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A functional peptidyl tRNA hydrolase, ICT1, has been recruited into the human mitochondrial ribosome

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1st Editorial Decision 11 January 2010

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office. The enclosed comments indicate a clear interest in your study that identifies ICT1 as mitochondrial ribosomal protein with peptidyl-tRNA hydrolase activity. As you will see all referee's request some revisions that are essentially aimed at increasing experimental support and impact of the study. Ref#2 asks for an essential specificity control for the knockdown effects (ref#2). Further, ref#3 requests better support/explanations for the hydrolysis assays, a more rational discussion of the evidence that ICT1 is part of the 39S subunit additional details on the ICT1 interactors found in the pull-downs. All in all, I am happy to invite submission of a modified version of your work that would attend to the comments of the referees. I also have to remind you that it is EMBO_J policy to allow a single round of revisions only, which means that the final decision on your work depends on the content within its final version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This manuscript deals with the mechanisms of protein synthesis that take place in mitochondria. Although in charge of synthesizing a restricted set of polypeptides, this process is of primary importance for mitochondrial function and biogenesis. Synthesis of mitochondrial polypeptides is unique when compared to the mechanisms employed by other ribosomes because it is tightly coupled with the adjacent events, many of them localized to the inner membrane. Despite its importance, this process has been poorly understood at the mechanistic level due to the technical restrictions, mainly an inability to reconstitute this process in vitro. In this manuscript, the authors present the identification of the ICT1 protein that is a member of the translation release factor family of prokaryotic origin. By the combination of elegant biochemical approaches, it is demonstrated that this essential protein has indeed an activity of the promiscuous peptidyl tRNA hydrolase. Importantly, and in contrast to other systems, this protein remains tightly bound to the mitoribosome, a feature not described before. Based on these results, the authors postulate a novel strategy employed in mitochondria in order to rescue stalled ribosomes and liberate tRNA molecules.

The manuscript is well written and data are of very high quality. Furthermore, it is a big step forward in order to understand how the mitochondrial ribosome operates and how mitochondrial translation is regulated. This significant contribution is interesting for both specialists in the field and a broad audience. I suggest that the authors address/discuss a few issues to strengthen the conclusions.

1. A model, which shows putative functions of ICT1, would clearly help non-specialists to fully appreciate the findings.

2. Is there any visible effect on the protein synthesis after a short time depletion of ICT1 (in a way that does not lead to a decrease in LSU)?

3. Does the overexpression of ICT1 affect the kinetics of mitochondrial protein synthesis? In the similar direction: is the overexpression of ICT1 able to substitute for the function of the regular mitochondrial release factor (or the recycling factor)?

4. To specify the function of ICT1, it would be helpful to test a possibility to detect stalled ribosomes charged with labeled nascent polypeptide chains in the absence of ICT1 (for example, by combination of the labeling followed by the centrifugation analysis). There is a certain chance that such things will be visible under normal conditions since ICT1 is required under normal conditions

Referee #2 (Remarks to the Author):

Understanding the mechanism of protein translation and quality control in mitochondria is an important area of study due to its relevance to disease and also contributing to our general understanding of evolutionary cell biology. Consequently many new findings have been uncovered in recent years and published in leading journals - including work from the authors that mitochondrial ribosome release factor (mtRF) 1a is required for translation termination of all mtDNA encoded proteins. In this manuscript, Richter et al report the characterization of another member of the release factor family, ICT1. The authors use a number of suitable approaches including pull-downs, in vitro reconstitution experiments and a novel knockdown/complementation approach in cells, to convincingly show that ICT1 is essential for cell viability, is found in mitochondrial ribosomes, and possesses peptidyl-tRNA hydrolase (PTH) activity that is dependent on a GGQ domain. The discussion is well balanced providing a good analysis of the previous literature on the topic plus some speculation regarding the function of ICT1. The hypothesis - that ICT1 is involved in recognition/hydrolysis of peptidyl-tRNAs that have been prematurely released from stalled mitochondrial ribosomes - appears well-founded and will be tested in future studies.

