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C9orf72 ALS/FTD dipeptide repeat protein levels are reduced by small molecules that inhibit PKA or enhance protein degradation

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Dear Alessandro,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from the comments, the referees find the analysis interesting. However, they also find that additional work is needed to consider publication in The EMBO Journal. Given the comments provided, we are interested in considering a revised version.

I think it would be helpful to discuss the raised points further and maybe it would be most helpful to do so via phone or skype. I will contact you in the next few days to discuss this further. I also know with the uncertainty of the COVID-19 situation and lab closures that working on the revision might not be so feasible, but I can easily extend the revision time to make it workable.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Looking forward to discussing the revisions further with you

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

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

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

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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Link Not Available

Referee #1:

The manuscript by Licata et al. starts by performing a screen in HEK cells using approximately 2500 compounds from different libraries on the GFP expression which is indicative for the expression of poly-GP from a GGGGCC repeat. The effects of the compounds on the GFP expression is compared to the RFP expression driven by an AUG. From these compounds, a number of compounds are studied in more detail and ultimately the manuscript is focusing on the effect of protein kinase A on the regulation of the DPR expression. Both by using H89, an inhibitor of protein kinase A as well as siRNA against the different isoforms of PKA it is convincingly shown that the Cbeta subunit plays an important role in the translation of the hexanucleotide transcript. Last but not least, treatment of transgenic flies overexpressing 36 GGGGCC repeats with H89 increased the motility of these flies as well as their survival, especially in the female flies. A similar effect was obtained by silencing the catalytic subunit of PKA. Overall, this is an interesting manuscript with novel and relevant data.

Major remarks
- The title doesn't cover the content of the manuscript. There is much more investigated/described than only the effect of the PKA catalytic subunit beta on (certain) DPRs. Or a more general title should be chosen, or the manuscript should be (much) more focused on the most interesting data (which I consider as the best option).
- Unfortunately, the description and presentation of the initial drug screen is not very clear. For instance, it is not clear when cycloheximide was exactly added. It seems likely that it is added at the same moment as the compounds, 3 h after transfection. In that case, it is weird that cycloheximide has no effect on the GP-GFP expression and the explanation that the stability of the DPRs is the cause of this lack of effect is unlikely as there will be not a lot of GP-GFP formed during this limited time frame. Much more information should be provided on this initial assay. In addition, the GP-GFP staining shown in Supplementary Figure 1d is not convincing as only a very limited number of cells show a bright green staining. Most cells show a very low signal. In addition, the quality of some of these pictures is low. A similar remark can be made about the figures shown in the confirmatory screening shown in Fig. 1b. What does a negative value for 'Average of cells positive' exactly means? What do the dotted lines represent? Despite all the information provided, it is also not clear on which basis the small molecules were selected. There also seem to be other compounds that show a similar effect and that were not selected. Overall, a much better and detailed explanation of this initial assay should be provided.
- The Drosophila data are very interesting. However, it is not clear whether the impressive survival effects are indeed due to an effect of the inhibitors or the siRNA on the expression of the DPRs. As a consequence, it is crucial that the effect of the compound as well as of the siRNA is shown on the expression level of the DPRs. Otherwise, it can't be excluded that the positive effects are due to an interference with a completely different pathway. Moreover, the question also arises whether forskolin will have the opposite effect and whether it will enhance the phenotype in the repeat-containing flies.

Minor remarks
- An abbreviation in the title should be avoided. Moreover, this abbreviation is also not defined in the abstract
- The added value of the negative results obtained in SH-SY5Y mentioned in the beginning of the result section (part of supplementary figure 1) is not very clear.
- Is the transfection efficiency for each of the constructs used in the assay always the same? Are always the same cells transfected with both constructs?
- The schematic representation of the construct doesn't contain information on what is upstream of the repeat. Are there no potential near-cognate start codons present upstream of the repeat in the GFP/GP reading frame? This upstream sequence information should be provided.
- The very important information in the scatter plot in Fig. 1b should be explained in more detail. What are the units on the X/Y axes? Where are the black dots (DMSO)? Legend refers to 'with dots'. Which compounds are considered as hits?
- Scale bars are missing on the photos shown on Supplementary figure 1.
- Were thapsigargin, tunicamycin and MG132 part of the screen? Otherwise references should be added.
- The identity of the selected compounds should be indicated on Fig. 1b.
- Supplementary Table 1 seems to contain raw data and these should be presented in a much better format making it clear what is exactly shown (with a legend). Names of compounds are not all readable.
- Not clear why the higher concentrations of the different compounds are used/shown as most of these compounds affect cell survival which will potentially compromise the interpretation of these data.
- Why is the effect of forskolin not dose dependent? At 0.5 µM the maximal effect is already reached.
- The abbreviation for geldanamycin could be confusing as one of the DPRs produced from C9 is GA.
- Statistics is lacking on panels d and e of Fig. 1. Moreover, t-tests are not the best way to determine significance in case more than two conditions are compared.
- Table 1: it is not clear at which concentration of the compounds the z scores are shown. Is it 5 µM? Same question for Fig. 2a.
- While erysolin was selected from the initial screen, follow-up experiments don't seem to confirm the effect of this compound. As a consequence, the question arises whether this was a false-positive hit. If so, this should be explicitly mentioned (or another explanation should be provided).

- While in the initial screen, a comparison is made with the effect on ATG mediated expression, this is not longer the case for H89. Was this tested? In addition, pictures comparable to the ones shown in Fig. 2a would be very helpful in visualizing the effect of the different PKA modulating drugs.
- Supplementary figure 7 only contains one panel and as a consequence it is not needed to add a label. However, it would be interesting to confirm the downregulation at the protein level.
- Typos should be corrected (e.g. line 126: 'many add an opposite effect')

Referee #2:

In this study, the authors performed a high-throughput screening of chemical compounds to identify modulators of C9of72 GGGGCC repeat associated DPRs levels. Multiple modulators targeting different cellular pathways were identified. Importantly, a key modulator that elevates cAMP level was found to increase DPRs expression levels. This suggested a role of cAMP/PKA pathway in regulating RAN translation. In support of this, a PKA inhibitor, H89, inhibits DPR production in cells and rescue toxicity in Drosophila carrying the G4C2X36 repeats. This result was further supported by genetic ablation of PKA catalytic subunits. Together, these findings established a new potential therapeutic target for ALS/FTD that can be studied further.

Overall, the present study is well performed and presentation is clear. However, a significant limitation that reduces its relevance of these findings is the lack of confirmation of effects on endogenous DPR levels in patient derived cells or correction of survival defects in mammalian systems. Beyond these major limitations, some additional experimental suggestions that can improve the current form of the manuscript are provided below.
1. Given the limitations noted above, some of the claims made in the abstract are inappropriate. For example "protein clearance and PKA-Cβ as new drug targets for C9ALS/FTD" and "Spironolactone, Geldanamycin and H89 could serve as tool compounds to develop new effective drugs" is a reach without any validation in a human or even mammalian neuronal system. Toning down these claims is highly recommended.
2. What is the exact sequence in front of the GP-GFP repeat here? This sequence has been shown to matter and I do not think these plasmids used in the screen here have the native sequence above the repeat (and neither do the flies, for that matter). The nLuc plasmids described do have this and yet are never used with nLuc in the paper (except in a control in the supplement without any of these treatments) to test any of their chemical hits. Instead they are doing westerns for a protein that is aggregate prone, which can be problematic. Why not use the nLuc as a much more quantifiable readout for RAN translation, as others have done?
3. The CUG start codon within the intron 1A of C9orf72 has been shown to be important for RAN translation. To evaluate whether the effect of these compounds was CUG independent, the authors used a 66xGA construct containing endogenous The CUG start codon within the intron 1A of C9orf72 has been shown to be important for RAN translation. To evaluate whether the effect of these compounds was CUG independent, the authors used a 66xGA construct containing the endogenous near-cognate CUG codon and the compounds still worked. However, they lack the right comparator to test this. Please also do this using the nLuc assay with and without the CUG (mutated to CCC or AAA) and see if this precludes the interaction in the GA and reading frames.
4. In general Figure descriptions should be more self-explanatory. For example - The way hits were selected from the screen as described in the method - 'Hits were defined as compounds capable to selectively reduce or increase the number of cells polyGP-GFP positive...'. However, representation of the results is not very clear in the description of figures.
a. e.g., in Fig 1c., what does the positive (+) or negative (-) population of cells represent? - cells that express RFP but not GFP? Are these Z-scores, total numbers of cells or percent of cells? For intensity, are the values normalized? Please clarify for both panels in the figure description.
5. The lack of CHX effect on GP is problematic, as is their explanation. "We observed that CHX did not decrease the fluorescence intensity or the number 109 of cells expressing polyGP-GFP (Supplementary Fig. 1f), possibly due to the stability of the DPRs in this time-frame." If there are major stability differences between AUG-RFP and their GP reporter then this could lead to mis-interpretation of their data.
a. Can you provide some data regarding the relative stability of these factors in your assay to support your model?
b. If stability is different, then I would be more concerned about these compounds/interventions impacting stability rather than RAN translation per se. As such, please look at whether stability is altered by your interventions.
c. An alternative explanation for the CHX result relates to the translation inhibitor used. Please consider trying a different inhibitor that works more cleanly on RAN constructs, such as Puromycin. Please see Kearse et al, 2019 Genes and Development and Kearse et al, 2016 Mol Cell for further explanation of this point. (as an aside, at least Kearse et al 2016 should be cited as well as this was the first mechanism paper on RAN translation and is thus relevant to this manuscript).
6. When testing effects of the compounds on autophagy markers, the authors conclude - 'None of the compounds induced neither the protein expression nor the transcripts level of autophagy markers....'. From the Fig 3C it seems FSK significantly changes TFEB mRNA level. Please include this in the results with proper explanations. In addition, it would be appropriate to evaluate the impact of these drugs/siRNAs on expression of your AUG-RFP control construct.
7. Authors tested effect of either PKACα or PKACβ on DPR production. Was there a greater effect if both subunits are knocked down together?
a. How many fly lines were used for each modifier? Generally, at least 2 independent siRNA or mutant lines are needed.
b. Please provide the number of flies (N=?) used for survival experiments.

c. Why does the survival of 36xGGGGCC flies alone (without any modifier or drugs) appears to be different in Fig 6 vs. Fig 7?
d. Why were no eye phenotypes observed or monitored? These are a prominent feature in this fly line.
8. A limitation of the work here is that they did not confirm the effects of PKA inhibition in drosophila were dependent on RAN translation. This could be done with publically available GR flies which initiate with an AUG and lack the repeat sequence.

Minor points -
Line 66-67. "Importantly, no small molecules are known to selectively modulate RAN translation..." This is not a correct statement! Need to re-write.
Line 76-77. DDX3X modulates FMR1 associated RAN translation in opposite way. Please mention recent paper by Lisalata, AE et al., (EMBO Report, 2019) as well.
Line 114. What is CAP-product?
Line 127-128. Compounds that induce cellular stress may also increase RAN, such as Thapsigargin. Is there any other compound?
Line 159. Along with Tabet, R. et al. (Ref 31) Green et al., (Ref 30) showed utilization of the CUG initiation.
Line 483. Addition not addiction
Figure Legend 1b. CHX treatment: please fix the color code mention in the legend
1d-e. Concentrations of drug in the represented as log [ ] in the figure. Is that correct?

