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A pseudouridine synthase module is essential for mitochondrial protein synthesis and cell viability

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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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

17 October 2016

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As I had already indicated in my earlier communication, the referee's opinions are divided. Two referees support publication of your study in EMBO reports with minor revisions, while referee 1, who is an expert in RNA editing, does not. In particular, this referee is concerned that the evidence for pseudouridylation of mitochondrial RNA is weak and points out that no direct enzymatic assay is provided. I discussed this point further with referee 3 who agreed in principle with referee 1 that a direct enzymatic assay would provide ultimate proof of the enzymatic function. However, this referee also concluded that overall, the findings presented in the manuscript are sufficiently strong in his/her opinion to support the claim that RPU4 modifies residue 3069 in the 16S rRNA.

Given the support by two referees and the constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Most of the referee comments can be addressed in writing except for the suggestion of referee 2 to test if 16S rRNA stability is affected in the absence of NGRN. I therefore think that a time frame of three weeks for the revision is reasonable.

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 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.

REFEREE REPORTS

Referee 1:

The present work expands on a recent report (by a different group) that suggested the possibility of pseudouridines in mitochondrial mRNAs. Here Antonicka et al. used the BioID proximity labeling approach to identify a protein module found in mitochondrial granules. Surprisingly, this module contained several homologs of pseudouridine synthases of unknown function. The work clearly shows that these newly identified proteins are important for mitochondrial function. For example, down-regulation of the expression of RPUSD4 leads to decreased levels of 16s rRNA, while depletion of each synthase leads to a decrease MRPL11 protein. Depletion of these synthases also led to OXPHOS complexes assembly defects presumably due to decrease synthesis of mt-encoded polypeptides. Furthermore, down-regulation of TRUB2 led to a decrease in ATP6 and ATP8 subunits but did not affect components of cox III or cox II. This is a well-written manuscript and in general the work presents nice data that may be of general interest. With this said, there are several issues (some major) with the experiments presented and the interpretation of the results.

- 1) Although as shown down regulation of the synthases leads to decrease in the levels of mito-encoded proteins, it is not clear whether this is a direct effect on mitochondrial translation. For example, MRPL11 also goes down, yet this protein is encoded in the nucleus. There are two issues at hand here, could it be that just like PUS1, the three new PseudoU synthases have functions in cytoplasmic translation? Was MRPL1 part of the FASTKD2 complex?
- 2) How significant are the reductions in mitochondrial proteins in terms of cell physiology? The authors should have provided direct measurements of standard mitochondrial activities; typically done in the field with any mitochondria study. For example, what happens to respiration? Membrane potential? Etc.
- 3) The statement is made several times about the synthases been implicated in mito translation. However, it is not clear how the authors rule out the possibility of mito protein stability instead. The mito translation effect could then be secondary to the main function of these proteins.
- 4) The argument that there is pseudoU in mito mRNAs is weak. Not clear why these authors get base skipping instead of "hard stops" at the positions of CMCT modification. If it is indeed pseudoU, then I suggest a more direct primer extension assay (see Bakin and Offengand) to validate their observations. This is particularly important because other modified nucleotides can also be targets of CMCT yet are resistant to mild alkali treatment, for example s4U.
- 5) Lastly, the statement is made in the introduction that this work has identified the enzymes responsible for pseudo formation in mito mRNAs. Here, I beg to differ, what the authors show is that the down-regulation of three different pseudoU synthase paralogs leads to decreases in what may be pseudoU in some RNAs. However, no direct evidence that these proteins can make pseudoU in those RNAs is provided. I strongly argue that to call something an enzyme requires biochemical evidence in the form of enzymatic assays that prove the point. Sorry but there is not such thing as guilt by association in enzymology.

Minor comment:

Page 5, line 8 from the top. It should read "thiolation" and for consistency, and to avoid redundancy, "taurine-addition."

Referee 2:

Antonicka et al report a study in which they continue their prior pioneering work of mitochondrial RNA-granule structure, and ask which proteins exist in close proximity to FASTKD2, to get knowledge of FASTKD2 function. They utilized a proximity-biotinylation assay, BioID, to search for interacting protein partners. They identified an interesting set of proteins, which were new in RNA-granules, including NGRN, WBSCR16 and three enzymes involved in pseudouridylation: TRUB2, RPUSD4 and RPUSD3. Functions of these proteins have been unknown.