The manuscript is well written and the experimental approaches are clear and generally well-controlled. A number of issues listed below should be addressed:

1. The authors convincingly show that knockdown of ICT1 is detrimental to cell viability. They go on to show that mitochondrial protein translation in compromised. While the experimental results shown in Fig 1E are consistent with the authors' conclusions, the decreased translation observed could be due to the general loss in cell viability from the knockdown. A control to show that total cellular protein synthesis is not affected would clarify that ICT1 knockdown specifically inhibits mitochondrial protein translation (i.e. pulse labeling of whole cells after 3-days knockdown).

2. Fig. 1D and 2F do not appear to be cited in the text.

3. Pg 6: The authors state that PTH activity of ICT1 is ribosome dependent but mention it as data not shown. This is an important aspect of the study (and stated in the abstract) and so the data should at least be shown as supplementary material.

Referee #3 (Remarks to the Author):

The manuscript by Richter et al. presents the very interesting observation that the protein ICT1 is a mitochondrial ribosomal protein in mammals that functions as a peptidyl-tRNA hydrolase. This protein belongs to the release factor family but does not appear to play a role in mitochondrial translational termination. The data are generally clean and present a convincing case that ICT1 is a mitochondrial ribosomal protein that has the ability to trigger hydrolysis of fMet-tRNA in a ribosome-dependent fashion. This work is quite important and adds significant new insights into our understanding of an important component of mammalian mitochondrial protein synthesis.

Points to consider:

The hydrolysis assay should be better described. The method refers back to the work by Tate but the legend (Fig. 3C) indicates that the details are presented in Methods. However, there is really no direct information in Methods here. The results here are intriguing. The assay measures fMet-tRNA hydrolysis using E. coli programmed ribosomes. In this experiment, ICT1 hydrolyzed the fMet-tRNA in a codon-independent manner. What is perplexing to this reviewer is how this could work on E. coli ribosomes with a protein that should be an integral part of the mitochondrial LSU. There are quite significant structural differences between bacterial and mammalian mitochondrial ribosomes and it is surprising that there would be an interaction at all, let alone that ICT1 would be able to carry out its function in this system. (Perhaps this makes the idea of ICT1 working through the PAS, which is not present in bacterial ribosomes less likely). The data in this figure should be presented in pmol so that the efficiency can be evaluated. Apparently non-limiting amounts of factor were used. It would be better to present data with limiting amounts of factor so that an assessment could be made of whether it is functioning catalytically. It would be useful to include the -ribosome control (p. 6) in Fig. 3C.

Fig. 2D and p. 5: The authors state here that ICT1 is a component of the 39S subunit based on its sedimentation profile after IP with ICT1-FLAG. The observations are certainly compatible with this idea. However, it is perhaps difficult to come to a definitive conclusion based on this data alone since ICT1 could be a protein that associated tightly with the large subunit. The sentence at the end of the long paragraph on p. 5 should be toned down to reflect the idea that this possibility must be considered. Regardless, the data in Fig. 2E are stronger showing that si-ICT1 leads to the destabilization of the large subunit and a decrease in the levels of LSU proteins as well.

Supplementary Table 1: The authors deal with the validity of the pull-downs with the ICT1 antibodies using the EMPAI scores. It would be helpful for most readers to have an indication of absolute scores and what ratios of control versus ICT1 EMPAI are indicative of a real interaction. For example, does the three-fold ratio for chaperonin represent a real interaction? Is the 5.4 to 4.4 ratio for LRPPRC indicative of a real interaction? The negative control antiserum here is not clearly defined nor is the presumed use of anti-FLAG-ICT1 clear (p. 12). It is possible that some of the hits in this table represent spurious interactions since ICT1-FLAG is over-expressed and soluble rather

than being incorporated into the ribosome. Some mention of this should be provided. This may be the case for the interaction seen with mitochondrial SSB and subunit II of cytochrome oxidase, for example. Alternatively, some of the hits could arise from proteins bound to the ribosome itself (e.g. elongation factor Tu).

Minor Points:

1. The title should use the term mitochondrial ribosome rather than the abbreviation mitoribosome.

2. p. 4: The authors should tell the reader that recombinant forms of ICT1 with and without the putative import signal were prepared and then introduce the delta29 nomenclature.