Referee #3:

In this study, Licata et al. performed a high-throughput drug screen based on the expression of polyGP RAN proteins in the cells transfected with GFP-tagged G4C2 repeat constructs. Several hits were identified that could decrease or increase the expression of polyGP-GFP fusion proteins by targeting the proteasome degradation or AC/PKA pathways. Further analyses shows that H-89, which inhibits PKA catalytic subunit β, down-regulates expression of C9 DPRs in transfected cells. Additionally, PKA inhibition behavior and survival phenotypes in a Drosophila model of C9 ALS/FTD. These data provide additional tools to understand the mechanisms of RAN translation and may contribute to developing therapeutic strategies for C9 ALS/FTD. Overall, this is a potentially interesting story, but a rigorous testing of the effects of these compounds and testing in alternative systems that don't grossly overexpress the proteins is needed to understand the potential impact of this story. Specific concerns and suggestions are listed below.

Major concerns:
1. Figure 2C - why does CHX not block protein synthesis and what was the rationale for the 3 hr assessment here vs. 24 hours for the other compounds. It doesn't seem like the positive control for reducing protein synthesis here is working.

2. Supplementary Figure 1A. An increasing body of evidence showed RAN translation across G4C2 repeat expansion is dependent on repeat length, flanking sequences and cell types. Does the (G4C2)58-GFP construct used for screening, which contains the upstream CUG contain additional endogenous human intron 1 C9orf72 sequence upstream and downstream of (G4C2)66 repeat expansion?

3. Different constructs were used to test if the identified compounds reduce polyGA-(HA) or polyGP using different cell types (HEK293T for GA) and (NSC34 for GP). Are similar results found when the cells used for these assays are swapped and are the reductions seen restricted to very specific cell types for each DPR type or are the drug effects more general across a range of different cell types.

4. Additionally, can polyGR be also decreased by these compounds?

5. Overexpression of relatively short G4C2 repeat constructs was used for both screening and validation of selected compounds. G4C2 repeat expansions are generally from a few hundreds to thousands in C9 patients and expression of DPRs are at much lower levels compared to overexpression of G4C2 repeat expansion in cells. Reduction of DPRs by these compounds should be tested in a C9 patient-derived cell line, such as iPSC line.

6. The authors claim that Geldanamycin and Spironolactone reduce levels of DPRs by enhancing UPS activity. It is not clear however if the DPRs are degraded through UPS. Are the DPRs ubiquitinated in the transfected cells? Do UPS inhibitors block the degradation of DPRs?

7. It is important to know if PKA inhibition differentially affects polysome profiling for canonical versus RAN translation. In figure 5g and 5h, GAPDH is not a proper control for the overexpression of a G4C2 construct. AUG-RFP should be used as a control for the polysome profiling experiments. Also, a C9 patient-derived-iPSC line could be used for the polysome profiling experiments.

8. The authors showed that inhibition of PKA reduces expression of DPRs, but little is known about the connections between the

PKA pathway and the translation of DPRs. Does PKA inhibition also affect canonical translation initiation? Does PKA inhibition modulate the phosphorylation of eIF2α?

9. One of the core findings in this study is that inhibition of PKA Cβ decreases RAN-translated DPRs across G4C2 repeat expansions. In figure 6 and 7, data showed PKA inhibition through both H89 administration and RNAi interference ameliorates motility deficits and survival in a Drosophila model of C9 ALS/FTD. But it is important to show the expression of DPRs in this model and also how PKA inhibition affects DPR expression.

Minor points:
1. The text on page 4 describing the reporter construct for S1A does not match the figure and needs revision.

2. Figure 1C should indicate the names of the compounds that were used for the various hits indicated by asterisks or use different symbols so that how the data appear in this figure can be linked to the specific compound.

3. Figure 2D - The GA abbreviation used for Geldanamycin to measure myc-tagged Gly-Ala protein also abbreviated GA is confusing. Please use a different abbreviation for the drug throughout the manuscript.

4. A brief introduction of the Adenylyl cyclase/Protein Kinase A (AC/PKA) pathway is needed to make the findings accessible to a broad readership.

5. The (G4C2)n should be consistently used through the manuscript including main text, figure legends, as well as in figures. Such as: line 37 on page2, G4C2X36 should be (G4C2)36; line 48 on page 3, GGGGCCn (also known as G4C2n) should be (GGGGCC)n, (G4C2)n.

6. Please clearly define the soluble and PBS-insoluble fractions for western blot and filter retardation assay. Such as line 166 on page 6: 'the soluble levels and the PBS-insoluble species of poly-GP'

7. In figure 2h, are there significant differences?

8. In supplementary figure 1b, the legend should describe the levels of RAN products.

9. Please define G4C2 (+1) and G4C2 (+2) mRNAs in supplementary figure 2b.

10. Line 9 on page 33: CHX 5μM (with dots). 'With' should be a color.

Dear Editor

We would like to thank all the Reviewers for the time and skill they devoted to our manuscript as well as for their constructive comments on our study. We have now addressed their specific concerns with the addition of new data, several additional figure panels and all requested changes.

Please find below, point-by-point responses to the specific comments and suggestions raised by the Reviewers. We are confident the new version of our manuscript helped us improving our work and strengthening our results, and we hope you may consider our paper suitable for publication in EMBO J.

Referee #1:

The manuscript by Licata et al. starts by performing a screen in HEK cells using approximately 2500 compounds from different libraries on the GFP expression, which is indicative for the expression of poly-GP from a GGGGCC repeat. The effects of the compounds on the GFP expression is compared to the RFP expression driven by an AUG. From these compounds, a number of compounds are studied in more detail and ultimately the manuscript is focusing on the effect of protein kinase A on the regulation of the DPR expression. Both by using H89, an inhibitor of protein kinase A as well as siRNA against the different isoforms of PKA it is convincingly shown that the C beta subunit plays an important role in the translation of the hexanucleotide transcript. Last but not least, treatment of transgenic flies overexpressing 36 GGGGCC repeats with H89 increased the motility of these flies as well as their survival, especially in the female flies. A similar effect was obtained by silencing the catalytic subunit of PKA. Overall, this is an interesting manuscript with novel and relevant data.

Major remarks

1-1) The title doesn't cover the content of the manuscript. There is much more investigated/described than only the effect of the PKA catalytic subunit beta on (certain) DPRs. Or a more general title should be chosen, or the manuscript should be (much) more focused on the most interesting data (which I consider as the best option).
R1Q1. We thank the Reviewer for the suggestion and changed the title as follows: “A small molecule screen identifies three modulators of C9orf72-derived dipeptide repeat proteins.”, as we think that all the molecules we are proposing deserve attention.

1-2) Unfortunately, the description and presentation of the initial drug screen is not very clear. For instance, it is not clear when cycloheximide was exactly added. It seems likely that it is added at the same moment as the compounds, 3 h after transfection. In that case, it is weird that cycloheximide has no effect on the GP-GFP expression and the explanation that the stability of the DPRs is the cause of this lack of effect is unlikely as there will be not a lot of GP-GFP formed during this limited time frame. Much more information should be provided on this initial assay.
R1Q2. We apologize for poor description of the drug screen. According to the Reviewer’s suggestion, we clearly state that Cycloheximide and the compounds were added simultaneously, after three hours of transfection. We thank the Reviewer for suggesting a recent work of Kearse *et al*., 2019 (PMID: 31171704) in which they showed that non-AUG

translation is resistant to the inhibitors of elongation including Cycloheximide. This is in line with the data we observed, being the polyGP-GFP without an AUG and with further observation related to translation-related factors that will be described in another manuscript. Accordingly, we removed the speculation about DPRs stability and reported this reference.

1-3) In addition, the GP-GFP staining shown in Supplementary Figure 1d is not convincing as only a very limited number of cells show a bright green staining. Most cells show a very low signal. In addition, the quality of some of these pictures is low. A similar remark can be made about the figures shown in the confirmatory screening shown in Fig. 1b. What does a negative value for 'Average of cells positive' exactly means? What do the dotted lines represent? Despite all the information provided, it is also not clear on which basis the small molecules were selected. There also seem to be other compounds that show a similar effect and that were not selected. Overall, a much better and detailed explanation of this initial assay should be provided.

R1Q3. We thank the Reviewer for raising this issue. We agree that the green staining has low intensity compared to a canonical IF experiment. The data were acquired using an automatic microscope Operetta High-Content Imaging System (Perkin Elmer) at 10x magnification on which the analyses was performed a well-established procedure for automatic image acquisition.

In the revised manuscript, we renamed the Y-axis of Fig 1B (HTS) as “Z-score of cells expressing AUG-RFP” and X-axis as Z-score of cells expressing polyGP-GFP. We also renamed the Y-axis of Fig 1C (confirmatory screening) as “Z-score of the cells expressing the reporters” meaning that for each compound it is possible to control its effect on both AUG-RFP and polyGP-GFP reporter expression. As specified in Y-axis, values are expressed as Z-score. As reported by Bray *et al*., 2017 (PMID: 23469374) the Z-score merges the measurement population distributions on each plate to a common distribution with zero mean and unit variance. Therefore, the Z-score is positive if the value lies above the average and negative if it lies below the average.

We thank the Reviewer for noticing the mistake of the threshold (the dotted lines) in Fig 1B. In the revised manuscript, we corrected Fig 1B and the dotted line in Fig 1C has been removed and replaced with a clearer explanation of the criteria used for selecting the five small molecules. This additional explanation is now in the text, M&M, and Dataset EV1 (previous Supplementary Table 1). Briefly, in the confirmatory screen we applied an arbitrary threshold to select compounds showing selective modulation of DPR levels. We agree that more small molecules could have be chosen. We reorganized the Dataset EV1 in a more easily readable format.

1-4) The Drosophila data are very interesting. However, it is not clear whether the impressive survival effects are indeed due to an effect of the inhibitors or the siRNA on the expression of the DPRs. As a consequence, it is crucial that the effect of the compound as well as of the siRNA is shown on the expression level of the DPRs. Otherwise, it can't be excluded that the positive effects are due to an interference with a completely different pathway. Moreover, the question also arises whether forskolin will have the opposite effect and whether it will enhance the phenotype in the repeat-containing flies.

R1Q4. We thank the Reviewer for the suggestion and pointing out this issue, which we agree is crucial to our work. In line with this, we did try to detect DPRs in *Drosophila* heads. Despite several attempts, we obtained faint and not always reproducible bands by

immunoblotting analysis. We did also change our protocols, using the methodology reported in Mizielinska *et al*., 2014 (PMID: 25103406). Unfortunately, DPRs were not detected. Given these results, we now stated in the discussion that we cannot exclude that the observed positive effects in *Drosophila* may depend on alternative pathways. However, the data obtained in C9orf72 patients derived iPSC-motor neurons are in line with the data derived from cell lines (please see R3Q5). Concerning the survival data, we reported the survival rate accompanying the climbing experiments. The experiments with H89 were performed three times, with a total n=15 each, total for each genotype, sex and treatment of individuals = 45, the experiments with SiRNA were performed twice with two different interfering agents (N=15 for each genotype and sex, total = 30). We agree that this number may not necessarily be satisfactory for a precise estimation of the survival rate. For this reason, we moved the survival curves to the expanded view figure (EV4A-D). We have performed climbing experiments using Forskolin and we did not observe an opposite effect. However, this data is to be considered preliminary and, in the graph below, please observe the Y-axis indicates the flies laying on the bottom of the vial.

