THE EMBO JOURNAL

microRNAs slow translating ribosomes to prevent protein misfolding in eukaryotes

Mohieldin Youssef, Olga Elisseeva, Takayuki Akimoto, Katsuhiko Suzuki, Takashi Ushida, Tadashi Yamamoto and Hiroaki SAKO
DOI: 10.15252/embj.2022112469

Corresponding author(s): Hiroaki SAKO (HiroakiSako@gmail.com), Tadashi Yamamoto (tadashi.yamamoto@oist.jp)

Review Timeline:

Submission Date:	25th Aug 22
Editorial Decision:	27th Oct 22
Appeal Received:	20th Mar 23
Accepted:	18th Jun 23

Editor: Stefanie Boehm / Kelly Anderson

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.)

Dr. Hiroaki SAKO
Okinawa Institute of Science and Technology Graduate University
1919-1 Tancha
Kunigami District 9040495
Japan

27th Oct 2022

Re: EMBOJ-2022-112469
microRNAs slow translating ribosomes to prevent protein misfolding

Dear Dr. Sako,

Thank you again for submitting your manuscript to The EMBO Journal and sending the preliminary response to the referee comments (included again below). As I had mentioned in my previous message, we are concerned that fully resolving the critical issues the referees raise would require extensive experimental work and time and include experiments of unknown outcome that affect main conclusions. We discussed your response amongst the editorial team and we were not fully convinced that the planed experiments would sufficiently resolve the issues. Thus, I contacted one of the initial referees for an expert opinion on your response. Unfortunately, the referee was also not convinced that the crucial issues would be resolved to a degree that would support publication. Specifically, this referee was in particular concerned about the experimental evidence for effects on the translate rate, as this is absolutely crucial for the model in the referee's view. Some of the individual issues the referee noted were that the ribosome profiling experiments and the discussion of the normalization point that referee # 2 brought up, as well as referee #3's issues with figures 3 and 4, did not appear as if they would be sufficiently resolved. Since journal policy only allows for a single round of major revision, we only invite a revision for manuscripts, where the required experimental work appears feasible in the time-frame and where the critical issues can likely be resolved without affecting the main conclusions of the study. We unfortunately do not find this to be the case here and thus cannot offer to formally invite a revision at this time.

However, as indicated before and also by the referees, we recognize the interest in the proposed model. Thus, if you are able to resolve the main issues- experimentally- in the future, we would be willing to reconsider a revised version of the manuscript. Please however note that in such cases, we again look at novelty and advance at the time of resubmission and may also involve alternative or additional reviewers, if needed. Please do not hesitate to contact us if you would like to further discuss this option.

I am sorry that I cannot be more positive on this occasion. Thank you again for giving us the opportunity to consider your manuscript.

Kind regards,

Stefanie Boehm

Stefanie Boehm
Editor
The EMBO Journal

****

Referee #1:

The paper by Sako et al describes an effect of miRNA on attenuating cotranslational protein folding. The authors perform ribosome profiling in eukaryotic cells to identify the ribosome distribution along the mRNA. By filtering data they estimate the relative codon-specific translation rate and show that miRNAs binding to the mRNA coding sequence induce ribosome pausing downstream of the binding site. They then test whether global or specific miRNA deficiency affect protein folding in cells using a reporter construct with an EGFP reporter fused to the NBD1 of CFTR (the latter is chosen because it is particularly aggregation-prone). They show that inhibition of NBD1-specific miRNA does not alter the total NBD1 production, but causes increased NBD1 aggregation. The reduction of aggregation can be also induced by the non-cleaving shRNAs targeting NBD1-mRNA. The authors quantify the insoluble proteome of CHX-treated cells and suggest that proteins with signal peptides, low complexity regions, intrinsically disordered domains and transmembrane domains are aggregation-prone. Finally, they have chosen insulin

as an example of such a protein and show that non-cleaving shRNAs prevent insulin misfolding. This is an exciting finding that can provide novel insights into the regulatory mechanisms that link translation speed and cotranslational protein folding. However, given the importance of the study, additional controls and conceptual considerations are necessary.

Major points:
1. My main concern is that the authors do not provide a clear quantification of the miRNA effect on the mRNA stability (except for Fig S4B, which presents a special case of non-cleaving shRNAs). Given that the speed of translation is supposed to be an important factor governing mRNA stability, this would be an essential control.
2. Concerning the misfolded proteins accumulating in puncta and in the aggregates: are they full-length or fragments resulting from translation of truncated mRNAs?
3. The model suggested by the authors predicts that in the absence of miRNAs, a sizable fraction of nascent proteins will aggregate. This should be checked at normal growth conditions, i.e., without CHX treatment, as according to their model mild CHX treatment should reduce misfolding.
4. Conceptually, it remains unclear how miRNAs should regulate folding in the sense that the amount of correctly folded protein is changed at certain conditions, for example by switching the miRNA production, which would improve the yield of soluble protein. Are these miRNAs expressed constitutively or are regulated? This should be discussed in more details.

