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Human TLR8 senses UR/URR-motifs in bacterial and mitochondrial RNA

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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 27 July 2015

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although all the referees find the topic of interest and in principle suitable for us, all referees request revision of the study before it can be considered for publicaiton here.

Referees 1 and 2 mainly refer to rewriting and improving the clarity, flow and coverage of the field (including the recent Eigenbrod et al, which they all mention impacts on the novelty of your work), as well as how your work advance the field with respect to that study. Referee 3 requests a number of experiments that will strengthen the work, which will need to be addressed for publication. Indeed, given that the bacterial RNA sensing capacity of TLR8 has been recently described, the stronger your study is, the more significant a contribution it will make to the field.

Given that all referees provide constructive suggestions on how to make the work more conclusive, I would like to give you the opportunity to revise your manuscript. If the referee concerns can be adequately addressed, we would be happy to accept your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review. In this case, all the referee concerns are pertinent and should be addressed. Please note that introducing subheadings into the Results&Discussion section, which is clearly needed in this case, will improve the structure and clarity of the work.

As timing is of essence in this case, and the article should be published this year to have the desired impact, please submit a revised version within the next 8 weeks. Please contact us if this deadline seems to be a problem.

Revised manuscripts must be submitted within three months of a request for revision unless previously discussed with the editor; they will otherwise be treated as new submissions. Revised manuscript length must be a maximum of 25,000 characters, including spaces and figure legends, but excluding references.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision)
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
- a separate PDF file of any Supplementary information (in its final format)

EMBO reports now accommodates the inclusion of extra figures (up to five) in the online version of the manuscript. These are presented in an expandable format inline in the main text so that readers who are interested can access them directly as they read the article. They are also provided for download in a separate typeset PDF to accompany the Article PDF. These should be those of particular value to specialist readers, but which are not required to follow the main thread of the paper (and not additional controls or reagent optimization). These should be labeled expanded view, and the rest supplementary.

We also encourage the publication of original source data -particularly for electrophoretic gels and blots, but also for graphs- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures and an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

Note:

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File 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."

REFEREE REPORTS

Referee #1:

In this MS Kruger and colleagues identify TLR8 as a human homologue of TLR13 that recognizes specific motifs in bacterial and mitochondrial RNA. The authors suggest that recognition of these RNA motifs is important for the inflammatory responses of human monocyte cells for Staph aureus and E coli infections. The MS presents a strong data set, but the text is lacking in clarity and confusing in places. Overall the text will need to be substantially re-written to make it easier to read and to to clarify the MS message.

1. What are the major claims and how significant are they?
The authors identify human TLR8 as functional homologue of mouse TLR13 and that this receptor contributes to driving inflammation in response to infection with Staph aureus and E coli. They conclude TLR8 could be a novel therapeutic target for treating sepsis.

2. Are the claims novel and convincing?
The data set presented are solid and convincing. A similar MS was recently published (TLR8 Senses Bacterial RNA in Human Monocytes and Plays a Nonredundant Role for Recognition of Streptococcus pyogenes.Eigenbrod T, Pelka K, Latz E, Kreikemeyer B, Dalpke AH. J Immunol. 2015 Aug 1;195(3):1092-9. doi: 10.4049/jimmunol.1403173. Epub 2015 Jun 22) which, unfortunately, impacts on the novelty of this paper. Clarification of the importance of this MS over the recently published paper would be very useful.

3. Are the claims appropriately discussed in the context of earlier literature?
The data are discussed within the context of the earlier literature except for the recent J Immunol paper described under question 2.

4. Who will be interested and why?
If TLR8 is proven to be a novel candidate for treating sepsis in people this MS will be of broad interest to clinicians and scientists, particularly those working in immunology.

5. Does the paper stand out in some way from the others in its field?
This is a careful and well performed study.

6. Are the experimental data of sufficient quality to justify the conclusions?
Yes

Referee #2:

In this manuscript, Krug et al identify that human TLR8 acts as a functional equivalent of murine TLR13 for sensing bacterial single stranded RNA by providing loss-of function genetic evidence (TLR8-/--3ddiTHP-1). This finding is consistent with several most recent reports (Eigenbrod et al., 2015, JI; Bergstrøm et al., 2015, JI; Cervantes JL et al., 2013, JLB). Unlike TLR13 that is known to recognize bacterial 23S ribosomal RNA with an exquisite sequence-specificity, the authors demonstrated that human TLR8 recognized its ligand promiscuously, such as a new sequence motif containing UR/URR derived from mammalian mitochondria 16S rRNA that was able to activate an immune response that is dependent on Myd88-, UNC93B- and TLR8-mediated pathway in human PBMC or human monocyte cell line THP1. Although some findings from this study are potentially interesting, there are a few concerns listed below.