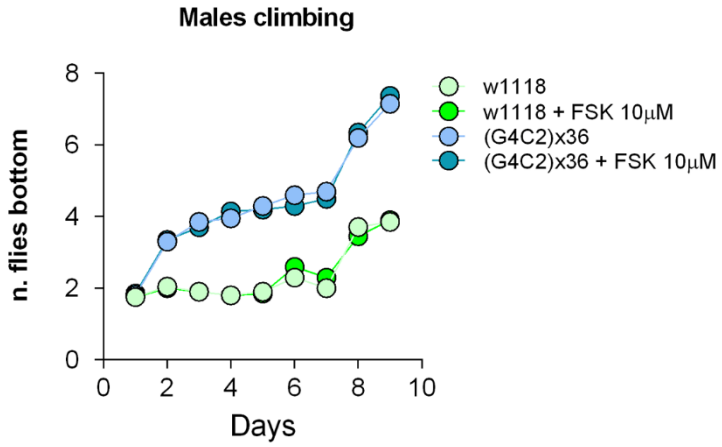


Figure 1. Graph showing the climbing ability flies carrying the *UAS-(G4C2)x36* construct in neurons using the *Elav-gal4* promoter upon treatment with FSK 10 µM diluted with 0.1 % of DMSO and 5% sucrose in PBS. The *wild-type* (w1118) line was used as control. No significant results were observed.

Minor remarks

-An abbreviation in the title should be avoided. Moreover, this abbreviation is also not defined in the abstract.
We thank the Reviewer for this comment we changed the title accordingly.

-The added value of the negative results obtained in SH-SY5Y mentioned in the beginning of the result section (part of supplementary figure 1) is not very clear.
We thank the Reviewer for this comment we removed the negative results from the text.

-Is the transfection efficiency for each of the constructs used in the assay always the same? Are always the same cells transfected with both constructs?
We apologize for not having been clear. Despite a batch-to-batch variation in the overall transfection efficiency (12%-35%), the data were normalized plate-wise and a minimum, but still a sufficient amount, of about 500 polyGP-GFP and about 1500 AUG-RFP transfected cells were analyzed (now specified in the Materials and Methods section).

-The schematic representation of the construct doesn't contain information on what is upstream of the repeat. Are there no potential near-cognate start codons present upstream of the repeat in the GFP/GP reading frame? This upstream sequence information should be provided.

We apologize for not having clearly specified it. No upstream sequence is present in the polyGP-GFP plasmid used for the screening. As reported in the text, all the information about the plasmid is contained in Freibaum *et al*., 2015 (PMID: 26308899). In the revised manuscript, more information is now reported in the caption of Fig 1A and in Materials and Methods.

-The very important information in the scatter plot in Fig. 1b should be explained in more detail. What are the units on the X/Y axes? Where are the black dots (DMSO)? Legend refers to 'with dots'. Which compounds are considered as hits?

We apologize for the poor description. In the revised manuscript, we improved the figure and explained in more detail this issue in the caption of Fig 1. Briefly, we counted polyGP-GFP or AUG-RFP positive cells according to the treatment and irrespectively of single or double transfection. The scale indicates the Z-score of cells expressing the reporters. In the revised manuscript, to improve the visualization of dots corresponding to DMSO treatment, we re-coloured the black dots as orange dots, and the white dots (we apologize for the typo) indicating CHX as purple dots. The majority of orange dots (DMSO) (now shown in Fig EV1D) are covered by the central cloud of ineffective compounds. Hit selection for the confirmatory screen, as described in the text, was based on two arbitrary thresholds represented by dotted lines (polyGP-GFP ±1.5 on the X-axis and ±1 for AUG-derived positive cells on the Y-axis) in the graph. The lack of horizontal threshold in the original version of the Fig 1B was a inattention from our part, for which we apologize

-Scale bars are missing on the photos shown on Supplementary figure 1.

We apologize for the omission. Scale bars has been now added in Supplementary Fig1, now renamed as Fig EV1A of the revised manuscript.

-Were thapsigargin, tunicamycin and MG132 part of the screen? Otherwise references should be added.

We apologize for not having been clear. The Reviewer is correct and the compounds were present in one of the compound-libraries used for the screen. In the revised manuscript, we highlighted them in Fig 1C and their presence is clearly stated in the text.

-The identity of the selected compounds should be indicated on Fig. 1b.

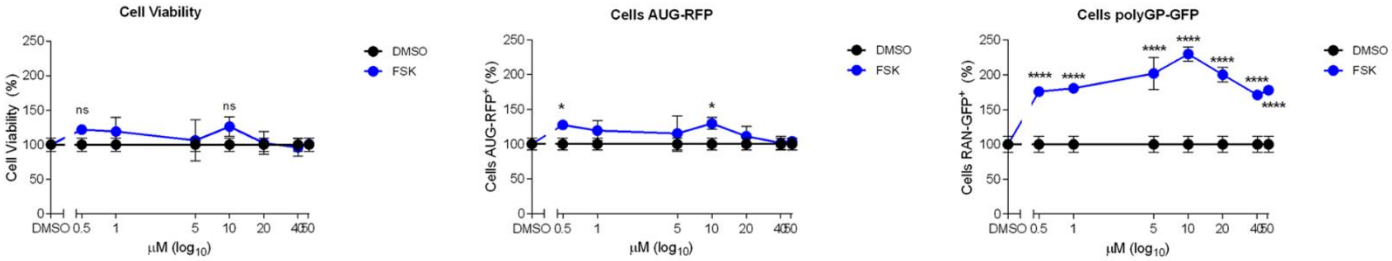
We added the identity of the selected compounds in Figure 1C for more clarity.

-Supplementary Table 1 seems to contain raw data and these should be presented in a much better format making it clear what is exactly shown (with a legend). Names of compounds are not all readable.

We modified the Supplementary Table 1 (now renamed Dataset EV1). Raw data of the primary and confirmatory screens have been left, but we created two further sheets in which we simplified the table. In the first sheet, (Confirmatory refined data) we kept columns with headings of Table 1, for easier interpretation of the data and the selected compounds are highlighted in yellow for easier identification. In the second sheet (Filtered compounds), parameters for compound selection are described.

-Not clear why the higher concentrations of the different compounds are used/shown as most of these compounds affect cell survival, which will potentially compromise the interpretation of these data.

We aimed at identifying a sort of "therapeutic index" for each compound to avoid cell death



effects. We then used non-toxic doses and that is the reason of excluding Helenin (HLN). We clearly specified in the text of the revised manuscript.

-Why is the effect of forskolin not dose dependent? At 0.5 μM the maximal effect is already reached.

We thank the Reviewer for highlighting this issue. We repeated the experiment (it is shown below) and the dose response is clear.

Figure 2. Dose-response analysis. HEK293T cells were co-transfected with polyGP-GFP and AUG-RFP and treated with FSK at 0.5, 1, 5, 10, 20, 40 and 50 μM for 24 h. Images and data were acquired used High-Content Imaging Sistem Operetta. Analysis from three biological replicates. Data are mean ± SD. 2-way ANOVA followed by Šídák's multiple comparisons test, *P < 0.05, **** P < 0.0001.

-The abbreviation for geldanamycin could be confusing as one of the DPRs produced from C9 is GA.

According to Reviewers suggestion, we substituted with GELD.

-Statistics is lacking on panels d and e of Fig. 1. Moreover, t-tests are not the best way to determine significance in case more than two conditions are compared.

We added the statistics in Fig 1D-E. We used 1-way ANOVA followed by Dunnett's multiple comparisons test.

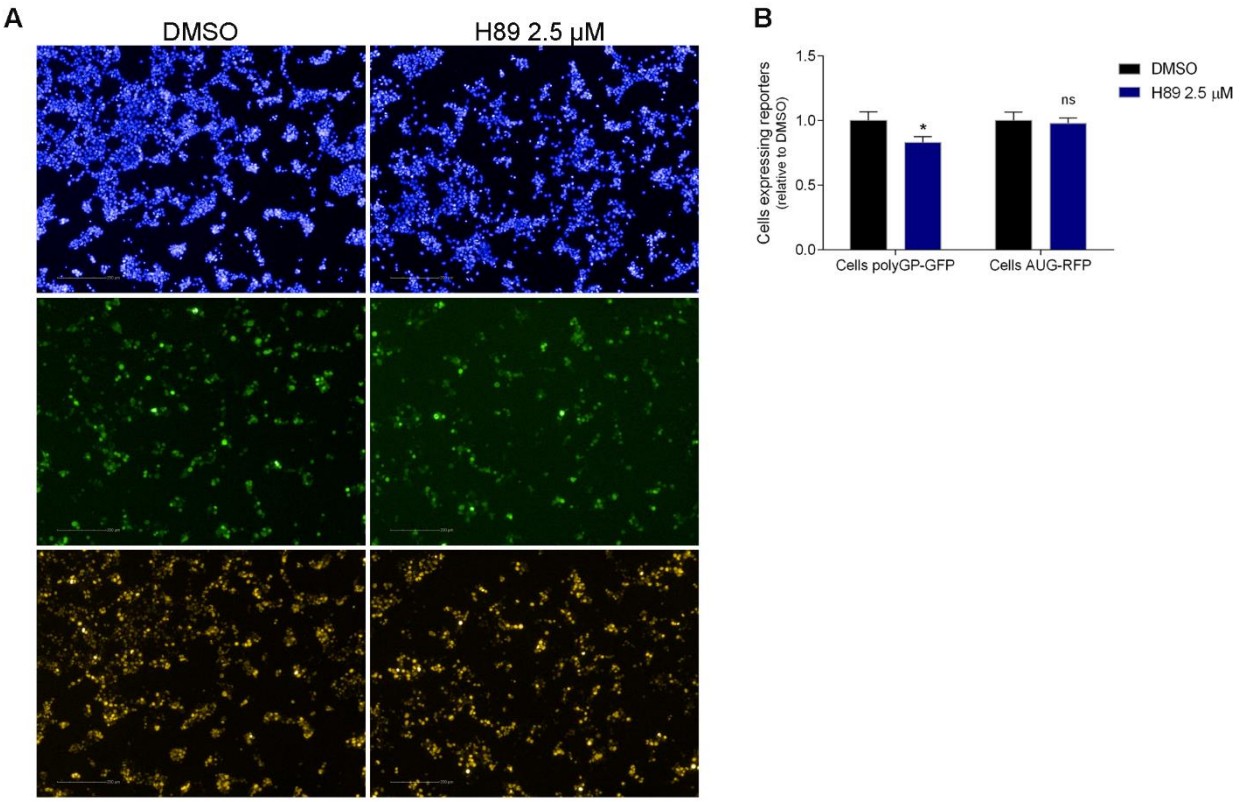
-Table 1: it is not clear at which concentration of the compounds the z scores are shown. Is it 5 μM? Same question for Fig. 2a.

We apologize for the imprecise description; we added this information into the captions of Fig 1 and Table 1. Compound-libraries and CHX were used at the final concentration of 5 μM.

-While erysolin was selected from the initial screen, follow-up experiments don't seem to confirm the effect of this compound. As a consequence, the question arises whether this was a false-positive hit. If so, this should be explicitly mentioned (or another explanation should be provided).

The Reviewer is correct and we apologize for not having clearly specified that. We consider Erysolin a false positive. In the revised manuscript, it is outlined in the text.

-While in the initial screen, a comparison is made with the effect on ATG mediated expression, this is not longer the case for H89. Was this tested? In addition, pictures comparable to the ones shown in Fig. 2a would be very helpful in visualizing the effect of the different PKA modulating drugs.



H89 was not present in the compound-libraries used for the screening. As reported in the manuscript, we performed immunoblot assay using an AUG-GFP plasmid (FigEV2A, B) and luciferase assay (Fig 4F) using plasmids containing the sequence above the repeat with the native CUG or mutated one. We obtained new data (Fig 3) consistent with the previous ones. We also add here pictures and related analysis of H89-treated cells. Taken these data all together, we show that H89 decreases the level of DPRs with significant, although not potent, efficacy in short time points as it is shown below.