3. p. 5 line 5 the "although" should be deleted.

4. p. 13: Additional information should be provided indicating how the 100 uL fractions were handled prior to Western analysis e.g. was the whole sample used, were the fractions concentrated etc. PAG should be defined.

5. This reviewer could not locate the supplementary procedures referred to in the legend to Fig. 2D. This information appears to be in methods.

6. There are several places where designations need to be checked (i.e. where si- or mt- is left out and the authors should go through the manuscript carefully to correct these problems.

7. The preparation of MRPL20-FLAG does not seem to be provided in Methods nor the details of the IP using this tagged protein indicated. The signal from the top of the gradients in Fig. 2G may not represent assembly intermediates but other interactions of over-expressed MRPL20-FLAG. Since no MRPL3 is found in these fractions, the signals from MRPL20 are unlikely to arise from assembly intermediates. It is a little strange that there is no signal from DAP3 in the 28S fractions in this run.

8. p. 6: The third sentence should simply refer to Fig. 3 since 3B presents the actual alignment indicating the release factor domains.

9. It would be interesting to have a sequence comparison or a comment on the alignment of ICT1 with the ribosome-independent peptidyl-tRNA hydrolase family of proteins.

10. It might be useful to other investigators to know how well ICT1 expressed in E. coli and if there were any problems with solubility.

11. The terminology PTH activity of the release factors (p. 6) is a bit confusing. These factors do not have direct PTH activity but rather are thought to trigger the hydrolysis of the bond through the action of peptidyl transferase. Perhaps clearer terminology would be useful here.

1st Revision - Authors' Response 15 January 2010

Referee 1:
1. *' A model which shows putative functions of ICT1 would clearly help non-specialists to fully appreciate the findings'.*
We have pleasure in including a schematic representation of our postulated mechanism for the working of ICT1 (new Fig 5 and associated figure legend). Obviously, this is still speculative at this time, but we hope this will adequately convey our thoughts.

2. *'Is there any visible effect on the protein synthesis after a short time depletion of ICT1.'*
We are a little puzzled by this suggestion and apologise to the reviewer for not understanding their point completely. We believe our data shows ICT1 is a component of the mt-LSU. Therefore, over a short time depletion the only effect would be a minor decrease in nascent mt-LSU. There is no

detectable free ICT1 in gradients of endogenous lysates and ICT1 is unable to promote ribosome-independent PTH. We hope this may help answer this question.

3. *'Does the overexpression of ICT1 affect the kinetics of mitochondrial protein synthesis' and 'is overexpression of ICT1 able to substitute for the function of regular mitochondrial release factor or recycling factor ?'*
Overexpression of ICT1 does not cause any detectable change in the rate of protein synthesis (this has been determined on previous occasions after 6 and 7 day induction of ICT1, evaluating synthesis after 15 min whilst rates are linear). However, ICT1 overexpression does cause a mild growth phenotype, even in glucose media (more profound in galactose media that forces cells to use oxidative phosphorylation). We believe this is due to free, uncomplexed ICT1 being able to compete, albeit poorly, with aminoacylated tRNA at the mitochondrial ribosomal A site, leading occasionally to premature hydrolysis. Although we agree with the suggestion of the reviewer in principle, this effect means it would be extremely difficult to determine whether ICT1 overexpression could rescue the growth phenotype we have published previously for mtRF1a- or mtRRF-depleted cells. We had not stated in the original text that ICT1 overexpression caused a mild growth defect, but it was also relevant to our experiments on overexpression of the GGQ-mutated ICT1, shown in Fig 4. It was essential to titrate the levels of ICT1 wild type and GSQ mutant overexpression by the addition of siRNA such that the ICT1 steady state level achieved was similar to the endogenous levels and did not result in any growth phenotype for the wild type ICT1 overexpression (as shown in Fig S2) . To clarify that ICT1 overexpression causes a mild growth phenotype, we have added the following text at the end of the results section 'A similar overexpression was also noted on induction of wild type ICT1-FLAG (Fig 4C, middle panel*), which in itself caused a mild growth phenotype.* What we can say about mtRRF is that the purified recombinant protein competes with ICT1 for the A site in our *in vitro* release factor assays which use *E.coli* ribosomes.

