA(Gln) by glutaminyl-tRNA synthetase: a complete identity set. *The EMBO journal*, 11(11), 4159–4165

Ibba, M., Hong, K. W., Sherman, J. M., Sever, S., & Söll, D. (1996). Interactions between tRNA identity nucleotides and their recognition sites in glutaminyl-tRNA synthetase determine the cognate amino acid affinity of the enzyme. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(14), 6953–6958.

Jahn, M., Rogers, M. J., & Söll, D. (1991). Anticodon and acceptor stem nucleotides in tRNA(Gln) are major recognition elements for E. coli glutaminyl-tRNA synthetase. *Nature*, 352(6332), 258–260

Khammanit, R., Chantakru, S., Kitiyanant, Y., & Saikhun, J. (2008). Effect of serum starvation and chemical inhibitors on cell cycle synchronization of canine dermal fibroblasts. *Theriogenology*, 70(1), 27–34

Larsson, O., Zetterberg, A., & Engström, W. (1985). Cell-cycle-specific induction of quiescence achieved by limited inhibition of protein synthesis: counteractive effect of addition of purified growth factors. *Journal of cell science*, 73, 375–387.

Li, V. C., Ballabeni, A., & Kirschner, M. W. (2012). Gap 1 phase length and mouse embryonic stem cell self-renewal. *Proceedings of the National Academy of Sciences of the United States of Americ*a, 109(31), 12550–12555

Liu, L., Michowski, W., Kolodziejczyk, A., & Sicinski, P. (2019). The cell cycle in stem cell proliferation, pluripotency and differentiation. *Nature cell biology*, 21(9), 1060–1067

Pardee A. B. (1989). G1 events and regulation of cell proliferation. Science (New York, N.Y.), 246(4930), 603–608

Perona, J. J., Swanson, R. N., Rould, M. A., Steitz, T. A., & Söll, D. (1989). Structural basis for misaminoacylation by mutant E. coli glutaminyl-tRNA synthetase enzymes. *Science*, 246(4934), 1152–1154

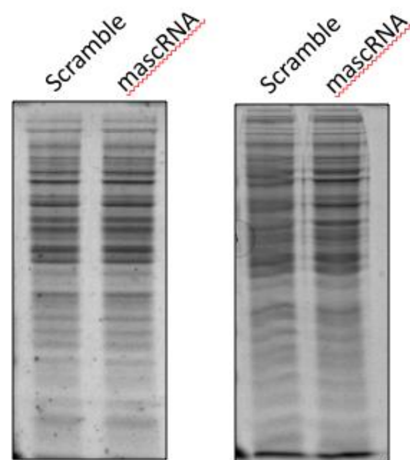
Rudkin, B. B., Lazarovici, P., Levi, B. Z., Abe, Y., Fujita, K., & Guroff, G. (1989). Cell cycle-specific action of nerve growth factor in PC12 cells: differentiation without proliferation. *The EMBO journal*, 8(11), 3319–3325.

Wilusz JE, Freier SM, Spector DL (2008) 3' end processing of a long nuclear-retained noncoding RNA yields a tRNA-like cytoplasmic RNA. *Cell* 135: 919-32

Yang, F., Yi, F., Han, X., Du, Q., & Liang, Z. (2013). MALAT-1 interacts with hnRNP C in cell cycle regulation. *FEBS letters*, 587(19), 3175–3181

Zhang, X., Neganova, I., Przyborski, S., Yang, C., Cooke, M., Atkinson, S. P., Anyfantis, G., Fenyk, S., Keith, W. N., Hoare, S. F., Hughes, O., Strachan, T., Stojkovic, M., Hinds, P. W., Armstrong, L., & Lako, M. (2009). A role for NANOG in G1 to S transition in human embryonic stem cells through direct binding of CDK6 and CDC25A. *The Journal of cell biology*, *184*(1), 67–82

Response Figure 1. Western blots of QARS in mascRNA and *MEN b* tRNA-like small RNA (menRNA) pulldown samples. β-tubulin was used as a loading control.



Response Figure 2. SDS-PAGE analysis and coomassie staining of total proteins in cells overexpressing mascRNA or expressing the scrambled RNA. Left, same number of cells were analyzed; right, same number of cells were cultured for 3 hours and then the total cells were analyzed.

1st Revision - Editorial Decision 5th Aug 2020

Dear Dr. Wang

Thank you for the submission of your revised manuscript. We have now received the comments from referees 1 and 3. Referee 1 has also assessed how well referee 2's concerns were addressed.

As you will see, while both referees acknowledge that most concerns have been addressed, they also both point out a few issues that have not yet been adequately addressed. I would like you to address all remaining points, and please let me know whether you would like to discuss any of these before starting the revisions.

A few other formal changes are also required:

- Please reduce the number of keywords to 5.

- Please correct the 'Declaration of Interests' to "Conflict of Interest" and call the experimental procedures "Materials and Methods".

- Our reference format has changed to Harvard style, please correct the references. A link to the new style can be found in our guide to authors online:
https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- Section B of the author checklist has not been completed. Given that you applied statistics, please complete this section and send us a new checklist.

- Please upload a single file per figure. Also, each figure must fit on a single page in portrait format.

- The Appendix table should be called Dataset EV1 and needs a legend or title added to the excel file (eg in the first tab). Please move the methods in the Appendix to the main manuscript file and delete the Appendix file.