1. The figure legends were written poorly and often times hard to follow. The authors did not include sufficient details on how they performed the individual experiment in all figures. In addition, the authors should clearly spell out the main conclusion of the main figure before going into the experimental details of subfigures.

2. The authors may need to consider reorganizing their data in order to make their story follow smoothly. For example, figure 1, the study on the identification and immune-stimulatory mechanism of Sal19-derivitives from mammalian mitochondria 16S rRNA could be rearranged to a separate figure or moved to Figure 3.

3. In Figure 1E, in contrast to S. aureus, 23s 16s and 5S rRNA of E. coli have little activity to induce IFNa. Then, how to explain the immune stimulating activity of E. coli total RNA?

Referee #3:

Krüger et al. "Human TLR8 senses UR/URR-motifs in RNA derived from bacteria and mitochondria"

This is a very thorough investigation of the role various oligoRNAs on TLR8/MyD88 signaling in PBMCs and differentiated human monocytoid THP-1 cells. A vast variety of tests were carried out to illustrate the specificity of ORNs and their extent of cytokine induction. The data are presented in a very compressed form. In general the results are convincing and represent also an important contribution TLR8 characterization, however, there are some questions and comments that should be considered.

The role of TLR7 is not convincingly addressed. TLR7 also recognizes single stranded RNAs as their natural ligand and as well as small synthetic molecules such as imidazoquinolines and nucleoside analogs. Stimulation studies with TLR7 and TLR7/8 (if available) k.o. cells would be important controls. It would be good to better comment the difference of RNA-ligands for TLR8 and TLR7.

What is the biological significance that the own mitochondrial rRNA is a ligand for TLR8. Is it also a ligand for TLR7?

The TLR7, 8, 9 and 13 are intracellular. The question arises and should be commented how extracellular oligoRNAs are taken up under natural conditions (not with the help of lipofectin)? Maybe TLR8 stimulation occurs mainly after phagocytosis?

The statement that TLR8 is the major bacterial/ mitochondria RNA sensor is not justified by the results. In this respect one would expect comparative stimulation studies with TLR8 and TLR2 k.o. cells with a) external applied ligands and b) with whole bacterial cells.

Why were bacteria only sensed when live but not when heat inactivated?

The very recent paper on TLR8 sensing of Staphylococcus aureus RNA should be quoted and discussed, as there are overlapping results:
Bergstrom B, Aune MH, Awuh JA, Kojen JF, Blix KJ, Ryan L, Flo TH, Mollnes TE, Espevik T, Stenvik J. 2015. TLR8 Senses Staphylococcus aureus RNA in Human Primary Monocytes and Macrophages and Induces IFN-beta Production via a TAK1-IKKbeta-IRF5 Signaling Pathway. Journal of immunology.

Expanded Fig. 1:
A) should shows only the 23 S rRNA, but not the mt 16SrRNA as written in Fig. legend;
B) it should not be N-terminal and C- terminal, but 5'- and 3' end.

1st Revision - authors' response 09 October 2015

Thank you very much for giving us the opportunity to revise our manuscript EMBOR-2015-40861 entitled **Human TLR8 senses UR/URR-motifs in RNA derived from bacteria and mitochondria** within 2 months and the reviewers very much for their constructive input by raising of points our addressing of which has improved the manuscript substantially. Moreover, addition of introducing subheadings in the Results & Discussion section also improved clarity of our work. Assuredly meeting the reviewer's specifications now, we hereby submit the resulting second manuscript version to The Journal while hoping for kind consideration of it for publication by you.

Referee #1:

In this MS Kruger and colleagues identify TLR8 as a human homologue of TLR13 that recognizes specific motifs in bacterial and mitochondrial RNA. The authors suggest that recognition of these RNA motifs is important for the inflammatory responses of human monocyte cells for Staph aureus and E coli infections. The MS presents a strong data set, but the text is lacking in clarity and confusing in places. Overall the text will need to be substantially re-written to make it easier to read and to to clarify the MS message.

