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Queuosine-tRNA promotes sex-dependent learning and memory formation by maintaining codon-biased translation elongation speed.

Cansu Cirzi, Julia Dyckow, Carine Legrand, Johanna Schott, Wei Guo, Daniel Perez-Hernandez, Miharu Hisaoka, Rosanna Parlato, Claudia Pitzer, Franciscus van der Hoeven, Gunnar Dittmar, Mark Helm, Georg Stoecklin, Lucas Schirmer, Frank Lyko, and Francesca Tuorto
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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Francesca,

Thank you for submitting your manuscript to The EMBO journal. Your study has now been seen by three referees and I am afraid that the overall opinion is not a positive one.

As you can see, the referees appreciate the insights gained from the tRNA aspect, but I am also afraid that they raise significant concerns that preclude publication here. As you can see from the comments below, both referees #2 and 3 raise concerns with the behavioral assays and neuronal phenotypes. Referee #1 is overall supportive of the work, but I should note that this referee comes from the RNA modification side and from that aspect things look good.

Given these comments from good experts in the field, I am afraid that I can't offer to consider publication in The EMBO Journal.

I thank you for the opportunity to consider this manuscript. I am sorry that we cannot be more positive on this occasion, but I hope nevertheless that you will find our referees' comments helpful.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

The role of the tRNA modification queuosine (Q) as an important translational regulator is currently emerging in different model systems. This study by the Tuorto Laboratory reveals that Q levels vary with tissue types with the highest levels observed in the brain, heart, and skeletal muscle but the main finding is that the Q absence in mice leads to learning and memory impairments, particularly in females. They reported hippocampal neuron composition and reduced dendritic outgrowth defects that must contribute to sex-dependent defects in learning and memory.
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Summary

The manuscript examines a large number of outcomes in a mouse that has lost the ability to modify tRNAs with Quenosine. Because of the large number of different outcomes, each examination is rather shallow and raises more questions than answers. There are problems with many of the experiments described. While it is very interesting to see the differences observed, very little insight into why these differences are observed is given. Moreover, there are some serious issues with many of the experiments that would have to be addressed (see below for specific comments).

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The decreased dendritic levels seen in the dentate gyrus could be due to many factors (decreased inputs from the inputs to the Dentate, degeneration, cell identify changes). Some follow up experiments are required to understand why these changes are seen.
Female animals change anatomy during the estrus cycle (Cooke and Woolley, 2005 PMID: 15884004) and it is not clear if this is being controlled for.

The myelin sheath changes in the proteome (fig. 7) are likely due to decreased myelination. This should be measured directly. This is likely to be due to changes in oligodendrocytes. Supplemental Fig. 5 examined astrocytes and microglia, but not oligodendrocytes. The number of oligodendrocytes and the extent of myelination needs to be shown. In these mice

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The authors here describe Queuosine-tRNA modification in the mouse hippocampus and link modification levels to the translatome and cognitive behavioral outcomes. They use a novel knockout mouse line with deletion of Qtrt1 for these experiments and compare male and female mice with or without deletion of this modification enzyme. They also provide a

comprehensive atlas of Q- modification for three tRNA families (tRNAAsp, tRNAAsn, tRNAHis) various mouse tissues. In the manuscript, the authors conclude there are sex differences between learning and memory tasks and proteomic outcomes that were likely linked, and that loss of Q-tRNA overall impairs learning and memory processes through changes in the translatome. The characterization of Q-tRNA across mouse tissues in conjunction with development of Qtrt1 KO mouse to assess phenotypic outcomes are excellent datasets and tools that are of immense importance to the fields of tRNA biology and neuroscience. While the molecular data (translatome, proteome, tRNA profiling, modification analysis) is excellent, the behavioral phenotyping is presented extremely poorly and conclusions drawn are speculative in nature.

Major comments:
1. No statistical analyses are presented for any behavioral or morphological comparisons between groups, which makes it impossible to draw any conclusions from the data shown(Figs 3-5).
2. The authors never provide a direct link between behavior and molecular outcomes but state in the paper that the changes in proteome are causing the cellular changes and behavioral alterations. However, there is no direct link between distinct protein abundance alterations and neuronal morphology and further to changes in MWM or FC.
3. The authors use Morris Water Maze and fear conditioning to assess learning and memory. It is unclear why these tasks were chosen and other types of learning and memory (i.e. working memory) were omitted. Additionally, as this is a new mouse line, it would be beneficial to conduct a full battery of behavioral tests, including motor performance ,anxiety-like, and depressive-like behavior.
4. There is a large amount of variability in the behavioral tests shown within each sex. The sample size should be increased to 8-10/sex/group to be able to achieve true statistical power with behavioral data.
5. The authors measure swimming strategies in the Morris Water Maze, which is not a conventional measure of cognitive behavior used in neurobiological and behavioral studies. Can the authors provide a rationale for looking at this behavior and what it would mean translationally for human behavior?
6. The authors make various claims throughout the manuscript based on behavioral data that are incorrect. For example, in line 201 they say they observe passive floating during the MWN and relate this to helplessness or frustration. It is important to avoid abstract claims like this that anthropomorphize the mice being tested, as "Frustration" isn't something that can be deduced from this test. Further, using passive floating during the MWM to obtain information about helplessness is not feasible, as the chamber is too large and they are able to escape via platform. The authors would have to use a forced swim test to be able to make this claim.
7. Fear conditioning data is shown in a very confusing manner that is not consistent with the norms of the field. It also appears the wildtype mice are not acquiring the memory sufficiently, making any comparisons impossible and suggesting there may be an issue with the performance of the tests and learning paradigms should perhaps be altered. Additionally, in Fig 3F, the authors show cued fear conditioning as a minute by minute bar graph, when it should really be shown as freezing at baseline and then after each CS.
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5. Supp. Fig 6C- in addition to using fluorescence intensity to measure Bassoon expression, it would be beneficial to confirm via western blot.
6. Line 300- Do the 327 differentially translated genes in females overlap at all with the 92 observed in males?

