EMBO reports

DDX3X and Specific Initiation Factors Modulate *FMR1* Repeat-Associated Non-AUG Initiated Translation

Alexander E. Linsalata, Fang He, Ph.D. Ahmed M. Malik, M. Rebecca Glineburg, Ph.D., Katelyn M. Green, Sam Natla, Brittany N. Flores, Ph.D., Amy Krans, MS, Hilary C. Archbold, Ph.D., Stephen J. Fedak, Sami J. Barmada, M.D., Ph.D., Peter K. Todd, M.D., Ph.D.

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Thank you for the transfer of your manuscript with the corresponding referee reports from The EMBO Journal to EMBO reports. Thank you also for providing a point-by-point response to the referee concerns.

As my colleague Anne Nielsen from The EMBO Journal indicated, we are interested in considering a manuscript that has been revised along the lines requested by the referees who evaluated your study for The EMBO Journal. With regards to the data you proposed to add, it will be sufficient to concentrate on the technical and validation aspects and it will not be essential to expand the screen for more factors. It is sufficient to include the explanation given in your response (i.e., that these were left out since they are expected to be lethal) in the manuscript itself. Moreover, it will not be essential to include a SUNSET assay to measure global translation but please include a few additional reporter constructs to broaden and substantiate the conclusion that is currently based on only one RAN-dependent and one RAN-independent reporter.

Taken together, please revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Your manuscript will be sent back to the same referees and acceptance will depend on a positive outcome of this second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied. Please also include scale bars in all microscopy images.

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When submitting your revised manuscript, we will require:

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- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
(In order to avoid delays later in the publication process please check our figure guidelines before preparing the figures for your manuscript:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf)
- a separate PDF file of any Supplementary information (in its final format)
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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The paper by Linsalata et al. from the Todd lab presents interesting data supporting a novel role of the helicase DDX3X in the regulation of Repeat-Associated Non-AUG (RAN) Translation. RAN is a form of translation which was previously shown by the Todd and other labs to be important for neurodegenerative and neurodevelopmental disorders, such as fragile X Syndrome and Huntington's disease. The Linsalata et al. manuscript further elucidates this mechanism by identifying 5 proteins that regulate RAN translation in Drosophila: eIF1, eIF5, eIF4B, eIF4H and DDX3X. The authors proceed to use human cells and cultured neurons to provide evidence supporting their hypothesis that the identified candidate proteins regulate RNA secondary structure and start-codon fidelity during RNA translation. I believe the findings of this study are of great interest and perhaps can reveal new mechanistic avenues for therapeutics however there are some major points that require clarification:
1) The rationale for choosing the 47 genes in the initial screen is unclear. The Todd lab has previously shown that RAN translation uses cap-dependent mechanisms (Kearse et al. Mol Cell 2017), thus I would have expected to see genes such as eIF4E and eIF4G in the UAS-GAL4 screen. Without dismissing the importance of the positive hits in his study, I think the dataset examined is not complete and might introduce bias into the mechanistic investigation of RAN translation. The authors need to convincingly explain their rationale for this or add more candidates in their screen. Moreover, Fig. 1 needs to include more information and not just the positive hits. While the study focuses on suppressors, in the results text it is mentioned that there 22 enhancers and of those 11 are specific to CGG repeats. This information needs to be accurately sumamrised in this Figure or an accompanying table.
2) RAN translation is 30-40% as efficient as canonical AUG translation (Kearse et al.). In FXS an increase in global translation was shown in several models and in patient cells by numerous labs. In Fig. 2 the authors conclude that global translation is not affected using GFP as a readout. The authors need to show an independent test for global translation (e.g. 35-S-Met incorporation) to clearly demonstrate that DDX3X knockdown only affects RAN translation. The same applies to other models used (cells, primary neurons).
3) The mechanistic investigation in this paper is lacking evidence downstream of DDX3X. Which mRNAs are preferentially translated during RAN? This would shed more light into this regulatory mechanism, but also confirm the findings obtained with reporter assays (Fig. 4).

Referee #2:

This study employs a genetic screen based on fly eye color of various initiation factors or helicases for ones that would inhibit RAN translation mediated by the repeated elements in the FMR 5'UTR of the FXTAS disease-causing allele. They identified the helicase Ddx3, eIF4B, eIF4H, and eIF1 as factors that are required for RAN translation, reducing the eye phenotype and, for Ddx3 and eIF4B at least, also mitigating the reductions in fly viability conferred by expression of the FXTAS allele. They go on to show that Ddx3 shRNA-knockdown in cells reduces expression of FXTAS reporter genes to a greater extent than other reporters, including one with the FMR 5'UTR but lacking repeated elements, suggesting a preferential effect on RAN translation. By contrast, knockdown of eIF4B or eIF4H did not have this preferential effect, reducing both reporters comparably. They show that overexpressing eIF1 reduces expression of the RAN reporter, provided it lacks an AUG codon, while overexpressing eIF5 has the opposite effect, in agreement with their effects on initiation at near-cognate start codons. They finish by showing evidence that knockdown of Ddx3 mitigates toxicity of the FXTAS construct in primary rodent neurons.

This paper is significant in using a creative genetic screen in Drosophila to identify Ddx3 as a factor that appears to be preferentially required for RAN translation both in flies and mammalian neurons. There are however several issues that need to be addressed.

Major comments:

The results on eIF1 presented are puzzling, as eIF1 was identified in the genetic screen as a factor presumably required for RAN translation, as its knockdown suppressed expression of the FXTAS

allele in the fly eye. However, they show in Fig. 6A that eIF1 overexpression suppresses expression of the FXTAS reporter. The latter results fit with expectations about eIF1 overexpression suppressing non-AUG initiation, which applies to RAN translation. Hence, it's difficult to understand how eIF1 knockdown suppressed expression of the FXTAS construct in fly eyes.

-At odds with their statement on p.7, the #2 siRNA against DDX3 reduced expression of the FMR AUG-NL reporter and was not specific for the FXTAS reporter in the results shown in Fig. 3A. This undermines the key claim that knockdown of Ddx3 preferentially impairs RAN translation.

Other comments:

-they didn't cite panels D-E of Fig.3.

-Regarding the statement on p. 8, "Similarly, insertion of an AUG codon in a strong Kozak context above the NRE in the GCG (+2) frame enhanced basal expression, but expression of this AUG-(CGG)100 (+2) NL-3xF reporter remained DDX3X-dependent." it's unclear what "above" means. They should include a schematic of the different RAN reporter constructs showing where the start codons in the +1 or +2 frames are located relative to the NREs, and how these constructs differ exactly from the control FMR AUG-NL reporter.

-The statement on p. 8 "DDX3X knockdown reduced their expression more than AUG-NL-3xF but less than (CGG)100 (+1) NL-3xF (Fig. 4F)" requires statistical analysis to be justified.

-On p.10, in "In total, these experiments demonstrate that manipulations that influence start-codon fidelity can bidirectionally regulate RAN translation at CGG repeats." the word bidirectionality is a poor choice.