1. Krüger et al. reply: Our study aiming at identification of a major human Gram-positive bacteria sensor potentially besides as such established toll-like receptor (TLR) 2 (the blockade of which did not efficiently inhibit human cell activity upon respective challenge)
started from observing responsiveness of human immune cells to challenge with TLR13 (not expressed in human) ligand and bacterial 23S rRNA contained Sa19, and low human RNA sequence specificity as compared to that of mice and choosing human (h) TLR8 as best TLR13 substitute RNA sensor candidate upon unbiased mRNA profiling to finish with a proposal of an anti sepsis pathology therapy with established compounds such as chloroquine to potentially prevent septic shock through specific TLR blockade.

Spinoffs were implication of "UR/URR" as TLR8 RNA ligand consensus motif expanding "UG/UGG" brought forward before by others which we now substantiated by showing another active Sa12 variant in which the A behind the inserted U was replaced by G additionally which is TLR8 stimulatory also (Expanded View Figure 3B) and a hypothesis on how abrogated TLR13 expression in the face of expression of comprehensively functional TLR8 by some Coelomata species including human might be explainable.

We have rewritten the text to improve its clarity and illustration of our study's coherence. For instance, clear representation of the close relation of bacterial and mitochondrial RNAs from a phylogenetic perspective already close to the text outset shall emphasize a potential property of the latter as "PAMP-like DAMP" (concentrated in the text as pathogen- and host- derived "danger associated molecular pattern", P-/DAMP) to justify the sequence of data illustrations throughout our manuscript.

1. What are the major claims and how significant are they?
The authors identify human TLR8 as functional homologue of mouse TLR13 and that this receptor contributes to driving inflammation in response to infection with Staph aureus and E coli. They conclude TLR8 could be a novel therapeutic target for treating sepsis.

2. Are the claims novel and convincing?
The data set presented are solid and convincing. A similar MS was recently published (TLR8 Senses Bacterial RNA in Human Monocytes and Plays a Nonredundant Role for Recognition of Streptococcus pyogenes.Eigenbrod T, Pelka K, Latz E, Kreikemeyer B, Dalpke AH. J Immunol. 2015 Aug 1;195(3):1092-9. doi: 10.4049/jimmunol.1403173. Epub 2015 Jun 22) which, unfortunately, impacts on the novelty of this paper. Clarification of the importance of this MS over the recently published paper would be very useful.

2. Krüger et al. reply: "Upon submission of this study four reports on Gram-positive bacterial RNA sensing by hTLR8 or on TLR13 structure have been published and largely summarized [26-30]. Thus, *S. aureus* as well as *Streptococcus pyogenes* and -*agalactiae* and *Listeria monocytogenes* total RNAs activate hTLR8 but not TLR7 [26,28]. Total RNAs of further Gram-positive and probiotic bacteria, as well as *Enterococcus faecalis* (EC-12) derived 23S and 16S rRNA drive TLR8 dependent, yet TLR7 independent IL-12 production [27]. Our results extended these and earlier findings by implicating 5S beyond 23S and 16S rRNA of *S. aureus* and also Gram-negative *E. coli* as well as mtRNA as immune stimulatory P-/DAMPs that activate hTLR8 with their UR/URR motif segments [11,13].
C*itations 11 and 13 had been intergrated into the original manuscript version already*" has been inserted as penultimate paragraph into the text.

3. Are the claims appropriately discussed in the context of earlier literature?
The data are discussed within the context of the earlier literature except for the recent J Immunol paper described under question 2.

4. Who will be interested and why?
If TLR8 is proven to be a novel candidate for treating sepsis in people this MS will be of broad interest to clinicians and scientists, particularly those working in immunology.

5. Does the paper stand out in some way from the others in its field?
This is a careful and well performed study.

6. Are the experimental data of sufficient quality to justify the conclusions?
Yes
Referee #2:

In this manuscript, Krug et al identify that human TLR8 acts as a functional equivalent of murine TLR13 for sensing bacterial single stranded RNA by providing loss-of function genetic evidence (TLR8-/--3ddiTHP-1). This finding is consistent with several most recent reports (Eigenbrod et al., 2015, JI; Bergstrøm et al., 2015, JI; Cervantes JL et al., 2013, JLB).

