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Reduced nuclear hnRNPA3 increases C9orf72 hexanucleotide repeat RNA levels and dipeptide-repeat protein deposition

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Review timeline:

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 15 December 2015

Thank you for your patience while your manuscript was peer-reviewed at EMBO reports. We have now received the full set of comments that is pasted below.

As you will see, while the referees acknowledge that the findings are potentially and conceptually interesting, they also point out that the data, especially the human patient data, are not sufficiently convincing. Both referees 1 and 3 remark that patient-derived fibroblasts or iPSC should be used to demonstrate an effect of hnRNPA3 on GA peptide levels. The referees also raise additional concerns and point out overstatements.

Given the potenital interest of the findings and the constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 25,000

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characters (including spaces but excluding materials and methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures, and the results and discussion section must be separate. In both cases all materials and methods must be included in the main manuscript files.

Regarding data quantification, please explain what you mean by "biological duplicate". If n=3 this means that three independent experiments were performed. May you mean technical duplicates?

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The identification of RNA binding proteins that directly interact with C9orf72 repeat RNA is of high importance to the field, and Mori et al. present evidence describing an interaction between hnRNPA3 and the C9orf72 repeats - that is reduction of hnRNPA3 increases the expression of C9 repeat RNA and GA dipeptide repeat proteins. They characterize this interaction in a cell culture model based on overexpression of the C9orf72 repeats in HeLa cells and primary rat neurons, and then attempt to extend their findings by looking for correlations between nuclear hnRNPA3 levels and GA deposition in C9-ALS/FTD patient brain tissue. Overall, the interaction presented in this manuscript is well characterized in cell culture models (lower levels or reduced nuclear hnRNPA3 corresponds to increased polyGA deposition), but the data seems to fall a bit short when looking closer at the patient data presented (see comments below). This functional interaction would indeed be interesting in regards to understanding the pathomechanisms of C9orf72 hexanucleotide expansion mutations in ALS/FTD, but might require more data to be thoroughly convincing.

Given the short format of EMBO Reports, we think that this manuscript and the careful analyses in cell culture and primary neurons could be interesting even without the preliminary human patient data, which are not strong enough to fully support the authors claims. We have the following suggestions and comments for the authors to consider.

Answers to specific questions:

1. Does this manuscript report a single key finding? Yes, the RNA-binding proteins hnRNPA3 negatively regulates expression of hexanucleotide repeat expansions in C9orf72, leading to decreased RNA and dipeptide repeat proteins.

2. Is the reported work of significance (YES), or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc (NO)? Yes and No. This work is significant because it describes a new regulator of C9orf72 mutant expression but it does not show data to support the authors claim that hnRNPA3 binds repeats and leads to their degradation.

3. Is it of general interest to the molecular biology community? This is potentially interesting to the molecular biology community because it reveals a way that C9orf72 mutant RNA expression is regulated.

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4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longerformat article (NO)? Yes, but only for the in vitro experiments (cell lines + primary neurons). The extension to human patient samples is preliminary and not convincing at this point.

Major concerns/comments:

The main concern with the manuscript lies in the analysis of the patient tissue data. This data might be too preliminary to include, or more data needs to be collected/more analysis needs to be done to be convincing.

The authors first state "strikingly in c9orf72 patients we frequently detected neurons with reduced nuclear hnRNPA3 (Figure 4A)" This claim needs to be backed by data that directly compares the nuclear hnRNPA3 levels between C9 and control cases, not just a few example micrographs. When looking at the data presented in the supplementary tables, it isn't clear that this is actually the case. In fact the controls and C9 cases look pretty similar in terms of nuclear A3 intensity. We used the data from the supplementary tables to plot the data and did not observe a significant correlation.

Maybe this difference comes from frequency of A3 negative nuclei or nuclear/cytoplasmic ratios? But this needs to be addressed and properly quantified.

Furthermore the analysis in Figure 4C needs to be more detailed. The authors should explain the choice of using the median nuclear A3 intensity as a basis for grouping High/Low cases to conclude if the main claim is a correlation between low nuclear A3 and high GA - a regression would show this correlation more clearly if it exists. Using the data in the supplementary table, we have performed a regression analysis and see no clear correlation between nuclear A3 intensity and GA positivity.

Also, it seems like a single outlier point in the low A3 group (Fig 4C) is driving the main difference in the data, which is already very small. If you perform a linear regression on the dataset looking at GA positivity compared to nuclear A3, there doesn't seems to be any clear correlation. This needs to be addressed.