-p. 10; they should verify that eIF1 and eIF5 are actually overexpressed considering that they each autoregulate their own expression.

-p. 10-11 and Fig.7: What is the logic behind using an EGFP reporter in which the AUG start codon has been replaced with a GGG codon (GGG-EGFP) as the negative control, as presumably there will be no EGFP expression at all from this construct. It would seem that the proper control would be a construct in which EGFP is expressed in the absence of the NRE-encoded polypeptide produced by RAN translation.

-p.12: the references (Kozel et al., 2016; Tang et al., 2017) are missing. Also, the Loughran et al paper PMCID: 3326321 is the appropriate one to cite here; and the Ivanov et al paper for eIF1.

Referee #3:

The manuscript by Linsalata et al. reports results from a candidate Drosophila screen for modifiers of RAN induced toxicity and highlights the identification of DDX3X, a helicase that reduces expression of RAN proteins in Drosophila and mammalian cells as a modifier of repeat toxicity and/or RAN translation. While the identification of modifiers of RAN translation is of high importance and the manuscript is potentially interesting, the data are not clearly presented and in some cases are overinterpreted. Overall a major focus of the paper is on the DDX3X RNA helicase gene as a modifier of RAN induced toxicity but the unclear nature of these effects, including that similar effects are found in mammalian cells expressing constructs that do not contain repeat expansions make the likelihood that the authors have identified a robust modifier of RAN translation unclear. Additional control experiment and data are needed to support the main conclusions on DDX3X and the other modifiers described in the manuscript.

The numbers of modifiers identified are not consistent throughout the paper:
Abstract says they identified five modifiers but figure 1 which summarizes the modifiers is inconsistent with the abstract. EIF5 is listed in the abstract but is colored grey (not clear what this means) as a modifier and RPS25, SF2, BGCN, eIF1 eIF2alpha are colored as modifiers in the figure but not mentioned in the abstract.

Some further explanation for what the various grey shapes on the ribosome represent is needed so the reader will know what they are looking at in figure 1.

A better summary of the data generated from the screens is needed. The manuscript says "47 candidate lines" were tested but the table shown in EV1A lists 64 lines with 12 acting as suppressors and 22 and enhancers of CGG toxicity but this is not well summarized in either the cited Figure 1 or Table EV1B. The authors should revise the paper to include a table with a complete summary of the effects of all of the fly lines that were tested including whether or not they had effects with and/or without the repeats.

All of the enhancers were checked as modifiers of flies expressing GMR-GAL4 as a control to screen for enhancers that require the repeat. The same screen should have been done for all of the suppressors.

It looks like 3 bel mutants not five as indicated in the text suppress the rough eye phenotype. A better description of these mutants is needed. Are they null mutants? What is being tested - loss of function/dominant negative effects? Are the flies heterozygous or homozygous for these mutations. The authors should better explain this experiment and why the effects of these mutations are not as striking as the shRNA results.

Why does shRNA suppression of Bel with shRNA 1 and shRNA 3, which showed similar reductions in FMR-polyGly have such different effects on survival in Fig 2C. Also the additional survival data shown in panel D for shRNA 3 do not look very different than controls - is the p value, which is shown as $p<0.0001$ correct?

The RNA transfection experiments provide only indirect evidence that DDX3X does not act on transcription or nuclear export. These comments should be removed from the results section or additional experiments supporting these claims should be added.

The repeat length independent effects of DDX3X from RNAs expressed in cells with plasmids but repeat dependent effects when RNA transfections are done is puzzling and additional data are needed to support this statement and if true to understand the molecular basis for these differences. Do the RNAs expressed from plasmids have a different localization patterns than the transfected RNAs in cells?

The paragraph on page 8 beginning with "One of the unique features..." and related data describing the effects of ATG and close cognate codons and DDX3X effects is confusing and should be written more clearly. Why do the authors claim that the transfections done with in vitro transcribed RNAs in EV3D and E are repeat length dependent without doing any statistical comparisons between the different repeat groups? Is the modest trend toward greater inhibition with longer repeats actually significantly different than the inhibition with shorter repeats? It seems the right comparisons were not performed to support these statements.

The precise flanking sequence can influence RAN translation and may also be important for understanding the DDX3X effects but the specific flanking sequences in the various plasmids used throughout the paper are not well described. A table should be added with all of the constructs used so that the reader can see exactly what has changed between the various plasmids.

The authors should comment on why they used only the +1 AUG like reporter construct for the in vitro translation experiments. Since the +1 frame has the AUG like sequence it would be interesting to compare the effects on the +2 reading frame.

Figure 4 RIP binding. Additional experiments should be performed to clarify if the binding site of DDX3X requires the repeat expansion or if it can bind to the upstream FMR RNA sequence that is included in repeat containing NL reporter. This is especially important because DDX3X does not require any CGG repeats to suppress NL reporter expression (as shown in Figure EV3B).

Figure 4F. The authors state that DDX3X reduces the NL reporter levels from the repeat containing plasmids more than from the close-cognate non-repeat containing transcripts but the authors show no statistical support for this statement by directly comparing these data sets to show they are

significantly different from each other.

Data summarized in EV3 panel B and elsewhere throughout the manuscript should be averaged and not selected from one of the three replicates or two of four replicates.

Panels with two replicates should be repeated so that there are three replicates. Figure EV3G

Minor:
Figure 3C - move legend for what colors mean to Figure 3C and indicate in figure legend that this key applies to both Figure 3C and 3D.

Page 6. The authors should just state that their knockdown of bel had no effect on steady state levels of CGG90-EGFP transcripts and not extend this to suggestions that it addresses transcription and turnover which this experiment does not.

Figure 3 labeling is confusing. The top and bottom parts of panel A should be placed side by side or given separate labels.

Page 7 the use of the phrase "mRNA copy number" is confusing and typically refers to DNA. This should be changed to "mRNA levels".

