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Transient ribosome slowdown during decoding triggers mRNA decay independent of Znf598 in zebrafish

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1st Editorial Decision **21st Jul 2021**

Dear Prof. Mishima,

Thank you for transferring your Review Commons manuscript with referee reports to The EMBO Journal. In light of the referees' comments and your revision plan, I am pleased to say that we would like to invite you to prepare and submit a revised manuscript.

As outlined in your revision plan, it will be important to address the reviewer's concerns, experimentally or by revising the manuscript, and to provide a careful response to each of their comments. We realize that the outcome of the experiments planned to address referee #1's major points 2 and 3 (Fig. 6), as well as referee #2' s major comment (ref#3- minor 10), are not predictable. In our view this data, in particular a validation for endogenous mRNAs, would strengthen the study, but a positive outcome will not be required for further consideration. However, regardless of the results, please discuss them, either in the manuscript or your response to the referees. Similarly referee #3's comments 2 and 4 can be addressed in the response or added to the manuscript discussion.

Please note that it is our policy to allow only a single round of major revision. Acceptance depends on a positive outcome of a second round of review and therefore on the completeness of your responses included in the next, final version of the manuscript. We realize that lab work worldwide is currently affected by the COVID-19/SARS-CoV-2 pandemic and that an experimental revision may be delayed. We can extend the revision time when needed, and we have extended our 'scooping protection policy' to cover the period required for a full revision. However, it is nonetheless important to clarify any questions and concerns at this stage, and that you contact us as soon as possible if any issues come up during the revision.

Please do not hesitate to contact me if you have any questions regarding this revision. Thank you for the opportunity to consider your work for publication, I look forward to receiving your revised manuscript.

Review #1 -

In this manuscript, Mishima et al., designed a reporter system (dubbed PACE, for Parallel Analysis of Codon Effects) to assess the effect of codon usage in regulating mRNA stability in a controlled sequence context. This reporter corresponds to a stretch of 20 repetitions of a given codon (to be tested for its effect on mRNA stability), each repetition being separated by one codon corresponding to each of the 20 canonical amino acids. This stretch is inserted at the 3' end of the coding sequence of a superfolder GFP flanked with fixed 5' and 3' untranslated regions. In vitro transcribed capped and polyadenylated RNAs are then produced from these reporters (each with a specific stretch of repetitions of a given codon), pooled together and injected into zebrafish zygotes to monitor their relative abundance at different time points upon injection.

Using the PACE reporter, the authors were able to obtain a quantitative estimation of the impact of 58 out of the 61 sense codons on modulating mRNA stability. Their results are in agreement with a previous report that estimated the effect of codon usage on mRNA stability using endogenous mRNAs and an ORFeome library (Bazzini et al., 2016). However, contrary to relying on endogenous mRNAs and ORFeome reporters, the advantage of the PACE strategy is that the effect of the codon to be studied can be probed in a defined context, thus avoiding the presence of other motifs or transcript features that could also regulate mRNA stability. Similarly to results from Bazzini et al., 2016, the authors show that blocking translation completely abrogates the effect of codon usage, indicating that translation is required to drive codon-dependent mRNA degradation from their reporters. Also, the extent of codon-dependent mRNA decay is correlated with tRNA abundance and occurs through a process involving mRNA deadenylation as previously described in the zebrafish (Mishima et al., 2016 and Bazzini et al., 2016).
Having validated their PACE protocol, the authors performed ribosome profiling to test whether ribosome occupancy on tested codons is correlated with their capacity to drive mRNA degradation. Their results indicate that, at least for polar amino acids, there is indeed an inverse correlation between ribosome occupancy at tested codons and mRNA stability thus suggesting that slow decoding of codons due to low levels of available cognate tRNA can induce mRNA degradation. The authors further validate this finding by reducing the levels of aminoacylated tRNAAsn (corresponding a polar amino acid) and showing that stability of the reporter RNA carrying a stretch of AAC codons (decoded by tRNAAsnGUU) is reduced. To test whether codon-dependent mRNA degradation in the context of slow ribosome decoding lead to ribosome stalling and collisions, the authors generated a mutant zebrafish strain with impaired expression of ZNF598 (an essential factor of the No-Go decay (NGD) pathway in yeast). They also integrated a known ribosome stalling sequence from hCMV (and a mutant version that does not trigger ribosome stalling) in their sfGFP reporter construct as a positive control for NGD in their assays. Their results indicate that although ZNF598 depletion impairs degradation of the hCMV reporter (as expected), it does not affect codon-dependent mRNA degradation, which appears to occur for most codons through a NGD-independent manner. Finally, through the use of a tandem ORF reporter assay separated by codon tags to be tested, the authors show that destabilizing codons do not stall ribosomes but only lead to their transient slowdown which induces mRNA deadenylation and degradation in a ZNF598-independent manner.

Overall, the manuscript is very well written and pleasant to read. The introduction is well documented and relevant to the study as it allows readers to place the study in the current

context of the field while highlighting open questions that have not been addressed yet. The results are clearly presented, the technical approaches are elegant and the conclusions convincing.

Below you will find some major and minor points that, in my opinion, should be addressed by the authors.

Major point:

- One interesting aspect of the PACE reporter assay is the possibility to monitor ribosome occupancy in parallel for all codon-tags tested, which the authors did in Figure 3. However, instead of using RNA-seq data to normalize ribosome footprints and obtain ribosome occupancy, the authors used an alternative normalization approach consisting, for each codon-tag, to calculate the number of ribosome footprints with test codons in the A site divided by the number of ribosome footprints with spacer codons in the A site. This approach is elegant and appears to work with codons corresponding to polar amino acids. However, it might have its limitations for other codons.

Indeed, ribosome dwell times (in yeast and mammals) have been shown to respond both to tRNA availability but also to other features such as the nature of the pair of adjacent codons, and the nature of the amino acid within the exit channel (Gobet C et al., 2020 PNAS; Gamble CE et al., 2016 Cell; Pavlov MY et al., 2009 PNAS). However, based on the work of "Buschauer R et al., 2020 Science", only ribosomes lacking an accommodated tRNA at the A site are able to recruit Ccr4-Not to mediate mRNA deadenylation and degradation. Other events that increase ribosome dwell time (and thus occupancy), such as slow peptidyl-transfer, do not lead to Ccr4-Not recruitment and are resolved by eIF5A. It is therefore possible that depending on the nature of the codon that is being tested, ribosome occupancy at test and spacer codons can be biased by the nature of codon-pairs and "dilute" the effects of tRNA availability.

If the authors performed RNA-seq together with the ribosome profiling experiment, it might be interesting to use the RNA-seq data to calculate ribosome occupancy on "tested" and "spacer" codons to check whether using this normalization, they do find a negative correlation between ribosome occupancy and PACE stability. A different approach would be to perform ribosome run-off experiments using harringtonine and estimate the elongation speed across the codon tag. However, I am aware that this experiment could be tedious an expensive.