Figure 3. HEK293T cells were co-transfected with polyGP-GFP and AUG-RFP and treated for 24 h. Images and data were acquired used High-Content Imaging Sistem Operetta. (A) Scale bars, 200 µm. (B) Analysis from three biological replicates. Data are mean ± SD. 2-way ANOVA followed by Šídák's multiple comparisons test, *P < 0.05.

-Supplementary figure 7 only contains one panel and as a consequence it is not needed to add a label. However, it would be interesting to confirm the downregulation at the protein level.

. In the revised manuscript, this figure has been renamed as Appendix Fig S7 and includes data related to the second PKA-RNAi line.

-Typos should be corrected (e.g. line 126: 'many add an opposite effect')

The paper has been reviewed by a native English speaker and all typos corrected.

Referee #2:

In this study, the authors performed a high-throughput screening of chemical compounds to identify modulators of C9orf72 GGGGCC repeat associated DPRs levels. Multiple modulators targeting different cellular pathways were identified. Importantly, a key modulator that elevates cAMP level was found to increase DPRs expression levels. This suggested a role of cAMP/PKA pathway in regulating RAN translation. In support of this, a

PKA inhibitor, H89, inhibits DPR production in cells and rescue toxicity in Drosophila carrying the G4C2X36 repeats. This result was further supported by genetic ablation of PKA catalytic subunits. Together, these findings established a new potential therapeutic target for ALS/FTD that can be studied further.

Overall, the present study is well performed and presentation is clear. However, a significant limitation that reduces its relevance of these findings is the lack of confirmation of effects on endogenous DPR levels in patient derived cells or correction of survival defects in mammalian systems. Beyond these major limitations, some additional experimental suggestions that can improve the current form of the manuscript are provided below.

2-1) Given the limitations noted above, some of the claims made in the abstract are inappropriate. For example "protein clearance and PKA-Cβ as new drug targets for C9ALS/FTD" and "Spironolactone, Geldanamycin and H89 could serve as tool compounds to develop new effective drugs" is a reach without any validation in a human or even mammalian neuronal system. Toning down these claims is highly recommended.
R2Q1. We agree with the Reviewer and in the revised manuscript, we toned down the conclusive paragraph.

2-2) What is the exact sequence in front of the GP-GFP repeat here? This sequence has been shown to matter and I do not think these plasmids used in the screen here have the native sequence above the repeat (and neither do the flies, for that matter). The nLuc plasmids described do have this and yet are never used with nLuc in the paper (except in a control in the supplement without any of these treatments) to test any of their chemical hits. Instead they are doing westerns for a protein that is aggregate prone, which can be problematic. Why not use the nLuc as a much more quantifiable readout for RAN translation, as others have done?
R2Q2. We thank the Reviewer and apologize for lack of precision. The polyGP-GFP plasmid used in the screening does not have the native sequence upstream the repeat. Indeed, we performed this screening before the importance of the CUG was discovered. In the revised manuscript, we reported these details in the text, in the caption of Fig 1A and Materials and Methods. All the information about the plasmid can be also found in Freibaum *et al*., 2015 (PMID: 26308899). The native sequence upstream the repeat is absent in flies that were already used in Mizielinska *et al*., 2014 (PMID: 25103406). We thanks to Professor Peter K Todd that kindly provided us with C9RAN NLuc reporters containing the native sequence above the repeat with the *wild-type* (CUG) and mutated (CCC) near-cognate start codon used in Green *et al*., 2017 (PMID: 29222490). In the revised manuscript, we added new data in Fig 2G and 4F of the revised manuscript with findings that are in line with the expected results. Indeed, while GELD, SPL and H89 decrease C9RAN NLuc signal, FSK increases it.

2-3) The CUG start codon within the intron 1A of C9orf72 has been shown to be important for RAN translation. To evaluate whether the effect of these compounds was CUG independent, the authors used a 66xGA construct containing endogenous The CUG start codon within the intron 1A of C9orf72 has been shown to be important for RAN translation. To evaluate whether the effect of these compounds was CUG independent, the authors used a 66xGA construct containing the endogenous near-cognate CUG codon and the

compounds still worked. However, they lack the right comparator to test this. Please also do this using the nLuc assay with and without the CUG (mutated to CCC or AAA) and see if this precludes the interaction in the GA and reading frames.
R2Q3. We appreciated and followed this insightful suggestion using the C9RAN NLuc reporters kindly provided by Professor Peter K Todd and used in Green *et al*., 2017 (PMID: 29222490). H89 did not lose its ability to decrease NLuc expression in either condition. These data are reported in Fig 4G of the revised manuscript.

2-4) In general Figure descriptions should be more self-explanatory. For example - The way hits were selected from the screen as described in the method - 'Hits were defined as compounds capable to selectively reduce or increase the number of cells polyGP-GFP positive...'. However, representation of the results is not very clear in the description of figures. a. e.g., in Fig 1c., what does the positive (+) or negative (-) population of cells represent? - cells that express RFP but not GFP? Are these Z-scores, total numbers of cells or percent of cells? For intensity, are the values normalized? Please clarify for both panels in the figure description.
R2Q4. Please see R1Q2 reply.

2-5) The lack of CHX effect on GP is problematic, as is their explanation. "We observed that CHX did not decrease the fluorescence intensity or the number 10^9 of cells expressing polyGP-GFP (Supplementary Fig. 1f), possibly due to the stability of the DPRs in this time-frame." If there are major stability differences between AUG-RFP and their GP reporter then this could lead to mis-interpretation of their data.
a. Can you provide some data regarding the relative stability of these factors in your assay to support your model?
b. If stability is different, then I would be more concerned about these compounds/interventions impacting stability rather than RAN translation per se. As such, please look at whether stability is altered by your interventions.
c. An alternative explanation for the CHX result relates to the translation inhibitor used. Please consider trying a different inhibitor that works more cleanly on RAN constructs, such as Puromycin. Please see Kearse et al, 2019 Genes and Development and Kearse et al, 2016 Mol Cell for further explanation of this point. (as an aside, at least Kearse et al 2016 should be cited as well as this was the first mechanism paper on RAN translation and is thus relevant to this manuscript).
R2Q5. We really appreciate and thank the Reviewer for this suggestion. Indeed, the alternative explanation he suggested is in keeping with our results. We have introduced the two references as suggested.

2-6) When testing effects of the compounds on autophagy markers, the authors conclude - 'None of the compounds induced neither the protein expression nor the transcripts level of autophagy markers....'. From the Fig 3C it seems FSK significantly changes TFEB mRNA level. Please include this in the results with proper explanations. In addition, it would be appropriate to evaluate the impact of these drugs/siRNAs on expression of your AUG-RFP control construct.
R2Q6. We thank the Reviewer for this comment. We performed new experiments (reported in the new Appendix Fig S3A-D) aimed to evaluate the modulation of TFEB and to better characterize its possible role in this context. We did not observe activation or the reduction in TFEB nuclear translocation not of its protein level. Together the results

indicate that none of the compounds significantly modulates the TFEB-mediated autophagy activation. We discussed the new results in the text of the revised result section. We also tested GELD, SPL, FSK and H89 to evaluate whether they modulated the protein level of AUG-GFP and we did not observe any effect (please see Fig EV2A, B of the revised manuscript).

2-7) Authors tested effect of either PKACα or PKACβ on DPR production. Was there a greater effect if both subunits are knocked down together?
a. How many fly lines were used for each modifier? Generally, at least 2 independent siRNA or mutant lines are needed.
R2Q7. We knocked down both subunits simultaneously and observed a stronger downregulation of DPRs expression (Appendix Fig S6H-K). Adult flies (15) were used for each genotype (each experiment was performed three times, n tot=45). Two independent Pka-C1-RNAi lines were used obtaining similar results (Please see Fig 6C and D, Fig EV4C and D and Appendix Fig S7B-E of the revised manuscript).
b. Please provide the number of flies (N=?) used for survival experiments.
We used 15 adult flies for each genotype and each experiment was performed three times, Ntot=45. In the revised manuscript, we added this information in the figures caption (Fig 6, Fig EV4 and Appendix Fig S7). Please see reply to Reviewer #1 R1Q4.
c. Why does the survival of 36xGGGGCC flies alone (without any modifier or drugs) appears to be different in Fig 6 vs. Fig 7?
We apologize for not having been clear; the two experiments (now in Fig 6A-D, Fig EV4A-D and Appendix Fig S7B-E) were performed with different settings. The genetic experiments was performed in complete food, so that flies survival is longer. The experiments with drug treatments were run using 5% sucrose in PBS. In this case, the fly's lifespan was reduced. We reported this information in the captions of Fig 6 and Fig EV4 and in Materials and Methods section.
d. Why were no eye phenotypes observed or monitored? These are a prominent feature in this fly line.
The Reviewer is correct but in our experiments we used the pan-neuronal *Elav-Gal4* driver to express C9orf72 with (G4C2)x36 (Mizielinska *et al*., PMID: 25103406) in neurons. This approach did not result in any visible morphological change of the eye. We never used the *GMR-Gal4* driver that instead gives a mild phenotype in the eye when C9orf72-(G4C2)x36 is expressed (Mizielinska *et al*., PMID: 25103406).

2-8) A limitation of the work here is that they did not confirm the effects of PKA inhibition in drosophila were dependent on RAN translation. This could be done with publically available GR flies which initiate with an AUG and lack the repeat sequence.
R2Q8. The Reviewer is correct; this is a limitation of the study. Unfortunately, we were not able to detect DPRs in fly heads. To provide additional and complementary data that support our conclusions, we used in HEK293T cells either the AUG-GFP plasmid (please see new results in Fig EV2C-F) and the AUG-GAx100 and AUG-GPx100 FLAG-tagged plasmids (Yamakawa *et al*., 2015; PMID: 25398948) that does not contain the repeat sequence (please see new results in Fig EV3A-E). The role of PKA is currently under further investigation in C9orf72 patient derived iPSC-motor neurons.

Minor points

- Line 66-67. "Importantly, no small molecules are known to selectively modulate RAN translation..." This is not a correct statement! Need to re-write.
We agree and apologize for this imprecision. We meant, “no clinically approved drug”. We changed the text in the revised manuscript.

- Line 76-77. DDX3X modulates FMR1 associated RAN translation in opposite way. Please mention recent paper by Lisalata, AE et al., (EMBO Report, 2019) as well.
We thank the Reviewer for this suggestion, we added the requested reference in the revised manuscript.

- Line 114. What is CAP-product?
We apologize this was an oversimplification. We replaced with AUG-products.

- Line 127-128. Compounds that induce cellular stress may also increase RAN, such as Thapsigargin. Is there any other compound?
We used a library of autophagy modulators. The entire list of small molecules used can be found in Dataset EV1. We highlighted the cell stressors that increased RAN products in Dataset EV1and we marked Thapsigargin, MG132 and Tunicamycin in Fig 1C of the revised manuscript.

- Line 159. Along with Tabet, R. et al. (Ref 31) Green et al., (Ref 30) showed utilization of the CUG initiation.
We thank the Reviewer for this suggestion and we added this reference.

- Line 483. Addition not addiction Figure Legend 1b. CHX treatment: please fix the color code mention in the legend 1d-e. Concentrations of drug in the represented as log [ ] in the figure. Is that correct?
We thank the Reviewer for this suggestion and we corrected the typo and the colour code. We confirm that concentrations are expressed in $\log_{10}$ [ ].

