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The tRNA methyltransferase Dnmt2 is required for accurate polypeptide synthesis during haematopoiesis

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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.)

Anne Nielsen

1st Editorial Decision

24 April 2015

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies for the rather unusual delay in the review process here. Your study has now been seen by three referees whose comments are shown below.

As you will see from the reports, all referees express interest in the findings reported in your manuscript; however, they do also raise a number of concerns that will need to be addressed before they can support publication of a revised version.

For the revised manuscript I would particularly ask you to focus your efforts on the following points:

-> Please establish whether the HSC phenotypes observed in Dnmt2-depleted mice are cell-intrinsic (as requested by ref #1).

-> Please clarify the statistical basis/reproducibility of the data presented in figs 1 and 2 (as pointed out by both refs #1 and #2)

-> Please alter the data presentation in fig 6 to address the concerns raised by ref #2

-> With regard to points 2 and 6 raised by ref #2 we appreciate the difficulty in fully linking the observations on translational fidelity to the observed phenotype for haematopoiesis; however, we would ask you to more extensively discuss a possible model for this.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised 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, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE COMMENTS

Referee #1:

Tuorto et al report a multifaceted analysis of Dnmt2 knock-out mice, revealing both cellular and molecular phenotypes. My own expertise is in blood stem cell biology. I can therefore only comment in detail on those aspects of the work. The experiments analysing the blood system appear to have been performed well. However, they are not definitive enough to support some of the conclusions being made. It may be important to bear in mind that the experiments on the blood system constitute just a portion of a larger study, that also looks at other tissues, and includes sophisticated molecular and proteomic assays. But, as stated above, I am really only qualified to comment on the experiments on the blood system.

Specific Comments:

1) The main issue with the blood phenotype is that it is not clear whether potential phenotypes seen at the HSC level are cell intrinsic or not. This is an important question, because the authors report that the bone marrow environment appears to be abnormal. Specifically this includes the trabecular zone where previous reports have reported HSCs to reside. There is a straightforward way to address this point, which is to perform reciprocal transplants (mutant HSCs into wt hosts, and wt HSCs into mutant recipients).

I realise that the colony assays support some cell intrinsic defects, but firstly these assays do not test HSC function, and secondly they do not exclude extrinsic influences.

2) Figure 1H: It appears this was done with pooled samples. But was it only done once? There are no error bars here? Some biological repeats of the whole experiment would seem required for top journals like EMBO J.

3) Figure 2I: The same applies as for Figure 1H. Also, the statement in text on line 126/127 isn't really supported by the data, because one could argue that a differentiation defect also exists at the early timepoint (because of the relative elevation of CD34⁻ cells with CD34⁺ cells being near normal).

Referee #2:

The paper by Tuorti et al. explores the effects of a tRNA methyl transferase Dnmt2 in vivo and in vitro. The authors use an impressive range of different techniques, from the analysis of effects in Dnmt2^{-/-} mice to effects of the modification on the stability of tRNA to effects on the proteome and translatoome in cells. Technically, the work is of high quality and well-conceived. Nevertheless, I have some doubts concerning the conceptual novelty beyond the descriptive side of the findings (see below). It would be essential that the authors make a strong point in this regard. Further points that remained unclear to me are the following.

1. Figs 1 and 2. The authors do not describe anywhere in the text why they have chosen to compare particularly phenotypic characteristics of the Dnmt2^{-/-} and wt mice. The reader gets an impression that the authors follow a preconceived idea on what should change and investigate those characteristics only. I am not saying that this is in fact the case and it is also not necessarily wrong to have a hypothesis, but the way the paper is written, the motivation and the choice of biological features to study do not come over clearly.

1a. The general reader of EMBO J, and particularly specialists in tRNA modifications, will hardly understand why "For comprehensive phenotypical examination, Dnmt2^{-/-} mice were backcrossed into the C57BL/6 background for more than 10 generations." (91-92). Please explain.

1b. "We therefore asked whether the ratio of bone marrow LSK cells was still also altered in Dnmt2^{-/-} 115 mice with increasing age" (114-115). Explain why and which parameters make LSK cells an important marker.