I would like to suggest a few changes to the abstract. Please let me know whether you agree with the following:

mascRNA is a small cytoplasmic RNA derived from the lncRNA MALAT1. After being processed by the tRNA processing enzymes RNase P and RNase Z, mascRNA undergoes CCA addition like tRNAs and folds into a tRNA-like cloverleaf structure. While MALAT1 functions in multiple cellular processes, the role of mascRNA is largely unknown. Here we show that mascRNA binds directly to the multi-tRNA synthetase complex (MSC) component glutaminyl-tRNA synthetase (QARS). mascRNA promotes global protein translation and cell proliferation by positively regulating QARS protein levels. Our results uncover a role of mascRNA that is independent of MALAT1, but could be part of the molecular mechanism of MALAT1's function in cancer, and provide a paradigm for understanding tRNA-like structures in mammalian cells.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is

550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript file.

I am looking forward to receiving the final manuscript as soon as possible. Please let me know if you have any questions or comments.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors have largely addressed my main concerns, but I have several additional minor suggestions. Point #1 requires an additional Western blot and should be included in the revised manuscript.

(1) Figure 6A: The authors write that this figure shows that "QARS was overexpressed in HEK cells" but, in reality, it only shows that a FLAG tagged version of QARS is present in cells. It does not show that overall QARS levels are increased compared to normal cells. An antibody to QARS needs to be used.

Formatting/Clarifications to Text:

(2) All items in a main figure should fit on a single page. As currently presented, Figures 2, 3, and 6 each take up two pages.

(3) Figure 2E: Please clarify if the 5S rRNA is from the input or IP. This is ambiguous in the current legend.

(4) Figure 3H: I do not find this figure helpful as drawn as it does not indicate where the identity elements of the Gln tRNA are located. Also, the figure may imply that mascRNA and Gln tRNA are more similar to one another than mascRNA is to other tRNAs. Is that true?

(5) Page 10: I found the writing at the top of this page to be confusing. The authors write that they did mascRNA overexpression from a plasmid, but then do not immediately tell reader what the result was on overall protein synthesis. They instead say "In an independent set of experiments.." It would be clearer to state the plasmid overexpression results, and then say that they confirmed the results by transfecting in vitro transcribed mascRNA.

(6) Figure 6P: The rescue effect is very weak.

(7) Figure EV5B: The text label is confusing. The authors should label each individual well rather than summarize the wells at the top.

(8) Figure 7F: Reducing GARS also abolishes the effect of mascRNA overexpression. This point should be added to the main text.

(9) Please include the data showing that the MEN b tRNA-like RNA does not bind QARS (Response Figure 1) in the manuscript. This result suggests that the two tRNA-like RNAs function differently and is worth highlighting.

(10) In the methods, there are places where more detail could be given. This includes:
- How much biotinylated RNA was used for the gel shift experiments?
- How much RNA was run on the Northern gels?
- How much protein was run on Westerns?
- MALAT1 shRNA sequence needs to be provided.
- Northern blot probe sequences should be provided.

New data not mandatory, but would be nice to include:

(11) Figure 7B: The authors should measure MALAT1 levels during differentiation, analogous to what they did for the starvation samples.

Referee #3:

In the revised version of their manuscript the authors have addressed some, but not all of my initial concerns.

For example, the authors did not convincingly show that the artificially overexpressed mascRNA is CCA-modified at the 3'end as was shown for the endogenous mascRNA after processing from endogenous MALAT1 (previous Comment #8). Referring to a Northern Blot of unknown nucleotide resolution is not really addressing this point. The CCA-modification might be relevant for the mechanism of action. Hence, it would be important to know, if the artificial expression system really mimics the endogenous situation or whether the effects seen might be also artificial, at least in part, and especially due to the lack of an appropriate mascRNA-knockout/knockdown model (e.g. CRISPR-mediated mascRNA removal or mutation) that could validate some of the findings by targeting the endogenous mascRNA.

Also, this Reviewer is well-aware of the standard effects of starvation and differentiation on cell cycle progression. Hence, this concern (previous Comment #4) was raised, because the authors failed (and still fail) to present data showing that these effects were also seen in their own hands using their own cells in their own lab. Thank you.

Previous Comment #5 was not sufficiently addressed. The higher protein turnover observed (see Figure 4K) is not explained and the provided Response Figure 2 lacks a quantification. This reviewer is not able to see a difference in the 3h time point analysis. Hence, the increased turnover is still not addressed nor explained. If the authors do not want to perform global quantitative mass spectrometry experiments (see previous Comment #3), which would help to address many of the

open questions of this study, they should at least check the abundance of relevant proteins for this study via Western Blot, e.g. proliferation marker (PCNA, Ki67) as well as proteasomal proteins.

September 18th, 2020

Esther Schnapp, PhD
Senior Editor
EMBO reports

Re: Cover letter for revised manuscript EMBOR-2019-49684V2

Dear Dr. Schnapp:

We would like to thank the editors and the reviewers again for the help and suggestions. We have done more experiments to address the remaining concerns, and the following are the responses to the issues raised.

Referee #1:

The authors have largely addressed my main concerns, but I have several additional minor suggestions. Point #1 requires an additional Western blot and should be included in the revised manuscript.

(1) Figure 6A: The authors write that this figure shows that "QARS was overexpressed in HEK cells" but, in reality, it only shows that a FLAG tagged version of QARS is present in cells. It does not show that overall QARS levels are increased compared to normal cells. An antibody to QARS needs to be used.
Response: We have added new data to Figure 6A, showing that QARS and GARS were overexpressed using QARS and GARS antibodies.