Specific comments:
1. The Abstract does not present the result in a logical way, the sentences should be shifted to achieve a smooth flow.
2. In the Introduction, the authors discuss the effect of translation kinetics in a somewhat oversimplified way, namely that slowing down translation leads to a better protein folding. This is by far not always the case, as correctly mentioned in the Discussion (refs 33 and others), but this information comes too late. The Introduction should be revised to provide a more complete picture, namely that efficient cotranslational folding requires a well-tune translation rate that has been optimized through evolution.
3. In many cases, statistical significance (the number of replicates, technical and biological replicates, etc) is not explained. This should be added to each Fig where relevant.
4. Based on data in Fig 1, the authors should estimate the kinetic effect of miRNAs on translation.
5. In all Figs, the curves showing normalized ribosome density at different conditions all have a very similar color, it is almost impossible to distinguish the results with, e.g., different miRNAs. The colors should be changed to allow a clear distinction.
6. P. 4, lines 99-101, the text is somewhat confusing, because the description of the translational slowdown at the 0-15 region is missing. The authors mention accumulation of ribosomes at codon 0 and the reduction at +20 to +25, but not the crucial region in between.
7. P. 4, lines 114-115. Please describe the rationale for using EGFP, SBP, P2A and mCherry in the reporter. While this may be obvious to the specialists in the field, the advantages of such reporter will not be immediately clear to the broader readership.
8. P. 4, line 119: why do the NBD1 puncta accumulate in the perinuclear space rather than elsewhere in the cytoplasm?
9. Fig 3E. The authors claim that miRNA binding to the mRNA does not bind the total protein production, however, Fig 3E shows a significant decrease in the NBD1 production upon treatment with miRNA inhibitors. The authors should clarify this contradiction.
10. Fig 4B and C: only one miRNA (covering the region around 1675) covers a similar region with cleaving and non-cleaving miRNA. The authors state that this has been designed this way to avoid suppressing the endogenous mRNAs, but this is not convincing, because the presence of endogenous cleaving miRNA and non-cleaving shRNA, plus miRNA inhibitors creates a very complex situation that is difficult to interpret unambiguously.
11. Fig S4B: add the label on the Y-axis.
12. The quality of data presented in Fig S5 is not of high quality, were these gels reproduced?

Referee #2:

Sako et al. present a series of experiments aimed at proving the role of miRNA-mediated translation slow down. Based on the following observations, this manuscript as written is not suitable for publication in EMBO J.
Major comments:
• The study is based on the observation of a correlation between ribosome footprint density enrichment, from RiboSeq data, and miRNA binding site (Fig1). However, the procedure to produce these results is rather confusing, with the methods section describing several normalizations that are hard to make sense of in which order they were performed. They justify the use of two different normalization procedures based on the detrimental effects that low-expressed genes might have on data assignment. However, the work is done only with highly expressed genes (Materials and Methods). In the "weight of 1" procedure (normalization of read counts in position i of gene j by total read counts of gene j), one risks of inflating the averages by giving equal weights to very dissimilar ratios. For example, a position with 10 read counts normalized by a total of 50 counts (low coverage) will yield a similar signal to a position of 300 counts normalized by 1500 (better coverage), therefore contributing equally to the signal. The "binary count" procedure is rather unusual in the context of RiboSeq signal processing as it gets rid of all sequencing depth and produces a one-hot encoding binary vector for each sequence ORF. The consequence of this is that, as coverage increases, the vectors are dominated by ones and all signal would be ablated (i.e. a highly transcribed gene with full coverage will be described by a uniform distribution). I consider that rather than reducing noise in the RiboSeq signal, these normalizations are getting rid of sequence depth and signal. Methods preserving the sequencing depth, such as normalizing by

translation level (e.g. TPM) or by mean gene translation level, should better reflect the biology of the observations.
• The authors need to rule out that the correlation between ribosome density and miRNA binding site is not a mere artefact of mRNA abundance. A similar metagene analysis to the one performed for Fig 1A and S1D should be carried out using total RNAseq data.
• Despite the correlations shown in the first part of the manuscript, a major question has to be addressed: are the ribosome-bound mRNAs miRNA loaded? It would be useful to explore this from a stochiometric point of view, i.e. comparing miRNA levels vs mRNA levels. Furthermore, the proposed model makes sense only in the light of monosme-mediated translation. In polysomes, the proposed model demands a rebinding of the miRNA between ribosomes, otherwise there would be a heterogeneous mix of folded and misfolded proteins. How likely is it that a miRNA will be rebind between monosomes, especially if the gene is heavily translated and the miRNA is not very abundant?
• Results from Figure 3 and 4 are based on the 3B panel. Here the authors report the identification of regions of faster translation in the absence of DROSHA and therefore of the corresponding miRNAs. However, there are actually more positions with increased density (i.e. slower translation). Following the interpretation of the text, this would suggest that miRNA depletion also induces ribosome pausing. How was this region chosen and how are those opposite observations reconciled?
• In figure 5 the authors identify that proteins containing a signal peptide and translating domains are less prone to misfolding by slowing down translation via CHX and proteasome inhibition. They use this to design miRNA-like molecules to slow down insulin translation. Giving the striking abundance of signal peptides and TMDs, the results could be explained by the interference of miRNA and miRNA-like molecules with the ER export process. How is this ruled out?