- Figure 6: Insertion of the Lys x8 AAA stretch in the tandem ORF reporter leads to a decrease in HA-DsRedEx expression compared to that of Myc-EGFP. However, results from "Juszkiewicz and Hedge, 2017" using a similar reporter in mammalian cells indicate that stretches of Lys AAA below 20 repetitions only elicit poor RQC (less than 10% of true ribosome stalling for 12 repetitions of the AAA codon). Instead, most of the loss in RFP signal results from a change in the reading frame of ribosomes due to the "slippery" translation of the poly(A) stretch. I therefore think that it could be important to perform the experiment in ZNF598 KO embryos to validate that the observed reduction in HA-dsRedEx does indeed result from stalling and RQC and not from a change in the reading frame of ribosomes.
On a similar note, how do the authors explain the decrease in signal of the Flag-EGFP and HA-DsRedEx observed when using the Flag-EGFP with non-optimal codons? I understand

that RQC occurring through NGD leads to ribosome disassembly at the stalling site and possibly mRNA cleavage (thus explaining the decrease in HA-DsRedEx signal compared to Myc-EGFP). However, I would assume that codon-mediated mRNA decay (even for ORF longer than 200 of non-optimal codons) should trigger mRNA deadenylation, followed by decapping and co-translational 5'to3' mRNA degradation, following the last translating ribosome. I would therefore expect not to see any change in the HA-DsRedEx/Myc-EGFP ratio even for the non-optimal Flag-EGFP reporter. Could the 200 non-optimal codons trigger some background RQC through NGD? Or could there be some ribosome drop-off? It might be interesting to test the optimal and non-optimal Flag-EGFP reporters in the ZNF598 KO background to check whether the observed decrease in the relative amount of HA-DsRedEx results from stalling-dependent RQC.

Minor comments:

- The color-coded CSC results from "Bazzini et al., 2016" presented at the bottom of panel B in figure 2 are misleading because many codons (such as PheUUU, AsnAAU, TyrUAC...) are lacking information. I have the impression that the authors used the combined data from the rCSCI (obtained from the reporter RNAs) and CSC (obtained from endogenous transcripts) corresponding to Figure 1F from Bazzini et al., 2016. This data set excluded all codons that were not concordant between the endogenous and reporter CSCs (which are those that are lacking a color code in Figure 2B from this study). However, in the scatter-plot of PACE Vs CSC (from Supplemental Figure 1D of this study), the authors used the complete set of CSC values from Bazzini et al .,2016. Could the authors please use the complete set of CSC values from Bazzini et al., 2016 to color code codons in their Figure 2B?
- Figure 4B. The charged tRNA measurements seem to have been done in a single biological replicate (there aren't any error bars in the chart). I understand that the procedure is tedious and requires a large amount of total RNA to begin with, but it would be preferable to perform it in three biological replicates.
- Supplementary Figure 2B. I do not understand what the figure represents. The legend is quite cryptic and states that the panel corresponds to the information content of each reading frame. More information should be given so that readers can understand how to interpret de figure and extract periodicity information.

Since the seminal work from Jeff Coller's laboratory in 2015 (Presnyak et al., 2015 Cell) showing a global and major role for codon optimality in determining mRNA half-lives in yeast, the role of codon usage in modulating translation and stability of mRNAs has been widely studied in different organisms (including zebrafish and mammals). As stated by the authors in the introduction, most studies have relied on correlation analyses between codon usage and mRNA half-lives from endogenous transcripts or from ORF libraries with fixed 5'UTR and 3'UTRs. This approaches could suffer from the presence of transcript features that can participate in other mRNA degradation pathways, which could limit their use when performing further mechanistic studies.

The work by Mishima and collaborators presents an original reporter assay that allows to evaluate the role of codon usage on regulating mRNA stability in a defined context, thus avoiding the impact of confounding factors that could bias the measurement of mRNA stability. Results obtained using this reporter are in good agreement with previous reports from Zebrafish (Bazzini et al 2016., and Mishima et al., 2016). From this validated reporter approach, the authors further show that codon-dependent mRNA degradation is directly related to tRNA availability and (at least partially) to ribosome occupancy (two factors

already suggested as being important for codon-mediated decay in zebrafish, although they were based on correlation analyses). Furthermore, the authors show that codon-mediated mRNA decay occurs during productive mRNA translation and that it is functionally distinct from RQC induced by ribosome stalling. As a consequence, codon-mediated mRNA degradation is independent from the RQC factor ZNF598 (which they also validate for the first time as an important RQC factor in zebrafish). This information is new within metazoans since only in yeast it has been clearly shown that codon-mediated mRNA decay is distinct from RQC induced by ribosome stalling and collisions.

Taken together, the reported findings will be of interest to the community working on mRNA metabolism and translation. It could also interest, more broadly, scientists working on translational selection and genome evolution.

Review #2 -

In this manuscript, Mishima et al aim to determine if the RNA-mediated decay determined by codon optimality is part of the ribosome quality control pathway, triggered by slowed codon decoding and ribosome stalling or it is an independent pathway.

To this end, the authors capitalize on their previous work to design a very elegant high-throughput reporter system that can analyze individually codon usage, ribosome occupancy and tRNA abundance. This reporter system, called PACE, is rigorously validated throughout the manuscript, because blocking translation with a morpholino blocking the AUG codon demonstrated that the effects no RNA stability are translation dependent.

When most of the available codons are tested using the PACE system, the authors recapitulate codon optimality profiles similar to the ones previously uncovered using transcriptome-wide approaches.

Thanks to the design of the reporter, which alternates repeats of a test codon with random codons, the authors can calculate how quickly a ribosome decodes the test codon on average. With this approach, the authors uncover a negative correlation between RNA stability and ribosome density on codons for polar amino acids and suggest that codon optimality is related to a slower decoding of the codons.

With the PACE reporter validated, the authors can interrogate the system to gain mechanistic insights of codon optimality. First, they test if RNA decay and deadenylation mediated by codon optimality is determined, in part, by the levels of aminoacylated tRNAs available. The authors use a very elegant approach, as they overexpress a bacterial enzyme (AnsB) in zebrafish that degrades asparagine, effectively reducing the levels of tRNA-Asn. The authors demonstrate that AnsB turns a previously optimal Asn codon, AAC, into a non-optimal one. This effect is translated into RNA destabilization and deadenylation, but this effect in not extended to other codons encoding amino acids not affected by Asn. These results provide a direct experimental validation of the previously published observation of tRNA levels and codon optimality.