Formatting/Clarifications to Text:

(2) All items in a main figure should fit on a single page. As currently presented, Figures 2, 3, and 6 each take up two pages.
Response: We have adjusted the figures and fit each one on a single page.
(3) Figure 2E: Please clarify if the 5S rRNA is from the input or IP. This is ambiguous in the current legend.
Response: The *5S* rRNA is from the IP. We have clarified it in the figure legend (Page 30).
(4) Figure 3H: I do not find this figure helpful as drawn as it does not indicate where the identity elements of the Gln tRNA are located. Also, the figure may imply that mascRNA and Gln tRNA are more similar to one another than mascRNA is to other tRNAs. Is that true?
Response: The identity elements of human Gln tRNA are not clear yet. We have shown that mascRNA binds specifically to QARS. QARS is the aminoacyl-tRNA synthetase of Gln tRNA, hence the comparison of the two RNAs. In addition, the figure shows that mascRNA does not have a conserved anticodon loop (Page 8, Line 10-12). mascRNA does share

more similarities with Gln tRNA than other tRNAs.

(5) Page 10: I found the writing at the top of this page to be confusing. The authors write that they did mascRNA overexpression from a plasmid, but then do not immediately tell reader what the result was on overall protein synthesis. They instead say "In an independent set of experiments.." It would be clearer to state the plasmid overexpression results, and then say that they confirmed the results by transfecting in vitro transcribed mascRNA.

Response: We have revised the writing (Page 8 line 21 - Page 9 line 3).

(6) Figure 6P: The rescue effect is very weak.

Response: The rescue effect of mascRNA overexpression on QARS knockdown is weak but statistically significant. mascRNA regulates QARS protein level at a post-transcriptional level, and the effect of QARS knockdown by shRNA is stronger than mascRNA overexpression. mascRNA overexpression in QARS knockdown cells slightly increased QARS protein level, resulting in a weak rescue effect on global protein translation.

(7) Figure EV5B: The text label is confusing. The authors should label each individual well rather than summarize the wells at the top.

Response: We have now labeled each well individually (Figure EV5C).

(8) Figure 7F: Reducing GARS also abolishes the effect of mascRNA overexpression. This point should be added to the main text.

Response: We have added this point in the main text (Page 11 Line 28-29).

(9) Please include the data showing that the MEN b tRNA-like RNA does not bind QARS (Response Figure 1) in the manuscript. This result suggests that the two tRNA-like RNAs function differently and is worth highlighting.

Response: We have added the data in Figure 2C and mentioned it in the main text (Page 6 Line 7)

(10) In the methods, there are places where more detail could be given. This includes:

- How much biotinylated RNA was used for the gel shift experiments?
- How much RNA was run on the Northern gels?
- How much protein was run on Westerns?
- MALAT1 shRNA sequence needs to be provided.
- Northern blot probe sequences should be provided.

Response: We have added the related information in the methods.

For gel shit experiments, 5 ng biotinylated RNA was used

For Northern blot, 6 ug RNA was loaded.

For western blot, 100 ug protein was loaded

For MALAT1 knockdown, shRNA targets 5′-ACGGAAGTAATTCAAGATCAA-3′

Northern blot probe sequences were listed as below:

Probe	sequence
mascRNA	5’AGACGCCGCAGGGATTTGAACCCCGTCCTGGAAACCAGGAGTGC CAACCACCAGCATC3’
5S rRNA	5’AAAGCCTACAGCACCCGGTATTCCCAGGCGGTCTCCCATCCAAGT ACTAACCAGGCCCGACCCTGCTTAGCTTCCGAGATCAGACGAGATC GGGCGCGTTCAGGGTGGTATGGCCGTAGAC3’
$tRNA^{Gly}_{CCC}$	5’TGCATTGGCCGGGAATTGAACCCGGGtCTCCCGCGTGGGAGGCG

	AGAATTCTACCACTGAACCACC3’
$tRNA^{Gln}_{UUG}$	5’AGGTCCCACCGAGCTCGGATCGCTGGATTCAAAGTCCAGAGTGCT AACCATTACACCATGGGACC3’
$tRNA^{Arg}_{ACG}$	5’CGAGCCAGCCAGGAGTCGAACCTGGAaTCTTCTGATCCGTAGTCA GACGCGTTaTCCATTGCGCCACTGGCCC’

New data not mandatory, but would be nice to include:

(11) Figure 7B: The authors should measure MALAT1 levels during differentiation, analogous to what they did for the starvation samples.
Response: We have added this data to Figure EV5B showing that MALAT1 levels also decreased during ESCs differentiation.

Referee #3:

In the revised version of their manuscript the authors have addressed some, but not all of my initial concerns.

For example, the authors did not convincingly show that the artificially overexpressed mascRNA is CCA-modified at the 3'end as was shown for the endogenous mascRNA after processing from endogenous MALAT1 (previous Comment #8). Referring to a Northern Blot of unknown nucleotide resolution is not really addressing this point. The CCA-modification might be relevant for the mechanism of action. Hence, it would be important to know, if the artificial expression system really mimics the endogenous situation or whether the effects seen might be also artificial, at least in part, and especially due to the lack of an appropriate mascRNA-knockout/knockdown model (e.g. CRISPR-mediated mascRNA removal or mutation) that could validate some of the findings by targeting the endogenous mascRNA.
Response: We agree with the reviewer that the previous data did not convincingly distinguish CCA-modified mascRNA (61 nt) and mascRNA transcript without the CCA tail (58 nt). To evaluate whether the exogenously expressed mascRNA is CCA-modified at the 3'-end just like the endogenous mascRNA, we resolved the RNA sample in a 16% TBE gel and detected mascRNA by northern blotting. As shown in Response Figure 1, CCA-modified mascRNA and mascRNA without CCA tail were separated by gel electrophoresis using the 16% TBE gel. Most mascRNA in mascRNA overexpressing cells were CCA-modified just like the endogenous mascRNA. The ratio of CCA-modified and unmodified mascRNA in mascRNA overexpressing cells was not significantly different from that in control cells. Therefore, the exogenously expressed mascRNA does mimic the endogenous RNA.