4. *'To specify the function of ICT1, it would be helpful to test a possibility to detect stalled ribosomes charged with labelled nascent polypeptide chains in the absence of ICT1. There is a certain chance that such things will be visible under normal conditions since ICT1 is required under normal conditions.'*
We thank this reviewer for the suggestion. We have formulated an hypothesis for the physiological function of ICT1 based on our results, but at this stage it is merely an hypothesis. We are currently considering different ways to falsify it. As shown in the manuscript, ICT1 depletion leads to a loss of fully assembled mitochondrial ribosomes and a concomitant loss of mitochondrial protein synthesis. Consequently, it is necessary to perform depletion studies in the ICT1^{GSQ} overexpressor, which allows the formation of normal levels of mitoribosome with non-functional ICT1. We would predict that peptidy tRNAs would be in greater abundance in these ICT1 wild type depleted cells. However, it has proved extremely difficult to trap and detect these intermediates, even under acid urea conditions. We are currently pursuing a molecular genetic approach to elucidate the physiological function, but even if successful this work is unlikely to be completed within a year.

Referee 2
1. *'A control to show that total cellular protein synthesis is not affected would clarify that ICT1 knockdown specifically inhibits mitochondrial protein translation.'*
The referee makes a suggestion that perhaps ICT1 may also function in the cytosol, or that its mitochondrial depletion may somehow signal cytosolic protein synthesis to be inhibited. Although this would be surprising, we believe our data already shows that ICT1 depletion does not affect cytosolic protein synthesis. At the levels of detection for western blot we find no evidence of ICT1 in the cytosol. Most convincingly, our experiments to show that depletion of ICT1 in rho0 cells (which have apparently normal cytosolic translation) has no significant affect on cell viability (Supp Fig 1), shows that depletion of ICT1 affects only mitochondrial gene expression. Third, all lanes from our ^{35}S metabolic labelling experiments are loaded with similar amounts of total cell protein (50 ug) as stated in the legend to Fig 1E. It is routine practice in our laboratory to stain the dried gel after exposure. This involves placing the dried gel and associated 3M paper into the same fixing solution, removing the paper and staining with CCB. We have therefore amended figure 1E to include coomassie staining of part of the gel, showing similar loading of total protein. Our data shows that there is a substantial decrease in mitochondrial compared to cytosolic protein synthesis. We have now reiterated the following statement to the Materials section under In vivo mitochondrial protein synthesis *'Aliquots (50 ug) of total cell protein were separated by 15% (w:v) SDS PAGE.'*

2. *'Fig 1D and 2F do not appear to be cited in the text.'*
We thank the reviewer for spotting our error. We have now added the reference in the revised text to Fig 1D on p.4 (…was confirmed by blotting of HeLa lysates *(Fig 1D.)*) and Fig 2F on p.5 (….FLAG-IP performed and eluates blotted (*Fig 2F*)).

3. *'The authors show that PTH activity of ICT1 is ribosome dependent but mention it as data not shown. This is an important aspect of the study and the data should be shown.'*
We agree with this reviewer. We have now added this data to Fig 3C.

Referee 3
'*The hydrolysis assay should be better described.'*
We have added a more detailed protocol for these assays in the Materials and Methods section.

'How could ICT1 hydrolyse fMet-tRNAMet when bound to the E.coli ribosome ?'
We do not believe that ICT1 integrates into the 70S *E.coli* ribosome in any analogous way to how we believe it operates in the mammalian mitochondrion. In the *in vitro* release activity assays, our data suggests that ICT1 enters the 70S particle via the A-site, as would a standard release factor, positioning the GGQ motif in a way that promotes hydrolysis of the fMet-tRNA^{Met} substrate. ICT1's activity is not restricted to a 70S particle loaded with only a particular A-site codon, because ICT1 lacks the domains defining sequence specificity. We are confident that ICT1 acts via the A-site in the *in vitro* assays with *E. coli* ribosomes as pre-incubation with mtRRF that binds in the A-site but cannot hydrolyse the substrate, inhibits release activity of ICT1 as entry to the A-site is blocked (data can be provided if requested).