As an alternative to analyzing correlations in post-mortem patients brains, it might be more relevant to directly manipulate hnRNPA3 levels in C9-patient cells, such as fibroblasts or iPSC models, and analyze changes in GA expression.

Does hnRNPA3 also bind the C9-antisense RNA repeats? If so, does knockdown of A3 have similar effects on the antisense dipeptides?

There seems to be a disconnect between Fig 2G and Supp. Fig. S3B. If lowering A3 levels increases the percentage of cells with double/triple DPR expression, and cells with GA/GR double reactivity have more TDP-43 mislocalization, you might predict more TDP-43 mislocalization upon A3 knockdown?

Other comments:

-It is an interesting finding that you can rescue the GA and repeat RNA expression phenotype with A2 overexpression rescue when A3 is knocked down. When you treat cells with both A3 and A2 siRNAs, it seems like A3 protein levels actually are increased compared to A3 siRNA alone (Western blot data FigS1-D). Is there some regulation here?

-Figure 1 in general is very busy and could be simplified or broken into more figures.

-Please provide a quantitative measure of the RNA foci increase observed upon A3 siRNA treatment in HeLa cells (Figure 1F). The foci measurements are not adding much and could even be removed entirely - this may de-clutter Figure 1 as well.

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-Was DNAse treatments were used for qPCR experiments, as you are measuring transcription from plasmid DNA?

-There are a couple examples (Fig2B and S3A) where co-expression of GA and GR DPRs correlates with a strange looking nuclear DAPI stain. Do you see this often or perhaps just a coincidence? If it does happen frequently, might the nuclear envelop be somehow disrupted?

-Including antibody dilutions used in these studies could be helpful for the field, especially for these types of quantitative histology experiments.

Referee #2:

In the paper by Mori et al., the authors show that a reduction of nuclear hnRNPA3 leads to an increase of the G4C2 repeat RNA as well as DPR proteins and deposition of DPR proteins in cultured cells and primary neurons. Furthermore, the authors showed that hippocampal neurons of C9orf72 patients are often time devoid of hnRNPA3 that correlates with increased DPR deposition, thus concluded that impaired nuclear import of hnRNPA3 in C9orf72 patients leads to increased levels of the repeat RNA and enhanced production and deposition of DPR proteins. In general, the work is well done and this work adds to our understanding of the role of G4C2 repeat binding protein in C9orf72 pathology.

Major Comments:

(1) The authors concluded, "Impaired nuclear import of hnRNPA3 in C9orf72 patients leads to increased levels of the repeat RNA and enhanced production and deposition of DPR proteins". This is overstated. "Impaired nuclear import of hnRNPA3" is implied because three published works showed that C9orf72 compromises nucleocytoplasmic transport, both import and export (Zhang et al., Nature 2015, Freibaum et al., Nature 2015, Jovičić et al., Nat Neurosci 2015), but none of these works showed that nuclear import of hnRNPA3 is compromised.

(2) Figure 1J-L: Based on their ΔM9 data, they suggest that the regulation of C9orf72 repeat requires the nuclear import of hnRNPA3 via the M9-NLS. However, the data is not satisfactory to make this statement since (1) the majority hnRNPA3 ΔM9 is found in the nucleus (Figure S1C) and hnRNPA3 ΔM9 expression still significantly blocks GA production (Figure 1J). Nuclear import inhibitor, M9M, has been shown to effectively block nuclear import of hnRNPs with PY-NLS (Cansizoglu et al., Nat Struct Mol Biol. 2007). Authors may see better effect by expression of M9M.

(3) Figure 4: Authors performed double immunostaining with anti-hnRNPA3 and anti-GA antibodies and demonstrated that in C9orf72 patients, neurons have low levels of hnRNPA3 that correlated with the deposition of poly-GA. Throughout the text, the authors state that nuclear hnRNPA3 reduction correlates with poly-GA accumulation. hnRNPA3 is a nuclear protein, and reduction of nuclear hnRNPA3 means the reduction of total hnRNPA3. Since the authors do not show that hnRNPA3 accumulates in the cytoplasm in C9orf72 patients, I would suggest being more general and call it reduction of hnRNPA3 rather than reduction of nuclear hnRNPA3.