Dear Karin,

We have now a substantially revised version of our manuscript (EMBOJ-2022-112507) ready that we ask you to reconsider for publication in The EMBO Journal. We think that we have now fully addressed the concerns of the referees regarding the behavioural assays and the neuronal phenotypes.

We have used an additional cohort of mice and performed more behaviour tests, thus increasing the sample size and the statistical power. Using three independent behavioural tests (MWM, IntelliCage and Fear conditioning), we show that female Q1 mice exhibit lower learning and memory performance compared to wild type littermates. Furthermore, additional tests, such as Home-cage observation, indicate that female Q1 mice are hyperactive.

We have performed NeuN staining, which confirms the reduction of post-mitotic neuronal density in the hippocampus of Q1 mice. This is also in agreement with the newly added Sholl analysis, showing an altered intersection of the pyramidal neurones in the dentate gyrus of Q1 mice.

We have checked oligodendrocyte density and myeline-associated proteins in the mutant mice and found no significant differences in respect to the previous data.

We have measured ubiquitination of ribosomal proteins by Western blot and the show increased ribosome quality control in the Q1 hippocampi, supporting our molecular model.

I will be ready to re-submit the manuscript next week (Wednesday) after the approval of all co-authors. Please let me know the best way to proceed with re-submission.

Best wishes,

Francesca

Dr. Francesca Tuorto
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Mannheim Institute for Innate Immunoscience (MI3)
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Web: https://www.umm.uni-heidelberg.de/biochemie/research/research-francesca-tuorto/ <https://www.umm.uni-heidelberg.de/biochemie/research/research-francesca-tuorto/>
Tel +49-621 38371439
Fax +49-621-38371435

Dear Francesca,

Thanks for your email outlining how you have responded to the referees' concerns.

Please use the link below to submit the revised version.

With best wishes

Karin

Karin Dumstrei, PhD
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Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

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See also guidelines for figure legends: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

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IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines
(https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

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Dear Karin,

Hereby, I submit a substantially revised version of our manuscript that we ask you to reconsider for publication in The EMBO Journal. We think that we have now fully addressed the concerns of the referees regarding the behavioral assays and the neuronal phenotypes. We thank the reviewers for the valuable suggestions that notably increased the quality of the revised study.

Here is a quick summary of the experiments that we added to the revised version, as requested after the first round of revision:

-We have used an additional cohort of mice and performed more behavioral tests, thus increasing the sample size and the statistical power. Using three independent behavioral tests (MWM, IntelliCage and Fear conditioning), we show that female Q1 mice exhibit lower learning and memory performance compared to wild type littermates. Furthermore, additional tests, such as Home-cage observation, indicate that female Q1 mice are hyperactive.

-We have performed NeuN staining, which confirms the reduction of post-mitotic neuronal density in the hippocampus of Q1 mice. This is also in agreement with the newly added Sholl analysis, showing reduced intersections of the pyramidal neurons in the dentate gyrus of Q1 mice.

-We have checked oligodendrocyte density and myelin-associated proteins in the mutant mice and found no significant differences with respect to the previous data.

-We have measured ubiquitination of ribosomal proteins by Western blot analysis. The result indicates activation of the ribosome quality control pathway (RQC) in the Q1 hippocampi, supporting our molecular model.

Major changes in the figures of the revised version:

- New Figure 3 and Figure EV1 show the additional and revised behavioral analyses.
- New panels in Figure 4 and EV2 show NeuN staining and Sholl analysis.
- New Figure EV5 shows eI2Fα phosphorylation in the liver of Q1 mice, the ubiquitination of ribosomal proteins and molecular functions dysregulated in the proteomic analyses.
- Appendix containing the information on the cohorts of animals used in the study, additional behavioral tests (Rotarod, Dark/lightest, Grip strength) and the settings for the behavioral assays for learning and memory.

A detailed point-by-point response to the referee's concerns is attached below.

Thank you for considering our revised manuscript.

With best wishes,

Francesca Tuorto (on behalf of all the authors)

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We thank the reviewer for the recognition of the importance of this work.

Minor Points:

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The manuscript examines a large number of outcomes in a mouse that has lost the ability to modify tRNAs with Quenosine. Because of the large number of different outcomes, each examination is rather shallow and raises more questions than answers. There are problems with many of the experiments described. While it is very interesting to see the differences observed, very little insight into why these differences are observed is given. Moreover, there are some serious issues with many of the experiments that would have to be addressed (see below for specific comments).