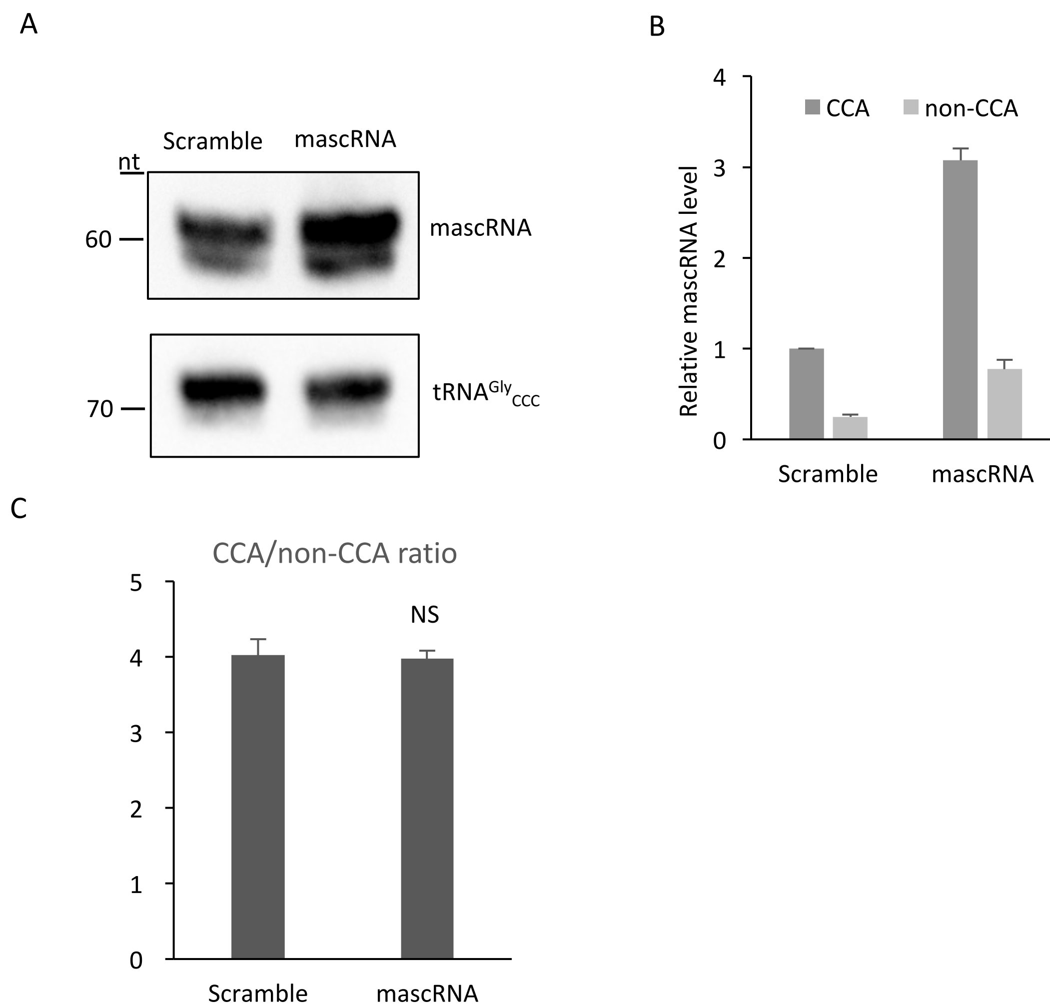
Also, this Reviewer is well-aware of the standard effects of starvation and differentiation on cell cycle progression. Hence, this concern (previous Comment #4) was raised, because the authors failed (and still fail) to present data showing that these effects were also seen in their own hands using their own cells in their own lab. Thank you.

Response: We thank the reviewer for the suggestion and have performed FACS analysis of cell cycle profiles to show the effect of starvation and differentiation on cell cycle. The results show that serum starvation decreased cell proliferation rate as the cell number in G1 phase increased from 42.81% to 49.66%, while that in the G2 phase decreased from 23.23% to 14.10% (Response Figure 2). Our results also show that cell proliferation rate decreased during ESCs differentiation as the G2 phase significantly decreased (Response Figure 3).

Previous Comment #5 was not sufficiently addressed. The higher protein turnover observed (see Figure 4K) is not explained and the provided Response Figure 2 lacks a quantification. This reviewer is not able to see a difference in the 3h time point analysis. Hence, the increased turnover is still not addressed nor explained. If the authors do not want to perform global quantitative mass spectrometry experiments (see previous Comment #3), which would help to address many of the open questions of this study, they should at least check the abundance of relevant proteins for this study via Western Blot, e.g. proliferation marker (PCNA, Ki67) as well as proteasomal proteins.

Response: The changes of total protein levels are not very big as 3 hours might not be enough for newly seeded cells to start dividing. Therefore, we analyzed total protein levels after 6 hours of culture to better address this point. A clearer difference was observed at this time point (Response Figure 4A-B).

We also examined several proliferation markers and proteasomal proteins by western blotting and observed increases of proliferation markers Ki67, PCNA and proteasomal proteins PSMB5 and PSMC3 in mascRNA overexpressing cells (Response Figure 4C).