2. Krüger et al. reply as above.
Unlike TLR13 that is known to recognize bacterial 23S ribosomal RNA with an exquisite sequence-specificity, the authors demonstrated that human TLR8 recognized its ligand promiscuously, such as a new sequence motif containing UR/URR derived from mammalian mitochondria 16S rRNA that was able to activate an immune response that is dependent on Myd88-, UNC93B- and TLR8-mediated pathway in human PBMC or human monocyte cell line THP1. Although some findings from this study are potentially interesting, there are a few concerns listed below.

1. The figure legends were written poorly and often times hard to follow. The authors did not include sufficient details on how they performed the individual experiment in all figures. In addition, the authors should clearly spell out the main conclusion of the main figure before going into the experimental details of subfigures.

3. Krüger et al. reply: We expanded figure legends according to this comment. The message of each main figure has been worded concisely also.

2. The authors may need to consider reorganizing their data in order to make their story follow smoothly. For example, figure 1, the study on the identification and immune-stimulatory mechanism of Sal19-derivitives from mammalian mitochondria 16S rRNA could be rearranged to a separate figure or moved to Figure 3.

4. Krüger et al. reply: Due to the phylogenetically relatively close relation of bacteria and mitochondria potentially qualifying their RNAs as “P-/DAMPs” we consider parallel analysis of both sorts of RNA as plausible. We express our respective motivation by clearer wording now attempting to convince this reviewer in respect to the current data illustration sequence. Please see also the first paragraph of the letter to the Editor and “1. Krüger et al. reply”.

3. In Figure 1E, in contrast to S. aureus, 23s 16s and 5S rRNA of E. coli have little activity to induce IFNa. Then, how to explain the immune stimulating activity of E. coli total RNA?

5. Krüger et al. reply: Out of total bacteria RNA, tRNA has been reported to drive via plasmacytoid DC TLR7 type I interferon (IFN) production
(citations 9 and 10) and please see “9. Krüger et al. reply” below for further information. To experimentally exclude (known) TLR7 activity interference we separated 5S from tRNA by preparative PAGE (Expanded View Figure 2A, EVF2A), which we stress more insistently now (through rewording). The respective text is now “While total bacterial RNA encompassing tRNA triggered substantial IFNα release, neither ..”.

Referee #3:

Krüger et al. "Human TLR8 senses UR/URR-motifs in RNA derived from bacteria and mitochondria"

This is a very thorough investigation of the role various oligoRNAs on TLR8/MyD88 signaling in PBMCs and differentiated human monocytoid THP-1 cells. A vast variety of tests were carried out to illustrate the specificity of ORNs and their extent of cytokine induction. The data are presented in a very compressed form. In general the results are convincing and represent also an important contribution TLR8 characterization, however, there are some questions and comments that should be considered.

The role of TLR7 is not convincingly addressed. TLR7 also recognizes single stranded RNAs as their natural ligand and as well as small synthetic molecules such as imidazoquinolines and nucleoside analogs. Stimulation studies with TLR7 and TLR7/8 (if available) k.o. cells would be important controls. It would be good to better comment the difference of RNA-ligands for TLR8 and TLR7.

6. Krüger et al. reply: Please see "9. Krüger et al. reply" below.

What is the biological significance that the own mitochondrial rRNA is a ligand for TLR8. Is it also a ligand for TLR7?

7. Krüger et al. reply: Taken as respectively applicable criteria that mtRNA or mtrRNA segments (16S mtRNA derived ORNs) did not activate murine macrophage TLR7 (EVF1D) and human PBMC did not produce typeI IFN upon mtRNA challenge (Fig. 1F), TLR7 is not activated by mtRNA. Please see "9. Krüger et al. reply" for further explanation of the underlying rationale.

The TLR7, 8, 9 and 13 are intracellular. The question arises and should be commented how extracellular oligoRNAs are taken up under natural conditions (not with the help of lipofectin)? Maybe TLR8 stimulation occurs mainly after phagocytosis?