We thank the reviewer for the critical and important comments. This is a multidisciplinary work and has the beauty of bringing together approaches from different angles. From the overall characterization of a novel transgenic mouse line to brain-specific phenotypes, we uncovered tRNA modification-dependent translation decoding in the murine brain and its effects on learning and memory. We would like to emphasize that details of each single aspect cannot be studied in depth within the scope of this single paper. In this regard, follow-up studies, which have been funded recently, will be performed. Regarding specific comments, please, see our answers below.

Major concerns
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Morris Water Maze (MWM). In the MWM, it appears that male mice did not learn the task. This is not usual, most male mice can learn the MWM, so this appears to be an experimental

problem. Normally, one shows all four quadrants in the probe trial and there needs to be a significant increase in the target quadrant to document learning. The data shown in Fig 3E is only for the time in the target quadrant and is measured in seconds (not percent of time) and other quadrants are not shown. This is not the normal way of describing this data. It is not clear from this data than any animal actually learned the task. The very low time spend by the female Qtr1 deficient mice seems lower than expected for no learning when the animals are expected to spend 25% of their time in all the quadrants.

We have re-analyzed the data as suggested by the reviewer and included new figure panels; Figure EV1 L and M. Both female and male wild type mice spent more than 25% of the time in the target quadrant during the probe trial. As suggested by the reviewer, this indicates that the wild type mice were mostly able to learn and remember the position of the platform, whereas both male and female Q1 mice, which spent less than 25% of the time in the target quadrant, showed impaired learning and memory capacity. Note that a clear indication for learning and memory capacity of wild type mice in the test, particularly of the male mice, is also the increase of success rate achieved over time (heat map in Figure 3B).

Fear conditioning

The protocol used here is not standard. For cued fear conditioning there are usually two chambers. The animal is habituated to chamber B. It is conditioned in chamber A and then cue conditioning happens in chamber B. Here, the experimenters had no habituation, but altered chamber A after learning into a different chamber. The methods do not say how it was remodeled. The animals are first extinguished in chamber A before the cued test in chamber B. All of this is not usual. Moreover, there is no control to measure whether the animals learned (no shock or better, non-paired shock for cue conditioning). This is also not appropriate. The figures are strange (Fig. 3F and supplemental figure 5C both show cued conditioning, but they are not the same numbers). Overall, it is not clear there is any cued learning in these cases. The description of how freezing behavior was measured is also quite non-standard. No reference is given for this definition of freezing. The level of freezing for this shock is quite small.

Increase of the sample size by the addition of a second cohort of mice gave us the statistical power to demonstrate the differences in freezing behavior and the defects in conditioning, contextual and cued FC of Q1 female mice. In addition, we now show the freezing data as minute-by-minute graphs as suggested by reviewer 3 (Figure 3G), which simplifies the visualization of the effects.

Of note, we performed this test as described previously and according to the references PMID: 32647155, PMID: 23019518, and similar to references PMID: 31444474, PMID: 33771871 PMID: 20953248. We used the same registration apparatus for all tests, whereas the chambers used in conditioning and contextual FC are profoundly different from the one used for cued FC test. For conditioning and contextual test, the chamber is a quadrat box, illuminated and in which the mice touch directly the metal grid. For the cued test, a flat plastic floor panel covers the grid and two black roof-shaped plastic panels are inserted, therefore, the chamber became a triangular box. In addition, the visible light is replaced by near-infrared and a source of perfume is added.
The setting of the test described above has been clarified in the Appendix (new appendix Figure 8) and Method sections (page 19, row 610).

Freezing behaviour was analyzed via a video camera connected to video tracking software. The absence of any movement excluding respiration was considered freezing and it was defined by freezing frame, as a change in <11 of 76800 pixels between adjacent frames (33 ms apart) over a time period of >1 s. This information has been added to the methods section.

2.Anatmoical observations
The changes in SytI mRNA levels make no sense. All neurons express either Syt I or Syt II. It is not clear whether the change in the number of neurons with a positive Syt I mRNA levels are due to transcriptional changes (Syt 2 replaces Syt 1), a loss of neurons (which should have been seen in DAPI and Nissl), or neurons about to die. This needs to be resolved. As of now, this makes no sense. The decreased dendritic levels seen in the dentate gyrus could be due to many factors (decreased inputs from the inputs to the Dentate, degeneration, cell identify changes). Some follow up experiments are required to understand why these changes are seen.
Female animals change anatomy during the estrus cycle (Cooke and Woolley, 2005 PMID: 15884004) and it is not clear if this is being controlled for.

We checked *Syt1* and *Syt2* mRNA levels using bulk RNA-Seq (Dataset EV1) and found no significant change in their expression in the Q1 mice. However, since we cannot exclude a local decrease of *Syt1* mRNA expression, we have now performed NeuN immunostaining as a marker of neuronal nuclei and found a decrease in neuronal density in the Q1 mutant mice (new Figure 4C and D and EV3A). These data, along with the reduced *Syt1* cell density and altered neuronal arborization observed by the Golgi staining, indicate an aberrant hippocampal cytoarchitecture in the Q1 mutant mice.

We thank the reviewer for pointing out this aspect. We did not check the estrous cycle of the female mice and this has now been clarified in the method section (page 15, row 491). Differently from Cooke and Woolley (PMID: 15884004) who describe changes in dendritic spine density during the estrus cycle, we did not observe any differences in dendritic spine density between control and mutant mice in our analyses (see Figure EV3D and E). However, we could detect other structural alterations in neuronal arborization. Indeed, we observe a reduced architectural complexity of the pyramidal neurons of the dentate gyrus and contextually a reduced cell density of these neurons with *Syt1* and NeuN staining. On parallel sections, we have also performed TUNEL assay (Figure EV2E) and activated Caspase 3 staining and Western blot analysis (data not shown), without observing any sign of cell death at the studied age.