Finally, the authors interrogate the relationship between the codon optimality pathways and the ribosome quality control pathways, that takes care of stalled ribosomes. The authors

generate a zebrafish mutant of Znf598, a vertebrate homolog of the yeast protein in charge of resolving stalled ribosomes. Using a maternal-and-zygotic mutant, the authors demonstrate that in these mutant's codon optimality proceeds as usual but ribosome stalling is not resolved, providing evidence for first time that Znf598 is involved in ribosome quality control in vertebrates.

Altogether, this manuscript presents work that builds on the previous findings of the authors and other labs but it is a qualitative leap forward rather than a marginal increment, because the body of work in the current manuscript i) establishes a reporter to dissect the mechanisms of codon optimality, ii) demonstrates that ribosome slow-down but not stalling is part of the trigger of RNA decay mediated by codon optimality, iii) demonstrates that this pathway is independent of ribosome quality control pathway and finally iv) demonstrates that vertebrate Znf598 is involved in the RNA decay mediated by ribosome stalling.

Due to these novel findings, and the rigor of the experimental design, this manuscript should be accepted for publication. The authors should first address the following comments:

Major comment:

1. The authors very elegantly demonstrate the impact of AnsB on the stability of the RNA reporter, and it is precisely the simplicity of the reporter that allows the authors to draw clear conclusions. Nevertheless, it would be interesting to determine if the reporter results in embryos injected with AnsB also translate to endogenous genes rich in AAC codons. The authors could perform a polyA-selected RNA-Seq in embryos treated with AnsB to determine if the transcripts rich in AAC codons are destabilized compared to wild-type, thus validating the reporter results in endogenous genes.

Minor comments:

2. In figure 5J the authors plot the normalized codon tag levels of the PACE reporter run in the MZznf598 mutant. The authors color code the labels in the x-axis following the PACE results in wild-type (figure 2B). The authors should also plot the wild-type values to have a direct visual comparison of the results trend in both genotypes.
3. The authors focus in the title on the role of Znf598 or the lack thereof in RNA decay induced by codon optimality. However, for the non-aficionados in codon-optimality, ZnF598 is an unknown protein and adds little information to the title. The authors should provide a more informative title, directly pinpointing that codon-optimality is independent of the ribosome quality control pathway.

This manuscript presents work that builds on the previous findings made by the authors and other laboratories but it is a qualitative leap forward rather than a marginal increment, because the body of work in the current manuscript i) establishes a reporter to dissect the mechanisms of codon optimality, ii) demonstrates that ribosome slow-down but not stalling is part of the trigger of RNA decay mediated by codon optimality, iii) demonstrates that this pathway is independent of ribosome quality control pathway and finally iv) demonstrates that vertebrate Znf598 is involved in the RNA decay mediated by ribosome stalling.

In addition to the conceptual findings, the authors establish a new high-throughput reporter system to evaluate the influence of codon optimality in RNA decay.

The work its done in zebrafish embryos, an in vivo model system where codon optimality has been extensively tested by the authors and others, following the stability of reporter and endogenous genes.

Review #3 -

Mishima et al. address a very timely topic of how the codon composition of the ORF and the associated translation elongation speed affect mRNA stability. Several studies have already shown a strong correlation between codon optimality and mRNA stability - meaning the more "optimal" the codons, the faster supposedly the elongation speed and the more stable the mRNA. Most of these studies were done by analyzing global expression data, with limited follow up, therefore being also impacted by other co-translational mRNA decay pathways and in addition these studies could also not test directly the effect of each single codon on mRNA stability. The authors took a systematic reporter-based assay approach, called PACE, which allowed them to test systematically the effect of codon composition on mRNA decay. By integrating also ribosome profiling data, the authors could nicely show that the speed of translation (measured by ribosome density) correlates with their determined mRNA stability effect of each codon and also the corresponding tRNA levels. However, interestingly this seems to be the case only for codons encoding polar amino acids, but not the ones that encode charged or non-polar amino acids. It will be very interesting to find out why that is? Finally, the authors address if some of the effects they see might be due to ribosome collisions and associated no go decay (NGD). For this they generated a Znf598 mutant by CRISPR-Cas9. Znf598 is the proposed homolog of Hel2, the protein in yeast that is essential for NGD. The authors go on to show that NGD is defective in this mutant, but that codon mediated decay, which is elongation dependent, is not to a large part not dependent on Znf598.

All minor comments:

1. It is intriguing why only polar AAs show a tRNA amount specific effect in the ribosome footprint data. Some hypothesis/discussion about this could be expanded further in the discussion or results.
2. On the same token some additional analysis might be helpful. For example, in Figure 2E, the authors group codons in weak, neutral and strong based on PACE measurements and then look at the tRNA expression range for each of the three groups. Could the authors do this also separately for the codons of polar, non-polar and charged amino acids? What do you see - still the same pattern as for all the codons or do again only polar amino acids show the trend?
3. Can the authors elaborate on the development of their PACE system? Why is it designed the way it is? What parameters did they test? For example, why the 20 amino acids tail, did you you test shorter sequences of the amino acid, spacer repeats, etc?
4. The next few questions are a bit more of a technical nature regarding the reporter construct used for PACE.
a. Did all AA pairs (Codon of interest + spacer codon) behave the same in the footprint assay? Does the data have enough information and resolution for this?
b. Was the order of the spacer codons always the same in all the constructs? Could the specific order, if it is consistent, have any unseen consequences (ie. interaction with the exit tunnel)? Did the authors test other orders?
c. Are the spacer codons optimized?