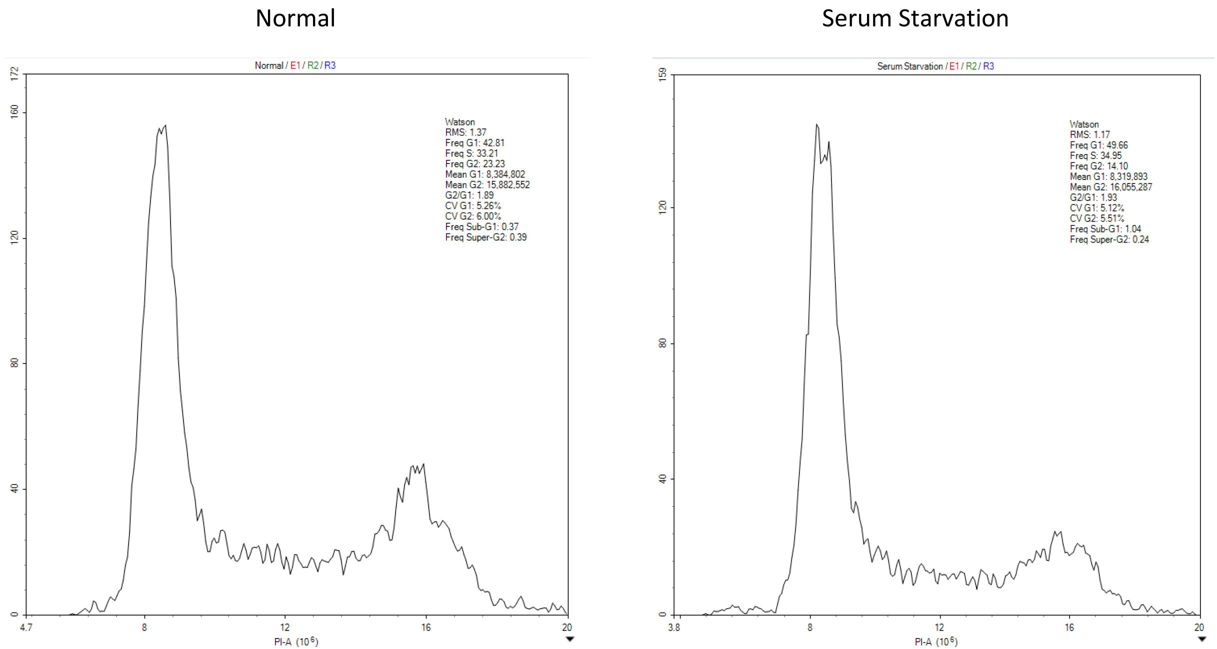
Response Figure 1. exogenously overexpressed mascRNA is CCA-modified

A. Northern blot analysis of mascRNA in mascRNA overexpressing HEK cells (mascRNA) and the cells expressing the scrambled RNA (Scramble). tRNA$^{Gly}_{CCC}$ was used as an internal control. RNA samples were resolved in a 16% TBE gel.

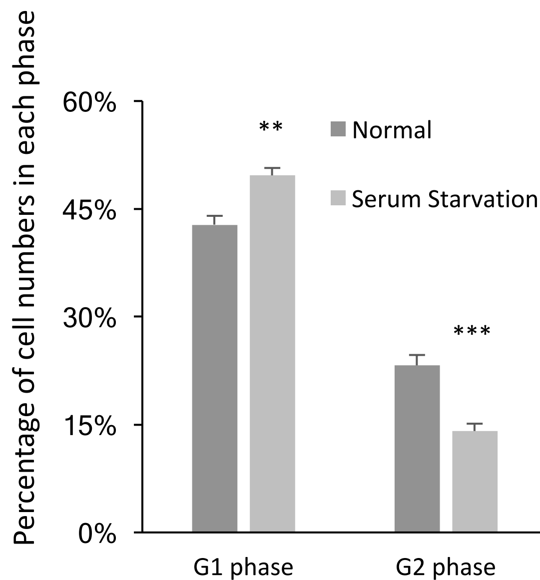
B. Quantification of the levels of the CCA-modified and non-CCA-modified mascRNA in Panel A.

C. Calculation of the ratio between CCA-modified and non-CCA-modified mascRNA in mascRNA overexpressing HEK cells (mascRNA) and the cells expressing the scrambled RNA (Scramble).