Minor Comments:

(1) Figure 1A: Does the control vector include 113 bp of the 5' flanking region of the human C9orf72 repeat and 3X TAG? In other word, is the only difference between (G4C2) expression and control vector the presence of (G4C2)80 repeat?

(2) Figure 2B, S3A: These results nicely demonstrate the correlation of DPR expression and redistribution of TDP-43. I would like to clarify couple of things though. Authors stated that poly-GA and poly-GR double positive cells, but not poly-GA single positive cells, frequently showed altered TDP-43 intracellular distribution. I see that all presented cells with TDP-43 mislocalization have a strong GA expression in aggregates. (1) Could it be the expression levels of GA (thus its localization) that affect TDP-43 localization rather than poly-GA and poly-GR double positiveness, or (2) could it be the solely GR-positiveness that determines TDP-43 localization? What are the

percentiles of poly-GA or poly-GR single positive cells?

(3) Images on Figure 4A and 4B are a lot more convincing than the quantification shown in 4C. What are the criteria to categorize each sample into "High" and "Low" hnRNPA3 expression group? In Figure 4C, If the nuclear hnRNPA3 intensity is plotted against the %GA positivity for each case, does it show negative correlation?

Referee #3:

The concept behind this work is interesting: that there is a destructive feedback loop which goes from c9 expression to impaired import of hnRNPA3 to more c9 dipeptide production. But the execution is rather disappointing. I would like to see confirmation in a more physiologic system such as iPSC cells. These are quite extensively available if the authors do not have them. At present one would worry about a heterologous promoter artefact.

I am not convinced (yet) by the genetic data implicating hnRNAPs

1st Revision - authors' response 18 May 2016

My colleagues and I would like to submit our extensively revised manuscript (EMBOR-2015-41724V1) entitled "**Reduced hnRNPA3 increases *C9orf72* repeat RNA levels and dipeptide-repeat protein deposition "** (please note that we adjusted/shortened the title according to the authors instructions of EMBO Reports). We have addressed all points raised by the reviewers. Importantly, we added data from patient derived fibroblasts (see entirely new Figures 5) and doubled the case number for neuropathological analyses to increase the confidence in our findings (see new Fig. 6C).

Taken together, we believe that we have carefully addressed all points raised.
In the name of my colleagues I want to thank you for considering our manuscript and for extending the revision time for the rather complicated experiments in patients derived cells and a large sample of additional brains from C9orf72 carriers.

Point-by-point response

General editorial points
Regarding data quantification, please explain what you mean by "biological duplicate". If n=3 this means that three independent experiments were performed. May you mean technical duplicates?
n= number of biochemical experiments performed in duplicates. Immunohistochemical experiments were replicated as indicated. This is now explained in the corresponding figure legends.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader.
This has been done as requested.

Referee #1
Major concerns and comments

The main concern with the manuscript lies in the analysis of the patient tissue data. This data might be too preliminary to include, or more data needs to be collected/more analysis needs to be done to be convincing.
We have now added data derived from 16 additional C9orf72 cases. Data analysis was replicated by an independent scientist and similar findings were observed. The extended analysis is now shown in the new Fig. 6C (for further information see below).

The authors first state "strikingly in c9orf72 patients we frequently detected neurons with reduced nuclear hnRNPA3 (Figure 4A)" This claim needs to be backed by data that directly compares the nuclear hnRNPA3 levels between C9 and control cases, not just a few example micrographs. When

looking at the data presented in the supplementary tables, it isn't clear that this is actually the case. In fact the controls and C9 cases look pretty similar in terms of nuclear A3 intensity. We used the data from the supplementary tables to plot the data and did not observe a significant correlation.
We greatly apologize for this error. Indeed hnRNPA3 levels are also reduced in some control cases. We only compared hnRNPA3 levels in *C9orf72* cases as only those have DPR deposits. This sentence has been deleted accordingly.
Together with the additional 16 cases we have now analyzed a total of 102 micrographs (see new Fig. 6C). This extensive neuropathological analysis confirms the significant increase of GA deposition in neurons expressing low levels of nuclear hnRNPA3.