Referee #3:

In this study, Licata et al. performed a high-throughput drug screen based on the expression of polyGP RAN proteins in the cells transfected with GFP-tagged G4C2 repeat constructs. Several hits were identified that could decrease or increase the expression of polyGP-GFP fusion proteins by targeting the proteasome degradation or AC/PKA pathways. Further analyses shows that H-89, which inhibits PKA catalytic subunit β, down-regulates expression of C9 DPRs in transfected cells. Additionally, PKA inhibition behavior and survival phenotypes in a Drosophila model of C9 ALS/FTD. These data provide additional tools to understand the mechanisms of RAN translation and may contribute to developing therapeutic strategies for C9 ALS/FTD. Overall, this is a potentially interesting story, but a rigorous testing of the effects of these compounds and testing in alternative systems that don't grossly overexpress the proteins is needed to understand the potential impact of this story. Specific concerns and suggestions are listed below.

Major concerns:

3-1) Figure 2C - why does CHX not block protein synthesis and what was the rationale for the 3 hr assessment here vs. 24 hours for the other compounds. It doesn't seem like the positive control for reducing protein synthesis here is working.
R3Q1. We used a higher dose of CHX than the other compounds in order to have a good technical positive control.

3-2) Supplementary Figure 1A. An increasing body of evidence showed RAN translation across G4C2 repeat expansion is dependent on repeat length, flanking sequences and cell types. Does the (G4C2)58-GFP construct used for screening, which contains the upstream CUG contain additional endogenous human intron 1 C9orf72 sequence upstream and downstream of (G4C2)66 repeat expansion?
R3Q2) We apologize again for not having been clear, please see R2Q2 in which we did an extensive reply. The (G4C2)x58-GFP construct does not contain the native sequence above the repeat.

3-3) Different constructs were used to test if the identified compounds reduce polyGA-(HA) or polyGP using different cell types (HEK293T for GA) and (NSC34 for GP). Are similar results found when the cells used for these assays are swapped and are the reductions seen restricted to very specific cell types for each DPR type or are the drug effects more general across a range of different cell types.
R3Q3) We used the same plasmids for the two cell lines but we swapped the antibodies. Specifically, the (G4C2)x66 construct (Gendron *et al*., 2013; PMID: 24129584) contains a specific tag in each frame (HA in the GA frame, His in the GP frame and FLAG in the GR frame). In HEK293T cells, since DPR levels was high, we preferred to detect GA by using an antibody against the HA tag, whereas in NSC34 cells, where the DPR levels was low, we used an antibody against GP. Therefore, we did not try the same antibodies on all cell lines.

3-4). Additionally, can polyGR be also decreased by these compounds?
R3Q4) Unfortunately, we have been not able to detect poly-GR. We tried using an antibody against GR (MABN778, Merk Millipore) and one against the FLAG tag (F1804, Sigma-Aldrich).

3-5). Overexpression of relatively short G4C2 repeat constructs was used for both screening and validation of selected compounds. G4C2 repeat expansions are generally from a few hundreds to thousands in C9 patients and expression of DPRs are at much lower levels compared to overexpression of G4C2 repeat expansion in cells. Reduction of DPRs by these compounds should be tested in a C9 patient-derived cell line, such as iPSC line.
R3Q5) We agree with the Reviewer that this is an important experiment. We have now treated three independent patient derived iPSC-lines with H89 and observed a significant decrease in both GA and GP, without affecting transcript levels or causing cytotoxicity. This provides compelling evidence that H89 reduces endogenously RAN translated DPRs, as now showed in the Figure 7 and associated Appendix Figures S8, S9 and S10 of the revised manuscript.

3-6). The authors claim that Geldanamycin and Spironolactone reduce levels of DPRs by enhancing UPS activity. It is not clear however if the DPRs are degraded through UPS.

Are the DPRs ubiquitinated in the transfected cells? Do UPS inhibitors block the degradation of DPRs?

R3Q6) We thank the Reviewer for this thoughtful comment. We followed this excellent suggestion and performed new experiments studying the effect of UPS and autophagy inhibition on the poly-GP degradation promoted by GELD and SPL. We observed that poly-GP is poly-ubiquitinated (Fig 3E). In addition, to provide additional results excluding a possible effect linked to translation modulation, we performed the experiments by overexpressing the poly-GP dipeptide utilizing the AUG-GPx100 plasmid. In this plasmid, poly-GP is encoded without repetitions and its translation start with a canonical AUG codon. The results of these experiments have been included in the new Fig 3F, G of the revised manuscript and show that the SPL effect on poly-GP is counteracted by autophagy inhibition, while GELD effect on poly-GP is counteracted by proteasome inhibition. We also confirmed that SPL does not alter the autophagy flux as showed in LC3 turnover experiment (Appendix Fig S3E) as well as the TFEB-mediated autophagy (Appendix Fig S3A-D). These results indicate that SPL does not play a role in autophagy activation, but it might increase the delivery of these aberrant cargoes to autophagy. This aspect is currently under investigation in our labs. We consequently modify the paragraph adding the new results and modifying the title accordingly.

3-7). It is important to know if PKA inhibition differentially affects polysome profiling for canonical versus RAN translation. In figure 5g and 5h, GAPDH is not a proper control for the overexpression of a G4C2 construct. AUG-RFP should be used as a control for the polysome profiling experiments. Also, a C9 patient-derived-iPSC line could be used for the polysome profiling experiments.

R3Q7) We agree that GAPDH is not the proper control for an overexpressed transcript, but, in our opinion, it is a good control for an endogenous one. We repeated the experiment with a pcDNA3-based, AUG-GFP plasmid (please see Fig EV2G-J of the revised manuscript) to be exactly comparable with the RAN-GFP plasmid. We did not observe any unloading of the transcripts from the heavier polysomal fractions. Unfortunately, due to massive work required and the technical difficulties, we could not perform the same experiment on C9orf72 patient-derived-iPSC motor neurons and we aim to perform this experiment in the next future.

3.8) The authors showed that inhibition of PKA reduces expression of DPRs, but little is known about the connections between the PKA pathway and the translation of DPRs. Does PKA inhibition also affect canonical translation initiation? Does PKA inhibition modulate the phosphorylation of eIF2α?

R3Q8) The Reviewer is correct, we are investigating this point and it will be presented in another publication.

3.9) One of the core findings in this study is that inhibition of PKA Cβ decreases RAN-translated DPRs across G4C2 repeat expansions. In figures 6 and 7, data showed PKA inhibition through both H89 administration and RNAi interference ameliorates motility deficits and survival in a Drosophila model of C9 ALS/FTD. But it is important to show the expression of DPRs in this model and also how PKA inhibition affects DPR expression.

R3Q9) The Reviewer is correct and we tried extensively to detect the DPRs level (please see R2Q8 for more details), but we failed. We discussed this point in the text of the new version of the manuscript.

Minor points:

1. The text on page 4 describing the reporter construct for S1A does not match the figure and needs revision.
We thank the Reviewer for reporting this omission. However, according to Reviewer #1 request (see its second minor remark), we removed these data.

2. Figure 1C should indicate the names of the compounds that were used for the various hits indicated by asterisks or use different symbols so that how the data appear in this figure can be linked to the specific compound.
We followed this suggestion and marked the selected small molecules and the cellular stressors with different symbols.

3. Figure 2D - The GA abbreviation used for Geldanamycin to measure myc-tagged Gly-Ala protein also abbreviated GA is confusing. Please use a different abbreviation for the drug throughout the manuscript.
The Reviewer is correct and we changed the abbreviation of Geldanamycin to GELD.

4 A brief introduction of the Adenylyl cyclase/Protein Kinase A (AC/PKA) pathway is needed to make the findings accessible to a broad readership.
We added a short description of the AC/PKA pathway prior to introduce the part relating H89 as requested.

5. The (G4C2)n should be consistently used through the manuscript including main text, figure legends, as well as in figures. Such as: line 37 on page2, G4C2X36 should be (G4C2)36; line 48 on page 3, GGGGCCn (also known as G4C2n) should be (GGGGCC)n, (G4C2)n.
The Reviewer is correct and we followed this suggestion and replaced with the form “(G4C2)xN" throughout the entire manuscript.

6. Please, clearly define the soluble and PBS-insoluble fractions for western blot and filter retardation assay. Such as line 166 on page 6: 'the soluble levels and the PBS-insoluble species of poly-GP'
We modified the sentence related to the PBS-insoluble fraction, and we added a sentence in M&M section, which better clarifies the significance of Filter Retardation Assay (FRA).

7. In figure 2h, are there significant differences?
We obtained significant results using 1-way ANOVA followed by Uncorrected Fisher's LSD FSK versus DMSO (P** = 0.0034). We reported this statistical analysis in the caption of the Fig 2H (renamed as Fig 2F in the revised manuscript).

8. In supplementary figure 1b, the legend should describe the levels of RAN products.
According to Reviewer #1 request (see the second minor remark), we removed the data.

9. Please define G4C2 (+1) and G4C2 (+2) mRNAs in supplementary figure 2b.
Primers used to amplify the RAN mRNAs have a forward primer in common downstream of repetition region but being part of the native gene sequence. In G4C2 (+1) the reverse primer is in the flag tag (amplified sequence 96bp); in G4C2 (+2) the reverse primer is in

HA tag (amplified sequence 124 bp). As PCR amplification gave similar results, we used only G4C2 (+1) couple for the following experiments.

10. Line 9 on page 33: CHX 5μM (with dots). 'With' should be a color.
We apologize for the typo; it should have been “white”. In the revised manuscript, we modified the colours of negative and positive controls (orange and purple, respectively) for better visualization

Dear Alessandro,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by referees #1 and 2.

As you can see from the reports, the revision received a bit of a mixed response from the referees and it is clear that the manuscript still needs work. I have discussed the raised issues further with the referees and everyone agrees that the there is a value to the reported findings. However, the manuscript needs to be re-organised and some further experiments are needed. It is OK if some of the results are not definitive, but make sure not to overstate the conclusions.

Should you be able to address the raised concerns in full then I am willing to consider a revised version. Let me know if we should discuss the specific revisions further.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

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

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

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline

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

Please see out instructions to authors
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 5th Jul 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

While the authors give the impression that all problems are solved, this is clearly not always the case. The very long answers do not always allow to exactly pinpoint what was exactly done. Remarkably, the answers to my questions also contain information not related at all to the questions which I asked (which makes it more difficult to find the exact answers). In addition, the formatting of the answers to the referees also doesn't help as figures and legends are separated and are inserted (more or less randomly) in the text.

Major comments
1-1) The new title contains an important typo! Moreover, the title is now extremely vague. What do these 'modulators' exactly do? Which three modulators? Not even completely clear after reading the abstract.... Overall, I am not convinced at all that this is a better title. On the contrary. Also, the running title is not very informative. Moreover, it is a pity that the manuscript is not (even) more focused on the most interesting findings.
1-2) The cycloheximide problem isn't solved (only explained in a better way). As far as I can judge, it can't be considered as a positive control and using/mentioning it as such remains confusing. It is indicated in the answers to the referees that I suggested the Kearse et al. citation. I didn't.
1-3) That the acquisition of the images was done with the Operetta High-Content Imaging system doesn't solve the issue that the GP-GFP staining in most cells is very low. Whether this is a well-established procedure or not is not really relevant and doesn't answer my question. I am also still struggling with the selection/definition of the 'arbitrary threshold'.
1-4) The authors agree that the interpretation of the Drosophila data could be different from what was stated in the original version of the manuscript (as they were not able to proof that there was indeed an effect of the siRNAs on the expression of the DPRs) and indicate this in their discussion. Not clear why so much information not related to my questions is presented in this answer. I am also puzzled by the absence of a negative effect of forskolin (which might be a major problem). As far as I can judge, this is not mentioned in the manuscript.