'The data [in fig 3C] should be presented in pmol so that efficiency can be evaluated.'
We have now altered the figure accordingly.

'It would be useful to include the – ribosome control.'
We agree (see point 3 referee 2) and now include this data in the new Fig 3C.

'…ICT1 could be a protein that associated tightly with the large subunit. The sentence at the end of the long paragraph on p.5 should be toned down to reflect this. Regardless, the data in Fig 2E are stronger showing that si-ICT1 leads to the destabilization of the large subunit and a decrease in the levels of LSU proteins as well.'
We believe the reviewer is suggesting that the data showing co-IP of mitoribosomal proteins with ICT1 is not the strongest evidence that ICT1 is a *bona fide* member of the mitoribosome. The data to show the decrease in mass of the mt-LSU on siRNA depletion of ICT1 is stronger. To reflect this, we follow the advice of the reviewer and modify the final sentence of the long para on p5, which now reads 'Therefore, ICT1 *behaves as* an integral member of the 39S mt-LSU and a component of the intact 55S monosome.' We have replaced 'confirmation' in the following sentence in the text with 'support' to read as follows "Further *support* that ICT1 is an integral component of the 39S subunit…'

'It would be helpful for most readers to have an indication of absolute scores and what ratios of control versus ICT1 emPAI are indicative of a real interaction.'
As described in the M&M section we used the following criteria to detect enriched MRP's:
*"MRPs were marked as enriched if (i) they were identified with at least 3 unique peptides and (ii) when emPAI values were at least two fold higher in the ICT1 IP sample compared to the negative control."*The reviewer has correctly identified that LRPPRC should not have been included, as the background emPAI value was substantial. We have also found several other examples, including four MRPs albeit of extremely low emPAI values that also do not meet this criterion. They have been removed from the final Supp Table S1 and we thank the reviewer. Our original intention with the LC MS/MS data was to try and provide a pared down table that gave immediately understandable data for the non-specialist. This is in part because in past submissions of manuscripts with proteomics data we have included all the original evaluations and have been asked by the editor to provide a more simplistic table. We understand that this reviewer is more of a specialist and we are happy to provide more detail. To this end, we have now included a more substantial table, which includes additional information that is described in the new Table S1.

'The negative control antiserum here is not clearly defined nor is the presumed use of anti ICT1-FLAG clear. It is possible that some of the hits in this table represent spurious interactions since ICT1-FLAG is overexpressed and soluble rather than being incorporated into the ribosome. Some mention of this should be provided.'
The negative control used lysate from untransfected wild type HEK293T cells subjected to the identical immunoprecipitation protocol, including identical incubation periods with the anti-FLAG beads. Eluate was produced by an identical treatment of the immunoprecipitated beads with FLAG peptide. Eluate was then subjected to an identical LC MS/MS protocol. This information has now been included in the Materials and Methods section. Non-specific interactions with over-expressed proteins in the mitochondrion in general was controlled for by analysis of proteins interacting with mtLuc-FLAG, however, it is always possible that some non-cognate interactions with free ICT1 may be detected.
Therefore, to respond to the reviewers comment about possible non-specific interaction with overexpressed protein, we have added in the text on p.5 *'…some of which may have been interacting in a non-specific manner with the overexpressed protein.'*

Minor points:
1. *'The title should use the term mitochondrial ribosome……'*
We have altered the title accordingly.

2. *'The authors should tell the reader that recombinant forms of ICT1 with and without the putative import signal were prepared and then introduce the delta29 nomenclature.'*
Two new sentences to introduce the recombinant proteins have been inserted near the end of the first paragraph on p4.

3. p5 line 5. 'Although' has now been deleted.

4. '*Additional information should be provided indicating how the 100ul fractions were handled prior to Western analysis.'*
Simply, 10 ul aliquots of each fraction were assessed in all cases. A short addition has been made in the materials and methods.

5. *Supplementary procedure referred to in the legend to Fig 2D is actually in the methods.'*
We thank the reviewer for finding this error. We have now correctly cross referenced this procedure to the Materials and Methods section.