We agree with the reviewer that it would be desirable to understand in greater detail at what age the changes in hippocampus architecture start, whether neurogenesis and proteostasis are affected, and what the exact molecular link to loss of Q is. This is mentioned in the discussion on page 14, row 465: “It is conceivable that a permanent imbalance in the speed of decoding leads to chronical translational stress, which may well lead to loss of Syt1and NeuN expressing neurons and alterations in the dendritic arborization as observed in the Q1 hippocampi (Figure 4). Therefore, exploring the link between Q modification, eIF2 signaling, neurodegeneration and neurogenesis appears to be a promising avenue for further research.”
Please also see our answer to reviewer 3 on point 2.

The myelin sheath changes in the proteome (fig. 7) are likely due to decreased myelination. This should be measured directly. This is likely to be due to changes in oligodendrocytes.

Supplemental Fig. 5 examined astrocytes and microglia, but not oligodendrocytes. The number of oligodendrocytes and the extent of myelination needs to be shown. In these mice

The oligodendrocyte density has now been evaluated by measuring *Plp1* mRNA levels using *in situ* hybridization, and the data have been added to Figure EV2D. No differences were detected between the two genotypes.
In addition, no differences were observed in the expression of MOG markers, both using immunohistochemistry and Western blot (shown in panel A and B below). Moreover, MBP (Myelin basic protein), which we found to be increased in the Q1 proteome (see volcano plot in panel C below), was confirmed to be increased in Q1 hippocampi by Western blot analysis (panel D), validating our proteomics data.

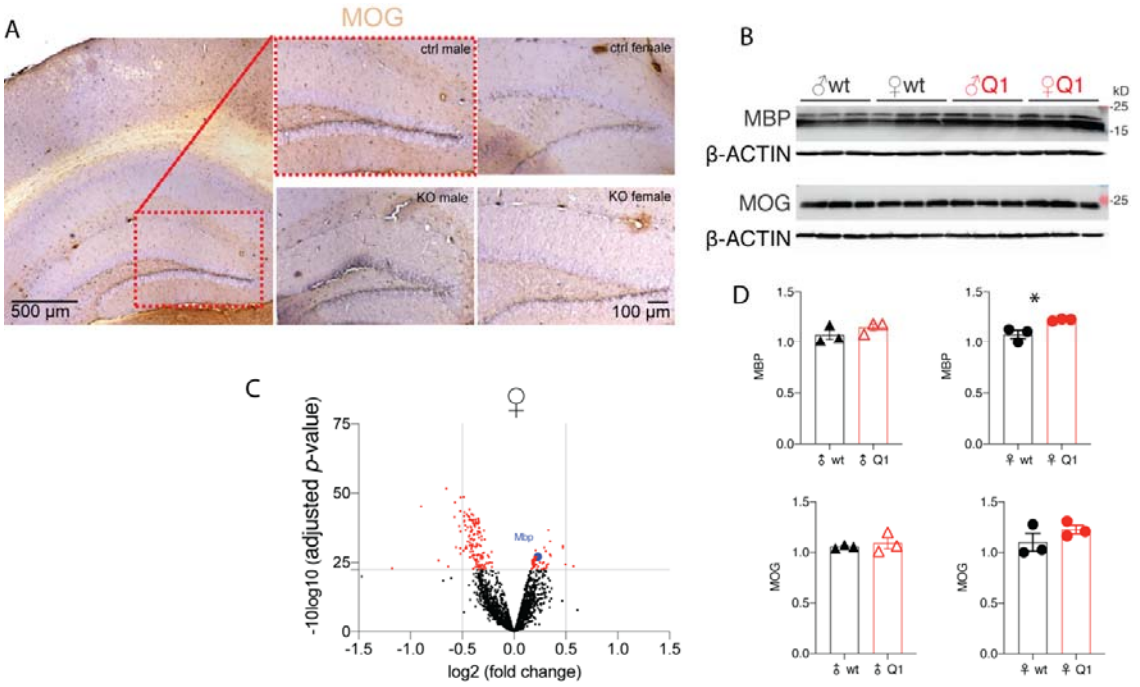

Figure for referees.
A. IHC staining of MOG in the hippocampus and DG of 7-month-old mice. **B.** Western blot analysis showing MBP and MOG expression upon loss of Q in the 8-month-old female Q1 hippocampi. β-ACTIN and global eIF2α levels were used as loading controls. **C.** Adapted from Figure 7B of the manuscript. Dimethyl-labelling analysis of 8 months old hippocampi. The red dots represent 188 deregulated proteins. The adjusted *p*-value < 0.05 is indicated by the horizontal line. MBP is indicated in blue. **D.** Quantification of the level of MBP (we quantified the bands corresponding to 21.5, 18.5, 17.2 and 14.0 kDa isoforms) and MOG proteins. The signal was normalized to β-ACTIN. Error bars: ±SD; (n=3), *=*p*-value < 0.05.