Minor comments
- It is indicated that ERY was not validated in the confirmatory screening (line 130-131). Why? When this compound is tested in the subsequent assay, it is now suddenly considered as a false positive (as an answer to my question). This remains confusing.
- I am a bit puzzled by the new results shown in Figure 3 in the answers to the referees. The effect of H89 on poly-GP-GFP is rather small. The effect is also hardly visible on the representative photos. The impression also exists that the background in both conditions is not the same. Isn't this a bit unexpected? Do the authors have an explanation for this relatively small effect of H89? Why are these results not incorporated in the revised manuscript (as supplementary material)?
- Despite the fact that the manuscript was corrected by a native speaker, it still contains (too) many mistakes (which do not always have to do with the English language). Just a few examples. The first sentence of the abstract contains a ',' that should not be there. What is exactly meant by 'The repeat-containing isoforms'? The repeats are present in introns and no longer in the C9orf72 isoforms. The abstract also indicates that there was knockdown of PKA-catalytic subunits, while only one subunit (Pka-C1) was genetically reduced. These are some examples from the abstract. The entire manuscript contains these kinds of 'mistakes' which significantly compromise the readability of the manuscript.
- The introduction is not very well written. A more up-to-date view on the current knowledge should be included. While it was initially indeed indicated that "pathological expansions of (G4C2)n reduce C9orf72 transcription causing loss of function of the C9ORF72 protein", the current view on the role of a loss of function is slightly different. Moreover, the C9ORF72 protein is still OK. There might be a little bit less (although not every study can confirm this).

Referee #2:

In this manuscript, Licata et al performed a screen for modulators of G4C2 RAN translation from linearized transfected RNAs lacking the native sequence context upstream of the repeat. These studies identified PKA pathways as important in the expression levels of GP-GFP. They further studied these pathways in drosophila and iPSC pathways.

Overall, this is an interesting and potentially important manuscript. The presentation of data across three levels (multiple reporters in different cell lines, drosophila and from endogenous sources) is considered a strength and I recognize that this represents a significant amount of work to date. I also appreciate that for the most part they did not over-interpret their data and findings. As PKA is a theoretically targetable pathway, the findings are potentially translatable. However, there are a number of problems that need to be addressed and which lower my enthusiasm.

1) The reporter used for their screen is problematic, as it contains the repeat out of its native sequence context and in a linear capped and poly-adenylated RNA that will rapidly transit to the cytoplasm. While the exact RNA species that undergoes RAN translation in C9orf72 is not yet definitively defined, it is definitely not this. Thus, some key aspects of its biology may be different from what happens in patients.
2) Please provide some more information on these reporters and their screen:
a. The actual sequences of the reporters are not provided- confounding interpretation. In many cases, these sequences were not included in the original publications of these reporters. Please include the sequences of all the reporters used in a supplementary table so that they can be compared and try to make the nomenclature clearer to that they can be kept straight.
b. Please show the relative expression levels of the GP-GFP reporter compared to the AUG-RFP signal by some measure

(western? Or at least GP-GFP to AUG-GFP alone?).
c. It appears that most of the cells are expressing either the RAN product or the AUG-RFP product rather than both together- is this correct? If so, were the dual labeled cells used, the single labeled cells, or both? As the repeats/DPRs can suppress global translation, this can make normalization in these contexts complicated, so the exact methodology used here is worth further description.
d. While CHX doesn't suppress RAN as well as for AUG initiated translation, there are other inhibitors that do (Puromycin, for example). Inclusion of this to achieve a suppression of signal from both reporters would help you establish a true floor for the RAN signal and a proper Z' calculation for RAN translation
3) There is a lot of hopping around between constructs. While this does add rigor, it can make comparisons difficult, especially when assays are done with only one of the potential constructs or drugs and not the others. For the central findings related to GLD, SPL, FSK and H89 treatment at a minimum, they should really evaluate all their constructs, including the AUG-GP and AUG-GA scrambled repeat constructs. Also make it clear in the figures that these are scrambled repeats (not obvious from your naming choices).
4) Please make sure that the AUG-GP and AUG-GA western results are in the linear range for detection and include measurement of both soluble and insoluble forms of the proteins.
5) Is FSK treatment activating stress pathways?
6) The polysome data is quite important, but lacks some important controls/experimental groups. First, the construct used for the initial screen (GP-GFP) should be included here, as it lacks the CUG which supports a lot of the translation from the G4C2 66 construct and thus may distribute differently in these preps. Second, the effects of H89 and FSK should be shown on these polysome profiles to confirm they have the effects predicted based on their end-product detection assays.
7) For the fly studies, they need to confirm that their manipulations are not just altering transgene expression at the transcription level. In general, the correct control for this kind of uas-siRNA manipulation is a non-targeting uas-siRNA (say, LacZ) as the presence of any additional UAS site can lower expression from the toxic transgene and thus give a false "suppression" signal. While the drug studies are not necessarily confounded by this, it suffers from its own limitations (see below).
8) Their iPSC data is important but is not very compelling. The lack of effect of FSK is quite striking given that this was the most robust effect in their screen assays. Second, they should be doing within-line comparisons as a paired t-tests rather than pooling all three lines, which currently makes the stats very hard to compare for the drug studies (the variance between lines is often greater than between treatment groups). Third, LDH release is inadequate as a readout here and there is a lack of H89 treatment on a control line to see the effect there and they saw discrepant effects between the 2 C9 lines they evaluated. As such, I am not compelled that H89 is really suppressing toxicity in any meaningful way. Fourth, the C9 repeat RNA levels are not measured here, just the mature product which lacks the repeats. Some assay to look at it (say, RNA foci counting) would be informative.
9) H89 is not PKA specific, yet it is really the only drug that shows effects in iPSCs. It inhibits at least 8 other kinases, while having a relatively large number of PKA-independent effects which may seriously compromise interpretation of data. At a minimum, this needs to be recognized as a limitation in the discussion.

Referee #1:
While the authors give the impression that all problems are solved, this is clearly not always the case. The very long answers do not always allow to exactly pinpoint what was exactly done. Remarkably, the answers to my questions also contain information not related at all to the questions which I asked (which makes it more difficult to find the exact answers). In addition, the formatting of the answers to the referees also doesn't help as figures and legends are separated and are inserted (more or less randomly) in the text.

We were sorry to read the comment of the Reviewer. In our first reply we tried to do our best to provide the best possible and complete answers to all points raised in the first round of revision. If our replies were not clear we sincerely apologize and we hope that in this second round of replies our comments will be clearer than before and are sufficiently detailed to fully reply to the reiterated and new questions raised by the Reviewer.

Major comments
1-1) The new title contains an important typo! Moreover, the title is now extremely vague. What do these 'modulators' exactly do? Which three modulators? Not even completely clear after reading the abstract.... Overall, I am not convinced at all that this is a better title. On the contrary. Also, the running title is not very informative. Moreover, it is a pity that the manuscript is not (even) more focused on the most interesting findings.

As suggested, we have done our best to find a very informative title, we are sorry that the reviewer did not like the second title. We hope that this third version will satisfy the requirement to be more informative; we changed the title to

"C9orf72 FTD/ALS dipeptide repeat protein levels are reduced by small molecules that inhibit protein kinase A or enhance protein degradation"

As running title, we changed into:

"Small molecule modulators of C9ALS/FTD-associated DPRs"

1-2) The cycloheximide problem isn't solved (only explained in a better way). As far as I can judge, it can't be considered as a positive control and using/mentioning it as such remains confusing. It is indicated in the answers to the referees that I suggested the Kearse et al. citation. I didn't.

We apologize, we realized that we erroneously attributed the suggestion to Reviewer #1; it was Reviewer #2. We also agree that stating CHX is a positive control was confusing. We have now amended the text to state that we used CHX to model inhibition of general translation and used it to optimize screening parameters for our canonical translation reporter. We hope this is clearer now. In our system, CHX does appear to specifically affect AUG initiated translation, as already shown by Kearse et al (Kearse et al., 2019). While this is of interest, we don't feel it is within the aims of our study to explore the mechanistic reasons of why CHX has different effects on RAN vs AUG initiated translation.

1-3) That the acquisition of the images was done with the Operetta High-Content Imaging system doesn't solve the issue that the GP-GFP staining in most cells is very low. Whether this is a well-established procedure or not is not really relevant and doesn't answer my question. I am also still struggling with the selection/definition of the 'arbitrary threshold'.

We understand the request of the reviewer, but as it is often the case for image-based screens, the quality of images is poorer than that obtained for single analyses using confocal microscopy. The entire procedure is now reported in Appendix M&M (see page 24). Shortly, we set thresholds at Z-score of ±1.5 for the AUG-RFP, since it allowed to discriminate DMSO treated control population from CHX population with a probability p<0.05% of behaving as false negative and of 0.78% as false positive (False positive risk calculation). Similarly, we used ±1.5 as thresholds for selecting compounds decreasing RAN-GFP with the risk of false negative hits was p<0.05%. Therefore, we obtained as robust thresholds as possible, but as with any screen, we then performed a range of different validation experiments, downstream of the primary screening to validate our findings.

1-4) The authors agree that the interpretation of the Drosophila data could be different from what was stated in the original version of the manuscript (as they were not able to proof that there was indeed an effect of the siRNAs on the expression of the DPRs) and indicate this in their discussion. Not clear why so much information not related to my questions is presented in this answer. I am also puzzled by the absence of a negative effect of forskolin (which might be a major problem). As far as I can judge, this is not mentioned in the manuscript.

We have investigated the toxicity of forskolin at two different doses (see Appendix Fig S8, 100 and 40 µM). Forskolin is toxic both in wild-type and in flies expressing the UAS-C9orf72 G4C2 (36) in neurons using the ELAV-Gal4 promoter. We did not observe a worsening effect of Forskolin on mutant flies, this is now reported in the manuscript (see line 297-300).

Minor comments

- It is indicated that ERY was not validated in the confirmatory screening (line 130-131). Why? When this compound is tested in the subsequent assay, it is now suddenly considered as a false positive (as an answer to my question). This remains confusing.

We apologize for the mistake; indeed, the compound was validated in the confirmatory screen but was not validated subsequently. The reason is reported in the text at line 215-217. We corrected the text at line 135.

- I am a bit puzzled by the new results shown in Figure 3 in the answers to the referees. The effect of H89 on poly-GP-GFP is rather small. The effect is also hardly visible on the representative photos. The impression also exists that the background in both conditions is not the same. Isn't this a bit unexpected? Do the authors have an explanation for this relatively small effect of H89? Why are these results not incorporated in the revised manuscript (as supplementary material)?

We have incorporated the results in Appendix Figure S5. We explain the relative low effect of H89 by the low level of DPRs and low activation of PKA in basal condition. Indeed, the effect of H89 is particularly evident in counteracting FSK activity as observed in Figure 4B-C.

- Despite the fact that the manuscript was corrected by a native speaker, it still contains (too) many mistakes (which do not always have to do with the English language). Just a few examples. The first sentence of the abstract contains a ',' that should not be there. What is exactly meant by 'The repeat-containing isoforms'? The repeats are present in introns and no longer in the C9orf72 isoforms. The abstract also indicates that there was knockdown of PKA-catalytic subunits, while only one subunit (Pka-C1) was genetically reduced. These are some examples from the abstract. The entire manuscript contains these kinds of 'mistakes', which significantly compromise the readability of the manuscript.

We are sorry for this inconvenience. We carefully revised the manuscript again hoping to have found all mistakes mentioned by the Reviewer.

- The introduction is not very well written. A more up-to-date view on the current knowledge should be included. While it was initially indeed indicated that "pathological expansions of (G4C2)n reduce C9orf72 transcription causing loss of function of the C9ORF72 protein", the current view on the role of a loss of function is slightly different. Moreover, the C9ORF72 protein is still OK. There might be a little bit less (although not every study can confirm this).