Minor comments:
• Mouse ribosome profiling data was produced with a CHX pre-treatment (Methods). This sort of treatment is known to bias the ribosome signal, as translation is inhibited and an uncertain number of rounds of elongation (~1) still happen after CHX binding. How does this affect the observed correlations in mouse vs human cells?
• Abstract needs reorganization, it is difficult to parse.
• Rationale for the study -line 56- is not well explained.
• Need to reference - line 53.
• Need to reference - line 69.
• Fig S1A is not intuitive.
• Figures S2G,H,I are missing.
• Supplement needs better organization, split multi-page figures if needed.

Referee #3:

This manuscript proposes a provocative role for microRNAs that bind mRNAs along their coding region. The authors propose, and provide some evidence, that miRNA binding in the coding region slows translating ribosomes and this is linked to more efficient protein folding. They test this idea using primarily a reporter protein consisting of a fusion with an aggregation prone domain of CFTR and later also looking at insulin.

The authors use two main assays to test their hypothesis. First, using ribosome profiling, they aim to detect slowing of ribosomes in specific regions of the mRNA, in relation to miRNA binding sites or their manipulation. Second, the authors assess the effect of manipulating miRNA binding sites on protein folding by quantifying the fractions of soluble and insoluble protein.

Before being able to assess the results in full, I need more information regarding the ribosome profiling experiments. Most importantly, how many replicates were performed and how the data are quantified to say that a specific signal is increased or decreased (as claimed in figures 3B and 4B, C). The authors spend quite some time explaining noise reduction methods but do not comment at all on the variability of the assay that makes this necessary, and the associated point of how many replicates are required to distill reproducible signals (which specific miRNA binding sites should produce). Without these data, the only assay the authors have is the measurement of protein solubility, which could in principle report on other effects of the different manipulations. In summary, I would like to see data from individual replicates and some form of quantification.

Related to what the quantitative signals in this assay mean, in figure 3B, it is surprising that there is still "slowing" of ribosomes even in the Drosha knock-out condition. This could be due to one of two things: i) technical: there are still miRNAs left (the authors should check whether the relevant miRNAs are still present under the precise experimental conditions used for this assay), or ii) biological: there is natural slowing in those regions even in the absence of miRNAs. The latter would make it more difficult to assess the role of miRNAs in slowing ribosomes, as it really would require very precise quantification to separate the effect.

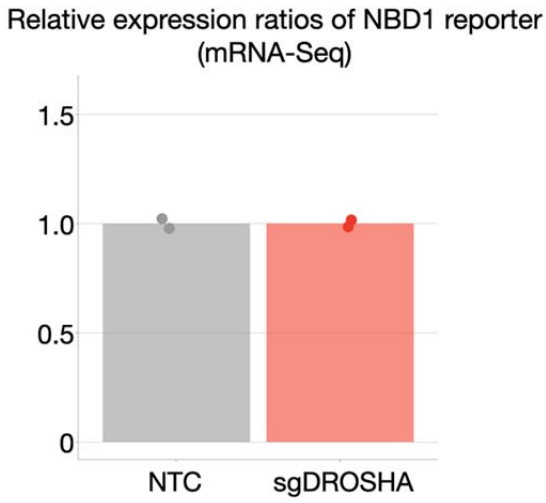
Another point that requires clarification is whether the total amount of expressed reporter protein is comparable across experiments. From the western blots in different figures it looks like the amount of reporter relative to tubulin varies quite a bit. I presume that aggregation of this reporter is likely concentration dependent? If the concentration across experiments is indeed variable, the authors should titrate the amount of reporter and assess whether indeed there is a concentration dependent effect on aggregation. This should be then taken into account in the interpretation of the other experiments.

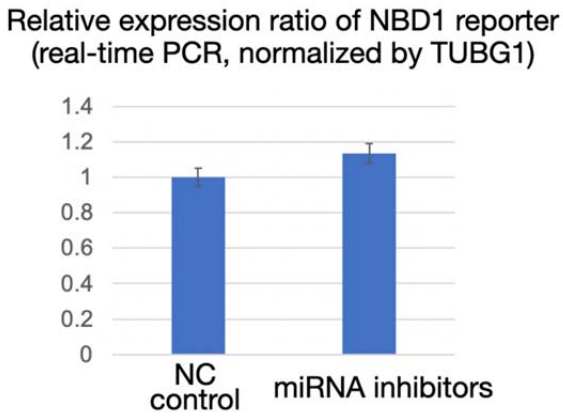
Minor points:
1. There is a typo in figure 1A, "nosie" instead of "noise"
2. In figure S2, panels G and I are missing?
3. In figure 3C, the axis that denotes miRNA abundance states log2, but it is unclear of what, is it RPM or some other measure?
4. In the insulin experiments at the end, is there more insulin?

We deeply appreciate the constructive comments from all the referees.

Referee #1 (Report for Author)
Major points:
1. My main concern is that the authors do not provide a clear quantification of the miRNA effect on the mRNA stability (except for Fig S4B, which presents a special case of non-cleaving shRNAs). Given that the speed of translation is supposed to be an important factor governing mRNA stability, this would be an essential control.

As shown below, we have the data on the effect of global miRNA loss by DROSHA knockout on NBD1 reporter mRNA, indicating that the global reduction of miRNAs has little effect on NBD1 reporter mRNA levels. As for the specific miRNA inhibitions, we carried out real-time PCR analysis (N = 3) of NC control and miRNA inhibitors used in Figure 3, indicating the little effect of miRNA inhibitions on NBD1 reporter mRNA levels.