8. Krüger et al. reply: The question for RNA take up is a very interesting one. Murine macrophages respond to untransfected Sa19 (unpublished) via TLR13 as if they actively take up the ORN. Transfection of the RNAs was necessary to trigger human PBMC activation, which indicates a species difference in cellular RNA processing. Possibly due to the low specificity of hTLR8 as compared to that of mTLR13 humans "can not afford" to sense naked RNA besides phagocytosed microbe borne RNA (because hTLR8 would be activated by self-mtRNA also). Our study in general and the last sentence, namely "The price to be paid, however, is reactivity towards endogenous ssRNA with which the immune system of hTLR8 transgenic mice can hardly cope [32,33]." specifically shall at least imply this consideration (space restriction largely wards us from respective expansion of the discussion). Our answer to the second question above is "yes" in any case.

The statement that TLR8 is the major bacterial/ mitochondria RNA sensor is not justified by the results. In this respect one would expect comparative stimulation studies with TLR8 and TLR2 k.o. cells with a) external applied ligands and b) with whole bacterial cells.

9. Krüger et al. reply: The title and the abstract of our original and present manuscript do not indicate TLR8 as "the major bacteria and mitochondria sensor". In the last paragraph of the introduction and at the end of the text we wrote "Our results identify TLR8 as major bacteria and mitochondria sensor .." and "Altogether, these data imply TLR8 as a major bacteria- and self-mitochondria sensor ..", respectively. To prevent any appearance of absoluteness we now position "one" in front of "major" in both of the latter sentences. Our implication for a non-involvement of TLR7 in recognition of designed ORNs, all bacterial rRNAs, and mtRNA bases on our observation of a failure of them to induce type I IFN release from PBMCs while they induce substantial proinflammatory cytokine and IL-8 release as indicated in Figure 1. This consideration accords with reports such as citations #19 and 14, which brought up and substantiated that ORNs such as RNA40 induce monocyte TLR8 for proinflammatory cytokine production and plasmacytoid DC TLR7 for type I IFN production in a mutually exclusionary manner. According to the consequent view ORNs can be classified as TLR7, TLR8, or TLR7/8 ligands by analyzing PBMC production of specific cytokines upon challenge such as has been exemplified by "Identification of RNA sequence motifs stimulating sequence-specific TLR8-dependent immune responses. Forsbach A, Nemorin JG, Montino C, Müller C, Samulowitz U, Vicari AP, Jurk M, Mutwiri GK, Krieg AM, Lipford GB, Vollmer J. J Immunol. 2008 Mar 15;180(6):3729-38". This concept (applied by us) is thoroughly illustrated by Figure 1 of the abovementioned, newly emerged, and now cited review citation #29.

Moreover, we addressed the point raised experimentally as implied by this referee. We knocked down TLR7 mRNA expression in both parental and *Tlr8*$^{-/-}$-THP-1 cells since differentiated THP-1 cells increased not only TLR8 but also TLR7 mRNA expression according to our transcriptome analyses as compared to controls (EVF2C). Accordingly differentiated parental, *Tlr8*$^{-/-}$-, and *Unc93b1*$^{-/-}$- THP-1 cells expressed TLR7 (protein) while only *Tlr8*$^{-/-}$ cells lacked TLR8 expression

according to newly performed experiments and included immunoblot analysis results (EVF2G now). Knockdown of TLR7 mRNA expression in parental THP-1 cells inhibited (per se weak) loxoribine driven TNF production as expected (EVF3A, middle panel). Notably, it significantly inhibited R848 and *E. coli* tRNA driven TNF release from *Tlr8*$^{-/-}$-THP-1 cells which indicated a capacity of TLR7 to mediate TNF production in specifically differentiated THP-1 cells (EVF3A, right panel). Type I IFN production by TLR7/8 specifically challenged THP-1 cells was not detected by us, which might indicate that the cell rather than the receptor determines cytokine production qualitatively. Accordingly, THP-1 cell (at least if differentiated) TLR7 expression contrasts with the lack of TLR7 expression in natural PBMC monocytes as described above. However, these results suggested a capacity of tRNA to activate human TLR8 besides TLR7 to largely similar degrees. Despite the presence of functional TLR7 in differentiated THP-1 cells, however, the exclusive TLR8 dependence of Sa19-like ORN recognition was unaffected by TLR7 mRNA knockdown.