Even in this case, *we are sorry for this inconvenience. We carefully revised the introduction again and update it to the current knowledge as much as possible.*

Referee #2:

In this manuscript, Licata et al performed a screen for modulators of G4C2 RAN translation from linearized transfected RNAs lacking the native sequence context upstream of the repeat. These studies identified PKA pathways as important in the expression levels of GP-GFP. They further studied these pathways in drosophila and iPSC pathways.

Overall, this is an interesting and potentially important manuscript. The presentation of data across three levels (multiple reporters in different cell lines, drosophila and from endogenous sources) is considered a strength and I recognize that this represents a significant amount of work to date. I also appreciate that for the most part they did not over-interpret their data and findings. As PKA is a theoretically targetable pathway, the findings are potentially translatable. However, there are a number of problems that need to be addressed and which lower my enthusiasm.

Major comments

1) The reporter used for their screen is problematic, as it contains the repeat out of its native sequence context and in a linear capped and poly-adenylated RNA that will rapidly transit to the cytoplasm. While the exact RNA species that undergoes RAN translation in C9orf72 is not yet definitively defined, it is definitely not this. Thus, some key aspects of its biology may be different from what happens in patients.

We agree with the Reviewer that the artificial construct allows a rapid translocation of the reporter mRNA. We decided to use this plasmid to avoid confusing effects due to additional mechanism that will introduce uncontrolled parameters into the analyses (e.g.: aberrant nuclear-cytoplasmic transport or intron splicing effects). However, it must be noted that we used the native intron sequence in the validation section of the study. We have pointed in the discussion that the reporter used for the screening is artificial (see line 340-344), and it does not mirror the situation in patients, and clarified that further validation studies of the screening results are required before a translation to the bedside is possible (see line 456).

2) Please provide some more information on these reporters and their screen:
a. The actual sequences of the reporters are not provided- confounding interpretation. In many cases, these sequences were not included in the original publications of these reporters. Please include the sequences of all the reporters used in a supplementary table so that they can be compared and try to make the nomenclature clearer to that they can be kept straight.

We added all the information we gathered about the plasmids used in this manuscript in Appendix Table 1 (summary of plasmid features) and 2 (nucleotide sequences).

b. Please show the relative expression levels of the GP-GFP reporter compared to the AUG-RFP signal by some measure (western? Or at least GP-GFP to AUG-GFP alone?).

The expressions of the GP-GFP and AUG-RFP reporters by western blots are now reported in Fig EV1B, line 96.

c. It appears that most of the cells are expressing either the RAN product or the AUG-RFP product rather than both together- is this correct? If so, were the dual labeled cells used, the single labeled cells, or both? As the repeats/DPRs can suppress global translation, this can make normalization in these contexts complicated, so the exact methodology used here is worth further description.

Reviewer touches a very important question that we explored during the screening set-up. We now describe and explain the reason of the choice in Appendix M&M (see page 24). Briefly, for Z'-factor calculation, all the transfection outcomes were counted. We found out a robust Z'-factor for AUG-RFP by counting the number of AUG positive cells irrespective of single of double transfection and using CHX as positive control. We did not obtain a significant Z' for RFP in double transfected only cells. Therefore, the readout of the primary screening, Z-score, was calculated irrespectively of whether plasmids were single or

double transfected into cells and of the intensity of the reporters. We agree this can be a limitation of our analysis, but we have been very stringent in the selection criteria of the hits. In addition, we relied on downstream validation using different type of plasmids and methods, which confirmed our initial observations.
Please see also reply to 1.3) question.

d. While CHX doesn't suppress RAN as well as for AUG initiated translation, there are other inhibitors that do (Puromycin, for example). Inclusion of this to achieve a suppression of signal from both reporters would help you establish a true floor for the RAN signal and a proper Z' calculation for RAN translation.

We made preliminary efforts in using puromycin as control, but it did not turn out to produce a reliable Z'-factor. However, in our screen, Puromycin reduced both the number of cells containing AUG and RAN initiated products, (Z-score green cells = -5.32 and Z-score red cells = -7.67).

3) There is a lot of hopping around between constructs. While this does add rigor, it can make comparisons difficult, especially when assays are done with only one of the potential constructs or drugs and not the others. For the central findings related to GLD, SPL, FSK and H89 treatment at a minimum, they should really evaluate all their constructs, including the AUG-GP and AUG-GA scrambled repeat constructs. Also make it clear in the figures that these are scrambled repeats (not obvious from your naming choices).

To help readers we prepared a table of plasmids with all the features inside (see Appendix Table 1). We added the missing western blots (see Figure EV1B).

4) Please make sure that the AUG-GP and AUG-GA western results are in the linear range for detection and include measurement of both soluble and insoluble forms of the proteins.

We performed this control; the experiments were performed in the linear range (15 µg for western blot and 8 µg for FRA). The Reviewer can find the linear range below.

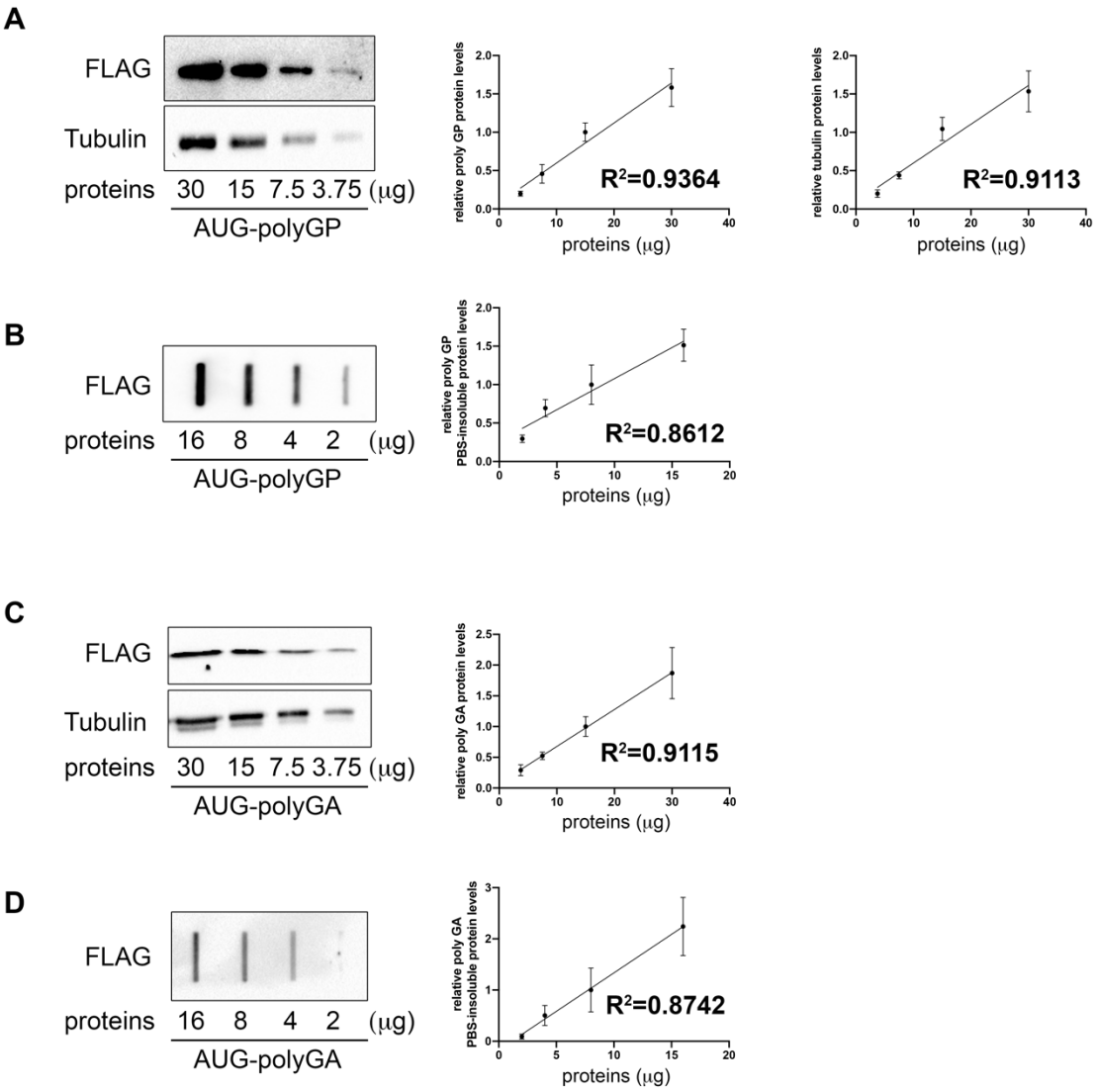

***Figure 3**: Anti-FLAG WB and FRA Linear range. We performed WB and FRA loaded with serial dilutions of cell lysate expressing AUG-polyGP (A and B) or AUG-polyGA (C and D). WB results (A and C) show a R^2 higher than 0.9 and FRA results show a R^2 in a range between 0.85 and 0.9. We can conclude that AUG-GP and AUG-GA western results are in the linear range of anti-FLAG antibody in our experimental condition.*

5) Is FSK treatment activating stress pathways?

It is an important point we are investigating in a different project, and we hope to provide the proper answer in a future manuscript devoted to these pathways; at present, we believe this characterization fall outside the scope of this manuscript.

6) The polysome data is quite important but lacks some important controls/experimental groups. First, the construct used for the initial screen (GP-GFP) should be included here, as it lacks the CUG which supports a lot of the translation from the G4C2 66 construct and thus may distribute differently in these preps. Second, the effects of H89 and FSK should be shown on these polysome profiles to confirm they have the effects predicted based on their end-product detection assays.

We agree these experiments are valuable, but they are not strictly necessary to clarify the mechanism of action as we focused on the molecular targets, i.e. the PKA catalytic

subunits. Similarly, H89 is not necessarily inhibiting PKA only, thus we focused on the genetic silencing, therefore we did not address the mechanism of H89 and forskolin via polysomes.

7) For the fly studies, they need to confirm that their manipulations are not just altering transgene expression at the transcription level. In general, the correct control for this kind of uas-siRNA manipulation is a non-targeting uas-siRNA (say, LacZ) as the presence of any additional UAS site can lower expression from the toxic transgene and thus give a false "suppression" signal. While the drug studies are not necessarily confounded by this, it suffers from its own limitations (see below).

We understand the Reviewer's concern and we agree that the correct control would have been a non-targeting UAS-siRNA. However, we have used many times UAS-Lacz-RNAi or UAS-Luc-RNAi and they did not change the viability or climbing of w1118 animals; on these bases, we are confident that the utilization of the w1118 alone could be used as control in the experiments.

8) Their iPSC data is important but is not very compelling. The lack of effect of FSK is quite striking given that this was the most robust effect in their screen assays.

We have shown all our data to be as open as possible. While there is no significant difference, the trend towards an increase is in the same direction as the other experiments in the manuscript. We thought it was interesting that the only consistent effect observed after 7-day treatment was this increase in DPRs caused by FSK. We also provided the data for each individual cell line (see Appendix Figure S11) and the increase in poly-GP is significant for two out of three C9 patient lines. As the difference is not significant when statistically testing across all lines, we have not made any claim in the manuscript, but felt it was worth including.

Second, they should be doing within-line comparisons as a paired t-tests rather than pooling all three lines, which currently makes the stats very hard to compare for the drug studies (the variance between lines is often greater than between treatment groups).