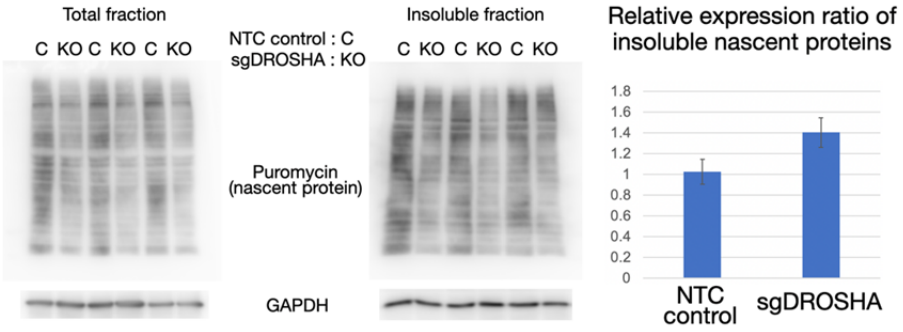


2. Concerning the misfolded proteins accumulating in puncta and in the aggregates: are they full-length or fragments resulting from translation of truncated mRNAs?

We assume that they are full-length. To produce truncated mRNAs, no-go decay (NGD) triggered by severe ribosomal stalls is required, leading to the degradation of the target mRNA. However, we have not observed the decreased reporter mRNA levels, as shown above, suggesting that NGD is not activated in the context and therefore the production of truncated reporter mRNA is unlikely.

3. The model suggested by the authors predicts that in the absence of miRNAs, a sizable fraction of nascent proteins will aggregate. This should be checked at normal growth conditions, i.e., without CHX treatment, as according to their model mild CHX treatment should reduce misfolding.

We quantified puromycin-incorporated nascent proteins in the insoluble (aggregated) fraction. NTC control or sgDROSHA cells were treated with 2ug/ml puromycin and 20uM MG132 for 3 hr before harvest. Total and insoluble protein fractions were prepared as described in the text.
Nascent protein (anti-puromycin) and GAPDH (loading control) were quantified by western blotting. Nascent protein was normalized by GAPDH in both protein fractions, followed by the normalization of insoluble nascent protein by the total fraction.



Although increased tendency was seen in the sgDROSHA, there was no statistical difference between NTC control and sgDROSHA. This could be because of that the relatively small proportion (~ 30 %, see Ref. 44 in the text) of nascent proteins undergo co-translational misfolding, and not all proteins can gain benefit from slow translation (see figure 6). We assume that this limitation made it difficult to see the effect of miRNA loss on the misfolding of the global nascent proteins.

4. Conceptually, it remains unclear how miRNAs should regulate folding in the sense that the amount of correctly folded protein is changed at certain conditions, for example by switching the miRNA production, which would improve the yield of soluble protein. Are these miRNAs expressed constitutively or are regulated? This should be discussed in more details.

We suppose that highly and constitutively expressed miRNAs are responsible for the miRNA-mediated slowing of ribosomes. Our data (Fig 3, Fig S3C) suggests that multiple miRNAs targeting a similar mRNA region are required for miRNA-mediated protein folding. Given that many miRNAs (39 miRNAs) target a similar mRNA region (Fig 3C), even if one miRNA expression decreases by external stimuli, another miRNA may compensate for the loss of the miRNA.

Specific comments:
1. The Abstract does not present the result in a logical way, the sentences should be shifted to achieve a smooth flow.

We corrected it.

2. In the Introduction, the authors discuss the effect of translation kinetics in a

somewhat oversimplified way, namely that slowing down translation leads to a better protein folding. This is by far not always the case, as correctly mentioned in the Discussion (refs 33 and others), but this information comes too late. The Introduction should be revised to provide a more complete picture, namely that efficient cotranslational folding requires a well-tune translation rate that has been optimized through evolution.

We added the information in the introduction.

3. In many cases, statistical significance (the number of replicates, technical and biological replicates, etc) is not explained. This should be added to each Fig where relevant.

We added these in the figure legends.

4. Based on data in Fig 1, the authors should estimate the kinetic effect of miRNAs on translation.

We experimentally evaluated the effects of miRNAs (non-cleaving shRNAs) on translation as shown in Figure 5 (newly added results).

5. In all Figs, the curves showing normalized ribosome density at different conditions all have a very similar color, it is almost impossible to distinguish the results with, e.g., different miRNAs. The colors should be changed to allow a clear distinction.

We adjusted the colors which can be distinguished by people with deuteranopia, protanopia, and tritanopia.

6. P. 4, lines 99-101, the text is somewhat confusing, because the description of the translational slowdown at the 0-15 region is missing. The authors mention accumulation of ribosomes at codon 0 and the reduction at +20 to +25, but not the crucial region in between.

We made it clear to explain the results.

7. P. 4, lines 114-115. Please describe the rationale for using EGFP, SBP, P2A and mCherry in the reporter. While this may be obvious to the specialists in the field, the advantages of such reporter will not be immediately clear to the broader readership.

We added the explanation for this point as below.

This NBD1 domain of CFTR is highly prone to misfolding during translation (2). When NBD1 is misfolded and aggregated, the aggregation can be observed as EGFP puncta. mCherry is expressed in a misfolding-independent manner and can be used as an internal control.