6. *'Designations should be checked, particularly where si- or mt- have been left out'*
We wonder whether there has been a small misunderstanding here. When using a statement such as 'mt-SSU and –LSU', we use the hyphen to indicate that both the subunits are mitochondrial. To prevent confusion, we have just added mt or si as a prefix to everything that is relevant.

7. *'The preparation of MRPL20-FLAG does not seem to have been provided in the Methods'*. We thank the reviewer again for identifying this omission. Indeed, it appears that we did not provide primers or the method for production of either the MRPL20-FLAG or MRPS27-FLAG. We have now added the relevant information to the Materials and Methods. Immunoprecipitation procedures were identical to all other IP expts.

'The signal from the top of the gradients in Fig 2G may not represent assembly intermediates but other interactions of over-expressed MRPL20-FLAG. Since no MRPL3 is found in these fractions, the signals from MRPL20 are unlikely to arise from assembly intermediates. It is a little strange that there is no signal from DAP3 in the 28S fraction in this run.'
It is a formal possibility that the reviewer is correct, but we believe the lack of MRPL3 in these fractions suggests that it has not yet integrated in the early intermediates that are associated with MRPL20. Further, contrary to the final statement where we think the reviewer may be mistaken, the absence of a 28S moiety with DAP3 is of course exactly what you would predict if MRPL20 was specifically IP-ing the 39S mt-LSU and monosome, alone. The absence of a signal from DAP3 in the earlier fractions suggests the immunoprecipitation via the mt-LSU is working as one would predict, without precipitating any contaminating mt-SSU.

8. p.6 reference to Fig 3 has been altered as suggested.

9. *'It would be interesting to have a sequence comparison or a comment on the alignment of ICT1 with the ribosome-independent peptidyl-tRNA hydrolase family.'*
The ribosome-independent peptidyl-tRNA hydrolases that the referee is referring to have a different 3D structure and are not homologous to the ribosome-dependent release factors. We can therefore not show an alignment. We have added two sentences in the legend to Fig 3b to clarify the difference between the ribosome-independent peptidyl-tRNA hydrolases and the ribosome-dependent peptidyl-tRNA hydrolases family that contains ICT1.

10. *'It might be useful to other investigators to know how well ICT1 expressed in E.coli'.* We have added a short section to the materials and methods in the section 'Production of FLAG- and GST-fusion constructs……'.
The purification of recombinant full length ICT1 was not easy, most of the overexpressed protein was in inclusion bodies, even at 16˚C. The deletion of 29 residues from the N-terminus increased the amount of soluble fusion protein and thus the purification of ICT1delta 29 was more efficient. Under the same conditions the yield after purification of recombinant ICT1delta 29 was 2-3 folds higher then of the full length ICT1. The purified protein is quite stable at 4˚C and even after freezing (with or without 50% Glycerol) it is still functioning in the release assay and is monodisperse. However the induction of ICT1delta29-GST causes some inhibition of bacterial growth, probably because of the spontaneous hydrolysis of peptidyl-tRNA in the 70S ribosome in a codon independent manner by ICT1. We believe this inhibition is partial mainly because the protein is being expressed as a fusion protein with GST.

11. *'The terminology PTH activity of the release factors is a bit confusing. Perhaps clearer terminology would be useful here.'*
We agree totally with this reviewer. We find there is substantial confusion in the literature about this. We have no doubt that ICT1 only possesses the indirect ribosome-dependent function that can promote peptidyl hydrolysis. We have tried not to confuse these two very different activities. We have therefore tried to clarify the indicated sentence on p.6 by altering to read as follows: 'To determine whether ICT1 possessed a direct, ribosome independent, PTH activity, or whether it functioned specifically to promote ribosome dependent hydrolysis…'

2nd Editorial Decision 25 January 2010

Your revised paper has been assessed from one of the original referees that fully supports publication in its current form.

The official acceptance letter together with the necessary paperwork will be with you shortly.

I'd like to congratulate you for such a nice piece of work.

Yours sincerely,

Editor
The EMBO Journal.