3. No examination of homeostasis and ribosome quality control

The authors appear to be explaining the increases in eIF2alpha to the increase seen from collided ribosomes. However, this should be associated with an increase in ribosome quality control pathways and ubiquitination of ribosomsal subunits. This should be examined.

We thank the reviewer for pointing out this aspect. The analysis of the ubiquitination of ribosomal proteins has now been added to Figure EV5A. Indeed, we observe increased ubiquitination of ribosomal proteins RPS10 and RPS20, indicating involvement of the ribosome quality control (RQC) pathway (Figure EV5A); page 11, row 345.

The specific mRNAs affected appear to be in the translation pathway and probably due to some homeostatic response to the alterations due to the loss of Q. This is not discussed or examined.

We agree with the reviewer that the translation pathway is affected in Q1 hippocampi. Our data suggest that this is due to the imbalance in the speed of codon-biased translation in the absence of Q-tRNA, which in turn activates RQC and eIF2 signaling. Both pathways are critical regulators of eukaryotic translation.

This point is discussed:
-In the results section, page 11 row 340: " PANTHER reactome analysis (Mi *et al*, 2021) revealed that genes translated differentially between wild type and Q1 hippocampi are enriched for functions in protein translation, ribosomal metabolism and non-sense mediated mRNA decay (NMD), especially in females (Figure 6C). This suggests that the imbalance in the translation speed due to the lack of Q causes profound perturbations in the synthesis and regulation of the translation machinery, as suggested by the increased ubiquitination of ribosomal proteins RPS10 and RPS20 observed in the Q1 mice hippocampi, indicating involvement of the ribosome quality control (RQC) pathway (Figure EV5A). In agreement with our previous observations in HeLa cells (Tuorto *et al.*, 2018), eIF2, a target of a number kinases during stress signalling…."
Also in the proteomic data, for which we have now added the deregulated molecular functions in Figure EV5C, page 11, row 365: "as well as molecular functions directly connected to dysregulated protein synthesis (Figure EV5C)".
-And in the discussion section, page 14, row 453: "An imbalance in the speed of codon-biased translation and RQC translational arrest are likely to have a considerable impact on the translatome and translation regulation giving rise to alterations in the proteome. In agreement with our previous observations in HeLa cells (Tuorto *et al.*, 2018), eIF2 signaling, a critical stress-induced regulator of eukaryotic translation (Bhat *et al*, 2015), was deregulated both in the translatome of the Q1 hippocampi and by Western blot (Figure 6C, 7A and EV7C). In fact, stalling of ribosomes was recently found to cause eIF2α phosphorylation (Wu *et al*, 2020), arguing that stalling at Q-decoded codons in Q1 mice may lead to direct activation of eIF2α signaling.".

All of the profiling was done in the nervous system, but translation in the nervous system is quite low compared to other tissues (see polysome distribution if Fig. S7B (there is no polysomal peak). To understand whether the differences in codon usage is nervous system-specific or seen in all tissues some other tissue needs to be examined. It will be interesting to see if the female- specific differences are restricted to the nervous system or are present animal wide.

We focus on the brain, particularly on the hippocampus, due to observed impairment of learning and memory. A remarkable number of neurodevelopmental disorders have been linked to defects in tRNA modifications that are expressed throughout the body (see PMID: 30529455), indicating that the central nervous system is particularly sensitive to alterations in the translation machinery. Our study fits very well with this general observation.

An extensive analysis of translation in another tissue in Q1 mice would have been very costly, and was not considered a priority given the neuronal phenotype. As an alternative, we checked eIF2α phosphorylation in mouse liver extracts and added these data to Figure EV5B. Similar to our observations in the brain, we have observed an increased eIF2α phosphorylation in the liver of female Q1 mice. As discussed in the manuscript, page 14, row 460, the difference between other tissues and brain could be explained by the fact that "eIF2alpha phosphorylation has important functions in brain physiology, beyond its essential role in stress-dependent regulation of translation initiation. Several reports have shown that an increase in eIF2α phosphorylation impairs learning and memory in mice and chicken (Batista *et al*, 2016; Costa-Mattioli *et al*, 2009; Sharma *et al*, 2020), and deregulated eIF2α phosphorylation is a common factor in many neurodegenerative diseases (Moon *et al*, 2018)".

Minor Concerns
Lines 180-188. There is no reason to report on the grooming phenotype; the point of the backcrossing is to eliminate any possible mis-targeting of the Crispr and remove differences due to the differing phenotypes of the mice used to make the cells and the recipient mice. If the behavior disappears with backcrossing, it is not a reproducible finding and should not be reported.

We have removed the grooming phenotype data and the figure from the manuscript. However, since we again observed increased grooming and loss of nose fur in 4 of the 10 Q1 female mice at the late age of 8 months in the last cohort of mice used for the revision, we kept a sentence on this in the results section: "Interestingly, during backcrossing, Q1 mice developed an excessive self-grooming behavior." In the future, we plan to assess the origin of this symptom more specifically, especially in older mice.