1c. The differences between the wt and ^{-/-} are very small and for many observables on the verge of statistical significance (Fig. 1 and 2). E.g. Fig. 2C - if error bars were shown in both directions, as they should be shown, it would be clear that the p5 point is not significantly different. The authors should be really careful when comparing small effects and perhaps comment on what they consider significant and why.

2. There is little connection between the phenotypic observations of Figs 1-3 and the more mechanistic part concerning tRNA modification and the changes in the translatoome and proteome. Basically nothing is really explained, the paper remains on the level of superficial phenomenology.

3. Fig. 4A: it is known that Dnmt² methylates C38. It is important to provide this control, but the finding is not new, it can go to the Supplement. Fig. 4B and C: the statistical significance is not clear, provide quantification. In addition, the notion is not new; this also can be removed to the Supplementary.

4. Fig. 5: Fig. 5A is dispensable (no effect) and can be moved to Supplement. Fig. 5F needs quantification and statistical analysis.

5. Fig. 6: In Fig. 6B it is absolutely essential to show the true error frequencies, rather than fold-change, and to provide statistics. Fig. 6C is not convincing. The authors should reconsider how to show the material for the ribosome profiling experiment - the data in the Supplementary file are more convincing, provided the statistics is explained. Minor point - figure labeling in Figs E6 and E7 is much too small, it is almost impossible to read.

6. Unfortunately, it remains unclear how and why ribosome stalling (or higher occupancy of particular codons) results in misreading of these codons, also here the authors remain rather descriptive and do not provide an appealing mechanistic explanation.

Referee #3:

The manuscript by Tuorto et al describes the effects of a knockout of the tRNA methyltransferase Dnmt2 in mice. The pathological examination of such mice, and the labeling mesenchymal stromal cells are beyond the expertise of this reviewer. Therefore, we concentrated on the RNA modification related experiments.

The function of Dnmt2 in several model organisms has been elusive. In this study the authors employed two key technologies: 1. Dynamic stable isotope labeling by feeding "heavy" amino acids feed to the cell culture, followed by proteomic mass spec sequencing. 2. Ribosome profiling to see longer occupancy at mis-translated codons. The rigorous application of these methods allowed the authors to unravel a function of C38 methylation in a subset of tRNAs.

The major findings are (i) downregulated proteins in Dnmt2-deficient cells have a high frequency of Lys(AAG) and Glu(GAG) codons. (ii) Significant Asp to Glu and Glu to Asp transitions through

near-cognate mistranslation have been observed. (iii) Ribosome profiling indicates longer transition time on Asp(GAU) and Glu(GAG) codons.

The major conclusion is supported by the experimental facts: C38 unmodified tRNA^{Asp}-GAC reduce the ability to compete with near-cognate tRNA and enhances near-cognate reading of GAG codon. Hence, C38 methylation of tRNA^{Asp}(GAC) is key for translational fidelity.

Minor points:

Page 2, lane 28: "Dnmt2 is a unique enzyme". The statement of novelty should be removed.

Page 2, lane 35: "dynamic SILAC analysis". SILAC needs to be written out.

Page 3, lane 67: Rephrase "are only beginning to be"

1st Revision - authors' response

15 June 2015

In response to the referee's and editor's comments we have made the following changes to the manuscript

Editor:

1. Please establish whether the HSC phenotypes observed in Dnmt2-depleted mice are cell-intrinsic (as requested by ref #1).

>> As requested by referee #1 (point 1) we have performed reciprocal transplantation experiments. The results show a cell autonomous defect with a significant disproportional increase in myeloid versus lymphoid differentiated cells in Dnmt2 mutants. We have included the new data in Fig. 2F. A minor extrinsic phenotype was also observed by the slight differentiation defect in reversed transplantation experiments (Fig. E1D).

Extrinsic defects are also supported by the observed niche alteration in newborn mice (see Fig. 1A,B; Fig. 3; Fig. 5). While the LSK number is reestablished with age (Fig. 1G) and the histology of adult mice recovers to the normal state (new Fig. E1B), an increase of self-renewal capacity versus differentiation was stably maintained through adulthood (Fig. 1G-H; Fig. 2C-E), which we again interpret as a cell-autonomous phenotype. This is now clarified in the text.