5. Are the codons affected in the NGD mutant the ones that are most different in the Bazzini data?
6. The authors inject directly mRNA into the embryos, therefore avoiding that the reporter mRNA is ever in the nucleus. However, there could be nuclear events (e.g. loading of particular proteins) that might affect the fate of an mRNA in the cytosol, among these the translation efficiency and also stability. Maybe some comment in the discussion as to the effect of missing nuclear factors would be welcome. This is not a criticism; it would just be nice to hear the authors' thoughts on that.
7. Page 6; final paragraph: "Finally, we compared the speed of the ribosome translating mRNA destabilizing codons to that of an aberrantly stalled ribosome."
Not sure the authors did that actually. They tested the effect of ribosome slowing down on protein production and mRNA levels and compared that to stalling ribosomes, but did not compare the "speed" directly and I am not even sure what they mean by that in this context. Probably good to rephrase.
8. Page 7, upper half: ".....by taking the positional effect of codon-mediated decay into account (Mishima and Tomari, 2016)."
This is my limited knowledge of the literature, but I think you should mention what this positional effect is and not just cite a paper.
9. Very minor, but on page 8 when PACE is introduced, the authors show the different destabilizing effects of the three Ile codons. While that is ok, in the section before, when the authors tested their construct by qRT-PCR, they focused on the two Leu codons. I would also mention them here and do a direct comparison of the qRT-PCR results with the pooled PACE result for these two codons. Based on the figure the two codons seem to behave qualitatively like expected, but I am not sure how good the quantitative behavior matches.
10. The AnsB experiment - the authors only mention data about one of the two Asn codons (AAC), but what about the second Asn codon (AAU) - do you also see an effect on that codon upon overexpression of AnsB as well? AAU is already a quite destabilizing codon and you might not see a further increase in destabilization, but it would be great to know if there was or not.
11. Page 13, second paragraph: More out of interest, but it is quite intriguing that GCG turned into a destabilizing codon (opposite of what one would expect if NGD would play a bit a role). Any speculation why?
12. Page 14, end of page and related to Figure 6C: AAU seems much more destabilizing than AAC. Therefore, I would have expected that the inserted sequence with the AAU codons would lead actually to downregulation of the mRNA and therefore the EGFP and DsRFP total protein signal relative to the construct with the AAC inserted in between, even if the ratio of EGFP/DsRed seems unchanged. However, based on the western blot in 6C the total protein levels seem very similar. Isn't that surprising? Although, AAU obviously allows translation to proceed it should still induce a stronger mRNA decay than AAC and therefore result in less total mRNA (and protein level as a consequence). Did the authors quantify the exact levels of the reporter proteins and mRNA and compared them between the two constructs?
13. Page 15, last sentence: Somehow for me the word "transient" is a bit hard to grasp in this context. What do you mean by that - do you really mean "impermanent" or "lasting only for a short amount of time"? Don't you simply mean "weaker", "less strong"?
14. Page 17, second sentence: I think the authors want to reference here Figure 2E and not Figure 2D.

All in all, I have to say that it was a real pleasure to read this manuscript. The authors were extremely thorough with their experiments and did nearly never overstate any of their conclusions. It is a very insightful story, which in my opinion will contribute greatly to the field of gene expression and posttranscriptional gene expression regulation in particular. The PACE assay, although a bit artificial, gave very clean results, which agree with the previous literature and could be very useful for future studies. Generating the Znf598 mutant and showing that the codon-dependent decay is independent from NGD is a great addition to this paper. Although it is a bit of a pity that we do not see more of a characterization of the Znf598 mutant in this paper, I do agree with the authors that this might take away a bit of the focus of this manuscript and that the mutant deserves actually its own story. I only have very minor comments/questions for the authors that they should be able to address easily. Finally, I can only repeat myself by saying: congrats on this great paper and I fully support publication.

Reviewer1

Major point:

Comment:

If the authors performed RNA-seq together with the ribosome profiling experiment, it might be interesting to use the RNA-seq data to calculate ribosome occupancy on "tested" and "spacer" codons to check whether using this normalization, they do find a negative correlation between ribosome occupancy and PACE stability. A different approach would be to perform ribosome run-off experiments using harringtonine and estimate the elongation speed across the codon tag. However, I am aware that this experiment could be tedious an expensive.

Response:

We thank the reviewer for suggesting this important analysis. Indeed, we obtained matched RNA-Seq data for ribosome footprint profiling. Unfortunately, however, the number of ribosome footprint reads was limited because most footprint reads were

derived from endogenous mRNAs. The footprint reads derived from PACE reporters are about 0.2 % of footprints derived from endogenous mRNAs. Therefore, we could not obtain enough footprint reads from all codon positions (we summarized the footprint distribution across codon tag sequences in Figure EV2F) (please also see our response to Reviewer 3, comment 4a). Hence, the normalization of ribosome density at each codon position using RNA-Seq data may not provide reliable means. This is one of the reasons why we summed test or spacer codon reads for a given codon tag transcript and calculated relative ribosome density on test codons, as shown in Figure 3A.

As an alternative approach to codon position wise analysis, we have instead calculated ribosome density on the entire codon tag sequence. To this end, the number of footprint reads from a given codon tag sequence was divided by the number of RNA-Seq reads from the same codon tag sequence. Although this normalization gives us an estimate of the ribosome density on each codon tag, the interpretation of the resultant value has a challenge. Since this calculation is akin to the classical translation efficiency (TE), a proxy for translation initiation flux, the value may also include the effect of initiation in addition to elongation. Below, we analyze the correlations between the normalized ribosome density and PACE or tRNA levels for three amino acid groups.

Figure for referees not shown.

While nonpolar codon tags show a significant negative correlation between PACE and the normalized ribosome density, the correlation is not simply attributable to the tRNA levels. Conversely, polar codon tags do not show a significant correlation between PACE and the normalized ribosome density. Therefore, although normalization using RNA-Seq might visualize a different aspect of PACE, it neither improves the results nor

allows a straightforward interpretation. We are happy to include these data in the Appendix figure upon request, although it seems difficult to integrate these data into the main text.

Comment:

Figure 6: Insertion of the Lys x8 AAA stretch in the tandem ORF reporter leads to a decrease in HA-DsRedEx expression compared to that of Myc-EGFP. However, results from "Juszkiewicz and Hedge, 2017" using a similar reporter in mammalian cells indicate that stretches of Lys AAA below 20 repetitions only elicit poor RQC (less than 10% of true ribosome stalling for 12 repetitions of the AAA codon). Instead, most of the loss in RFP signal results from a change in the reading frame of ribosomes due to the "slippery" translation of the poly(A) stretch. I therefore think that it could be important to perform the experiment in ZNF598 KO embryos to validate that the observed reduction in HA-dsRedEx does indeed result from stalling and RQC and not from a change in the reading frame of ribosomes.

Response:

We agree with the reviewer that reduced translation downstream of the Lys AAA stretch could result from the mixed effects of ribosome stall, RQC, and ribosome slippage/frameshift. In the revised manuscript, we performed new experiments to clarify this point (Figure 6 and Figure EV5). In these experiments, we switched the ORFs of the tandem reporter from fluorescent proteins to luciferases to enable a more sensitive and quantitative measurement of the signals (Figure 6A). Inserting Lys AAG×8 or Lys AAA×8 into this luciferase-based reporter resulted in stronger repression of the downstream ORF (Fluc) than the previous fluorescent reporter (Figure 6B). Although the exact reason for this difference is unclear, strong repression by a relatively short AAA stretch (AAA×9) was also reported in a previous study using mammalian cells (Arthur et al., Science Advances, 2015). We assume that the degree of repression by an A-stretch depends on the sequence context of the reporters.