1st Revision - authors' response 15 March 2019

Specific Responses to reviewers

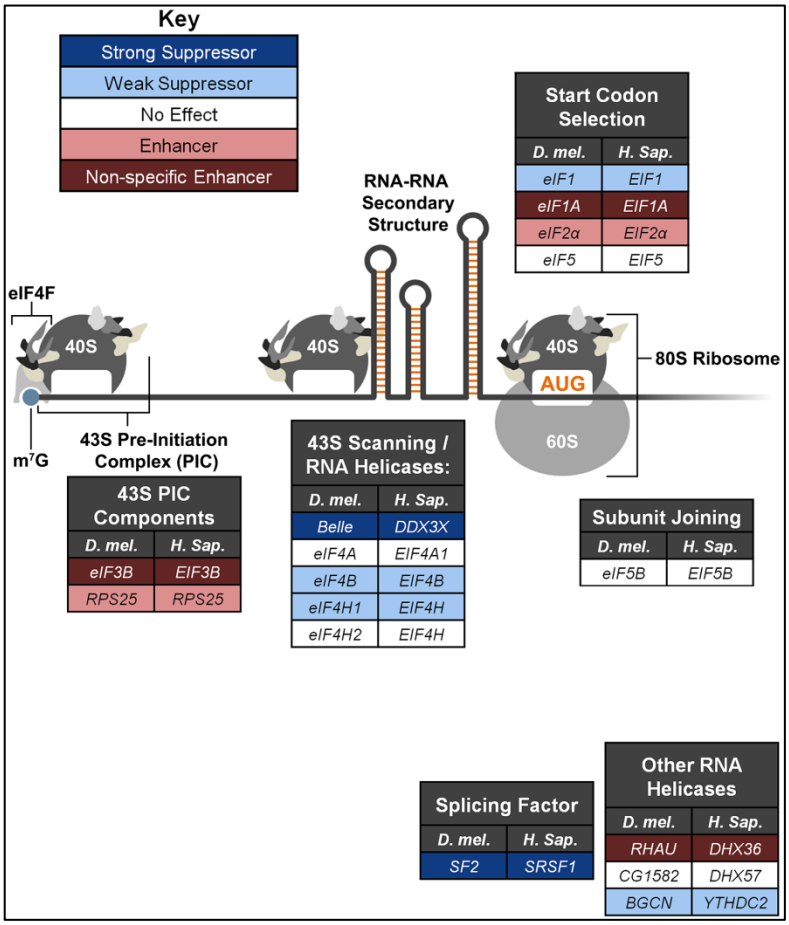
Ref #1:
The paper by Linsalata et al. from the Todd lab presents interesting data supporting a novel role of the helicase DDX3X in the regulation of Repeat-Associated Non-AUG (RAN) Translation. RAN is a form of translation which was previously shown by the Todd and other labs to be important for neurodegenerative and neurodevelopmental disorders, such as fragile X Syndrome and Huntington's disease. The Linsalata et al. manuscript further elucidates this mechanism by identifying 5 proteins that regulate RAN translation in Drosophila: eIF1, eIF5, eIF4B, eIF4H and DDX3X. The authors proceed to use human cells and cultured neurons to provide evidence supporting their hypothesis that the identified candidate proteins regulate RNA secondary structure and start-codon fidelity during RNA translation. I believe the findings of this study are of great interest and perhaps can reveal new mechanistic avenues for therapeutics however there are some major points that require clarification:

1) The rationale for choosing the 47 genes in the initial screen is unclear. The Todd lab has previously shown that RAN translation uses cap-dependent mechanisms (Kearse et al. Mol Cell 2017), thus I would have expected to see genes such as eIF4E and eIF4G in the UAS-GAL4 screen. Without dismissing the importance of the positive hits in his study, I think the dataset examined is not complete and might introduce bias into the mechanistic investigation of RAN translation. The authors need to convincingly explain their rationale for this or add more candidates in their screen. Moreover, Fig. 1 needs to include more information and not just the positive hits. While the study focuses on suppressors, in the results text it is mentioned that there 22 enhancers and of those 11 are specific to CGG repeats. This information needs to be accurately summarized in this Figure or an accompanying table.

RESPONSE:

This candidate screen focused on factors known to be involved in start codon fidelity, non-canonical translation initiation, or RNA helicases implicated in protein translation. These categories were chosen based on our previous studies *in vitro* and educated guesses related to the mechanism underlying RAN translation. It is an admittedly and purposely incomplete list of initiation factors. Given the nature of the screen, in which we sought to identify factors involved in RAN translation specifically, we avoided most factors whose loss we predicted would markedly impair global translation. eIF4E and eIF4G were excluded exactly because previous work (as the reviewer points out, Kearse *et al*., 2016) had demonstrated that RAN translation at *FMR1* is cap- and scanning-dependent. We did not feel that re-establishing this in the fly model was needed. In addition, because eIF4E and eIF4G are required for translation of the majority of mRNAs, we feel that they would not represent good candidates for drug therapies, as their effects on global protein synthesis would be significant. **We have now made this explicit in the text**.

We also agree that the information in Figure 1 and the associated supplemental table can be made more clear and more information related to the "hits" can be included.



To this end, we have:

i) Improved Figure 1 by including labels of the major protein- and RNA-containing complexes at each stage of initiation.

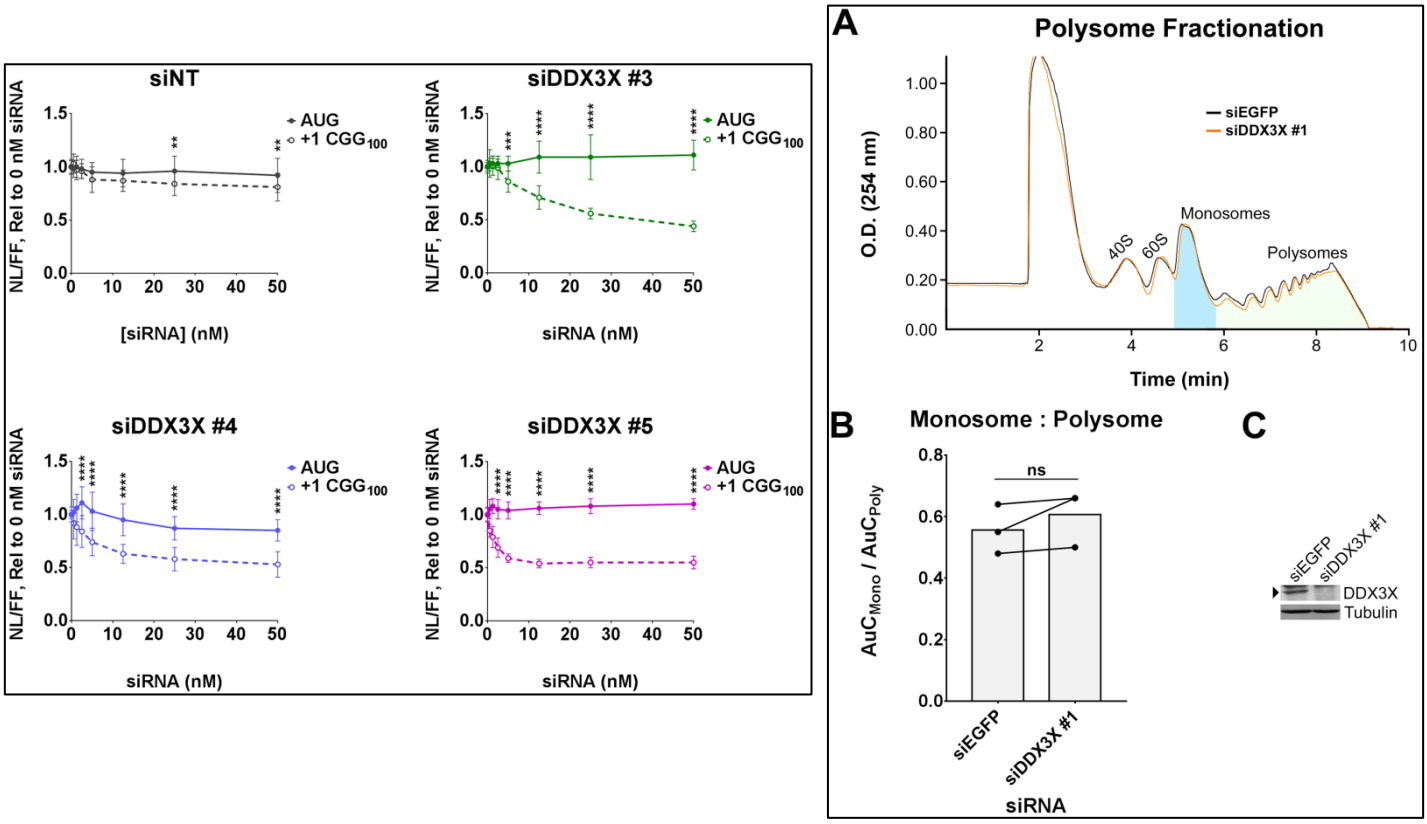
ii) Increased the size of the key, which describes how the candidates are indicated as suppressors, enhancers, without effect, or non-specific modifiers.