Referee #3:
The authors here describe Queuosine-tRNA modification in the mouse hippocampus and link modification levels to the translatome and cognitive behavioral outcomes. They use a novel knockout mouse line with deletion of Qtrt1 for these experiments and compare male and female mice with or without deletion of this modification enzyme. They also provide a comprehensive atlas of Q- modification for three tRNA families (tRNAAsp, tRNAAsn, tRNAHis) various mouse tissues. In the manuscript, the authors conclude there are sex differences between learning and memory tasks and proteomic outcomes that were likely linked, and that loss of Q-tRNA overall impairs learning and memory processes through changes in the translatome. The characterization of Q-tRNA across mouse tissues in conjunction with development of Qtrt1 KO mouse to assess phenotypic outcomes are excellent datasets and tools that are of immense importance to the fields of tRNA biology and neuroscience. While the molecular data (translatome, proteome, tRNA profiling, modification analysis) is excellent, the behavioral phenotyping is presented extremely poorly and conclusions drawn are speculative in nature.

We thank the reviewer for their recognition of this work. We have added a second cohort of mice and performed additional behavioral tests, which provided more reliable results and clearly strengthened our study.

Major comments:

1. No statistical analyses are presented for any behavioral or morphological comparisons between groups, which makes it impossible to draw any conclusions from the data shown(Figs 3-5).

We have performed statistical analyses for all behavioral, histological and molecular parameters in mice of both sexes. To account for the strong sex bias of our phenotype, all comparisons were conducted separately for male and female mice. In addition, we have tested the effect of sex on genotype differences showing that female mice are significantly more affected than male mice.

Statistical significance was analyzed using:
- two-tailed unpaired t-test to compare the number of cells of female wt and Q1 or alternatively male wt and Q1 in Figure 4B, D, E, etc. The same test was used to compare protein expression levels in female wt and Q1 or alternatively male wt and Q1 in Figure EV 5A, B, etc.
- two-way ANOVA followed by Benjamini and Hochberg post hoc test was used to show genotype effects and individual time *p*-values for female wt and Q1 or alternatively male wt and Q1 for the MWM test (Figure 3A) and for the Sholl analysis.
- for data sets depicting percentages, we made use of beta regression as an alternative test that is compatible with non-normal distribution (Figure 3B, D,F and G). In addition, we tested for these datasets the effect of sex on genotype differences and included the p-values in the figure legends.
- The Wilcoxon rank sum test was used as a nonparametric test for Figure 5B and C and Figure EV1L.

2. The authors never provide a direct link between behavior and molecular outcomes but state in the paper that the changes in proteome are causing the cellular changes and behavioral alterations. However, there is no direct link between distinct protein abundance alterations and neuronal morphology and further to changes in MWM or FC.

To pin-point a single gene or protein whose dysregulation directly explains all cellular and behavioral differences in Q1 mice would be desirable, yet finding this molecular culprit may require many years of work. Also, it is far from certain that a single gene or protein is responsible for the phenotype. Given that we observe a profound imbalance in the speed of translation decoding in Q1 mice (Figure 5 and 6), we favor the hypothesis that wide-spread dysregulation of protein synthesis causes the phenotype, as we observe activation of stress-related pathways such as ribosomal quality control (RQC, Figure EV5A) and eIF2α phosphorylation (Figure 7A). In fact, as discussed on page 14 row 460 “elevated eIF2α phosphorylation has important functions in brain physiology, beyond its essential role in stress-dependent regulation of translation initiation. Several reports have shown that an increase of eIF2α phosphorylation impairs learning and memory in mice and chicken (Batista *et al*, 2016; Costa-Mattioli *et al*, 2009; Sharma *et al*, 2020), and that eIF2α phosphorylation is a common factor in many neurodegenerative diseases (Moon *et al*, 2018)”.

It remains a mystery why lack of Q-tRNA affects female mice much more strongly than male mice. The sex-dependent aspect will be subject of a follow-up study, which has been funded recently.

3. The authors use Morris Water Maze and fear conditioning to assess learning and memory. It is unclear why these tasks were chosen and other types of learning and memory (i.e.

working memory) were omitted. Additionally, as this is a new mouse line, it would be beneficial to conduct a full battery of behavioral tests, including motor performance ,anxiety-like, and depressive-like behavior.

We agree with the reviewer that a full battery of behavioral tests would give a better overview of all the defects of Q1 mice. We were able to perform Open field, Home-cage observation, MWM, Fear conditioning and IntelliCage, which are now all included in Figures 3 and EV1. In addition, we also performed Grip strength, Dark/Light test and Rotarod, which gave no significant differences and are shown in the appendix (see Table 1, Figures 4 and 5 in the Appendix). Since Rotarod and Grip strength tests excluded deficiencies in motor functions, and also due to limited resources, we decided to focus on a single brain region for molecular analyses and related behavioral tests. The hippocampus was chosen because of its major role in cognitive functions such as learning and memory.
This is now stated in the results section page 6, row 184: " Therefore, in the final C57BL/6J background, Q1 adult mice were subjected to a comprehensive battery of behavioral tests (Appendix Table 1), with a focus on learning and memory functions of the hippocampus."
Among the numerous types of mazes to test hippocampal spatial learning, the MWM is one of the most widely used paradigms and fear conditioning was included as an additional test, since the formation of fear memory to a discrete sensory cue is hippocampus-dependent. Lastly, we included IntelliCage, because this system can monitor spatial hippocampal learning in an unbiased home cage setting. This is now stated on page 6, row 201: "Next, we used three behavior methods on two cohorts of mice (I. cohort n=6, II. cohort n≥8) to assess hippocampal-dependent learning and memory functions by Morris water maze (MWM), IntelliCage and Fear conditioning."