8. P. 4, line 119: why do the NBD1 puncta accumulate in the perinuclear space rather than elsewhere in the cytoplasm?

As shown in the text,

Misfolded proteins are transported to distinct subcellular compartments for processing, with aggregates found in the perinuclear region being degraded by proteasomes (25)

9. Fig 3E. The authors claim that miRNA binding to the mRNA does not bind the total protein production, however, Fig 3E shows a significant decrease in the NBD1 production upon treatment with miRNA inhibitors. The authors should clarify this contradiction.

Figure 3E agrees with our model. We assume that total NBD1 can be stabilized due to the miRNA-mediated correct folding of the nascent proteins. The loss of such miRNAs (miRNA inhibitors) can decrease the level of total NBD1 as shown in Figure 3E.

10. Fig 4B and C: only one miRNA (covering the region around 1675) covers a similar region with cleaving and non-cleaving miRNA. The authors state that this has been designed this way to avoid suppressing the endogenous mRNAs, but this is not convincing, because the presence of endogenous cleaving miRNA and non-cleaving shRNA, plus miRNA inhibitors creates a very complex situation that is difficult to interpret unambiguously.

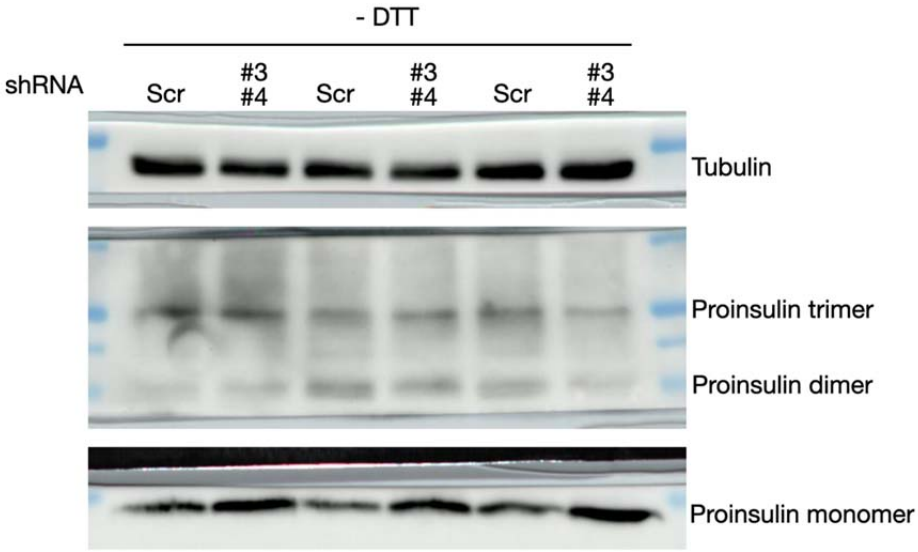
We would like to clarify this point. Non-cleaving shRNA and miRNA inhibitor were not present at the same time. The binding positions of non-cleaving shRNA were designed to minimize the overlap with the binding regions of endogenous miRNAs.

11. Fig S4B: add the label on the Y-axis.

We added the label, “Fold change”.

12. The quality of data presented in Fig S5 is not of high quality, were these gels reproduced?

Yes, they were reproduced. One example is shown below which is one of the other trials of Fig S5C.



Referee #2 (Report for Author)
Major comments:
• The study is based on the observation of a correlation between ribosome footprint density enrichment, from RiboSeq data, and miRNA binding site (Fig1). However, the procedure to produce these results is rather confusing, with the methods section describing several normalizations that are hard to make sense of in which order they were performed. They justify the use of two different normalization procedures based on the detrimental effects that low-expressed genes might have on data assignment. However, the work is done only with highly expressed genes (Materials and Methods). In the "weight of 1" procedure (normalization of read counts in position i of gene j by total read counts of gene j), one risks of inflating the averages by giving equal weights to very dissimilar ratios. For example, a position with 10 read counts normalized by a total of 50 counts (low coverage) will yield a similar signal to a position of 300 counts normalized by 1500 (better coverage), therefore contributing equally to the signal. The "binary count" procedure is rather unusual in the context of RiboSeq signal processing as it gets rid of all sequencing depth and produces a one-hot encoding binary vector for

each sequence ORF. The consequence of this is that, as coverage increases, the vectors are dominated by ones and all signal would be ablated (i.e. a highly transcribed gene with full coverage will be described by a uniform distribution). I consider that rather than reducing noise in the RiboSeq signal, these normalizations are getting rid of sequence depth and signal. Methods preserving the sequencing depth, such as normalizing by translation level (e.g. TPM) or by mean gene translation level, should better reflect the biology of the observations.

The “weight of 1” method is designed to reveal small effects on ribosome speed which are often masked by larger effects, such as highly structured RNA. The basic concept of the weight of 1 method was also described to call a ribosomal stall (Romika K,… Pavel Baranov, 2018 RNA). Moreover, the robustness of the “weight of 1” method was validated in Fig 1B and Fig S1B. In the experiments, due to the arginine depletion, ribosomes are supposed to stall at arginine codons. However, without noise reduction, the effect of arginine starvation was not distinguishable from other ribosomal peaks (masked by other larger effects). But the “weight of 1” method successfully captured the effect of arginine starvation even with the time-dependent effect. This is not achieved by TPM or similar normalization methods.
We stopped using binary count method because of the “unusual” approach. We focused on the “weight of 1” method and analyzed Ribo-Seq using all the genes detected in mouse skeletal muscle (Figure 1C).