Using this new reporter system, we dissected the repressive effect of Lys AAA×8 into three parts: 1) Lys AAG×8 caused an ~40% reduction of in Fluc activity, likely representing ribosome retardation by a positively charged nascent peptide that interacts with the ribosome exit tunnel. 2) Lys AAA×8 further reduced Fluc activity, suggesting that strong ribosome stalling was caused by a combination of a positively charged nascent peptide and consecutive AAA codons (~90% reduction in Fluc activity). 3) Lys AAA×8

caused frameshifting, which accounted for an ~14% reduction in Fluc activity (Figure EV5A). Hence, frameshifting indeed occurred and contributed to the reduced Fluc activity observed with the Lys AAA×8 reporter but to a limited extent. We also tested the effect of a naturally occurring poly AAA stall site of ZCRB1 (Arthur et al., Science Advances, 2015). The ZCRB1 stall site caused an ~35% reduction in Fluc activity, and approximately 20% of the reduction was attributable to frameshifting (Figure EV5C and D).

To analyze the contribution of RQC to the reduction in Fluc activity, we repeated experiments in *znf598* mutant embryos as suggested by the reviewer. We did not observe significant recovery of Fluc activity with Lys AAA×8 or ZCRB1 reporters in MZ*znf598* embryos (Figure EV5E and F). This result was slightly unexpected, as it indicates that a loss of RQC does not increase readthrough at the stall site in the context of early zebrafish embryos. As previously reviewed (Meydan and Guydosh, Current Genetics, 2020), the consequence of ZNF598 depletion on ribosome stalling may be variable among organisms or transcripts. Although interesting, further clarification of this point will require a significant amount of work, and therefore, we would like to address this issue in our future study.

The most crucial point is that most if not all of the observed reduction in Fluc activity by consecutive Lys AAA codons is attributable to ribosome stalling. In addition, our assay also detected ribosome slowdown by consecutive proline codons that are inhibitory to peptide bond formation (Figure 6C). Under the same experimental conditions, nonoptimal codon reporters did not cause a significant reduction in Fluc activity (Figure 6D and E). Hence, these new data support our original argument: translation of nonoptimal codons does not result in strong ribosome stall or slowdown. We appreciate the reviewer encouraging us to perform out these experiments, which clarified and improved our original results.

Comment:

On a similar note, how do the authors explain the decrease in signal of the Flag-EGFP and HA-DsRedEx observed when using the Flag-EGFP with non-optimal codons? I understand that RQC occurring through NGD leads to ribosome disassembly at the stalling site and possibly mRNA cleavage (thus explaining the decrease in HA-DsRedEx signal compared to Myc-EGFP). However, I would assume that codon-mediated mRNA decay (even for ORF longer than 200 of non-optimal codons) should trigger mRNA deadenylation, followed by decapping and co-translational 5'to3' mRNA degradation,

following the last translating ribosome. I would therefore expect not to see any change in the HA-DsRedEx/Myc-EGFP ratio even for the non-optimal Flag-EGFP reporter. Could the 200 non-optimal codons trigger some background RQC through NGD? Or could there be some ribosome drop-off? It might be interesting to test the optimal and non-optimal Flag-EGFP reporters in the ZNF598 KO background to check whether the observed decrease in the relative amount of HA-DsRedEx results from stalling-dependent RQC.

Response:

We thank the reviewer for his or her careful examination of the data. As the decrease in Flag-EGFP and HA-DsRedEx was very modest and statistically insignificant, we argued that ribosome slowdown at the nonoptimal EGFP ORF, if such exists, should be weaker than the ribosome stall caused by a consecutive Lys AAA sequence. To confirm this statement more quantitatively and to address the reviewer's concern about RQC, we performed a series of analyses using luciferase reporters in wild-type and *znf598* mutant embryos. As a result, no significant decrease in Fluc activity was detected with a reporter mRNA containing a nonoptimal EGFP ORF (Figure 6D, 6E, EV5G, and EV5H). We apologize for our confusing description. We have rephrased the description for this part in the revised manuscript.

Minor comments:

Comment:

The color-coded CSC results from "Bazzini et al., 2016" presented at the bottom of panel B in figure 2 are misleading because many codons (such as PheUUU, AsnAAU, TyrUAC...) are lacking information. I have the impression that the authors used the combined data from the rCSCI (obtained from the reporter RNAs) and CSC (obtained from endogenous transcripts) corresponding to Figure 1F from Bazzini et al., 2016. This data set excluded all codons that were not concordant between the endogenous and reporter CSCs (which are those that are lacking a color code in Figure 2B from this study). However, in the scatter-plot of PACE Vs CSC (from Supplemental Figure 1D of this study), the authors used the complete set of CSC values from Bazzini et al .,2016. Could the authors please use the complete set of CSC values from Bazzini et al., 2016 to color code codons in their Figure 2B?

Response:

As this reviewer mentioned, the color-coded data at the bottom of Figure 2B were combined data from the rCSCI and CSC in Bazzini et al., 2016. In the revised manuscript, we have substituted the color code with the complete set of CSC values from Bazzini et al., 2016. We truly appreciate the reviewer's excellent suggestion.

Comment:

Figure 4B. The charged tRNA measurements seem to have been done in a single biological replicate (there aren't any error bars in the chart). I understand that the procedure is tedious and requires a large amount of total RNA to begin with, but it would be preferable to perform it in three biological replicates.

Response:

We thank the reviewer for raising this point with a deep understanding of the experimental procedure. We have confirmed the reproducibility using three biological replicates in the revised manuscript.

Comment:

Supplementary Figure 2B. I do not understand what the figure represents. The legend is quite cryptic and states that the panel corresponds to the information content of each reading frame. More information should be given so that readers can understand how to interpret de figure and extract periodicity information.

Response:

We apologize for the lack of information regarding Supplemental Figure 2B (now Figure EV2B). The analysis evaluated the codon periodicity by calculating the relative entropy of information content (Kullback–Leibler divergence). Please see below for an example. We have added information about this analysis in the main text and legend.

Figure for referees not shown.

Reviewer2

Major comment:

Comment:

1. The authors very elegantly demonstrate the impact of AnsB on the stability of the RNA reporter, and it is precisely the simplicity of the reporter that allows the authors to draw clear conclusions. Nevertheless, it would be interesting to determine if the reporter results in embryos injected with AnsB also translate to endogenous genes rich in AAC codons. The authors could perform a polyA-selected RNA-Seq in embryos treated with AnsB to determine if the transcripts rich in AAC codons are destabilized compared to wild-type, thus validating the reporter results in endogenous genes.

Response:

We are pleased to learn that the reviewer found the AnsB experiment elegant and clear. To address this reviewer's comment and comment 12 by reviewer 3, we performed RNA-Seq using embryos with the PACE library and AnsB expression. As a result, PACE confirmed that AnsB conferred mRNA destabilizing effects to Asn codons (EV Figure 3). However, the effect of AnsB on endogenous mRNAs was not apparent (please see below).