Furthermore the analysis in Figure 4C needs to be more detailed. The authors should explain the choice of using the median nuclear A3 intensity as a basis for grouping High/Low cases to conclude if the main claim is a correlation between low nuclear A3 and high GA - a regression would show this correlation more clearly if it exists. Using the data in the supplementary table, we have performed a regression analysis and see no clear correlation between nuclear A3 intensity and GA positivity.
Also, it seems like a single outlier point in the low A3 group (Fig 4C) is driving the main difference in the data, which is already very small. If you perform a linear regression on the dataset looking at GA positivity compared to nuclear A3, there doesn't seems to be any clear correlation. This needs to be addressed.
We categorized the samples into "low" and "high" by splitting them in the median, since we observed overall differences in hnRNPA3 expression levels. This is now explained in detail in the Experimental Procedures. Moreover, we increased the number of brains extensively. The new data analysis also excludes the possibility that a single outlier affects the conclusion (see new Fig. 6C).
In addition, we followed the reviewers suggestion " As an alternative to analyzing correlations in post-mortem patients brains, it might be more relevant to directly manipulate hnRNPA3 levels in C9-patient cells, such as fibroblasts or iPSC models, and analyze changes in GA expression" (see next point).

As an alternative to analyzing correlations in post-mortem patients brains, it might be more relevant to directly manipulate hnRNPA3 levels in C9-patient cells, such as fibroblasts or iPSC models, and analyze changes in GA expression.
We followed this suggestion of the reviewer and investigated fibroblasts derived from three independent patients with C9orf72 repeat extensions. Since we never observed accumulation of DPR proteins in patient fibroblasts and iPSC derived neurons using an array of highly specific monoclonal DPR antibodies against all five species we investigated the number of pathologically equally important RNA foci after hnRNPA3 knockdown. In line with our analysis in HeLa cells (see Fig. 1F and new Fig. 1G), we found that the number of RNA foci significantly increased upon either siRNA or lentiviral mediated knockdown of hnRNP A3 (completely new Fig. 5).

Does hnRNPA3 also bind the C9-antisense RNA repeats? If so, does knockdown of A3 have similar effects on the antisense dipeptides?
This is difficult to address in our experimental paradigm as the expressions vectors used do not allow antisense RNA transcription. However, when we analyzed RNA foci in the three fibroblast cell lines derived from the *C9orf72* patients, we observed an even stronger increase of anti-sense RNA foci than sense-RNA foci.

There seems to be a disconnect between Fig 2G and Supp. Fig. S3B. If lowering A3 levels increases the percentage of cells with double/triple DPR expression, and cells with GA/GR double reactivity have more TDP-43 mislocalization, you might predict more TDP-43 mislocalization upon A3 knockdown?
This question relates to the TDP-43 mislocalization in the GA/GR positive "control" cells (fourth bar in EV3 B). Here we selected only the GA/GR double positive cells, which in line with the hnRNPA3 knockdown cells also show a similar redistribution of TDP-43. However, as shown in Fig. 3G the number of double positive cells significantly increases upon hnRNPA3 knockdown due to enhanced levels of the repeat RNA.

Other comments
It is an interesting finding that you can rescue the GA and repeat RNA expression phenotype with A2 overexpression rescue when A3 is knocked down. When you treat cells with both A3 and A2

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siRNAs, it seems like A3 protein levels actually are increased compared to A3 siRNA alone (Western blot data FigS1-D). Is there some regulation here?
We believe that this tiny difference is due to experimental variations, but we cannot exclude a compensatory mechanism.

Figure 1 in general is very busy and could be simplified or broken into more figures.
We have split Fig. 1 after the new Fig. 1G. The second half moved to Fig. 2, which now only shows the "rescue" experiments, while the shortened new Fig. 1 shows the initial characterization of the hnRNPA3 knockdown and its consequences on RNA foci and GA accumulation.

Please provide a quantitative measure of the RNA foci increase observed upon A3 siRNA treatment in HeLa cells (Figure 1F).
This is now provided in the new Fig. 1G. The quantitative analysis revealed a significant increase of RNA foci upon siRNA mediated knockdown of hnRNPA3 (see also new Fig. 5, which shows the increase of RNA foci upon hnRNPA3 knockdown in patient derived fibroblasts).

Was DNAse treatments were used for qPCR experiments, as you are measuring transcription from plasmid DNA?
Yes, this is mentioned in the Experimental Procedures.

There are a couple examples (Fig2B and S3A) where co-expression of GA and GR DPRs correlates with a strange looking nuclear DAPI stain. Do you see this often or perhaps just a coincidence? If it does happen frequently, might the nuclear envelop be somehow disrupted?
Yes, the reviewer is right. We almost always observed altered nuclear morphologies in cells expressing GA and GR. This is now mentioned in the Results section.