• The authors need to rule out that the correlation between ribosome density and miRNA binding site is not a mere artefact of mRNA abundance. A similar metagene analysis to the one performed for Fig 1A and S1D should be carried out using total RNAseq data.

The ribosome density plots were already normalized by mRNA-seq data (Fig 1C). Therefore, an artifact of mRNA abundance is unlikely.

• Despite the correlations shown in the first part of the manuscript, a major question has to be addressed: are the ribosome-bound mRNAs miRNA loaded? It would be useful to explore this from a stochiometric point of view, i.e. comparing miRNA levels vs mRNA levels. Furthermore, the proposed model makes sense only in the light of monosme-mediated translation. In polysomes, the proposed model demands a rebinding of the miRNA between ribosomes, otherwise there would be a heterogeneous mix of folded and misfolded proteins. How likely is it that a miRNA will be rebind between monosomes, especially if the gene is heavily translated and the miRNA is not very abundant?

It is highly likely that miRNAs rebind between monosomes. Given that: i) miRNAs find target binding sites by sliding over mRNA ~70 nt within 0.1 sec (Chandradoss S,… Chirlmin Joo, 2015 Cell), and ii) there is 10 sec on average for a ribosome to collide with

a leading ribosome (see the discussion, line 253 - 262), there is sufficient space and time for miRNAs to rebind between monosomes.

• Results from Figure 3 and 4 are based on the 3B panel. Here the authors report the identification of regions of faster translation in the absence of DROSHA and therefore of the corresponding miRNAs. However, there are actually more positions with increased density (i.e. slower translation). Following the interpretation of the text, this would suggest that miRNA depletion also induces ribosome pausing. How was this region chosen and how are those opposite observations reconciled?

If a ribosome stalls for a while, downstream of the stall site will have no ribosome, resulting in lowered ribosome density. Similarly, if a stalled ribosome collides a trailing ribosome, another peak can be found upstream of the stalled one. In addition, ribosome density within a stalled ribosome region (~ 30 nt) can be low (i.e., ~ 30 nt upstream region of a ribosomal peak signal).
Therefore, in Fig 3B panel, ribosome density was low (NTC sample from 1630 to 1650 and from 1660 to 1673 nt) due to the upstream stall sites (NTC sample at ~1625 and ~1655 nt) compared with sgDROSHA, which makes ribosomes seemingly slower in sgDROSHA in the regions.

• In figure 5 the authors identify that proteins containing a signal peptide and translating domains are less prone to misfolding by slowing down translation via CHX and proteasome inhibition. They use this to design miRNA-like molecules to slow down insulin translation. Giving the striking abundance of signal peptides and TMDs, the results could be explained by the interference of miRNA and miRNA-like molecules with the ER export process. How is this ruled out?

We have confirmed the specificity of non-cleaving shRNA targeting mouse insulin. If there were interference between miRNAs and non-cleaving shRNAs and non-cleaving shRNA inhibited endogenous miRNAs targeting insulin, all the proinsulin monomer, dimer, and trimer expressions would be increased. However, we have not observed such increases. Therefore, we can rule out such interference between miRNAs and non-cleaving shRNAs.

Minor comments:
• Mouse ribosome profiling data was produced with a CHX pre-treatment (Methods). This sort of treatment is known to bias the ribosome signal, as translation is inhibited and an uncertain number of rounds of elongation (~1) still happen after CHX binding. How does this affect the observed correlations in mouse vs human cells?

To address the bias of CHX, we used a human dataset without CHX pre-treatment and reached a similar outcome compared with the one with CHX pre-treatment (Fig 1C and S1H). Importantly, a recent paper discussing such bias concluded CHX pre-treatment

had no effect on ribosome occupancy (Sharma P, … Sebastian Leidel, 2021 Nat. Commun.).

• Abstract needs reorganization, it is difficult to parse.

We corrected it.

• Rationale for the study -line 56- is not well explained.

We added as below.

The inhibitory activity is lost if the miRNA binding sites are moved from the 3´ UTR to the CDS (20), suggesting that ribosomes can detach CDS-targeting miRNA before it induces an inhibitory effect.

• Need to reference - line 53.

We added the references.

• Need to reference - line 69.

We added the references.

• Fig S1A is not intuitive.

We deleted it.

• Figures S2G,H,I are missing.

We corrected it.

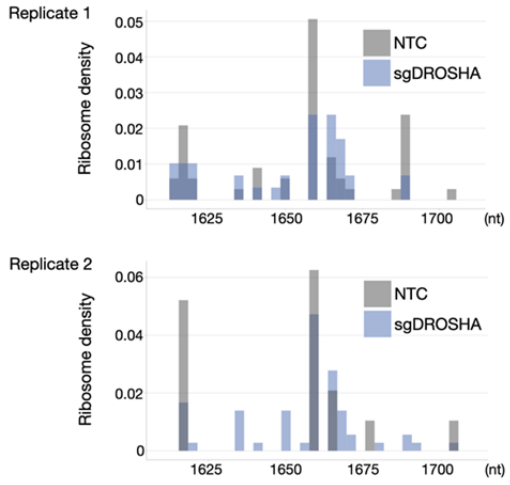
• Supplement needs better organization, split multi-page figures if needed.