iii) Included a table which summarizes the raw phenotype data (Figure EV1) in an easy-to-read format.

2) RAN translation is 30-40% as efficient as canonical AUG translation (Kearse et al.). In FXS an increase in global translation was shown in several models and in patient cells by numerous labs. The authors need to show an independent test for global translation (e.g. 35-S-Met incorporation) to clearly demonstrate that DDX3X knockdown only affects RAN translation. The same applies to other models used (cells, primary neurons).

RESPONSE:

We do not claim that *DDX3X* knockdown only effects RAN translation. Our data does, however, demonstrate that it preferentially effects RAN translation over AUG initiated translation across multiple different reporter systems. To expand on this specificity, we have included data showing preferential effects of 3 additional, independent anti-*DDX3X* siRNAs on RAN translation. In addition, we have performed polysome fractionation in cells transfected with anti-*DDX3X* siRNAs. These data do not support a global inhibition of protein synthesis following *DDX3X* knockdown**. These data are now included in the revised manuscript as Figures EV3 and EV4.**



In addition, we think that the reviewer may be confusing Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) and Fragile X Syndrome (FXS). We apologize for not making this difference clearer in the introduction, and we have included edits making this more explicit. FXTAS results from a moderate expansion of the CGG repeat in *FMR1* to 50-200 CGGs. This repeat is transcribed into RNA and can be translated into toxic proteins via RAN translation. The result is an age-related neurodegenerative condition that can be modeled by expression of the CGG repeat in isolation in model systems, as we have done here. It is not associated with loss of the fragile X protein, FMRP. In contrast, FXS occurs when the repeat gets very large (>>200 CGG repeats). This results in transcriptional silencing of the Fragile X locus, resulting in no *FMR1* RNA (and thus no CGG repeat RNA expression) and complete loss of FMRP. In Fragile X syndrome, complete loss of FMRP (as modeled in mice by knocking out the gene) results in a global increase in protein translation as FMRP suppresses protein synthesis. As the mechanistic underpinnings of these two allelic disorders are quite different, we would not anticipate that, in FXTAS or the FXTAS models evaluated in our study, there would be a global increase in translation as occurs in FXS. Indeed, there is actually some evidence that overexpression of CGG repeats leads to a global decrease in protein translation (see Green *et al*., Nature Communications, 2017). Others have previously demonstrated that loss of FMRP does not influence the CGG repeat associated toxicity in Drosophila (Jin *et al*., 2003, Neuron) and we have independently confirmed this result (data not shown).

3) The mechanistic investigation in this paper is lacking evidence downstream of DDX3X. Which mRNAs are preferentially translated during RAN? This would shed more light into this regulatory mechanism, but also confirm the findings obtained with reporter assays (Fig. 4).

RESPONSE:

This study is focused on RAN translation at CGG repeats. We agree that it would be of interest to characterize the downstream consequences of selectively blocking RAN translation on *FMR1* on the expression of FMRP, as this would inform how RAN translation impacts the expression of other open-reading frames (ORFs) on *FMR1* transcripts in *cis*. However, these downstream consequences of RAN translation are not the focus of this study and we feel this extends well beyond the scope of this manuscript. Of note, other groups have published studies detailing the changes in global translation that occur with knockdown of *DDX3X* or its yeast

homologue, *Ded1* (Sen *et al*., Genome Research, 2015; Sen *et al*., PNAS, 2016; Guenther *et al*., Nature 2018, Gupta *et al*., eLife 2018; Valentin-Vega *et al*., Scientific Reports, 2016) and thus repeating those studies here would not, we feel, add significant new data to the field. **We have been sure to explicitly state this and cite this published work more clearly in the revised manuscript.**

Ref #2:

This study employs a genetic screen based on fly eye color of various initiation factors or helicases for ones that would inhibit RAN translation mediated by the repeated elements in the FMR 5'UTR of the FXTAS disease-causing allele. They identified the helicase Ddx3, eIF4B, eIF4H, and eIF1 as factors that are required for RAN translation, reducing the eye phenotype and, for Ddx3 and eIF4B at least, also mitigating the reductions in fly viability conferred by expression of the FXTAS allele. They go on to show that Ddx3 shRNA-knockdown in cells reduces expression of FXTAS reporter genes to a greater extent than other reporters, including one with the FMR 5'UTR but lacking repeated elements, suggesting a preferential effect on RAN translation. By contrast, knockdown of eIF4B or eIF4H did not have this preferential effect, reducing both reporters comparably. They show that overexpressing eIF1 reduces expression of the RAN reporter, provided it lacks an AUG codon, while overexpressing eIF5 has the opposite effect, in agreement with their effects on initiation at near-cognate start codons. They finish by showing evidence that knockdown of Ddx3 mitigates toxicity of the FXTAS construct in primary rodent neurons.

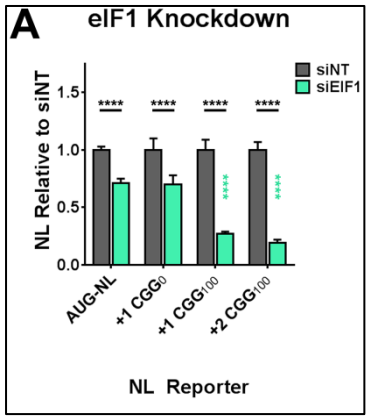
This paper is significant in using a creative genetic screen in Drosophila to identify Ddx3 as a factor that appears to be preferentially required for RAN translation both in flies and mammalian neurons. There are however several issues that need to be addressed.

Major comments:

1) The results on eIF1 presented are puzzling, as eIF1 was identified in the genetic screen as a factor presumably required for RAN translation, as its knockdown suppressed expression of the FXTAS allele in the fly eye. However, they show in Fig. 6A that eIF1 overexpression suppresses expression of the FXTAS reporter. The latter results fit with expectations about eIF1 overexpression suppressing non-AUG initiation, which applies to RAN translation. Hence, it's difficult to understand how eIF1 knockdown suppressed expression of the FXTAS construct in fly eyes.