We analyzed the effect of asparagine deprivation on endogenous mRNA stability in an unbiased way as follows. First, we identified genes whose mRNA levels were reduced by more than 4-fold from 2 hpf to 6 hpf in wild-type or AnsB-expressing embryos (2917 and 3356 genes, respectively, with 2619 genes overlapping). mRNAs that fall in this category are expected to be enriched with maternally supplied mRNAs that are degraded after fertilization in a codon usage-dependent manner (Mishima and Tomari Mol Cell. 2016). Analysis of wild-type data revealed that codon enrichment/depletion in downregulated genes compared to the background gene set (all genes analyzed, 12703 genes) correlated well with PACE (please see figure below, left panel). As expected, Asn AAC and AAU codons were depleted and enriched in the downregulated genes, respectively. Hence, this codon enrichment/depletion in downregulated genes reflects the codon effects on endogenous mRNAs. We then compared codon enrichment/depletion in downregulated genes between wild-type and AnsB-expressing embryos. As shown in the plot on the right, the enrichment/depletion of Asn codons in downregulated genes was unchanged between wild-type and AnsB-expressing embryos. We also analyzed the data by applying different fold-change cutoffs and criteria. However,

we could not observe any evident change in Asn codon enrichment.

Figure for referees not shown.

Considering the difference between the endogenous mRNAs and in vitro transcribed reporter mRNAs (we commented on this point in the discussion section in response to Reviewer 3, Point 6), the effect of asparagine deprivation on endogenous mRNAs could be more complex than PACE. Therefore, a more sophisticated experimental setup is required to address this question further. As the reviewer indicated, these data further emphasized that the simplicity of the codon tag reporter allowed us to capture the specific change in the effect of the Asn codons after asparagine deprivation. Therefore, we would like to focus on PACE reporters in the current study. Since the impact of tRNA and amino acid availabilities on the stability of endogenous mRNAs is an important topic, we will analyze this issue in our future study.

Minor comments:

Comment:

2. In figure 5J the authors plot the normalized codon tag levels of the PACE reporter run in the MZznf598 mutant. The authors color code the labels in the x-axis following the PACE results in wild-type (figure 2B). The authors should also plot the wild-type values to have a direct visual comparison of the results trend in both genotypes.

Response:

We have included wild-type values in the plot in Figure 5J. We appreciate the reviewer's thoughtful comment.

Comment:

3. The authors focus in the title on the role of Znf598 or the lack thereof in RNA decay induced by codon optimality. However, for the non-aficionados in codon-optimality, ZnF598 is an unknown protein and adds little information to the title. The authors should provide a more informative title, directly pinpointing that codon-optimality is independent of the ribosome quality control pathway.

Response:

We agree with the reviewer that not all readers are familiar with Znf598, and ribosome-associated quality control or no-go decay make it easier for readers to understand the meaning of the title at a glance. On the other hand, we are afraid that it might be an overstatement because the definitions of RQC and, in particular, NGD in vertebrates are not as clear as those in yeast. Therefore, we feel that the use of Znf598 in the title better represents what we showed in the manuscript. We are happy to discuss this point with the reviewers and editors.

Reviewer 3

All minor comments:

Comment:

1. It is intriguing why only polar AAs show a tRNA amount specific effect in the ribosome footprint data. Some hypothesis/discussion about this could be expanded further in the discussion or results.

Response:

We thank the reviewer for suggesting this important discussion. One possibility is that nascent peptides enriched with polar amino acids do not slow ribosome movement, allowing precise measurement of the influence of tRNA availability on ribosome density. We discussed this possibility by focusing on codons for nonpolar and charged amino acids.

Comment:

2. On the same token some additional analysis might be helpful. For example, in Figure 2E, the authors group codons in weak, neutral and strong based on PACE measurements and then look at the tRNA expression range for each of the three groups. Could the authors do this also separately for the codons of polar, non-polar and charged amino acids? What do you see - still the same pattern as for all the codons or do again only polar amino acids show the trend?

Response:

We thank the reviewer for suggesting this analysis. We have examined the correlation between PACE and the tRNA levels for each amino acid group.

Figure for referees not shown.

It seems that the trend is maintained in all three groups, but the trend becomes less clear in part due to the small sample size and large variation in the tRNA data. We are happy to include this data in the Appendix figure, if requested.

Comment:

3. Can the authors elaborate on the development of their PACE system? Why is it designed the way it is? What parameters did they test? For example, why the 20 amino acids tail, did you you test shorter sequences of the amino acid, spacer repeats, etc?

Response:

We thank the reviewer for raising these points. We decided the number of test codons based on our previous study, Mishima and Tomari Mol Cell. 2016. In this paper, we substituted 21 Leu CUG codons in codon-optimized EGFP with Leu CUA and showed that the synonymous codon substitution altered the deadenylation rate. Based on these data, we thought that 20 codons should be sufficient to detect the codon's effect on mRNA stability. Although we did not test this extensively, reporters with shorter 5 or 10 test codons did not exhibit clear differences in deadenylation and degradation. We included spacer codons 1) to avoid generating highly consecutive nucleotide and amino acid sequences, 2) to stably maintain the sequences during plasmid construction, and 3) to obtain unique reads in RNA-Seq and ribosome footprint profiling. We have described this information in the Materials and Methods and included a notice in the main text.

Comment:

4. The next few questions are a bit more of a technical nature regarding the reporter construct used for PACE.

a. Did all AA pairs (Codon of interest + spacer codon) behave the same in the footprint assay? Does the data have enough information and resolution for this?

Response:

We agree with the reviewer that our PACE footprint data may help understand ribosome movements on codon or amino acid pairs. Unfortunately, however, the number of the ribosome footprint reads was not sufficient to address this point partly due to the low yield of footprint reads in the library (approximately 25% of total reads) with

contamination of unrelated RNA fragments, such as rRNA. This is a general issue in ribosome profiling experiments. Additionally, we had a high background of footprint reads from endogenous mRNAs, as we injected a minimum amount of PACE reporter mRNAs to avoid overloading the translational machinery in the embryo. The footprint reads derived from PACE reporters are approximately 0.2% of footprints derived from endogenous mRNAs. As a result of these technical limitations, not all codon positions yielded footprints. For an overview of the data, we summarized the footprint distribution along codon tag sequences in Figure EV2F.

Comment:

b. Was the order of the spacer codons always the same in all the constructs? Could the specific order, if it is consistent, have any unseen consequences (ie. interaction with the exit tunnel)? Did the authors test other orders?

Response:

Yes, we used the same order of the spacer codons for all constructs in PACE. In our initial attempt, the order of the spacer codons was not adjusted, and there was a region where the local hydrophobicity was extremely high. When test codons encoded hydrophobic amino acids, those constructs significantly reduced the protein output, possibly inducing cotranslational aggregation or interaction between the nascent peptide and the ribosome exit tunnel. Therefore, we adjusted the order of spacer codons to average the local hydrophobicity. We have described this information in the Materials and Methods.