We re-organized it.

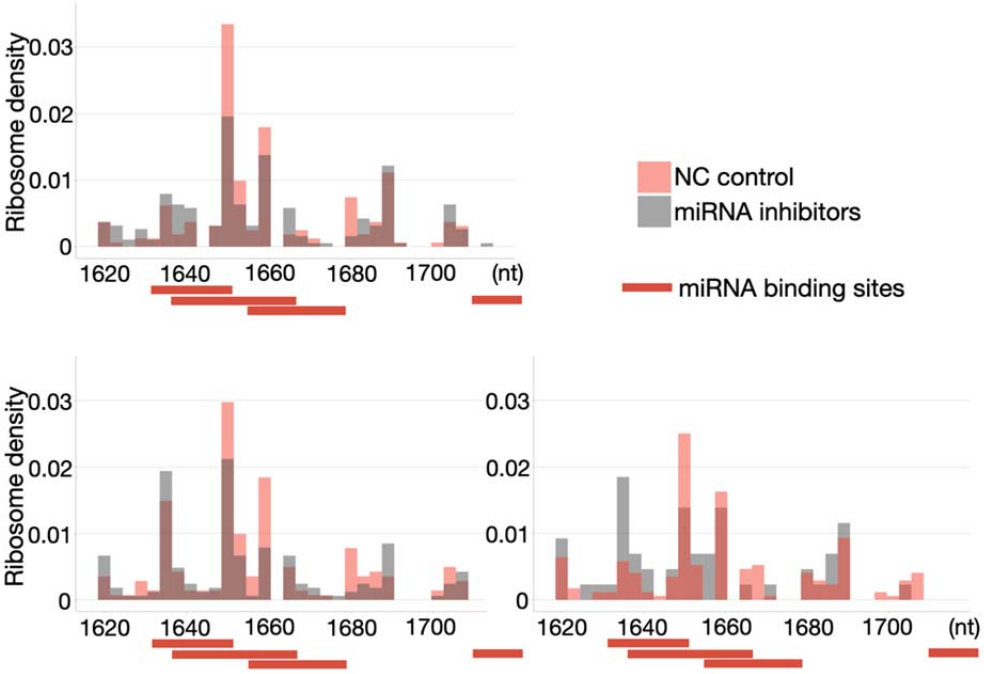
Referee #3 (Report for Author)

Before being able to assess the results in full, I need more information regarding the ribosome profiling experiments. Most importantly, how many replicates were performed and how the data are quantified to say that a specific signal is increased or decreased (as claimed in figures 3B and 4B, C). The authors spend quite some time explaining noise reduction methods but do not comment at all on the variability of the assay that makes this necessary, and the associated point of how many replicates are required to distill reproducible signals (which specific miRNA binding sites should produce). Without these data, the only assay the authors have is the measurement of protein solubility, which could in principle report on other effects of the different manipulations. In summary, I would like to see data from individual replicates and some form of quantification.

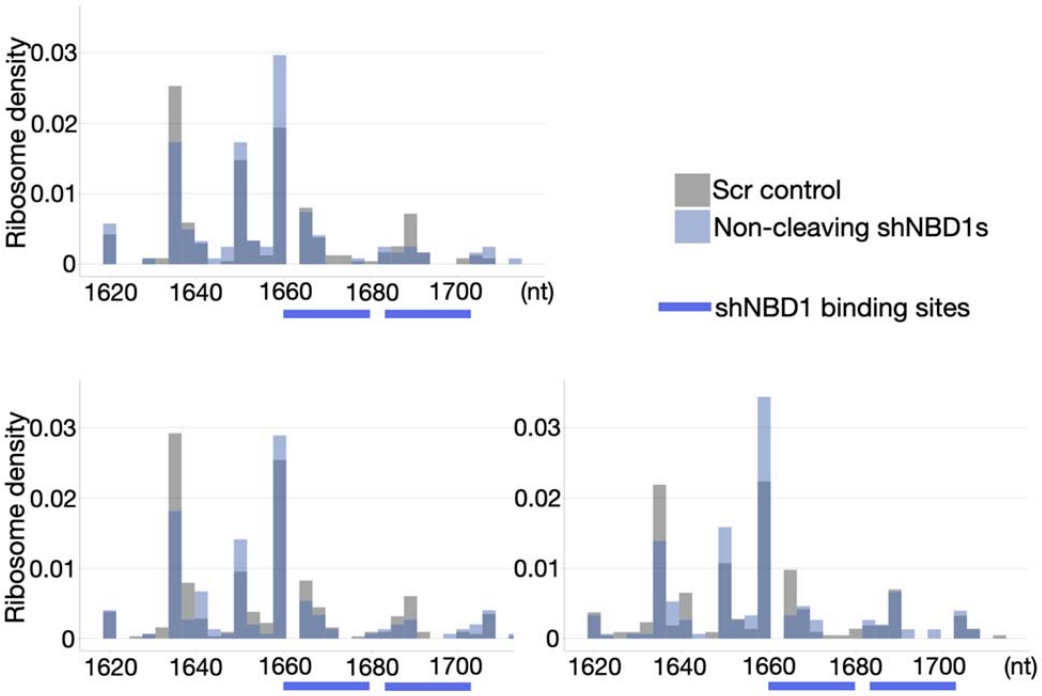
We selected the target region for the downstream analyses (miRNA inhibitions and shRNA). Briefly, the ribosome signals on the NBD1 domain of CFTR were manually screened for the region showing a higher peak in NTC compared with sgDROSHA and the downstream showed “dip” in NTC compared with sgDROSHA, reflecting lowered ribosome density derived from the upstream ribosomal stall.
Here are the replicates for Fig 3B (n = 2).