A



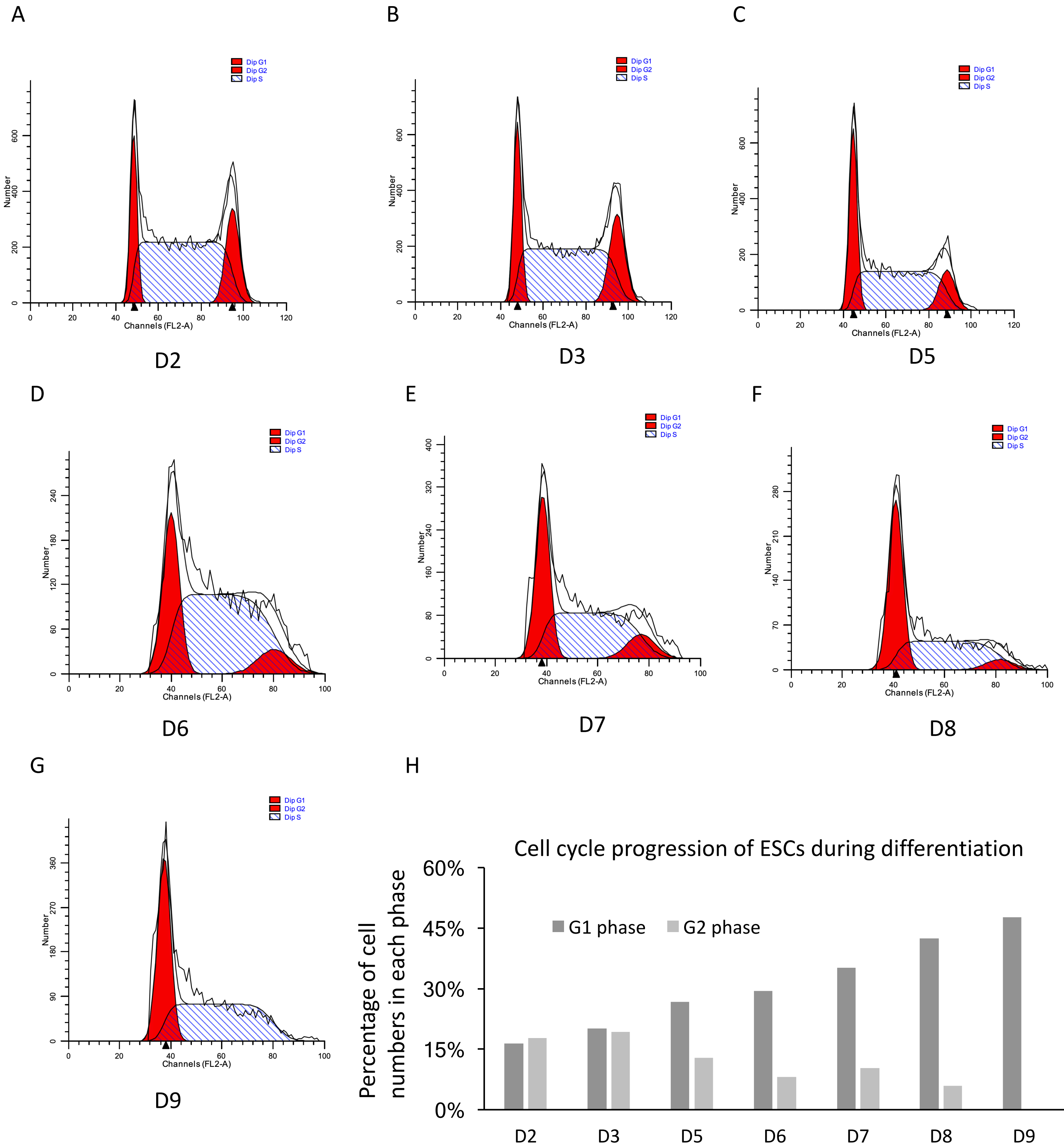
B



Response Figure 2. Effects of serum starvation on cell proliferation

A. Cell cycle profiles of HEK cells cultured in normal medium (Normal) and the medium without FBS (Serum Starvation).

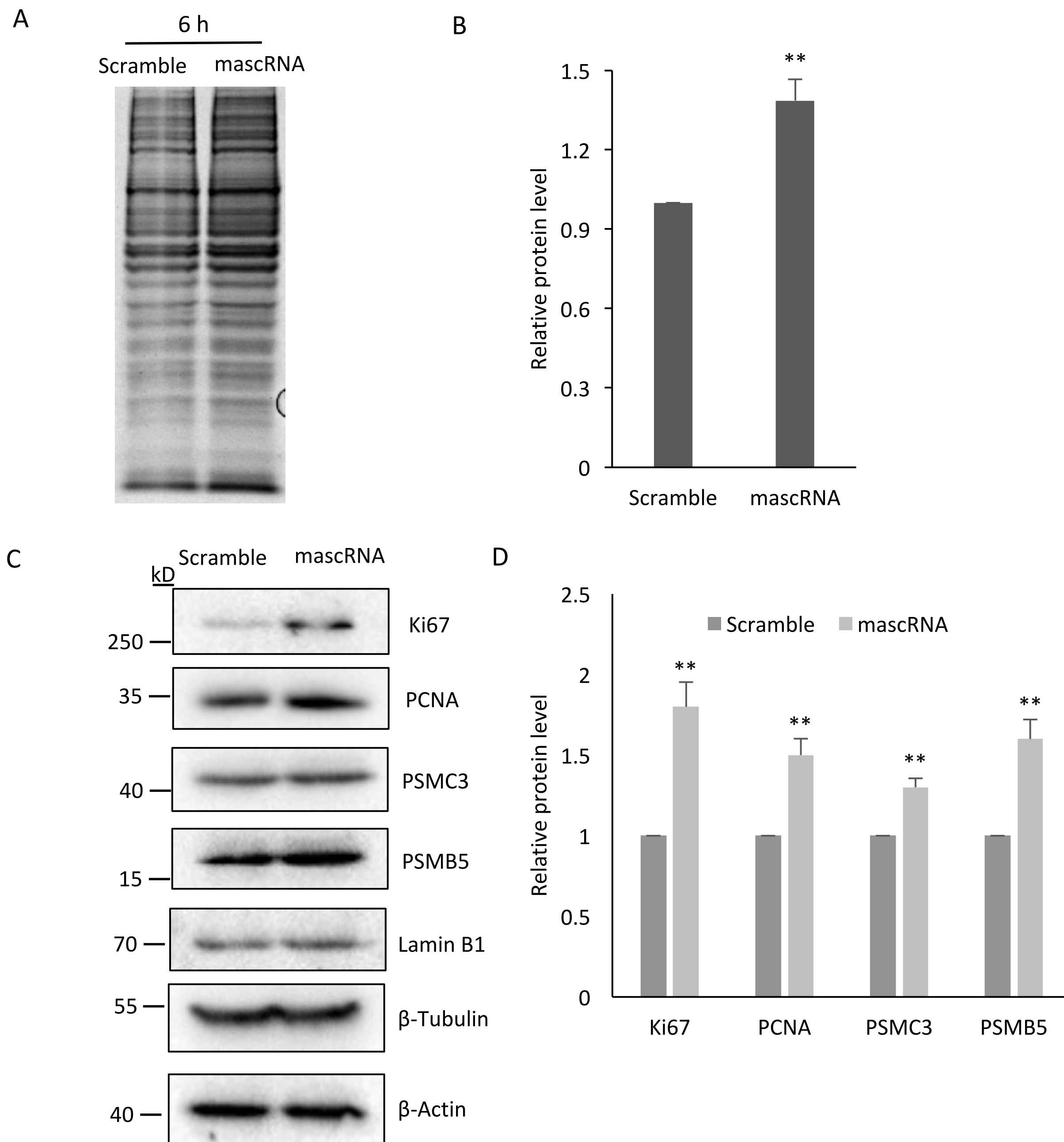
B. Quantification of the G1 phase and G2 phase cell numbers in normal medium (Normal) and the medium without FBS (Serum Starvation) in Panel A.