Including antibody dilutions used in these studies could be helpful for the field, especially for these types of quantitative histology experiments.
Antibody dilutions were added for all experiments (western blots and immunofluorescence) in the Experimental Procedures.

Referee #2
Major concerns

The authors concluded, "Impaired nuclear import of hnRNPA3 in C9orf72 patients leads to increased levels of the repeat RNA and enhanced production and deposition of DPR proteins". This is overstated. "Impaired nuclear import of hnRNPA3" is implied because three published works showed that C9orf72 compromises nucleocytoplasmic transport, both import and export (Zhang et al., Nature 2015, Freibaum et al., Nature 2015, Jovičić et al., Nat Neurosci 2015), but none of these works showed that nuclear import of hnRNPA3 is compromised.
This was indeed overstated. We have corrected this accordingly.

Figure 1J-L: Based on their ΔM9 data, they suggest that the regulation of C9orf72 repeat requires the nuclear import of hnRNPA3 via the M9-NLS. However, the data is not satisfactory to make this statement since (1) the majority hnRNPA3 ΔM9 is found in the nucleus (Figure S1C) and hnRNPA3 ΔM9 expression still significantly blocks GA production (Figure 1J). Nuclear import inhibitor, M9M, has been shown to effectively block nuclear import of hnRNPs with PY-NLS (Cansizoglu et al., Nat Struct Mol Biol. 2007). Authors may see better effect by expression of M9M.
Nuclear import inhibitors such as M9M are not selective and will affect many proteins with PY-NLS (see also our previous publication Dorman et al., EMBO J 2012). Therefore, we believe that our mutant is more selective. However, due to the PY-NLS independent nuclear import (see Fig. S1C) we indeed observed some repression of GA and the repeat RNA. Nevertheless, the deltaM9 mutant clearly fails to efficiently rescue repression as compared to the wt protein (see Fig. 1 and Fig. S1). This is now explained in more detail in the Results section.

Figure 4: Authors performed double immunostaining with anti-hnRNPA3 and anti-GA antibodies and demonstrated that in C9orf72 patients, neurons have low levels of hnRNPA3 that correlated with the deposition of poly-GA. Throughout the text, the authors state that nuclear hnRNPA3 reduction correlates with poly-GA accumulation. hnRNPA3 is a nuclear protein, and reduction of

nuclear hnRNPA3 means the reduction of total hnRNPA3. Since the authors do not show that hnRNPA3 accumulates in the cytoplasm in C9orf72 patients, I would suggest being more general and call it reduction of hnRNPA3 rather than reduction of nuclear hnRNPA3.
We followed the reviewers suggestion and changed the text as requested.

Minor Comments

Figure 1A: Does the control vector include 113 bp of the 5' flanking region of the human C9orf72 repeat and 3X TAG? In other word, is the only difference between (G4C2) expression and control vector the presence of (G4C2)80 repeat?
The control vector indeed lacks only the GGGGCC repeat but still contains the 5' flanking region and 3x TAG. This is now explained in the figure legend.

Figure 2B, S3A: These results nicely demonstrate the correlation of DPR expression and redistribution of TDP-43. I would like to clarify couple of things though. Authors stated that poly-GA and poly-GR double positive cells, but not poly-GA single positive cells, frequently showed altered TDP-43 intracellular distribution. I see that all presented cells with TDP-43 mislocalization have a strong GA expression in aggregates. (1) Could it be the expression levels of GA (thus its localization) that affect TDP-43 localization rather than poly-GA and poly-GR double positiveness, or (2) could it be the solely GR-positiveness that determines TDP-43 localization? What are the percentiles of poly-GA or poly-GR single positive cells?
Based on our intensive immunohistochemical analysis, we indeed speculate that the additional accumulation of GR stimulates TDP-43 re-localization (see also newly added lower panels in Fig EV3A). However, this is difficult to address quantitatively since GR single positive cells are very rarely observed. Nevertheless, we adjusted Fig. EV3 B to better explain this point and discuss this issue within the Results section.

Images on Figure 4A and 4B are a lot more convincing than the quantification shown in 4C. What are the criteria to categorize each sample into "High" and "Low" hnRNPA3 expression group? In Figure 4C, If the nuclear hnRNPA3 intensity is plotted against the %GA positivity for each case, does it show negative correlation?
Similar questions were raised by reviewer 1 (please also see answer above). We categorized the samples into "low" and "high" by splitting them at the median. This is now described in more detail in the Experimental Procedures. Moreover, we doubled the sample size by the addition of 16 brains with C9Orf72 repeat extensions. This further strengthened our findings (see new Fig. 6C).