We have now transfigured Expanded View Figure 2G and added data illustrated by the new Expanded View Figure 3 to put our respective statement on human TLR8 into relation in respect to the point raised by the reviewer. The new EVF3C also indicates a likely TLR7 driven type I IFN production upon bacterial infection since in contrast to TNF production it was entirely TLR2 independent and thus comprehensively inhibited by chloroquine alone (Fig. 4B). Moreover, the former title of Figure 4 “Inhibition of TLR8 activation upon both, Gram-positive and Gram-negative bacterial infection by blockade of lysosomal function is effective ex vivo.” has been exchanged by “Lysosomal function inhibition affecting TLR8 activity is anti-inflammatory upon both, Gram-positive and Gram-negative bacterial infection ex vivo.”

The phylogenetic tree previously representing EVF3 is now shifted toward EVF4 and substantiated further by EVF3E indicating hog and macaque PBMC’s human like (and mouse unlike) point-methylated bacterial 23S rRNA derived ORN Sa19 recognition.

Why were bacteria only sensed when live but not when heat inactivated?

10. Krüger et al. reply: A major subject of Figure 4 is infection with *S. aureus* and *E. coli* and antibiotic treatment 1 h later in parallel with challenge with heat inactivated (hi) bacteria, as well as analysis of consequent THP-1 cell and whole blood activation. In general, results of respective challenges accord well with each other (from our perspective). Moreover, Fig. 1A, Fig. 2B,C und Fig. 3C as well as EV2F display hi bacteria driven cell activations.

The very recent paper on TLR8 sensing of Staphylococcus aureus RNA should be quoted and discussed, as there are overlapping results: Bergstrom B, Aune MH, Awuh JA, Kojen JF, Blix KJ, Ryan L, Flo TH, Mollnes TE, Espevik T, Stenvik J. 2015. TLR8 Senses Staphylococcus aureus RNA in Human Primary Monocytes and Macrophages and Induces IFN-beta Production via a TAK1-IKKbeta-IRF5 Signaling Pathway. Journal of immunology.

11. Krüger et al. reply: Please see “2. Krüger et al. reply” and “9. Krüger et al. reply”.

Expanded Fig. 1:
A) should shows only the 23 S rRNA, but not the mt 16SrRNA as written in Fig. legend;

12. Krüger et al. reply: Despite substantial size reduction from bacterial as compared to mitochondrial largest rRNAs, generally 23S and frequently 16S rRNA, respectively, the domain string is identical in both holo RNA molecules. Extended Data Figure 3 in citation #18 is a respectively largely informative illustration. Thus the Expanded View Figure 1A sketch shall represent both of the two RNA molecules. The red marks, however, represent localizations of ORN segments in 16S mtrRNA only.

B) it should not be N-terminal and C- terminal, but 5'- and 3' end.

13. Krüger et al. reply: Thank you very much for raising our respective mistake. We changed EV Figure 1A accordingly.

2nd Editorial Decision 09 October 2015

Dear Prof. Kirschning

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the enclosed reports from two of the three referees that were asked to assess it. Given that both referees support publication of the revised manuscript without further revision and as timing is of essence in this case, I decided to go ahead with these two reports.

From the editorial side, there are a few things that we need before we can proceed with the official acceptance of your study.

As the articles by Eigenbrod et al and Bergstrom et al from 2015 impact on the novelty of your work, the article should be published this year to have the desired impact. In order to appear in the December issue the deadline for acceptance is October 15. Therefore, please submit the revised material by Monday, 12.10.

- Regarding data quantification, can you please specify the statistical test used to calculate the p-value in all figure legends?

- Every EMBO reports paper now includes a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version and they are freely accessible to all readers. The synopsis includes a short standfirst text, summarizing in 2 sentences the study (max. 205 characters) as well as 2-4 one sentence bullet points that summarize the highlights. These should be complementary to the abstract, i.e., not repeat the same text. This will be accompanied by a thumbnail image and a Synopsis image (500 x 400 pixel) of your choice. Could you please provide the standfirst text, bullet points and a synopsis image?

REFEREE REPORTS

Referee #1:

The authors have addressed all my concerns

Referee #2:
In this revised version, the authors have by and large answered my inquires, especially, the figure legends of this manuscript have been improved substantially. Thus, I would recommend the Journal to consider accepting the manuscript.

Referee #3:

In the revised manuscript, EMBOR-2015-40861V2, and rebuttal, all the points raised by me were satisfactorily addressed. In my opinion, the paper is now acceptable.

2nd Revision 13 October 2015

The author made the necessary editorial changes.

3rd Editorial Decision 15 October 2015

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.