Response Figure 3. Effect of ESCs differentiation on cell proliferation

A-G. Cell cycle profiles of human embryonic stem cells on different days of differentiation (D2, D3, D5, D6, D7, D8, D9).

H. Quantification of the G1 phase and G2 phase cell numbers on different days of differentiation in Panel A-G.



Response Figure 4. Effect of mascRNA overexpression on total protein, proliferation marker and proteasomal protein levels

A. Coomassie staining of total proteins in mascRNA overexpressing HEK cells (mascRNA) and the cells expressing the scrambled RNA (Scramble) after the same number of cells were cultured for 6 hours.

B. Quantification of the total protein levels in mascRNA overexpressing HEK cells (mascRNA) and the cells expressing the scrambled RNA (Scramble) in Panel A.

C. Western blot analysis of proliferation markers Ki67, PCNA, and proteasomal proteins PSMC3 and PSMB5. Lamin B1, β-Tubulin and β-Actin were used as loading controls.

D. Quantification of the protein levels in Panel C.

September 18th, 2020

Esther Schnapp, PhD
Senior Editor
EMBO reports

Re: Cover letter for revised manuscript EMBOR-2019-49684V2

Dear Dr. Schnapp:

We would like to thank the editors and the reviewers again for the help and suggestions. We have done more experiments to address the remaining concerns, and the following are the responses to the issues raised.

Referee #1:

The authors have largely addressed my main concerns, but I have several additional minor suggestions. Point #1 requires an additional Western blot and should be included in the revised manuscript.

(1) Figure 6A: The authors write that this figure shows that "QARS was overexpressed in HEK cells" but, in reality, it only shows that a FLAG tagged version of QARS is present in cells. It does not show that overall QARS levels are increased compared to normal cells. An antibody to QARS needs to be used.
Response: We have added new data to Figure 6A, showing that QARS and GARS were overexpressed using QARS and GARS antibodies.

Formatting/Clarifications to Text:

(2) All items in a main figure should fit on a single page. As currently presented, Figures 2, 3, and 6 each take up two pages.
Response: We have adjusted the figures and fit each one on a single page.
(3) Figure 2E: Please clarify if the 5S rRNA is from the input or IP. This is ambiguous in the current legend.
Response: The *5S* rRNA is from the IP. We have clarified it in the figure legend (Page 30).
(4) Figure 3H: I do not find this figure helpful as drawn as it does not indicate where the identity elements of the Gln tRNA are located. Also, the figure may imply that mascRNA and Gln tRNA are more similar to one another than mascRNA is to other tRNAs. Is that true?
Response: The identity elements of human Gln tRNA are not clear yet. We have shown that mascRNA binds specifically to QARS. QARS is the aminoacyl-tRNA synthetase of Gln tRNA, hence the comparison of the two RNAs. In addition, the figure shows that mascRNA does not have a conserved anticodon loop (Page 8, Line 10-12). mascRNA does share

more similarities with Gln tRNA than other tRNAs.

(5) Page 10: I found the writing at the top of this page to be confusing. The authors write that they did mascRNA overexpression from a plasmid, but then do not immediately tell reader what the result was on overall protein synthesis. They instead say "In an independent set of experiments.." It would be clearer to state the plasmid overexpression results, and then say that they confirmed the results by transfecting in vitro transcribed mascRNA.

Response: We have revised the writing (Page 8 line 21 - Page 9 line 3).

(6) Figure 6P: The rescue effect is very weak.

Response: The rescue effect of mascRNA overexpression on QARS knockdown is weak but statistically significant. mascRNA regulates QARS protein level at a post-transcriptional level, and the effect of QARS knockdown by shRNA is stronger than mascRNA overexpression. mascRNA overexpression in QARS knockdown cells slightly increased QARS protein level, resulting in a weak rescue effect on global protein translation.

(7) Figure EV5B: The text label is confusing. The authors should label each individual well rather than summarize the wells at the top.

Response: We have now labeled each well individually (Figure EV5C).

(8) Figure 7F: Reducing GARS also abolishes the effect of mascRNA overexpression. This point should be added to the main text.

Response: We have added this point in the main text (Page 11 Line 28-29).