Referee #3

The concept behind this work is interesting: that there is a destructive feedback loop which goes from c9 expression to impaired import of hnRNPA3 to more c9 dipeptide production. But the execution is rather disappointing. I would like to see confirmation in a more physiologic system such as iPSC cells. These are quite extensively available if the authors do not have them. At present one would worry about a heterologous promoter artefact.
We followed the suggestion of the reviewer and investigated fibroblasts derived from three independent patients with C9orf72 repeat extensions. Since we never detect DPR accumulation in patient derived cells (including neurons derived from iPSCs) despite extensive tries using a number of different monoclonal antibodies, we investigated the number of the pathologically equally important RNA foci after hnRNPA3 knockdown. In line with our analysis in HeLa cells (see Fig. 1F and new Fig. 1G), we found that the number of RNA foci significantly increased upon either siRNA (completely new Fig. 5A-E) or lentivirus mediated knockdown of hnRNP A3 (completely new Fig. 5F and G).
Moreover, we also included in the original version control experiments using EGFP expression under the very same promotor (see Fig. 1). Note that EGFP expression was not significantly affected by the knockdown of hnRNAPA3.

I am not convinced (yet) by the genetic data implicating hnRNAPs
The genetic data were published as an article in Nature:

Kim, H.J., Kim, N.C., Wang, Y.D., Scarborough, E.A., Moore, J., Diaz, Z., MacLea, K.S., Freibaum, B., Li, S., Molliex, A.*, et al.* (2013). Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. Nature 495, 467-473.
To our knowledge there are no contradictory data published. Therefore it is a bit difficult for us to address this comment. Replications of the genetic data may be difficult, since these mutations seem to be extremely rare. However, a D314N mutation in hnRNPA1 was identified in a Japanese cohort with inclusion body myopathy:
Izumi et al., (2015) Isolated inclusion body myopathy caused by a multisystem proteinopathy–linked *hnRNPA1* mutation**.** Neurol Genet. , 3, e23.
Moreover, in the original study segregation of the mutations was observed in three independent families over several generations.

2nd Editorial Decision 30 June 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

REFEREE REPORTS

Referee #1

I read through the revised manuscript and the authors' point-by-point response and in my opinion the authors have addressed my original concerns appropriately and I recommend publication of this work, which reveals a new mechanism facilitating the localization and translation of C9orf72 repeat encoded RNAs.

Referee #3

This is an extremely interesting and through report showing that knocking down hnRNPA3 increases the RAN translated c9 transcript and increases the dipeptide deposition. This will be of considerable and rather general interest to the field both for mechanistic and eventually and more speculatively for treatment reasons. I liked the paper: it is well presented and very clear with the appropriate controls. It will be of interest to all those working on FTD/ALS.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Christian Haass and Kohji Mori
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2015-41724V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	for neuropathological anlyses we chose brain sections from 34 carriers, which is extremely high with respect to the low frequency of the mutation. Fibroblast cell lines were available from three clinically defined patients.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Demographic information of each case were blinded until data collection and analysis.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
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.	Data distribution was assumed to be normal but was not tested.
Is there an estimate of variation within each group of data?	Most graphs are presented as mean ±SEM or maen ±95%CI. No further analysis were performed for variance estimation.
Is the variance similar between the groups that are being statistically compared?	Most graphs are presented as mean ±SEM or maen ±95%CI. No further analysis were performed for variance estimation.

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).	Deteiled information is provided in Experimental Procesures. In addition, our RNAi experiments and experiments with fusion proteins obtained expected results.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Absence of mycoplasma contamination was confirmed with DAPI staining.

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

D- Animal Models

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

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Information is provided in "Patient derived fibroblast" and "Human brain samples" sections in Experimental Procedures.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Information is provided in "Patient derived fibroblast" sections in Experimental Procedures. All cases provided by the Neurobiobank Munich, Ludwig-Maximilians-University (LMU) Munich and the University of British Columbia were collected and distributed according to the guidelines of the local ethical committee. Brain autopsy was performed on the basis of informed
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Some demographic information about postmortem human brain section was not available. This is stated in Table EV1.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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