Comment:

c. Are the spacer codons optimized?

Response:

Yes, we referred codon usage and avoided using uncommon codons at spacer codon positions. We described this information in the Materials and Methods.

Comment:

5. Are the codons affected in the NGD mutant the ones that are most different in the Bazzini data?

Response:

We did not see such a trend. For example, two tyrosine codons and an Ile AAU codon were evaluated as destabilizing codons in PACE, while they were stabilizing codons in the CSC analysis in Bazzini et al. EMBO J. 2016. These three codons did not change their effects in *znf598* mutant embryos. Val GUG and GUU were also inconsistent between the two studies: the two Val codons were classified as destabilizing codons in CSC but showed a modest stabilizing effect in PACE. Although Val GUG was further stabilized in the *znf598* mutant, this result does not attribute the inconsistency between the two studies to NGD. The revised manuscript shows the complete set of CSC data in Figure 2B and both wild-type and MZ*znf598* PACE data in Figure 5J. We hope these changes help readers compare the codon effects between different datasets.

Comment:

6. The authors inject directly mRNA into the embryos, therefore avoiding that the reporter mRNA is ever in the nucleus. However, there could be nuclear events (e.g. loading of particular proteins) that might affect the fate of an mRNA in the cytosol, among these the translation efficiency and also stability. Maybe some comment in the discussion as to the effect of missing nuclear factors would be welcome. This is not a criticism; it would just be nice to hear the authors' thoughts on that.

Response:

We thank the reviewer for raising this important point. Factors that bind to mRNAs in the nucleus, such as the exon-exon junction complex, are missing in the in vitro transcribed mRNA. These factors may have additional effects on codon-mediated mRNA decay directly or indirectly. We have mentioned this point in the discussion.

Comment:

7. Page 6; final paragraph: "Finally, we compared the speed of the ribosome translating mRNA destabilizing codons to that of an aberrantly stalled ribosome."

Not sure the authors did that actually. They tested the effect of ribosome slowing down on protein production and mRNA levels and compared that to stalling ribosomes, but did not compare the "speed" directly and I am not even sure what they mean by that in this context. Probably good to rephrase.

Response:

We appreciate the reviewer's careful examination of the text. By incorporating this suggestion, we rephrased the sentence as follows:

"Finally, we compared the effect of ribosome slowdown at destabilizing codons on protein production to that of other ribosome stalling events."

Comment:

8. Page 7, upper half: ".....by taking the positional effect of codon-mediated decay into account (Mishima and Tomari, 2016)."

This is my limited knowledge of the literature, but I think you should mention what this positional effect is and not just cite a paper.

Response:

We apologize for the lack of information. In Mishima and Tomari, Mol Cell. 2016, we showed that the relative position of nonoptimal codons in the ORF is one of the determinants of the efficiency of codon-mediated decay. Nonoptimal codons placed at the 3´ end of the ORF induced more robust mRNA deadenylation than those placed at the 5´ end. We have explained this finding in the revised manuscript.

Comment:

9. Very minor, but on page 8 when PACE is introduced, the authors show the different destabilizing effects of the three Ile codons. While that is ok, in the section before, when the authors tested their construct by qRT-PCR, they focused on the two Leu codons. I would also mention them here and do a direct comparison of the qRT-PCR results with the pooled PACE result for these two codons. Based on the figure the two codons seem to behave qualitatively like expected, but I am not sure how good the quantitative behavior matches.

Response:

We thank the reviewer for his or her careful assessment of the data. Indeed, the two Leu codons behaved similarly in qRT–PCR and PACE, as shown in Figure EV1E and F. We added a notice about Leu codons in the main text.

Comment:

10. The AnsB experiment - the authors only mention data about one of the two Asn codons (AAC), but what about the second Asn codon (AAU) - do you also see an effect on that codon upon overexpression of AnsB as well? AAU is already a quite destabilizing codon and you might not see a further increase in destabilization, but it would be great to know if there was or not.

Response:

We thank the reviewer for this interesting suggestion. As mentioned in our response to reviewer 2, we performed PACE with AnsB (Figure EV3) encouraged by the reviewer comment. We found that AnsB conferred mRNA destabilizing effects not only to the Asn AAC codon but also to the Asn AAU codon. As the reviewer predicted, the effect of AnsB on Asn AAU was smaller than that of Asn AAC.

Comment:

11. Page 13, second paragraph: More out of interest, but it is quite intriguing that GCG turned into a destabilizing codon (opposite of what one would expect if NGD would play a bit a role). Any speculation why?

Response:

We are glad to know that the reviewer shares the same interest with us. One possibility is that loss of Znf598 indirectly activates an mRNA-degradation mechanism that binds to the GCG-containing motif that appears in the GCG codon tag sequence. Alternatively, tRNA or amino acid levels were altered in the Znf598 mutant. These are just speculation and might be a path to a new project.

Please also note that since we included an additional wild-type replicate, the PACE results in the wild type have slightly changed in the revised manuscript.

Comment:

12. Page 14, end of page and related to Figure 6C: AAU seems much more destabilizing than AAC. Therefore, I would have expected that the inserted sequence with the AAU codons would lead actually to downregulation of the mRNA and therefore the EGFP and DsRFP total protein signal relative to the construct with the AAC inserted in between, even if the ratio of EGFP/DsRed seems unchanged. However, based on the western blot

in 6C the total protein levels seem very similar. Isn't that surprising? Although, AAU obviously allows translation to proceed it should still induce a stronger mRNA decay than AAC and therefore result in less total mRNA (and protein level as a consequence). Did the authors quantify the exact levels of the reporter proteins and mRNA and compared them between the two constructs?

Response:

We thank the reviewer for his or her careful assessment of the data. We were also aware of this point that codon-mediated mRNA decay should reduce the total protein output from Asn AAU reporter mRNA at later timepoints. Therefore, we performed a time-course analysis of the two Lue codon reporter mRNAs to identify a time point where the difference in the mRNA amount between optimal and nonoptimal constructs was minimal.

Figure for referees not shown.

Based on this result and a detailed time course analysis of codon-mediated deadenylation in our previous study (Mishima and Tomari, Mol Cell. 2016), we decided to measure protein levels at three hours postfertilization, the time point before the significant progression of mRNA decay. This is one of the reasons why nonoptimal codons did not significantly reduce the total protein output in the previous Figure 6. It is also true that mRNA injection experiments can be technically more variable than in vitro and cell culture experiments, making the comparison of absolute protein output from different reporters challenging.