The exact same set of RNA-granule proteins was characterized by Mootha laboratory, with a completely different approach (Crispr/Cas9 death screen), in a paper which is online in preprint format in Cell Metabolism currently, indicating solid replication of the results in these two simultaneously ongoing studies. However, the functional characterization in the current paper goes into much more depth. Mootha group concluded that the whole RNA-granule binding protein sextet is required for 16S rRNA stability, and the current results challenge that conclusion with an elegant functional study. Especially to be applauded in Antonicka paper is the detailed focus in pseudouridylation - a little studied field, but essential for RNA stability in mitochondria.

The authors validate their BioID results in detail, utilizing siRNA depletion in 143B cells, every protein individually, and assess the effects of these RNA-granule proteins on each other, and on oxphos protein synthesis, assembly and function. They note specific effects of TRUB2 in synthesis of ATP6 and 8, Trub2 and RPUSD4 for ribosome assembly. They found that RPUSD4 knockdown was the only one to decrease 16S rRNA, and propose that this is the pseudouridylyase for 16S rRNA absolutely required for its stability. However, they found no effects of TRUB2 or RPUSD3 to RNA stability of abundance, which is differing from Mootha-lab finding (who proposed that all of the RNA-granule proteins are involved in 16S stability). The current paper used 143B cells, whereas Mootha-lab used K562, HeLa and HEK293T cells. Whether the partial discrepancy in findings is due to cell type remains to be seen. However, technically the current paper is state-of-the-art, and conclusions novel.

NGRN in Mootha paper was found to be required for 16S stability. I suggest the authors to test whether they find the same, or is just RPUSD4 needed in their model.

The authors motivate their study to learn more of FASTKD2 function. However, they focus in their results and discussion in the functions of the interacting partners. Did the study bring new knowledge of FASTKD2? A sentence or two of this would be warranted.

Minor questions:

Figure EV1: it appears that the different RNA-granule proteins are only partially overlapping with GRSF1. The authors do not comment this, but they could discuss a bit of the background: could some dynamics of the identified proteins occur e.g. stimulated by respiratory activity?

Some typos exist, for example, second subheading of results: OHPHOS --> oxphos

Referee 3:

This is a timely manuscript on an interesting topic. It deals with the discovery of three pseudouridine synthases acting on mitochondrial RNA. The manuscript is well written and the data technically sound, helping to establish the roles of the enzymes in modifying specific target RNAs. Before acceptance, the authors should address these minor points:

1. The method on how this pseudouridine synthase module was discovered is not well described in the text. A short explanation should be added to clarify what AP-MS and BioID are.
2. In a recent publication by Vamsi Mootha and colleagues, the same module was identified and characterized. It would be good to shortly discuss their findings in the current manuscript.

We thank the referees for their overall positive comments on the manuscript. All referees suggested we refer to the recently published study from Mootha's group on the same protein module and discuss differences between the two studies, which we have now done in the text. Answers to specific concerns/questions appear below.

Referee 1:

I will deal with the most important criticism first – which is that the evidence for pseudouridine (ψ) modification is weak. We completely disagree with this statement. I will focus my comments on RPUSD4 and the 16S rRNA, which we feel is the most important finding. Let me reiterate the evidence:

(1) The ψ modification at position 3069 is the only known ψ in the human mitochondrial 16S rRNA (ref 4). There is universal agreement on that point.

(2) The assay we used to detect ψ is widely accepted and has been published in pioneering studies from the Gilbert lab in Nature (ref 7) (amongst others). It is no different in principle than the primer extension assay referee #1 proposes, but it has two major advantages: it allows one to study many RNA species at once, rather than one at a time, and in addition, it permits one to estimate the number of molecules that are modified because one can count the number of reads. This is not possible with the primer extension assay, because it is very difficult to determine a denominator in that case. In fact, we have used the primer extension assay in preliminary experiments in which we silenced FASTKD2 and TRUB2, looking for evidence of the 16S rRNA modification. FASTKD2 knock-down lead to on average 50% decrease in the level of 16S rRNA (ref 9), thus any changes in the intensity of the detected band by primer extension needed to be normalized to the level of the RNA, which was hard to determine as the RNA samples are “degraded” during the alkaline treatment. However, we saw no evidence for TRUB2 involvement in 16S rRNA modification (consistent with the results we now report), but decided to use the pseudouridine-seq, which permits a good estimate of the relative proportion of modifications independent of the effect on the level of the transcript.