Please clarify the statistical basis/reproducibility of the data presented in figs 1 and 2 (as pointed out by both refs #1 and #2)

>> Additional biological replicates were analyzed to improve the statistical power of Fig. 1 and Fig. 2. The revised Figures include at least one and often two additional biological replicates for each genotype and the differences are statistically significant. Also, the FACS analysis was improved by changing the gating to analyze only living single cells.

3. Please alter the data presentation in fig 6 to address the concerns raised by ref #2

>> As requested by referee #2, we replaced figure 6B with a plot showing the true error frequencies and we applied statistical testing to confirm the differences. Figure 6C represents a standard way to illustrate bulk polysome codon occupancy (Zinshteyn & Gilbert, 2013). As requested by referee #2, we provided the statistics in the figure. Additionally, part of figure 6C was moved to the supplementary information and replaced with graphs that include statistics in the visualization of codon occupancy of Dnmt2 target codons (Fig 6D). We also increased the size of the labeling in Figs E6 and E7.

4. With regard to points 2 and 6 raised by ref #2 we appreciate the difficulty in fully linking the observations on translational fidelity to the observed phenotype for haematopoiesis; however, we would ask you to more extensively discuss a possible model for this.

>> We have created a model and included it as figure 7. Also we included a new point in the discussion that explains the role of Dnmt2 in adapting tRNAs to codons during differentiation, thus suggesting a role as a „canalizer“ of translation (last paragraph of the discussion).

Referee #1:

1) The main issue with the blood phenotype is that it is not clear whether potential phenotypes seen at the HSC level are cell intrinsic or not. This is an important question, because the authors report that the bone marrow environment appears to be abnormal. Specifically this includes the trabecular zone where previous reports have reported HSCs to reside. There is a straightforward way to address this point, which is to perform reciprocal transplants (mutant HSCs into wt hosts, and wt HSCs into mutant recipients). I realise that the colony assays support some cell intrinsic defects, but firstly these assays do not test HSC function, and secondly they do not exclude extrinsic influences.

>> As requested by the referee we have performed reciprocal transplantation experiments. The results show a cell-autonomous defect within a significant disproportional increase in myeloid versus lymphoid Dnmt2 differentiated cells. We have included the new data in Fig 2F. A minor extrinsic phenotype was also observed by the slight differentiation defect in reversed transplantation experiments (Fig. E1D).

Extrinsic defects are also supported by the observed niche alteration in newborn mice (see Fig. 1A,B; Fig. 3; Fig. 5). While the LSK number is reestablished with age (Fig. 1G) and the histology of adult mice recovers to the normal state (new Fig. E1B), an increase of self-renewal capacity versus differentiation was also observed in adult mice (Fig. 1G-H; Fig. 2C-E), which we again interpret as a cell-autonomous phenotype. This is now clarified in the text.

2) Figure 1H: It appears this was done with pooled samples. But was it only done once? There are no error bars here? Some biological repeats of the whole experiment would seem required for top journals like EMBO J.

>> Additional biological replicates were analyzed to improve the statistical power and the differences are statistically significant.

3) Figure 1I: The same applies as for Figure 1H. Also, the statement in text on line 126/127 isn't really supported by the data, because one could argue that a differentiation defect also exists at the early timepoint (because of the relative elevation of CD34- cells with CD34+ cells being near normal).

>> Additional biological replicates were analyzed to improve the statistical power and the differences are statistically significant. Also we have changed the text according to the reviewer's suggestions.

Referee #2:

Figs 1 and 2. The authors do not described anywhere in the text why they have chosen to compare particularly phenotypic characteristics of the Mnmt2-/- and wt mice. The reader gets an impression that the authors follow a preconceived idea on what should change and investigate those characteristics only. I am not saying that this is in fact the case and it is also not necessarily wrong

to have a hypothesis, but the way the paper written, the motivation and the choice of biological features to study do not come over clearly.

>> We have chosen the haematopoietic system as a paradigm of cellular proliferation and differentiation and we have clarified this point in the introduction (84-85).

1a. The general reader of EMBO J, and particularly specialists in tRNA modifications, will hardly understand why "For comprehensive phenotypical examination, Dnmt2^{-/-} mice were backcrossed into the C57BL/6 background for more than 10 generations."(91-92). Please explain.