Considering these points, we utilized luciferases instead of western blotting to detect protein outputs at three hours postfertilization in the revised manuscript and focused on the Fluc/Rluc ratio (Figure 6 and EV5). Luciferase assays confirmed our initial findings that were observed by western blotting.

Comment:

13. Page 15, last sentence: Somehow for me the word "transient" is a bit hard to grasp in this context. What do you mean by that - do you really mean "impermanent" or "lasting only for a short amount of time"? Don't you simply mean "weaker", "less strong"?

Response:
We apologize our unclear description. We have rephrased the last sentence as follows.
“These results showed that the ribosome slowdown required for inducing codon-mediated decay is weaker than the ribosome stall events that elicit specific rescue systems.”

Comment:
14. Page 17, second sentence: I think the authors want to reference here Figure 2E and not Figure 2D.

Response:
Yes, this is our mistake. We thank the reviewer for pointing out this error, which has been fixed.

Thank you for submitting your revised manuscript which had been reviewed at Review Commons. We sent the revised version back to the initial referees and have now received their reports (please see comments below). I am happy to say that they find that their concerns have been addressed and now recommend publication. Therefore, I would now ask you to prepare a final version of the manuscript addressing a number of editorial and formatting issues that are listed in detail below.

Please feel free to contact me if you have further questions regarding the revision or any of the specific points listed below. Thank you again for giving us the chance to consider your manuscript for The EMBO Journal.

Referee #1:

This is a revision of the manuscript by Mishima et al., that interrogates whether the codon optimality RNA control pathway is part or not of the ribosome quality control pathway triggered by ribosome slowdown or stalling.

The previous version of the manuscript was already very robust, introducing a very elegant reporter system to measure codon optimality, ribosome occupancy and tRNA abundance. With this high-throughput reporter method, combined with other genetic tools, the authors were able to determine that Znf598, a homolog of the yeast protein in charge to resolve ribosome stalling, is involved in ribosome quality control in vertebrates but not in codon optimality mediated RNA decay.

In this revision the authors address thoroughly one major comment and two minor comments that I raised in the previous version. In particular, I inquired about the possibility that the overexpression of AnsB, that impairs the ability of tRNAs loaded with Asparagine, would affect the stability of endogenous mRNAs. The authors already showed that AnsB overexpression altered the codon optimality control of their reporter system. In this version of the manuscript, the authors conduct RNA-Seq experiments on AnsB injected embryos but they could not observe any codon enrichment or depletion in endogenous transcripts. The authors postulate that the inherent differences in the biogenesis of their reporter system and the endogenous transcripts (injected vs nuclear-transcribed) could influence the outcome of codon optimality. To disentangle the nuances of these two types of mRNA regarding codon optimality would be part of a whole new project and I am satisfied with the answers of the reviewers, who also acknowledge this discrepancy in the Discussion section.

In addition, the authors improve the figure 5J showcasing the PACE results according to my previous suggestion. By displaying both the PACE result both in WT and Znf598 mutant and adjusting the color codes, now it is self-evident that Znf598 is not involved in the control of RNA stability mediated by codon optimality in vertebrates.

Overall, the authors addressed all my questions and also included additional improvements as suggested by other reviewers, making an even more robust and impactful manuscript. This manuscript should be accepted for publication in EMBO Journal as it addresses the timely question of the mechanisms governing RNA stability mediated by codon optimality and its elegant reporter system in a whole organism makes clear contributions to the advancement of the field.

Referee #2:

The authors have addressed all of my concerns and I fully support acceptance of the manuscript as currently submitted. This manuscript was a pleasure to read.

Referee #3:

Dear Editor,

The authors have addressed all my questions and concerns in a thorough and elegant manner.
I particularly appreciate the new PACE experiment performed in the presence of AnsB, which clearly shows that the effect is mainly restricted to Asn codons. The differences observed between the PACE reporter and endogenous mRNAs upon AnsB expression clearly highlight the interest of their reporter, which allows to focus on the direct impact of a given codon independently from other transcript cis-acting features that could act as confounding factors.
New results obtained with the luciferase reporters are an improvement over the previous version with fluorescent proteins and raise many questions that will be interesting to address in a follow up work.

I consider the work suitable for publication.

***

Rev_Com_number: RC-2021-00814
New_manu_number: EMBOJ-2021-109256R
Corr_author: Mishima
Title: Transient ribosome slowdown during decoding triggers mRNA decay independent of Znf598 in zebrafish

Dear Editor,

Thank you very much for considering our manuscript (EMBOJ-2021-109256R) “Transient ribosome slowdown during decoding triggers mRNA decay independent of Znf598 in zebrafish”. Below we summarize changes made in the final version. Changes are made using the track change option.

1) Please update availability section so that it complies with the journal's formatting requirements (please add the direct link) and ensure the datasets are set to public.

We have modified the data availability, and set the data available for public.

2) Please update the Appendix pdf file to the final version (this file will not be typeset and be published as you submit it):
a) Page 1 is a table of contents with page numbers, please add page numbers.
b) Please remove the legends for Appendix Table S1 and S2 from the main manuscript.

We have made changes as requested.

3) Please add the header "EV Figure Legends" to separate these from the main figures in the manuscript text.

We have made changes as requested.

4) Please update the order of the sections in the manuscript to the journal's standard.

We have made changes as requested.

5) For the source data:
- Please check the source data labeling for Fig. 1 A, it seems to refer to 1B.
- Please check if the file submitted for Fig. 2 DE is correct and add a brief legend/explanation to how the figure was calculated (i.e. is a tab in the excel file).

We apologize for the mistake, the source data labeling for Fig. 1 A should refer to 1B. We have changed the labeling.
The source data for Fig. 2 DE was divided in Fig2 D and E with a brief explanation.

6) Our data editors have raised queries with the data descriptors in the figure legends, which you will find as comments in the Word document EMBOJ-2021-109256_figure_QC.docx (as part of your submission and attached to this message).

We have addressed the queries.

Thank you for submitting the final revised version of your manuscript. As we finalize the files for transfer to the publisher Wiley, you will hear from me again regarding some suggestions for changes in wording, but for now I am pleased to inform you that we have accepted the study for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Yuichiro Mishima
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2021-109256

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For zebrafish experiments, n=3 was chosen empirically.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For zebrafish experiments, n=3 was chosen empirically.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No sample was excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Luciferase assay samples were measured at random order.
For animal studies, include a statement about randomization even if no randomization was used.	When possible, zebrafish embryos were collected randomely from a sample pool.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was performed.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used both pearson correlation (parametric) and spearman rank test (non-parametric) to analyze correlation.
Is there an estimate of variation within each group of data?	No.

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Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Details are described in the Method section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell line was used.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Described in the Method section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Described in the Method section.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Done.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	GEO accession numbers for RNA-seq data are provided in the Data Availability section.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.