(3) Strong stops/pausing/skipping at the modified U will depend on the exact nature of the reverse transcriptase used in the assay. These are constantly being improved to read through RNA secondary structures. The base skipping we observed happened at the precise site of the known ψ modification in the 16S rRNA, and in no other place in this molecule. Thus there is really no doubt that our assay recognized the modified base correctly. This was also seen in ref 7, although using their kits they reported stops. The assay, by its nature, is not quantitative, and as we mention in the paper a positive control with 100% ψ , returned only 43% stops (ref 22). We found that 32% of reads produced base skipping, so it is likely that the modification occurs in the vast majority, if not all, of the 16S rRNAs.

(4) We only saw a specific and strong reduction in the proportion of ψ 's in the 16S rRNA when we knocked down RPUSD4, a protein with a known pseudouridine synthase module, and which localizes entirely to mitochondria, largely in RNA granules, established centres for posttranscriptional RNA modification. RPUSD4 cannot be completely deleted as it is a core fitness enzyme, so we do not expect to reduce the ψ modification to zero.

(5) The loss of the ψ modification is associated with a dramatic reduction in the level of 16S rRNA, an inability to assemble mitochondrial ribosomes, resulting in a severe protein synthesis defect, and a concomitant inability to assemble the oxidative phosphorylation complexes – all convincingly demonstrated in the manuscript.

So although we have not directly measured the activity of the enzyme, when we knock it down we observe a strong, specific and statistically significant reduction in the only ψ site in the 16S rRNA, with clear and severe defects in mitochondrial gene expression. We do not feel that developing an *in vitro* assay is necessary to prove the point. We would be willing to qualify our conclusions and state that suppression of the pseudouridine synthase proteins is associated with significant decreases in pseudouridylation in mitochondrial RNAs, suggesting that are directly involved in this base modification.

I will briefly address the other points raised by this reviewer:

(1) We demonstrate by pulse-labeling experiments that there is in fact a direct effect on mitochondrial translation when we knockdown the proteins, which in this comment the referee refers to as synthases. MRPL11 is indeed encoded in the nucleus, and we have consistently observed a decrease in this protein when we suppress the levels of mitochondrial RNA granule proteins. It is extremely unlikely that any of the three synthases have cytosolic functions, as they all appear to localize to the mitochondrial compartment exclusively. In fact we show this by immunofluorescence studies.

(2) As addressed above, there are very clear effects on assembly of the OXPHOS complexes, so obviously severe effects on the ability of the cell to carry out oxidative phosphorylation. As mentioned in the manuscript, two of the synthases are core fitness enzymes. All were picked up in Mootha's screen (albeit at different FDR's). So there are clearly crucial for survival of mammalian cells that rely on oxidative phosphorylation for survival. Measurement of the so-called standard mitochondrial activities would not provide any further insight into the function of the synthases. The data we present provide much more mechanistic insight than would, for instance, measurements of respiration or membrane potential.

(3) As stated above we used a pulse translation assay to evaluate mitochondrial protein synthesis. This directly measures initial rates of synthesis.

(4) We completely disagree with this comment (see above).

(5) Again, we are ready to qualify our conclusion as indicated above.

Minor comments: We have corrected these as requested.

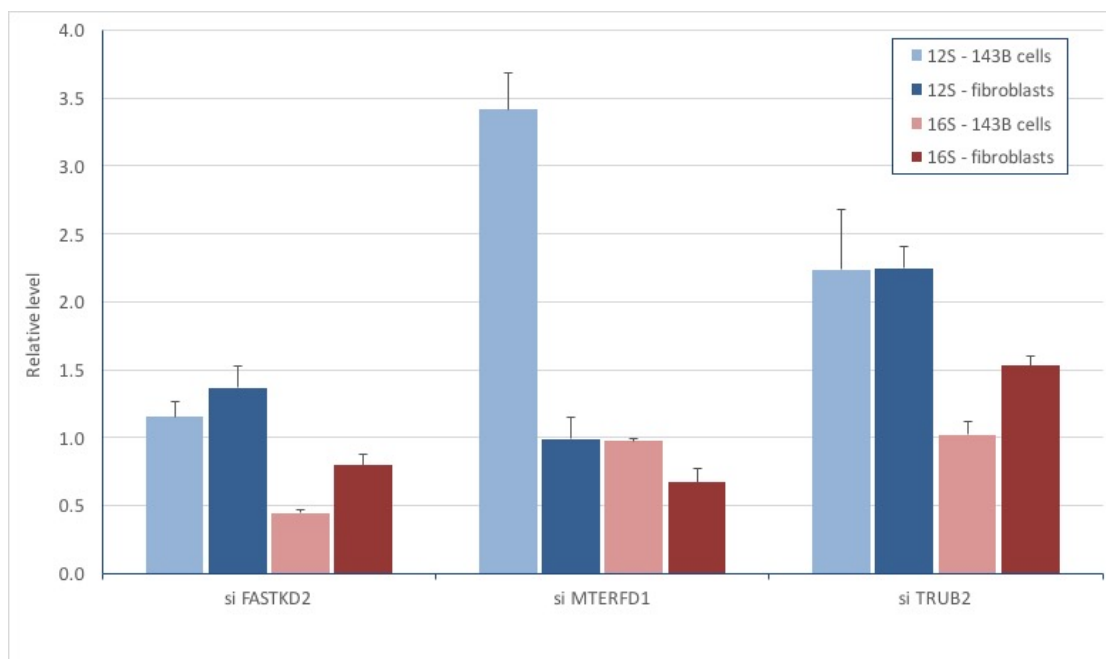
Referee 2:

The major concern was that we found no difference in 16S rRNA stability when we suppressed TRUB2 or RPU5D3, whereas the study by Mootha's group did. We have now done experiments in which we have suppressed NGRN (as requested) and also WBSCR16, and we also find no statistically significant differences in the stability of the 16S rRNA (Fig. EV2C). We suspect, as suggested by the reviewer, that this may have to do with differences in the cells used in the two studies and have now mentioned this in the text. Certainly this requires further investigation, but we have some evidence for this in the figure below in which we show quite large differences between 143B cells and immortalized human fibroblasts.

In this study we did not learn more about the molecular details of FASTKD2 function, but we think that we have now put it in a larger context within a protein module with other proteins required for posttranscriptional modifications that we show are essential for mitochondrial ribosome biogenesis.

Minor questions:

Figure EV1. It is certainly possible that the proteins that we investigate here transit in to and out of the granules in response to different physiological signals, and that could be the reason why the overlap with GRSF1 is only partial. We have not yet directly investigated that possibility, but we now mention it in the text.



Referee 3:

1. We provide a detailed explanation of AP-MS and BioID in the text (Results/Methods).
2. As mentioned in the opening paragraph above we have now referenced the Mootha paper and discussed the differences between our findings and those reported by his group.

2nd Editorial Decision – part 1

21 November 2016

Thank you for your patience while we have reviewed your revised manuscript. It has been sent to three referees, and today we have received the report from referee 1 who is now also positive about your manuscript. As two of the referees recommend publication in EMBO reports, I would like to proceed with the revision of your manuscript. Please note that this is a preliminary decision made in the interest of time, and that it is subject to change should the third referee offer very strong and convincing reasons for this, which I do not expect based on the first report. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed.

At the moment there is only one small editorial change we would require. We have noticed that part of the data on the sucrose gradient centrifugation is shown twice, once in Figure 3A and again in Figure EV3. It appears that Fig. EV3A shows additional data from the same experiment depicted in Fig. 3A, top panel. While this is in principle no problem, I suggest to add a statement in the legend of figure EV3A to avoid any confusion.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

REFeree REPORTS

Referee 1:

I would like to thank the authors for the clarifications in this round of reviews. My biggest concern was the fact that no direct biochemical evidence was provided for enzymatic activity of RPU5D4

and as such it is misleading to claim that it is the enzyme responsible for the activity. The authors for the most parts have done an excellent job of correcting this. Still; however, in the abstract the statement that it "pseudouridyates..." should be changed to "it plays a role in the pseudouridylation..." Sorry for being picky but one must err on the side of being safe. Otherwise, an excellent piece of work.

Referee 3:

The authors have addressed all the points we had on the initial version and we recommend publication of this solid manuscript.

2nd Editorial Decision – part 2

21 November 2016

Please find attached also the report of referee 2, which just came in. As already outlined in my earlier mail, we only need the minor modification to the figure legend before official acceptance.

Referee 2:

The authors have convincingly responded to all my concerns, and I have no further comments.

2nd Revision - authors' response

21 November 2016

We attach a revised version of the manuscript entitled "A pseudouridine synthase module is essential for mitochondrial protein synthesis and cell viability." We have made the editorial changes you and referee #1 requested and hope that you will now find the manuscript suitable for publication in EMBO Reports.

3rd Editorial Decision

22 November 2016

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

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Eric A. Shoubridge
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-43391V1

Reporting Checklist for Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n < 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/ varied/ perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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C- Reagents

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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines have been studied in our laboratory for years. We routinely test for mycoplasma contamination before every experiment

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

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8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	not applicable
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	not applicable
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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	not applicable
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	not applicable
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	not applicable

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	RNAseq data will be deposited in a publically available database
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