>> We have clarified this issue and provided an explanation in the text (94-95): "... thus establishing a homogeneous genetic background for the evaluation of specific aberrant phenotypes."

1b. "We therefore asked whether the ratio of bone marrow LSK cells was still also altered in Dnmt2^{-/-} 115 mice with increasing age" (114-115). Explain why and which parameters make LSK cells an important marker.

>> We have clarified the importance of LSK cells. Lin⁻ Sca-1⁺ c-kit⁺ (LSK) has been generally used as a canonical marker set for HSC enrichment. We now explain that they represent the most primitive hematopoietic cells that have self-renewal capacity and can give rise to all mature cell types found in the blood and provide a reference (115-118). Nevertheless, the LSK fraction is heterogeneous, including long- term self-renewing HSC, short-term non-self-renewing HSC and lineage-committed progenitors. We therefore used the CD34 marker (Osawa et al, 1996) to further distinguish between these two populations. This is also explained in the text (129-132).

1c. The differences between the wt and ^{-/-} are very small and for many observables on the verge of statistical significance (Fig. 1 and 2). E.g. Fig. 2C - if error bars were shown in both directions, as they should be shown, it would be clear that the p5 point is not significantly different. The authors should be really careful when comparing small effects and perhaps comment on what they consider significant and why.

>> We have included additional biological replicates to improve the statistical power of figures 1 and 2, also see our response to referee 1, points 2 and 3. Two additional biological replicates were included in Fig 2C and we replaced the growth curves with population doubling levels from multiple experiments. Observed differences were statistically significant in all cases.

2. There is little connection between the phenotypic observations of Figs 1-3 and the more mechanistic part concerning tRNA modification and the changes in the translatome and proteome. Basically nothing is really explained, the paper remains on the level of superficial phenomenology.

>> We respectfully disagree with the reviewer's opinion. To better explain the novel mechanistic insight provided by our study, we have created a model and included it as figure 7. Also we included a new point in the discussion that explains the role of Dnmt2 in adapting tRNAs to codons during differentiation, thus suggesting a role as a „canalizer“ of translation (see last paragraph of the discussion).

3. Fig. 4A: it is known that Dnmt² methylates C38. It is important to provide this control, but the finding is not new, it can go to the Supplement. Fig. 4B and C: the statistical significance is not clear, provide quantification. In addition, the notion is not new; this also can be removed to the Supplementary.

>> We think that this figure should remain in main text because of its important "bridging" function

between phenotype (Figs. 1-3), published molecular data and new mechanistic data (Figs. 5-7). Also, the increase of fragmentation of tRNA in non-stressed tissue is a new finding. Finally, Figure 4C represents an orthogonal, quantitative approach to Fig. 4B.

4. Fig. 5: Fig. 5A is dispensable (no effect) and can be moved to Supplement. Fig.5F needs quantification and statistical analysis.

>> We agree with the reviewer and moved Fig. 5A to the supplement. We also added quantification and statistical analysis on immunofluorescence and included the results as Fig. 5F.

5. Fig. 6: In Fig. 6B it is absolutely essential to show the true error frequencies, rather than fold-change, and to provide statistics. Fig. 6C is not convincing. The authors should reconsider how to show the material for the ribosome profiling experiment - the data in the Supplementary file are more convincing, provided the statistics is explained. Minor point - figure labeling in Figs E6 and E7 is much too small, it is almost impossible to read.

>> We replaced figure 6B with the true error frequencies, which were calculated as the number of aberrant peptides over the total number of identified peptides, and we applied Fisher's Exact test to confirm the statistical significance of the differences. Part of Figure 6C was moved to the supplementary information and replaced with a graph that includes statistics. We also increased the size of the labeling in Figures EV6 and EV7.

6. Unfortunately, it remains unclear how and why ribosome stalling (or higher occupancy of particular codons) results in misreading of these codons, also here the authors remain rather descriptive and do not provide an appealing mechanistic explanation.

>> See point 2 above.

Referee #3:

Page 2, lane 28: "Dnmmt2 is a unique enzyme". The statement of novelty should be removed.

Page 2, lane 35: "dynamic SILAC analysis". SILAC needs to be written out.