For full transparency we provided both combined stats (see Fig. 7) and the within-line comparisons (see Appendix Figures S11 and S12). The correct statistical test to use is not

necessarily individual t-tests; we would certainly not use t-tests for standard stable cell lines, where this would be inappropriate, and the test would be performed on the three (or more) biological replicates. We therefore sought statistical advice from Dr Vincent Plagnol, an expert in statistics. His suggestion was to use a linear effects model which takes into account the correlation structure (i.e. that the replicate measurements are coming from separate cell lines). This allows the technical replicates to be taken into account but is much more stringent than performing a t-test on pooled replicates. We feel that as we have provided both analyses we have been as open as possible. All three lines show a decrease (to differing degrees) with H89 treatment, so we think the statistical analysis matches the observed data.

Third, LDH release is inadequate as a readout here and there is a lack of H89 treatment on a control line to see the effect there and they saw discrepant effects between the 2 C9 lines they evaluated. As such, I am not compelled that H89 is really suppressing toxicity in any meaningful way.

We made no claim that H89 is suppressing toxicity, but we mentioned it as a possibility in the discussion. We agree that H89 treatment is required on a control line too. We therefore removed this speculation from the discussion. LDH was primarily used to show that H89 is not exerting toxicity, which is a standard read-out.

Fourth, the C9 repeat RNA levels are not measured here, just the mature product which lacks the repeats. Some assay to look at it (say, RNA foci counting) would be informative.
We have now counted RNA foci in H89-treated iPSC-neurons and there is no effect (see Appendix Figure S13C-D).

9) H89 is not PKA specific, yet it is really the only drug that shows effects in iPSCs. It inhibits at least 8 other kinases, while having a relatively large number of PKA-independent effects which may seriously compromise interpretation of data. At a minimum, this needs to be recognized as a limitation in the discussion.

We have already outlined this aspect either in the results and in the discussion sections (see line 251-253 and 394-396). In addition, we performed a PKA activity assay in iPSC-MNs treated with H89 and we observed that PKA is significantly inhibited by H89 (see line 334 Appendix Figure S13E). So, at least this enzyme is inhibited by the treatment in this cell model. Of course, we cannot exclude other kinases are inhibited as well (now reported in line 434).

Dear Alessandro,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the two referees and their comments are provided below. As you can see both referees appreciate that the core findings have been strengthen. Referee #2 mentions some limitations of the current analysis but taking everything into consideration I am pleased to let you know that we will accept the manuscript for publication here. Before sending you the formal acceptance letter there are a few things that needs to be resolved:

- please address the remaining comments of referee #1

- we need 3-5 keywords

- We also need a Data Availability section. Should be placed after Materials and methods and before Acknowledgements. This is the place to enter accession numbers etc. If no data needs to be entered into a public database please state: This study includes no data deposited in external repositories

- Please remove the figure legends from the figure files.

- The list of ORCIDs should be removed from the Manuscript file.

- In the author contribution section, please distinguish between the two APs (APo and APr). Also remove the institutions.

- Please use the correct reference format.

- In Fig EV2 there are 2 I panel labels and K panel label is mising. The legend needs correcting as well.

- Please check figure callouts to: Fig EV1E, Fig EV2I+L, Fig EV3F, Fig EV4E, Fig EV5, Appendix table S1, Appendix Fig S6L, Appendix Fig S7F-K , Appendix Fig S8 and Appendix Figs S11-S13.

- There are callouts to panels Fig 5G+H, Appendix Fig S5D-M, Appendix Fig S9F and Appendix Fig S10 but I don't think the panels exist. Please double check

- In the appendix file label tables as 'Appendix Table S#'. Please also correct callouts in text.

- The EV source data needs splitting to 1 file per figure.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

- I have asked our publisher to do their publication checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

That should be all!

You can use the link below to upload the revised version. Let me know if you have any questions.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 1st Dec 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors did a lot of efforts to answer my questions and to rewrite the manuscript accordingly. I also like the new titles as these are indeed much more informative. As a consequence, I have no further major remarks.

Minor remarks
- In Fig.6, the x-axis shows the 'Age' in (days).
- It is a bit weird that the scale of the x-axis is so different in the treatment experiment (A, B) in comparison to the crossbreeding experiment (C, D) of both figures. As a consequence, I only noticed now for the first time that the motility and the survival of the w118 males is significantly different in both experiments (all control male flies died before day 20 in the treatment experiment, while almost all male w118 flies are still alive at that age in the crossbreeding experiment). What is the explanation for this difference?
- Is the genetic background of all the fly lines the same? This is important (and should be mentioned in the M&M section as mixing different genetic backgrounds can affect results.
- The significance for the fly data in Fig. 5 is always reported at a moment when the values of the 'controls' are (very close to) 0. As a consequence, it is a bit misleading to come up with a p value lower than 0.0001 (while the absolute difference is rather low at that moment). Isn't it possible to choose a better time point (e.g. when 50% of the 'controls' fail)?
- Fig. 6: For the crossbreeding experiment, 'days after treatment' are indicated in the legend of the figure while these flies were not treated with an inhibitor.
- Appendix Figure S9. What is the difference between panels B,C and panels D,E. From the information provided it looks as if these are exactly the same experimental conditions. Is this correct?
- I would suggest to remove the disclaimer at the end of the discussion (=the last sentence) as this is very obvious (and true for every preclinical study).
- The formatting of the references should be checked as there are at least three citations (19, 51 & 66) with a weird first author name.

Referee #2:

The authors have conducted additional experiments and clarified/addressed some of my concerns. I appreciate all of the work the authors have done on this project and I commend their inclusion of data that does not support their central conclusions. The use of complementary systems is also commendable and the inclusion of their iSPC-MN work is important. However, the manuscript still has moderate limitations. It has not established a mechanism by which PKA activation boosts RAN translation or even established definitively that it does so endogenously. The H89 compound that they lean heavily on has too many off-target potential effects to be considered definitive. This means that while the manuscript has value and is generally scientifically sound, the work as a whole represents a more modest advance in our understanding of how these repeats cause toxicity.

Editorial questions:
- we need 3-5 keywords
We added: C9ALS/FTD; C9orf72; DPR; protein clearance; PKA

- We also need a Data Availability section. Should be placed after Materials and methods and before Acknowledgements. This is the place to enter accession numbers etc. If no data needs to be entered into a public database please state: This study includes no data deposited in external repositories
We added: This study includes no data deposited in external repositories

- Please remove the figure legends from the figure files.
Removed

- The list of ORCIDs should be removed from the Manuscript file.
Removed

- In the author contribution section, please distinguish between the two APs (APo and APr). Also remove the institutions.
Corrected

- Please use the correct reference format.
Changed in EMBO style

- In Fig EV2 there are 2 I panel labels and K panel label is missing. The legend needs correcting as well.
We checked and corrected

- Please check figure callouts to: Fig EV1E, Fig EV2I+L, Fig EV3F, Fig EV4E, Fig EV5, Appendix table S1, Appendix Fig S6L, Appendix Fig S7F-K , Appendix Fig S8 and Appendix Figs S11-S13.
We checked and corrected

- There are callouts to panels Fig 5G+H, Appendix Fig S5D-M, Appendix Fig S9F and Appendix Fig S10 but I don't think the panels exist. Please double check
As agreed this comment is not pertinent to our paper.

- In the appendix file label tables as 'Appendix Table S#'. Please also correct callouts in text.
Corrected

- The EV source data needs splitting to 1 file per figure.
Done.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.
Included.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.
Included.

- Appendix tables should be labeled Appendix Table S1 (you need to add an S). Please re-label and correct callout in text.
Corrected

- Please zip the source data for the EV individual figures in one zip. The source data for regular figures please keep as single files.
Done

- Looking at the source data - please look at 3F. Do you show the right lanes? I think the markings are different between the source data and figure.
We apologize, we changed accordingly.

- Also did you respond to our publishers' comments regarding the figure legends?
Yes, please see above

Referee #1:

The authors did a lot of efforts to answer my questions and to rewrite the manuscript accordingly. I also like the new titles as these are indeed much more informative. As a consequence, I have no further major remarks.

Minor remarks

- In Fig.6, the x-axis shows the 'Age' in (days).
  We thank you for suggesting, we corrected the x-axis.

- It is a bit weird that the scale of the x-axis is so different in the treatment experiment (A, B) in comparison to the crossbreeding experiment (C, D) of both figures. As a consequence, I only noticed now for the first time that the motility and the survival of the w118 males is significantly different in both experiments (all control male flies died before day 20 in the treatment experiment, while almost all male w118 flies are still alive at that age in the crossbreeding experiment). What is the explanation for this difference?
  The difference observed in w1118 males between the treatment (Fig 6B) and the crossbreeding experiment (Fig 6D) depends on different experimental conditions. Indeed, the fly experiments with pharmacological treatments were performed in 5% sucrose, while the crossbreeding ones in complete food. Consequently, flies fed with 5% sucrose have the half-life shorter in comparison to the ones fed with complete food.

- Is the genetic background of all the fly lines the same? This is important (and should be mentioned in the M&M section as mixing different genetic backgrounds can affect results.
  Yes they have, it is now mentioned in the M&M.

- The significance for the fly data in Fig. 5 is always reported at a moment when the values of the 'controls' are (very close to) 0. As a consequence, it is a bit misleading to come up with a p value lower than 0.0001 (while the absolute difference is rather low at that moment). Isn't it possible to choose a better time point (e.g. when 50% of the 'controls' fail)?
  Following your suggestion, we repeated the analysis of flies' data choosing as better time point immediately before 50% of "control" fails. The significance of data remains unchanged.

- Fig. 6: For the crossbreeding experiment, 'days after treatment' are indicated in the legend of the figure while these flies were not treated with an inhibitor.
  We thank you for suggesting the mistake we corrected it.

- Appendix Figure S9. What is the difference between panels B,C and panels D,E. From the information provided it looks as if these are exactly the same experimental conditions. Is this correct?
  Experimental conditions are the same, graphs in Fig B and C show motility and survival of female flies, while in Fig D and E are reported the graphs of males flies. Since experimental condition do not change, we have re-written the caption of panels merging them.

- I would suggest to remove the disclaimer at the end of the discussion (=the last sentence) as this is very obvious (and true for every preclinical study).
  We removed the last sentence as suggested.

- The formatting of the references should be checked as there are at least three citations (19, 51 & 66) with a weird first author name.
  We thank you for reporting it. We checked the formatting of the references.

Referee #2:

The authors have conducted additional experiments and clarified/addressed some of my concerns. I appreciate all of the work the authors have done on this project and I commend their inclusion of data that does not support their central conclusions. The use of complementary systems is also commendable and the inclusion of their iPSC-MN work is important. However, the manuscript still has moderate limitations. It has not established a mechanism by which PKA activation boosts RAN translation or even established definitively that it does so endogenously. The H89 compound that they lean heavily on has too many off-target potential effects to be considered definitive. This means that while the manuscript has value and is generally scientifically sound, the work as a whole represents a more modest advance in our understanding of how these repeats cause toxicity.

3rd Revision - Editorial Decision **12th Oct 2021**

Dear Alessandro,

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

Congratulations on a nice study

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Corresponding Author Name: Alessandro Provenzani, Angelo Poletti
Journal Submitted to: EMBO Journal
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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 iustified
- 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?	Reported in M&M
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Reported in M&M
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	Reported in M&M, first experiment out of three was with randomized animals to treatment
For animal studies, include a statement about randomization even if no randomization was used.	Reported in M&M, first experiment out of three was with randomized animals to treatment
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.	Reported in M&M, in the first experiment out of three the researcher was blind
4.b. For animal studies, include a statement about blinding even if no blinding was done	Reported in M&M, in the first experiment out of three the researcher was blind
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.	No methods used
Is there an estimate of variation within each group of data?	Yes

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

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Reported
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Reported

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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.	Reported
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E- Human Subjects

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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
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