(9) Please include the data showing that the MEN b tRNA-like RNA does not bind QARS (Response Figure 1) in the manuscript. This result suggests that the two tRNA-like RNAs function differently and is worth highlighting.

Response: We have added the data in Figure 2C and mentioned it in the main text (Page 6 Line 7)

(10) In the methods, there are places where more detail could be given. This includes:

- How much biotinylated RNA was used for the gel shift experiments?
- How much RNA was run on the Northern gels?
- How much protein was run on Westerns?
- MALAT1 shRNA sequence needs to be provided.
- Northern blot probe sequences should be provided.

Response: We have added the related information in the methods.

For gel shit experiments, 5 ng biotinylated RNA was used

For Northern blot, 6 ug RNA was loaded.

For western blot, 100 ug protein was loaded

For MALAT1 knockdown, shRNA targets 5′-ACGGAAGTAATTCAAGATCAA-3′

Northern blot probe sequences were listed as below:

Probe	sequence
mascRNA	5’AGACGCCGCAGGGATTTGAACCCCGTCCTGGAAACCAGGAGTGC CAACCACCAGCATC3’
5S rRNA	5’AAAGCCTACAGCACCCGGTATTCCCAGGCGGTCTCCCATCCAAGT ACTAACCAGGCCCGACCCTGCTTAGCTTCCGAGATCAGACGAGATC GGGCGCGTTCAGGGTGGTATGGCCGTAGAC3’
$tRNA^{Gly}_{CCC}$	5’TGCATTGGCCGGGAATTGAACCCGGGtCTCCCGCGTGGGAGGCG

	AGAATTCTACCACTGAACCACC3'
$tRNA^{Gln}_{UUG}$	5'AGGTCCCACCGAGCTCGGATCGCTGGATTCAAAGTCCAGAGTGCT AACCATTACACCATGGGACC3'
$tRNA^{Arg}_{ACG}$	5'CGAGCCAGCCAGGAGTCGAACCTGGAaTCTTCTGATCCGTAGTCA GACGCGTTaTCCATTGCGCCACTGGCCC'

New data not mandatory, but would be nice to include:

(11) Figure 7B: The authors should measure MALAT1 levels during differentiation, analogous to what they did for the starvation samples.
Response: We have added this data to Figure EV5B showing that MALAT1 levels also decreased during ESCs differentiation.

Referee #3:

In the revised version of their manuscript the authors have addressed some, but not all of my initial concerns.

For example, the authors did not convincingly show that the artificially overexpressed mascRNA is CCA-modified at the 3'end as was shown for the endogenous mascRNA after processing from endogenous MALAT1 (previous Comment #8). Referring to a Northern Blot of unknown nucleotide resolution is not really addressing this point. The CCA-modification might be relevant for the mechanism of action. Hence, it would be important to know, if the artificial expression system really mimics the endogenous situation or whether the effects seen might be also artificial, at least in part, and especially due to the lack of an appropriate mascRNA-knockout/knockdown model (e.g. CRISPR-mediated mascRNA removal or mutation) that could validate some of the findings by targeting the endogenous mascRNA.
Response: We agree with the reviewer that the previous data did not convincingly distinguish CCA-modified mascRNA (61 nt) and mascRNA transcript without the CCA tail (58 nt). To evaluate whether the exogenously expressed mascRNA is CCA-modified at the 3'-end just like the endogenous mascRNA, we resolved the RNA sample in a 16% TBE gel and detected mascRNA by northern blotting. As shown in Response Figure 1, CCA-modified mascRNA and mascRNA without CCA tail were separated by gel electrophoresis using the 16% TBE gel. Most mascRNA in mascRNA overexpressing cells were CCA-modified just like the endogenous mascRNA. The ratio of CCA-modified and unmodified mascRNA in mascRNA overexpressing cells was not significantly different from that in control cells. Therefore, the exogenously expressed mascRNA does mimic the endogenous RNA.

Also, this Reviewer is well-aware of the standard effects of starvation and differentiation on cell cycle progression. Hence, this concern (previous Comment #4) was raised, because the authors failed (and still fail) to present data showing that these effects were also seen in their own hands using their own cells in their own lab. Thank you.

Response: We thank the reviewer for the suggestion and have performed FACS analysis of cell cycle profiles to show the effect of starvation and differentiation on cell cycle. The results show that serum starvation decreased cell proliferation rate as the cell number in G1 phase increased from 42.81% to 49.66%, while that in the G2 phase decreased from 23.23% to 14.10% (Response Figure 2). Our results also show that cell proliferation rate decreased during ESCs differentiation as the G2 phase significantly decreased (Response Figure 3).

Previous Comment #5 was not sufficiently addressed. The higher protein turnover observed (see Figure 4K) is not explained and the provided Response Figure 2 lacks a quantification. This reviewer is not able to see a difference in the 3h time point analysis. Hence, the increased turnover is still not addressed nor explained. If the authors do not want to perform global quantitative mass spectrometry experiments (see previous Comment #3), which would help to address many of the open questions of this study, they should at least check the abundance of relevant proteins for this study via Western Blot, e.g. proliferation marker (PCNA, Ki67) as well as proteasomal proteins.

Response: The changes of total protein levels are not very big as 3 hours might not be enough for newly seeded cells to start dividing. Therefore, we analyzed total protein levels after 6 hours of culture to better address this point. A clearer difference was observed at this time point (Response Figure 4A-B).

We also examined several proliferation markers and proteasomal proteins by western blotting and observed increases of proliferation markers Ki67, PCNA and proteasomal proteins PSMB5 and PSMC3 in mascRNA overexpressing cells (Response Figure 4C).