Page 3, lane 67: Rephrase "are only beginning to be"

>> We have changed the text according to the reviewer's suggestions.

2nd Editorial Decision

02 July 2015

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees whose comments are shown below.

As you will see they both find that all experimental criticisms have been sufficiently addressed. However, ref#2 asks for additional text revision in the discussion as well as modifications to the model in fig 7 to better reflect the possible (if currently open) link between altered translational fidelity and the hematopoietic system.

Based on this input I would therefore invite you to submit a final revised version of the manuscript incorporating the points made by ref #2. In addition, I would ask you to address the following editorial points:

-> Please make sure that all figure legends contain information on the number of replicates and nature of error bars when depicting statistical analysis.

-> We can currently only accommodate up to 5 figures to be displayed as expanded view. I would therefore ask you to either move two of the current EV figures into the appendix (if possible) or to alternatively move all 7 EV figures to the appendix (as traditional supplemental figures). I would be happy to discuss the layout of this further, if necessary.

-> We generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your revision.

REFEREE COMMENTS

Referee #1:

I am happy to see that the authors carried out the reciprocal transplant experiments which I had suggested. This strengthens the paper substantially, and also appears to open up avenues for future investigations.

Overall, I am happy with the way the authors have responded to the issues I had raised.

Referee #2:

The authors generally answered most of my questions, except for the issue that they attempted to illustrate in Fig. 7. I appreciate that it is difficult to provide an experimental link between the phenotypic observations in mice, tRNA modifications and the changes in the transcriptome and proteome. The aim of my comment was to encourage the authors to provide a more comprehensive explanation for the potential link, even if it is a hypothetical one. The new Figure 7 does not fulfill this task, and even more, it is a disaster. It is not clear what is shown and Fig legend does not provide the explanation. There are no "T"s in the mRNA or tRNA, it is a "U", this is a real mistake (also writing the anticodon upside-down is not helpful). Furthermore, the references in the respective part of the Discussion (lines 388-422) are largely inaccurate (see below). The Figure should be revised to illustrate the link between fidelity, ribosome pausing and in vivo phenotypes, the text of Figure legend should be added (there is essentially no explanation now) and factual mistakes have to be corrected.

Here are a few examples of inaccurate citations; in addition, the authors should go throughout the text and carefully check the suitability of all citations.

395 "Misincorporation of amino acids leads to the production of mistranslated or aborted nascent polypeptides (Blanco et al, 2014; Moghal et al, 2014; Zaher & Green, 2011)" Reference Blanco is not suitable in this context, because it does not say anything about mistranslation itself. This is a citation to original publication, which is inappropriate to mix with reviews; two other citations are indeed reviews, whether these are the most appropriate ones, is a matter of taste.

404 "the so-called wobble base (Crick, 1996)". Use the reference for the extended wobble rules, the old reference is not appropriate since the extended wobble rules (including tRNA modifications!) were described.

407 "modified U34 maintains proteome integrity in yeast and in *C. elegans* (Nedialkova & 408 Leidel, 2015)". The authors should cite earlier papers which show the role of U34, e.g. Rezgui et al., 2014 and others.

409 "Furthermore, the ribosome discriminates between correct and incorrect aminoacyl-tRNAs according to the match between anticodon and mRNA codon in the A site of the ribosome (Manickam et al, 2014; Marquez et al, 2004)." Both references are entirely unsuitable. The statement can be found in every textbook and does not require citations, but if the authors prefer to have a reference than they should use a decent classical review.

413 "Consequently, a two-step process, consisting of initial selection and subsequent proofreading, has been proposed (Blanchard et al, 18 2004; Gromadski & Rodnina, 2004; Jenner et al, 2010; Johansson et al, 2012)". Again, the references are unsuitable, because this is known since the 70s-80s of last century. Again, find an influential review on decoding and cite it. These facts (as the statement in 409) were not discovered in 2004 or 2012.

420 "thereby reducing ribosomal proofreading" The authors misunderstand the meaning of "proofreading", which is the 2nd step of tRNA selection (as they correctly state in line 414), rather than the ability of the ribosome to discriminate (which is probably what the authors mean).