RESPONSE:

We agree that the differences between our results in flies and in cells following *eIF1* manipulation are somewhat difficult to interpret. First, in flies, it appears that the degree of *eIF1* knockdown influences its phenotypic effects. Complete loss of *eIF1* is lethal in all eukaryotic organisms, and some of our *eIF1* knockdown lines had significant toxic phenotypes in flies in the absence of CGG repeats (Figure EV1). We predict that the rescue observed with some shRNA lines results from partial loss of *eIF1*. Partial loss is anticipated to impair global translation, decreasing both RAN and canonical translation and suppressing the phenotype. This is consistent with previous data demonstrating global translational inhibitors suppress repeat associated toxicity (Mizielinska *et al*., Science, 2014).



Consistent with this hypothesis, knockdown of *eIF1* in mammalian cells suppresses expression of both RAN- and canonical-translation reporters. **We now include this data in the revised manuscript as Figure S6A.**

2) At odds with their statement on p.7, the #2 siRNA against DDX3 reduced expression of the FMR AUG-NL reporter and was not specific for the FXTAS reporter in the results shown in Fig. 3A. This undermines the key claim that knockdown of Ddx3 preferentially impairs RAN translation.

RESPONSE:

We agree that the anti-*DDX3X* siRNA #2 reduces expression of our AUG-NL-3xF construct, and we appreciate the reviewer's scrutiny. However, the effect on the +1 CGG_{100} construct is significantly greater (two-way ANOVA with Tukey's post-hoc, $P<0.0001$ for siRNA

effect, P<<0.05 for each data point above 0.04 nM siRNA).

To confirm this result, we have tested 3 additional, independent siRNAs against *DDX3X* (Figure EV3—see Reviewer #1's comments). Two of these elicited similar responses as siDDX3X #1: robust inhibition of +1 $(CGG)_{100}$ NL-3xF with no effects on AUG-NL-3xF. The third siRNA elicited a similar response as siDDX3X #2: slight effects on AUG-NL-3xF, but significantly greater inhibition of the RAN-translation reporter. We have included these dose-response curves as an additional supplemental figure in the manuscript. **Given that all of these siRNAs effectively reduce DDX3X expression, we conclude that any modest effects on AUG-NL-3xF are significantly overshadowed by a strong selectivity for RAN translation.**

Other comments:
i)They didn't cite panels D-E of Fig.3.

RESPONSE:

This has now been corrected.

ii) Regarding the statement on p. 8, "Similarly, insertion of an AUG codon in a strong Kozak context above the NRE in the GCG (+2) frame enhanced basal expression, but expression of this AUG-(CGG)100 (+2) NL-3xF reporter remained DDX3X-dependent." it's unclear what "above" means. They should include a schematic of the different RAN reporter constructs showing where the start codons in the +1 or +2 frames are located relative to the NREs, and how these constructs differ exactly from the control FMR AUG-NL reporter.

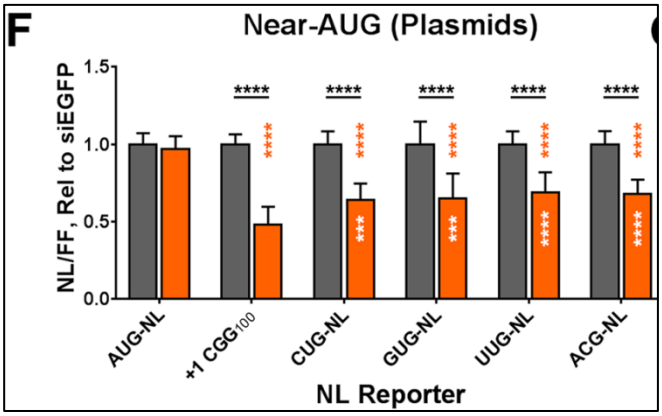
RESPONSE:

"Above" refers to 5' to the repeat. We have amended the text to make this specific issue clearer and also included a schematic of all reporters used in this study (Figure S1; Figure S2).

iii) The statement on p. 8 "DDX3X knockdown reduced their expression more than AUG-NL-3xF but less than (CGG)100 (+1) NL-3xF (Fig. 4F)" requires statistical analysis to be justified.

RESPONSE:

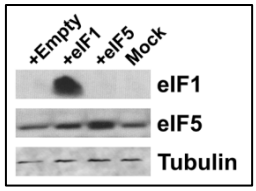
Analysis of NL expression of siDDX3X-treated cells with comparisons to +1 CGG_{100} NL is now included [two-way ANOVA with Tukey's post-hoc; white asterisks indicate statistical comparisons with +1 $(CGG)_{100}$ NL-3xF].



iv) On p.10, in "In total, these experiments demonstrate that manipulations that influence start-codon fidelity can bidirectionally regulate RAN translation at CGG repeats." the word bidirectionality is a poor choice.

RESPONSE:

We agree. This phrase has been changed to "In total, these experiments demonstrate that manipulation of factors that influence start-codon fidelity can up- or down-regulate RAN translation at CGG repeats."



v) p. 10; they should verify that eIF1 and eIF5 are actually overexpressed considering that they each auto-regulate their own expression.

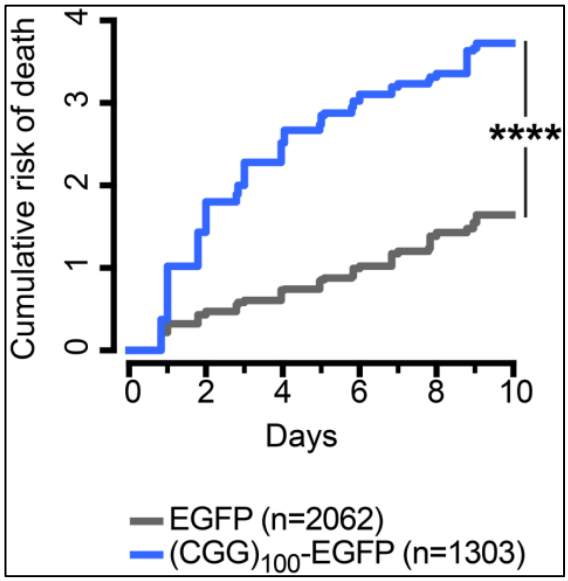
RESPONSE:

We have provided western blot data to confirm that *eIF1* and *eIF5* are over-expressed in this system (Figure S6B).

vi) p. 10-11 and Fig.7: What is the logic behind using an EGFP reporter in which the AUG start codon has been replaced with a GGG codon (GGG-EGFP) as the negative control, as presumably there will be no EGFP expression at all from this construct. It would seem that the proper control would be a construct in which EGFP is expressed in the absence of the NRE-encoded polypeptide produced by RAN translation.