Obviously, we cannot exclude that other brain regions, as well as other organs, may be altered in the Q1 mice. However, it is important to remember that the mutant mice are viable and fertile without obvious changes in overall development or growth (Appendix Figure 3, stated on page 6, row 181). We believe that our choice of analyzing the hippocampus is well justified given the behavioral phenotype, and validated by the cellular and architectural alterations we observe in this tissue. We agree that tests on working memory would be interesting and could implicate molecular and histological alterations of the prefrontal cortex, yet this aspect will have to be addressed in future studies.

4. There is a large amount of variability in the behavioral tests shown within each sex. The sample size should be increased to 8-10/sex/group to be able to achieve true statistical power with behavioral data.

We have added a second cohort of mice to the study with 8-10 mice/sex/group (appendix Table 1) as suggested by the reviewer. This notably improved the statistical power of the analyses.

5. The authors measure swimming strategies in the Morris Water Maze, which is not a conventional measure of cognitive behavior used in neurobiological and behavioral studies. Can the authors provide a rationale for looking at this behavior and what it would mean translationally for human behavior?

We agree with the reviewer and have removed the swimming strategies in Morris Water Maze from the manuscript.

6. The authors make various claims throughout the manuscript based on behavioral data that are incorrect. For example, in line 201 they say they observe passive floating during the MWN and relate this to helplessness or frustration. It is important to avoid abstract claims like this that anthropomorphize the mice being tested, as "Frustration" isn't something that can be deduced from this test. Further, using passive floating during the MWM to obtain information about helplessness is not feasible, as the chamber is too large and they are able to escape via platform. The authors would have to use a forced swim test to be able to make this claim.

As above (point 5), we have removed the swimming strategies in Morris Water Maze from the manuscript.

7. Fear conditioning data is shown in a very confusing manner that is not consistent with the norms of the field. It also appears the wildtype mice are not acquiring the memory sufficiently, making any comparisons impossible and suggesting there may be an issue with the performance of the tests and learning paradigms should perhaps be altered. Additionally, in Fig 3F, the authors show cued fear conditioning as a minute by minute bar graph, when it should really be shown as freezing at baseline and then after each CS.

As suggested by the reviewer, we now show the freezing data as a minute-by-minute graph (Figure 3G). By increasing the sample size with the addition of a second cohort of mice, we now have sufficient statistical power to demonstrate the differences in freezing behavior and the defects in context-dependent learning and memory of Q1 female mice.

8. For data representing hippocampal morphology (Fig 5), did the authors use Sholl analysis? Additionally, was dendritic complexity measured? This would be beneficial in addition to dendritic length.

Yes, we used Sholl analysis; a graph depicting intersections of dendrite branches was added to Figure 4F.

Minor comments.

1. Fig 1: Figures should show individual data points
-Done.
2. Fig 2: It is difficult to see the light gray error bars in this figure, they should be darkened to enhance contrast for the reader
-Done.
3. Can the authors describe what breeding scheme was used to generate these litters of mice?
-This information has been added to the Appendix Table 1.
4. Supp. Fig 6C- figure says p=0.5, unsure if this is a typo.
-This figure has been removed, see below.
5. Supp. Fig 6C- in addition to using fluorescence intensity to measure Bassoon expression, it would be beneficial to confirm via western blot.
- We could not reproduce the difference of Bassoon staining by Western blot, mainly due to the difficulty to blot efficiently and detect quantitative differences for such a big protein (420 kDa). Therefore, we have decided to remove this part from the manuscript.
6. Line 300- Do the 327 differentially translated genes in females overlap at all with the 92 observed in males?

The overlap between differentially translated genes in male and female Q1 hippocampi has been added to Figure EV4G. and in the results section, page 10, row 320.

Dear Francesca,

Thank you for submitting your revised manuscript to The EMBO Journal. Your revision has now been seen by the original three referees and their comments are provided below. As you can see the referees appreciate that the revised version has addressed the initially raised concerns and are supportive of publication of the revised version in The EMBO Journal. Referee #2 suggests adding the Morris Water Maze data to the appendix. Can we discuss this further?

When you submit the revised version will you also take care of the following points:

- Double check Wei Guo - in manuscript file is says Guo Wei vs. Wei Guo in eJP

- Please add funding information to acknowledge section.

- We need 3-5 keywords

- Data Availability Section should be placed before Acknowl. Section

- COI needs to be renamed to "Disclosure Statement and Competing Interests"

- Please remove the Authors Contributions from the manuscript. The 'Author Contributions' section is replaced by the CRediT contributor roles taxonomy to specify the contributions of each author in the journal submission system. Please use the free text box in the 'author information' section of the manuscript submisssion system to provide more detailed descriptions (e.g., 'X provided intracellular Ca++ measurements in fig Y')

- The appendix should have a ToC and page numbers.

- We need a synopsis text => a summary statement plus 3-5 bullet points describing the key findings of the MS.

- We also need a synopsis image => 550 wide by [200-400]

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Take a look at the word file and the comments regarding the figure legends and respond to the issues.

- Also, we don't encourage showing statistic when N=2. Please simply show both data points.

- Could you also provide source data for EV4 Figure EV4a tRNA His & tRNA Asp as the blots look very similar.

That should be all, let me know if you need further input from me.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

I had no issues with molecular components last time and the minor issues I had raised have been fixed. The main issues were about the behavioral data pointed out by the other reviewers that had more expertise on that aspect of the work.