2nd Revision - authors' response

13 July 2015

In response to the editor's and referee's comments we have made the following changes to the manuscript:

Editor:

As you will see they both find that all experimental criticisms have been sufficiently addressed. However, ref#2 asks for additional text revision in the discussion as well as modifications to the model in fig 7 to better reflect the possible (if currently open) link between altered translational fidelity and the hematopoietic system.

>> We changed the model according to the reviewer's suggestions and we corrected the mistakes. Moreover, we provided additional detail in the figure legend. We also re-checked the suitability of all citations and made additional changes according to the reviewer's suggestions.

Please make sure that all figure legends contain information on the number of replicates and nature of error bars when depicting statistical analysis.

>> We have re-checked the figure legends accordingly.

We can currently only accommodate up to 5 figures to be displayed as expanded view. I would therefore ask you to either move two of the current EV figures into the appendix (if possible) or to alternatively move all 7 EV figures to the appendix (as traditional supplemental figures).

>> We moved two EV figures into the appendix.

We generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

>> We provided the source data for the Western blot in Figure 2B and the Northern blots in Figure 4B.

Referee #2:

The new Figure 7 does not fulfill this task, and even more, it is a disaster. It is not clear what is shown and Fig legend does not provide the explanation. There are no "T"s in the mRNA or tRNA, it is a "U", this is a real mistake (also writing the anticodon upside-down is not helpful). Furthermore, the references in the respective part of the Discussion (lines 388-422) are largely inaccurate (see below). The Figure should be revised to illustrate the link between fidelity, ribosome pausing and in vivo phenotypes, the text of Figure legend should be added (there is essential not explanation now) and factual mistakes have to be corrected.

>> We simplified and clarified the model according to the reviewer's suggestions and we corrected the mistakes. We also added a detailed legend to the model.

395 "Misincorporation of amino acids leads to the production of mistranslated or aborted nascent polypeptides (Blanco et al, 2014; Moghal et al, 2014; Zaher & Green, 2011)" Reference Blanco is not suitable in this context, because it does say anything about mistranslation itself. This is a citation to original publication, which is inappropriate to mix with reviews; two other citations are indeed reviews, whether these are the most appropriate ones, is a matter of taste.

>> We removed changed the citations for this sentence: "Misincorporation of amino acids leads to the production of mistranslated or aborted nascent polypeptides, which triggers lysine ubiquitination, proteasome activation and degradation of mistranslated proteins (Drummond & Wilke, 2008; Lee et al, 2006)."

404 "the so-called wobble base (Crick, 1996)". Use the reference for the extended wobble rules, the old reference is not appropriate since the extended wobble rules (including tRNA modifications!) were described.

>> We added a reference for extended wobble rules (Agris et al., 2007).

407 "modified U34 maintains proteome integrity in yeast and in C. elegans (Nedialkova & 408 Leidel, 2015)". The authors should cite earlier papers which show the role of U34, e.g. Rezgui et al., 2014 and others.

>> We added the citation to the text.

409 "Furthermore, the ribosome discriminates between correct and incorrect aminoacyl-tRNAs according to the match between anticodon and mRNA codon in the A site of the ribosome (Manickam et al, 2014; Marquez et al, 2004)." Both references are entirely unsuitable. The statement can be found in every textbook and does not require citations, but if the authors prefer to have a reference than they should use a decent classical review.

>> We removed the citations from the text.

413 "Consequently, a two-step process, consisting of initial selection and subsequent proofreading, has been proposed (Blanchard et al, 18 2004; Gromadski & Rodnina, 2004; Jenner et al, 2010; Johansson et al, 2012)". Again, the references are unsuitable, because this is known since the 70s-80s of last century. Again, find an influential review on decoding and cite it. These facts (as the statement in 409) were not discovered in 2004 or 2012.

>> We removed the 2 citations from the text and cited an influential review (Rodnina & Wintermeyer, 2001).

420 "thereby reducing ribosomal proofreading" The authors misunderstand the meaning of

"proofreading", which is the 2nd step of tRNA selection (as they correctly state in line 414), rather than the ability of the ribosome to discriminate (which is probably what the authors mean).

>> We replaced "ribosomal proofreading" with "translational fidelity".