RESPONSE:

We feel that this is the right control, as the constructs we are expressing contain this sequence 3' to the inserted *FMR1* 5'UTR sequence and not an AUG-driven EGFP. To confirm that the choice of control was not a factor here, we have included supplementary data demonstrating that expanded CGG repeats are also toxic compared to AUG-EGFP (Figure EV6).



vii) p.12: the references (Kozel et al., 2016; Tang et al., 2017) are missing. Also, the Loughran et al paper PMCID: 3326321 is the appropriate one to cite here; and the Ivanov et al paper for eIF1.

RESPONSE:

We apologize for the oversight and have made these corrections.

***Ref #3**:*

The manuscript by Linsalata et al. reports results from a candidate Drosophila screen for modifiers of RAN induced toxicity and highlights the identification of DDX3X, a helicase that reduces expression of RAN proteins in Drosophila and mammalian cells as a modifier of repeat toxicity and/or RAN translation. While the identification of modifiers of RAN translation is of high importance and the manuscript is potentially interesting, the data are not clearly presented and in some cases are overinterpreted. Overall a major focus of the paper is on the DDX3X RNA helicase gene as a modifier of RAN induced toxicity but the unclear nature of these effects, including that similar effects are found in mammalian cells expressing constructs that do not contain repeat expansions make the likelihood that the authors have identified a robust modifier of RAN translation unclear. Additional control experiment and data are needed to support the main conclusions on DDX3X and the other modifiers described in the manuscript.

1) The numbers of modifiers identified are not consistent throughout the paper: Abstract says they identified five modifiers but figure 1 which summarizes the modifiers is inconsistent with the abstract. EIF5 is listed in the abstract but is colored grey (not clear what this means) as a modifier and RPS25, SF2, BGCN, eIF1 eIF2alpha are colored as modifiers in the figure but not mentioned in the abstract.

RESPONSE:

We have changed the text of the Abstract and Results sections to include all of the factors that were hits in the *Drosophila* screen. We have also made it clearer what each color means in Figure 1 and provided an additional table summarizing this information in the supplement (Figure EV1; see Reviewer #1's comments).

2) Some further explanation for what the various grey shapes on the ribosome represent is needed so the reader will know what they are looking at in Figure 1.

RESPONSE:

We have updated Figure 1 to include labels of the major protein- and RNA-containing complexes at each stage of initiation.

3) A better summary of the data generated from the screens is needed. The manuscript says "47 candidate lines" were tested but the table shown in EV1A lists 64 lines with 12 acting as suppressors and 22 and enhancers of CGG toxicity but this is not well summarized in either the cited Figure 1 or Table EV1B. The authors should revise the paper to include a table with a complete summary of the effects of all of the fly lines that were tested including whether or not they had effects with and/or without the repeats.

RESPONSE:

We have replaced Figure EV1 (in the original manuscript) with a table, as requested, and revised the text and Figure 1 to more clearly represent the findings from our primary screen.

4) All of the enhancers were checked as modifiers of flies expressing GMR-GAL4 as a control to screen for enhancers that require the repeat. The same screen should have been done for all of the suppressors.

RESPONSE:

We apologize for not making this clearer in the text. All tested lines (suppressors, enhancers, and those with no effects on CGG_{90} toxicity) were also crossed to GMR-GAL4 flies to test for baseline effects on phenotype (Figure EV1). We have amended the Results section to clarify this point and will highlight these results more clearly.

5) It looks like 3 bel mutants not five as indicated in the text suppress the rough-eye phenotype. A better description of these mutants is needed. Are they null mutants? What is being tested - loss of function/dominant negative effects? Are the flies heterozygous or homozygous for these mutations. The authors should better explain this experiment and why the effects of these mutations are not as striking as the shRNA results.

RESPONSE:

In total, five bel mutants suppressed CGG_{90} toxicity. Fig. 2A, B includes two mutants (bel^{6} and bel^{EKE}) that suppressed, while Fig. EV2A, B includes four different mutants, three of which (bel^{L4740}, bel^{74407}, and $bel^{cap\text{-}1}$) suppressed toxicity. The flies are indeed heterozygous, which might explain why these alleles suppress to less of an extent than the shRNA constructs. **The text has been amended to clarify these experiments.** Information on the mutant alleles used is as follows:

Bel^{6}: premature termination codon (EMS)
Bel^{EKE}: unclear, though complementation assays indicate loss-of-function (Johnstone et al, Developmental Biology, 2005)
Bel^{L4740}: P-element insertion (Spradling et al, Genetics, 1999)
Bel^{74407}: P-element insertion (Poulton et al, Development, 2011)
Bel^{47110}: P-element insertion (Poulton et al, Development, 2011)
$Bel^{cap\text{-}1}$: P-element insertion (Castrillon et al, Genetics, 1993; Spradling et al, Genetics, 1999)

6) Why does shRNA suppression of Bel with shRNA 1 and shRNA 3, which showed similar reductions in FMR-polyGly have such different effects on survival in Fig 2C. Also the additional survival data shown in panel D for shRNA 3 do not look very different than controls - is the p value, which is shown as $p<0.0001$ correct?

RESPONSE:

There are inherent background effects across fly lines (even on the same background strain) that can influence the degree of effect observed with the assays used here. We have included ALL of the publicly available *Bel* shRNA lines and ALL of the publicly available insertion/deletion lines as we feel that this makes the data significantly more robust and broadly interpretable/replicable between investigators.

We thank the reviewer for pointing out a typo; in Fig. 2D, we reported the Gehan-Breslow-Wilcoxon P value for shBel 4 (which has the smallest effect). The correct value, for the Log Rank (Mantel-Cox) test, is P=0.0009 (n=147 shCherry flies, n=299 shBel 4 flies). The P value for shBel 3 is correct (n=147 shCherry flies; n=297 flies).

7) The RNA transfection experiments provide only indirect evidence that DDX3X does not act on transcription or nuclear export. These comments should be removed from the results section or additional experiments supporting these claims should be added.

RESPONSE:

We agree that these data do not preclude a contribution of this factor to transcription and nuclear export. However, our studies in cell extracts confirm a direct impact by DDX3X on RAN

translation. An additional contribution from transcriptional or nuclear export effects cannot be entirely ruled out, and we have amended the text accordingly.

8) The repeat length-independent effects of DDX3X from RNAs expressed in cells with plasmids but repeat dependent effects when RNA transfections are done is puzzling and additional data are needed to support this statement and if true to understand the molecular basis for these differences. Do the RNAs expressed from plasmids have a different localization patterns than the transfected RNAs in cells?