Here are the replicates for Fig 4B (n = 3).



Here are the replicates for Fig 4C (n = 3).



To further examine the effect of miRNA (or non-cleaving shRNA), we designed another experiment (Figure 5 was newly added), quantitatively showing that non-cleaving shRNAs can slow translation.

Related to what the quantitative signals in this assay mean, in figure 3B, it is surprising that there is still "slowing" of ribosomes even in the Drosha knock-out condition. This could be due to one of two things: i) technical: there are still miRNAs left (the authors should check whether the relevant miRNAs are still present under the precise experimental conditions used for this assay), or ii) biological: there is natural slowing in those regions even in the absence of miRNAs. The latter would make it more difficult to assess the role of miRNAs in slowing ribosomes, as it really would require very precise quantification to separate the effect.

As referee #2 pointed out in the 4th major comment, if a ribosome stalls for a while, downstream of the stall site will have no ribosome, resulting in lowered ribosome density. We used this phenomenon to screen the ribosomal peaks in the NBD1 domain to select the target regions for the downstream analyses (miRNA inhibitions and shRNA experiments).

Another point that requires clarification is whether the total amount of expressed reporter protein is comparable across experiments. From the western blots in different figures it looks like the amount of reporter relative to tubulin varies quite a bit. I presume that aggregation of this reporter is likely concentration dependent? If the concentration across experiments is indeed variable, the authors should titrate the amount of reporter and assess whether indeed there is a concentration dependent effect on aggregation. This should be then taken into account in the interpretation of the other experiments.

To keep the NBD1 reporter expression constant, we used a clonal line stably expressing the NBD1 reporter. If the observed aggregation were concentration-dependent, the total NBD1 and insoluble NBD1 levels should have been correlated in Fig 2C, D, 3D, E, 4D, E, and F. However, our data showed an inverse correlation, indicating that the observed NBD1 aggregation was concentration independent.

Minor points:
1. There is a typo in figure 1A, "nosie" instead of "noise"

We corrected it.

2. In figure S2, panels G and I are missing?

We corrected it.

3. In figure 3C, the axis that denotes miRNA abundance states log2, but it is unclear of what, is it RPM or some other measure?

Yes, it is RPM. We added it.

4. In the insulin experiments at the end, is there more insulin?
We couldn't detect mature insulin for quantification.

Dear Hiroaki,

Congratulations on an excellent manuscript, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal. Thank you for your comprehensive response to the referee concerns and for providing detailed source data. It has been a pleasure to work with you to get this to the acceptance stage.

I will begin the final checks on your manuscript before submitting to the publisher next week. Once at the publisher, it will take about 3 weeks for your manuscript to be published online. As a reminder, the entire review process, including referee concerns and your point by point response, will be available to readers.

I will be in touch throughout the final editorial process until publication. In the meantime, I hope you find time to celebrate!

Kind regards,
Kelly

Kelly M Anderson, PhD
Scientific Editor
The EMBO Journal
k.anderson@embojournal.org

Referee #1:

The authors have answered my concerns in a satisfactory way.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Your manuscript will be processed for publication in the journal by EMBO Press. Manuscripts in the PDF and electronic editions of The EMBO Journal will be copy edited, and you will be provided with page proofs prior to publication. Please note that supplementary information is not included in the proofs.

You will be contacted by Wiley Author Services to complete licensing and payment information. The required 'Page Charges Authorization Form' is available here: https://www.embopress.org/pb-assets/embo-site/tej_apc.pdf - please download and complete the form and return to embopressproduction@wiley.com

EMBO Press participates in many Publish and Read agreements that allow authors to publish Open Access with reduced/no publication charges. Check your eligibility: https://authorservices.wiley.com/author-resources/Journal-Authors/open-access/affiliation-policies-payments/index.html

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

EMBO Press Author Checklist

Corresponding Author Name: Hiroaki Sako, Tadashi Yamamoto
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2022-112469R-Q

USEFUL LINKS FOR COMPLETING THIS FORM

The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

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?	Yes	Material and Methods

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	Material 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, Table

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.	Yes	Material and Methods
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.	Yes	Material and Methods

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 Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
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?	Yes	Acknowledgments section

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Material and Methods
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	Material and Methods
Include a statement about **blinding** even if no blinding was done.	Yes	Material 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.	Yes	Material and Methods
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	Material 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	Material and Methods
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Material and Methods

Ethics

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	Material and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

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)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Yes	Material and Methods
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	Availability of data and materials section
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**.	Yes	Material and Methods, reference sections