Referee #2:

The manuscript has been improved significantly in a number of ways and most of my issues have been addressed. I think the manuscript makes an important contribution to the role of queuosine in neuronal translation and since it may be a limiting factor based on the gut microbe, this could be of importance. A number of interactions between the gut microbe and nervous system development have been recently shown and thus, this study could be of some importance.

The last outstanding issue is the Morris Water Maze. The lack of learning in the male mice indicates problems in the protocol. Escape latency is the only data shown in the main figure, and this is a poor measurement of learning in this task. Since there is considerable other evidence for hippocampal learning deficits in the intellicage experiment and the contextual fear conditioning, I would suggest just removing the MWM data or moving all of it into supplemental data and briefly mention in the text showing a deficit in female learning, but no learning in either WT or Q males.

Referee #3:

The Authors responded adequately to previous review. I do not have additional comments.

Dear Karin,
Hereby, I submit the last version of our manuscript.

Thank you for considering our manuscript for publication to The EMBO Journal.

With best wishes,
Francesca Tuorto (on behalf of all the authors).

Editor questions:
When you submit the revised version will you also take care of the following points:

- Double check Wei Guo - in manuscript file is says Guo Wei vs. Wei Guo in eJP
Done and corrected.

- Please add funding information to acknowledge section.
Done.

- We need 3-5 keywords
***Keywords**: queuosine, tRNA modifications, protein translation, hippocampus, sex bias, learning and memory.*

- Data Availability Section should be placed before Acknowl. Section
Done.

- COI needs to be renamed to "Disclosure Statement and Competing Interests"
Done.

- Please remove the Authors Contributions from the manuscript. The 'Author Contributions' section is replaced by the CRediT contributor roles taxonomy to specify the contributions of each author in the journal submission system. Please use the free text box in the 'author information' section of the manuscript submisssion system to provide more detailed descriptions (e.g., 'X provided intracellular Ca++ measurements in fig Y')
Done.

- The appendix should have a ToC and page numbers.
Done.

- We need a synopsis text => a summary statement plus 3-5 bullet points describing the key findings of the MS.
Summary statement:
The loss of Q-tRNA modification in Q1 mice causes an imbalance of the overall protein translation speed and homeostasis. This translational stress results in alterations of the hippocampal cellular architecture, hampering learning and memory formation in a sex-dependent manner.

Bullet points:

- *Q-tRNA levels vary considerably between murine tissues, with the brain showing a high*

queuosinylation level.

- *Loss of Q results in learning and memory deficits, with females being more affected than male mice.*
- *Q1 knockout mice exhibit altered neuronal cytoarchitecture in the hippocampus.*
- *Loss of Q leads to ribosome stalling on Q-decoded codons and alteration of overall protein translation homeostasis.*
- *Translational stress and increased eIF2Œ± phosphorylation impair neuronal cognitive functions in Q1 female mice.*

- We also need a synopsis image => 550 wide by [200-400]
Uploaded.

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Take a look at the word file and the comments regarding the figure legends and respond to the issues.
Done. I accepted and commented in the final manuscript file "EMBOJ-2022112507R2-Related_manuscript_File_QC_FT.

- Also, we don't encourage showing statistic when N=2. Please simply show both data points.
Removed.

- Could you also provide source data for EV4 Figure EV4a tRNA His & tRNA Asp as the blots look very similar.
Provided and I also upgraded the source data of the new Figure 3.

That should be all, let me know if you need further input from me.

With best wishes

Karin

****

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The last outstanding issue is the Morris Water Maze. The lack of learning in the male mice indicates problems in the protocol. Escape latency is the only data shown in the main figure, and this is a poor measurement of learning in this task. Since there is considerable other evidence for hippocampal learning deficits in the intellicage experiment and the contextual fear conditioning, I would suggest just removing the MWM data or moving all of it into supplemental data and briefly mention in the text showing a deficit in female learning, but no learning in either WT or Q males.

As agreed with the editor, we move the Morris Water Maze data to the EV figures. The data clearly show a deficit in learning of female mice. We agree with the reviewer on the fact that both male mice, wt and Q1, similarly performed less. We don't think this is due to an error in the protocol that should have necessarily affected both sexes, but that it is due to a difference in behavior and motivation of the males to perform the tasks.
Therefore, we reduced and changed the text (in line with what the reviewer suggested) as follows:

"During trials with the hidden platform, both learning (Figure EV1H-I) and success rate (Figure EV1J) were decreased in Q1 female mice that showed also a notable reduction in the time spent in the quadrant containing the former platform in the probe trial on day 12 (Figure EV1K-M). However, male mice, both wt and Q1, were in general less performant, which renders the evaluation of their learning capacity difficult (Figure EV1I-M).

Referee #3:

The Authors responded adequately to previous review. I do not have additional comments.

2nd Revision - Editorial Decision **28th Jul 2023**

Dear Francesca,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a look at everything and all looks good! I am very pleased to accept the study for publication here.

Congratulations on a nice study!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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 χ2 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Material and Methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Material and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Material and Metohods

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Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

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If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	In Figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	In Materials and Methods
Include a statement about **blinding** even if no blinding was done.	Yes	In Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	In Materials and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	In Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	In Figure legends

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Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	In Materials and Methods
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Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
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Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	In Data Availability
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	