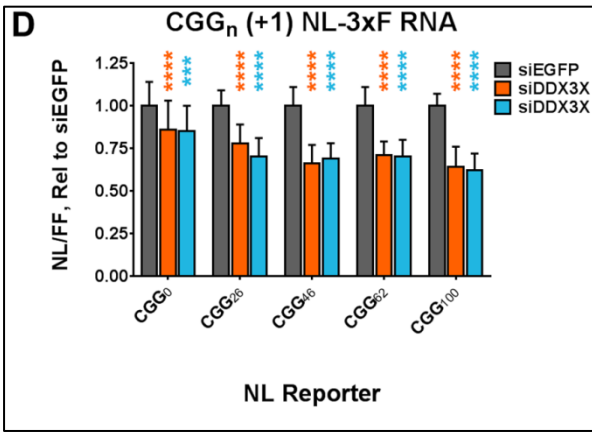
RESPONSE:

After we pooled data across several independent replicates, the repeat-dependency of *DDX3X* knockdown's efficacy was markedly reduced for transfected RNA reporters (Figure S3D, E, data below). As such, these comments have been removed from the text.

9) The paragraph on page 8 beginning with "One of the unique features..." and related data describing the effects of ATG and close cognate codons and DDX3X effects is confusing and should be written more clearly. Why do the authors claim that the transfections done with in vitro transcribed RNAs in EV3D and E are repeat length dependent without doing any statistical comparisons between the different repeat groups? Is the modest trend toward greater inhibition with longer repeats actually significantly different than the inhibition with shorter repeats? It seems the right comparisons were not performed to support these statements.

RESPONSE:

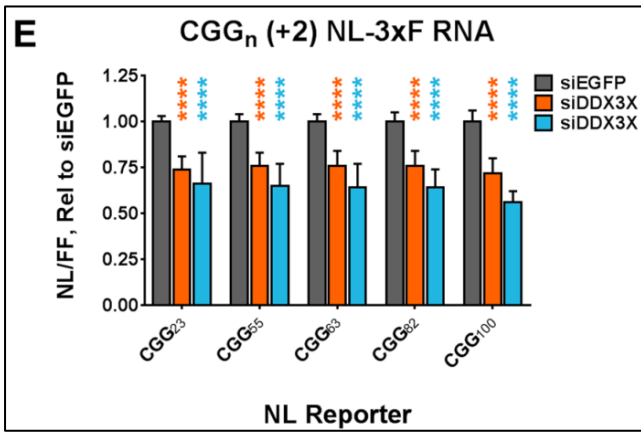
We have now edited this. See above response and data.



10) The precise flanking sequence can influence RAN translation and may also be important for understanding the DDX3X effects but the specific flanking sequences in the various plasmids used throughout the paper are not well described. A table should be added with all of the constructs used so that the reader can see exactly what has changed between the various plasmids.

RESPONSE:

A schematic is now included (Figure S1, Figure S2).



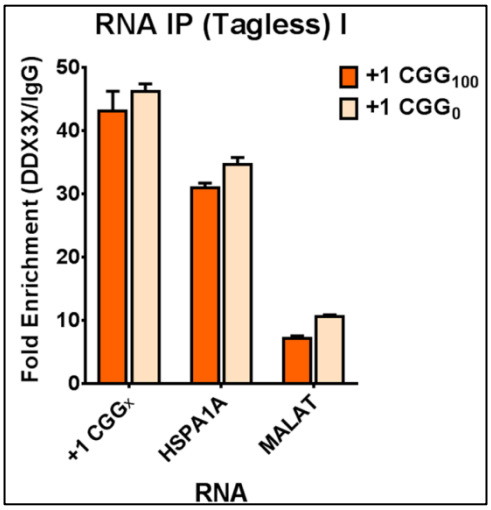
11) The authors should comment on why they used only the +1 AUG like reporter construct for the in vitro translation experiments. Since the +1 frame has the AUG like sequence it would be interesting to compare the effects on the +2 reading frame.

RESPONSE:

We agree that this an interesting experiment. We have performed the *in vitro* translation experiments using the +2 reporters. These have largely yielded results similar to the +1 reporters, but with less consistency. Because we are not as confident in the results of our experiments with the +2 reporters, we decided to exclude these results at this time.

13) Figure 4 RIP binding. Additional experiments should be performed to clarify if the binding site of DDX3X requires the repeat expansion or if it can bind to the upstream FMR RNA sequence that is included in repeat containing NL reporter. This is especially important because DDX3X does not require any CGG repeats to suppress NL reporter expression (as shown in Figure EV3B).

RESPONSE:



To address this concern, we have repeated this mRNA-protein binding assay (RIP) using the 5'UTR of *FMR1* both with and without the CGG repeat (Figure S5B, C, and right). The results are unchanged. These data, along with our other studies, suggest that DDX3X is not acting to inhibit translation solely through repeat binding.

14) Figure 4F. The authors state that DDX3X reduces the NL reporter levels from the repeat containing plasmids more than from the close-cognate non-repeat containing transcripts but the authors show no statistical support for this statement by directly comparing these data sets to show they are significantly different from each other.

RESPONSE:

This analysis has now been included, demonstrating that the changes are statistically significantly different between the repeat containing plasmid and the close-cognate non-repeat transcripts. See response to Reviewer #2's comments (minor comment iii) above for details.

15) Data summarized in EV3 panel B and elsewhere throughout the manuscript should be averaged and not selected from one of the three replicates or two of four replicates.

RESPONSE:

We have provided the data in this requested format and revised all relevant figures.

16) Panels with two replicates should be repeated so that there are three replicates. Figure EV3G

RESPONSE:

This is done.

Minor:
i) Figure 3C - move legend for what colors mean to Figure 3C and indicate in figure legend that this key applies to both Figure 3C and 3D.

RESPONSE:

This is done.

ii) Page 6. The authors should just state that their knockdown of bel had no effect on steady state levels of CGG90-EGFP transcripts and not extend this to suggestions that it addresses transcription and turnover which this experiment does not.

RESPONSE:

This is done.

iii) Figure 3 labeling is confusing. The top and bottom parts of panel A should be placed side by side or given separate labels.

RESPONSE:

This is done.

iv) Page 7 the use of the phrase "mRNA copy number" is confusing and typically refers to DNA. This should be changed to "mRNA levels".

RESPONSE:

This is done.

2nd Editorial Decision 20 May 2019

Thank you for the submission of your revised manuscript to EMBO reports and for your patience while it was under review. We have now received the reports from former referee #1 and referee #3 (now #2), which are copied below.

As you will see, both referees acknowledge that the manuscript has been improved during revision. However, referee 2 remains unconvinced that you made a strong case for the role of DDX3X as a suppressor of RAN translation. Upon further discussion of these concerns, also referee 1 agreed with this concern and I therefore ask you to carefully phrase and tone down your conclusions in the abstract and main manuscript to most accurately describe your findings.

Both referees considered it helpful to have only one merged file of Supplementary information. Since we allow to have these two sets of Supplementary information as such (Expanded View and Appendix), I will leave this decision to you as to whether you want to supply all Supplementary information merged into one Appendix or as to whether you want to keep some key figures in the Expanded View format. Both options are fine for us. But please keep this general information on formatting in mind:

- Expanded View: The legends of these figures are part of the main manuscript in a section called 'Expanded View Figure Legends', which comes after the main Figure Legends section.
- Appendix: All figures, tables and their legends are merged into single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

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

- Author contributions: Amy Krans is missing and Stephen Fedak is listed a SFJ. Please double-check and correct/add.

- Table 1 is currently in color. Please note that our journal style accommodates only black-and-white or greyscale tables.

- Please note that our journal policies do not permit citation of "Data not shown". All data refereed to in the paper should be displayed in the main or supplemental information. You currently refer to "Data not shown" on page 6 of your manuscript ("over-expression of bel neither suppressed nor enhanced this phenotype"). Please include the relevant data in the Appendix.

- Please update the references to the numbered format of EMBO reports. The abbreviation 'et al' should be used if more than 10 authors. You can download the respective EndNote file from our Guide to Authors
https://drive.google.com/file/d/0BxFM9n2lEE5oOHM4d2xEbmpxN2c/view

- Our data editors from Wiley have already inspected the Figure legends for completeness and accuracy. Please see their suggested changes in the attached Word file.
- I noticed that you state in some legends "... were independently replicated at least twice" or you indicate that the data comes from duplicates: Fig. 2, Fig. 4C, Fig. 5, Fig. EV1, Fig. EV4, Fig. S4C. Please note that statistics shall only be applied to datasets obtained from at least three independent replicates. If n=2, the data should be shown as scatter blots without any statistical evaluation.

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (width x height). You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

****

REFEREE REPORTS

Referee #1:

The authors have addressed all of my 3 major concerns raised in the first round of revisions, thus I am now convinced that the study in its current form should be published in EMBO reports.

Referee #2:

While the authors have made improvements in the manuscript the effects of DDX3X as a suppressor of RAN translation are still oversold. While the data do show that DDX3X can suppress FXTAS RAN, these effects are mostly CGG repeat independent (frames +1 and +2) and do not preferentially affect non-AUG versus AUG and close-cognate AUG-like translation. Since a repeat and the lack of an AUG initiation codon are core features of RAN translation, the DDX3X effects should be more accurately described as suppressors of translation requiring upstream non-CGG repeat sequences unique to the FMR1 locus. This is not the message that comes through in the Abstract or the Title or most of the paper and should be revised.

The two sets of supplemental figures (EV and S) make the figures and manuscript hard to follow. Combining these data into one set of additional material would improve the readability of the manuscript.

The two sets of supplemental figures (EV and S) make the figures and manuscript hard to follow. Combining these date into one set of additional material would improve the readability of the manuscript.

2nd Revision - authors' response 4 June 2019

1) As you will see, both referees acknowledge that the manuscript has been improved during revision. However, referee 2 remains unconvinced that you made a strong case for the role of DDX3X as a suppressor of RAN translation. Upon further discussion of these concerns, also referee 1 agreed with this concern and I therefore ask you to carefully phrase and tone down your conclusions in the abstract and main manuscript to most accurately describe your findings.
Response: As requested, we have modified the language in our abstract and manuscript to more accurately reflect our findings related to RAN translation and DDX3X. This includes using the phrase "FMR1 RAN translation" to reflect that these findings are most directly relevant to the FMR1 locus. We also have a discussion of our findings and their meaning which we think captures the nuance of how each factor impacts non-AUG initiated translation. This is provided in two forms- one with all changed accepted, and one with line numbers and all changes tracked.

2) Both referees considered it helpful to have only one merged file of Supplementary information. Since we allow to have these two sets of Supplementary information as such (Expanded View and Appendix), I will leave this decision to you as to whether you want to supply all Supplementary information merged into one Appendix or as to whether you want to keep some key figures in the Expanded View format. Both options are fine for us. But please keep this general information on formatting in mind:
Response: We have converted all figures to Appendix figures, as we think that having all of these merged as a single PDF is the most useful approach in this context. We now include a Table of contents as well as legends for each Appendix figure or table. These are provided as single items and as a merged PDF file.

3) Author contributions: Amy Krans is missing and Stephen Fedak is listed a SFJ. Please double-check and correct/add.

Response: This has been addressed.

4) Table 1 is currently in color. Please note that our journal style accommodates only black-and-white or greyscale tables.
Response: This has now been converted to appendix figure 1.

5) Please note that our journal policies do not permit citation of "Data not shown". All data referred to in the paper should be displayed in the main or supplemental information. You currently refer to "Data not shown" on page 6 of your manuscript ("over-expression of bel neither suppressed nor enhanced this phenotype"). Please include the relevant data in the Appendix.
Response: This statement has been removed and the sentence it referred to has been modified to only refer to the data in the manuscript.

6) Please update the references to the numbered format of EMBO reports. The abbreviation 'et al' should be used if more than 10 authors
Response: This has now been done.

7) Our data editors from Wiley have already inspected the Figure legends for completeness and accuracy. Please see their suggested changes in the attached Word file. I noticed that you state in some legends "... were independently replicated at least twice" or you indicate that the data comes from duplicates: Fig. 2, Fig. 4C, Fig. 5, Fig. EV1, Fig. EV4, Fig. S4C. Please note that statistics shall only be applied to datasets obtained from at least three independent replicates. If n=2, the data should be shown as scatter blots without any statistical evaluation.
Response: All N's are now clearly specified. All experiments reflect a minimum of three independent replicates.

8) Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (width x height). You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.
Response: These are now included in the manuscript (for points A and B) and as a separate synopsis image (for point C).

3rd Editorial Decision 12 June 2019

Thank you for your patience while we have assessed your revised manuscript. I am now writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed..

3rd Revision - authors' response 19 June 2019

The authors performed all minor editorial changes.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Peter K Todd, MD, PhD
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-47498V2

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	A minimum of three independent replicates were performed for all experiments to ensure the results were replicable. For experiments in fruit flies, a prespecified number of >25 was used for all genotypes analyzed, although n far in excess of 25 were often obtained. A power calculation was not performed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For fruit-fly experiments, a prespecified number of >25 was used for all genotypes analyzed, although n far in excess of this were often obtained. For neuronal survival experiments using longitudinal microscopy, a minimum of 2000 neurons/condition were acquired and tracked over 3 replicate experiments.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For cell-based reporter assays, wells with <10% the reporter expression of identically-treated wells within the experiment were excluded, as the transfection was unsuccessful. This criterion was pre-established.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done for fruit-fly experiments, but additional, independent researchers verified the data presented in Figure 2A, B. Quantification of neuronal survival was performed using an automated system blind to condition.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	A normal distribution was assumed for most continuous measures. For data with discrete measurements, non-parametric statistical analyses were applied.
Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Yes. Provided in the Materials & Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines were recently re-obtained from ATCC to make sure their identity was accurate. Mycoplasma was routinely tested for before, while, and after all the experiments were conducted. All tested lines were negative, and most recently within the past 6 months.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Timed pregnant Long-Evans Rats were obtained from Charles River.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal protocols were reviewed and approved by the University of Michigan Institutional Animal Care & Use Committee (IACUC).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Our work is in compliance with the ARRIVE guidelines.

E- Human Subjects

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

F- Data Accessibility

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

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA