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Sam68 sequestration and partial loss of function are associated with splicing alterations in FXTAS patients

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1st Editorial Decision 11 July 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees, whose comments are enclosed below, have now evaluated your manuscript. As you will see from their reports the referees express potential interest in role of Sam68 in FXTAS, however, they provide mixed recommendations and it is clear that significant further experimental analysis is required to support the proposed role of the splicing factor in the disease and to make it suitable for publication in the EMBO Journal.

Although the referees find the main conclusions of the potentially interesting they each also raise major concerns about several aspects of the study. Referee #1 finds that some of the experiments should be repeated with endogenous proteins and would like some insight (not necessarily the full mechanism) into how Sam68 is recruited into foci and how it recruits additional proteins. A major concern of all three referees is the effect of Sam68 sequestration on splicing, and the data currently does not support the main conclusion of the important role of Sam68 in the disorder, quantitative analysis of the changes in splicing and statistical analysis should be provided. Referee #3 would also like to see key experiments including analysis of the splicing to be repeated in neuronal cell lines and analysis of the splicing pattern in Sam68 -/- mice, finally, s/he asks if treatment with tautomycin rescues the splicing defects.

I realize that the referees ask for a lot of work and the outcome of the experiments especially the importance of the effects of Sam68 sequestration on splicing are uncertain. This makes a decision on the study difficult and not a straightforward one to ask for a revised manuscript. However, given the interest in the potential disease role of Sam68, should you be able to address these criticisms, we could consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a

single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. I do realize that addressing all of the referees' criticisms might require a lot of additional time and effort and be technically challenging, and I would also understand it if you were to rather decide to publish the manuscript rapidly and without any significant changes elsewhere. If you decide to thoroughly revise the manuscript and submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors report on a number of RNA binding proteins that are sequentially recruited on CGG repeats and analyze their potential role in the onset or progression of Fragile X-associated Tremor/Ataxia Syndrome. Among other already known proteins that mark CGG aggregates, the authors identify Sam68 as a novel component of these aggregates and suggest that its presence is essential for the localization of several hnRNPs in the FXTAS foci. In addition, the authors found that Sam68 is enriched in aggregates in FTXAS biopsies, and that the splicing events modulated by Sam68 are impaired in the disease. Most of the work is based on overexpression studies and the experimental results are often presented in the form of single-cell images, with no specific information on the frequency and reproducibility of the results. Although the data presented in the paper might unveil a novel contribution of Sam68 in a human disease and therefore be of very general interest, the experiments presented in the manuscript are too preliminary and incomplete to warrant publication in the EMBO Journal.
Specific comments are listed below:

1. Sam68 dimerizes and forms complexes in vivo whereas its transient overexpression induces its accumulation in large nuclear foci and causes cell death in many cell types; this tendency to aggregate might explain why the authors detect Sam68 before the other RNA-binding proteins tested in their study and experiments with endogenous proteins would be much more informative. In line with this criticism, the authors showed immunofluorescence /FISH analyses of some of the proteins identified by mass spectrometry and claim that there was no co-localization with the CGG repeats 24 hours after transfection. However, from the pictures presented it seems that there is co-localization also at this early stage of transfection, although the GFP localization is also diffuse. The dimension of the CGG repeat foci is also very different upon the transfection of the different RNA binding proteins, indicating that overexpression experiments alter the normal formation of such aggregates. It is possible that the transfection of some of them can induce an increase of these foci. Thus, to avoid this problem, the authors should perform the same CGG transfection experiments and look at the localization of the endogenous MBNL1, hnRNPA2B1, hnRNPG and Sam68. Similarly, the RNA aggregates formed by CUG and AUUCU repeats in Figure 3 are much smaller than the CGG ones shown and this might justify the reduced recruitment of Sam68 to these foci. Indeed, in the CUG-transfected cell, the largest aggregates shown contain Sam68 staining. Open field images for clear representation of the results and a summary showing statistics and number of cells examined for each treatment should be included in all descriptive figures of the manuscript.
2. The authors claim that Sam68 acts as a nucleation site for the accumulation of additional RNA binding proteins in the CGG foci. Since the RNA binding activity of Sam68 is dispensable whereas the N-terminus is required, they put forward the hypothesis that Sam68 is recruited to the aggregates by protein-protein interactions. This is puzzling, because if Sam68 acts as a nucleation trigger, it should be recruited first. Nevertheless, there is no attempt to identify the mechanism by which Sam68 is recruited to the foci nor how it recruits additional proteins. However, the lack of a

mechanism throughout the study strongly weakens the hypothesis proposed in the present manuscript.
3. The dimension of the CGG repeat foci is different upon transfection of different portion of Sam68. The authors should explain how recombinant portion of Sam68 can trigger the accumulation of the CGG repeats.
4. In Figure 4 the authors showed that upon Sam68 knockdown, the recruitment of MBNL1, hnRNPA2 and hnRNPG in the CGG repeats, is greatly impaired. The authors should determine whether without transfection of the CGG repeats, the absence of Sam68 is sufficient to modulate the localization of these hnRNPs. The authors should also show by Western blot the level of downregulation of Sam68 protein. In addition, in panel A and C there is plenty of residual Sam68 that does not accumulate in the aggregates, which again suggests that Sam68 recruitment is partially due to overexpression.
5. Figure 5C: why is Sam68 not detected in the nucleus of normal brain cells? Sam68 is abundantly expressed in neurons and glial cells and staining of the nucleoplasm is expected. The lack of specific controls for the immunohistochemistry makes it difficult to evaluate this result.
6. The authors claimed that in their hands Sam68 promotes the long form of Bcl-x without showing the data; since this effect is different from what previously reported, they should show the result of the experiment.
7. The choice of exon 28b of ATP11B is unclear. There are many brain-specific splicing events (see Yeo et al., 2004); what kind of bioinformatic search have the authors done? Why ATP11B attracted the authors as a potential target of Sam68?
8. The results of Figure 8 should be better documented by adding the number of the patients and the description of the different stages of the disease.
9. Figure 9 is very preliminary; no real correlation between the treatments and the state of phosphorylation of Sam68 is presented. TPA increases the size of the aggregates even though it is known to induce ERK1/2-mediated phosphorylation and activation of Sam68 (Matter et al., 2002). Similarly, the other inhibitors likely affect a multitude of substrates in the cell. Can the authors really attribute the observed effect on the aggregates and protein recruitment to changes in Sam68 phosphorylation?

Minor points

1. Figure 1C, Figure 2A, Figure 9, Supp Figure 2 and B it is labeled Cy3-CCG instead of Cy3-CGG.
2. Figure 5C is not clear without indicating the magnification used. Magnification of the Sam68 positive cells should be shown.
3. Figure 9C should have the same magnification as the others panels. The enlargement presented is not understandable.

Referee #2 (Remarks to the Author):

In this manuscript, the authors present evidence supporting RNA-gain of function mechanism in FXTAS pathogenesis. They show that the expanded CGG repeats (60 or more) accumulate in dynamic intranuclear RNA structures that expand over time in several cell lines transfected with a construct containing 60 CGG-repeat. They identified Sam68 as a protein that co-localizes with CGG aggregates at each time point after transfection (24-48-72 hr) and show that Sam68 is required for the subsequent aggregation of other RNA-binding proteins such as MBNL1, hnRNP A, and hnRNP G into CGG aggregates. By analogy to the model of MBNL1 sequestration by CUG-repeat in myotonic dystrophy, the authors propose that Sam68 is sequestered by CGG-repeats and as a result, its normal function in splicing regulation of several target genes tested is lost. Furthermore, they show that Sam68's phosphorylation status modulates its aggregation with CGG aggregates and identified a compound, Tautomycin, that not only prevents Sam68 co-localization in CGG aggregates but also abolishes CGG aggregates formation. Overall the studies are interesting and have the potential to lead to understanding FXTAS mechanism. However, several key points need to be addressed before publication can be considered.

1) The data regarding Sam68 sequestration resulting in mis-regulation of pre-mRNA splicing are weak and insufficient to support the strong and main conclusion presented in the title. As Figures 7 and 8 show, the CNS specific exon 28b of the ATP11B minigene (not previously known to be the

target of Sam68) is slightly more included (from 46% to 62%) when Sam68 is overexpressed and slightly more skipped (from 46% to 30%) when Sam68 is knocked down by shRNA or sequestered by CGG repeat overexpression. A similar degree of effect on ATP11B exon 28b splicing was observed in FXTAS patients. Additionally, as stated in the text, splicing events previously known to be regulated by Sam68 show either no change (Ktn1 and Sgce2) or only a modest change (Bin1, Dncic2, Clasp2) in FXTAS patients. These are subtle changes that need to be quantified by real-time PCR to validate their statistical significance.

2) The assertion that Sam68 is the only protein that co-localizes with CGG aggregates is not justified. It is detected sooner than the other proteins tested - that is all that can be said. Data on the proteomic analysis have not been provided.

3) In Figure 4, shRNA knockdown of Sam68 does not seem to work efficiently and varies from cell to cell, as significant amount of Sam68 immunostaining signals still remains after shRNA treatment. Western blot analysis of Sam68 protein level needs to be included in Figures 4 and 7 to demonstrate the overexpression and efficiency of shRNA knock down of Sam68.

Referee #3 (Remarks to the Author):

Summary:
Fragile X-associated tremor/ataxia syndrome (FXTAS) is a neurodegenerative disorder caused by the expansion of 55-200 CGG repeats in the 5' UTR of the fragile X mental retardation 1 (FMR1) gene. FXTAS is thought to be caused by a toxic 'gain-of-function' of the expanded CGG-repeat in the FMR1 mRNA. The paper by Sellier et al., highlights the role of RNA binding protein Sam68 and provides insight to the dynamic intranuclear CGG aggregate formation that recruits several of the RNA binding proteins. The author also provides evidence of the role of Sam68 as a regulator of splicing, sequestration of which leads to the splicing defect. There is also evidence of regulation of Sam68 activity by phosphorylation and tautomycin prevents both the Sam68 localization and CGG RNA aggregate formation suggestive of a target route of intervention.

Major comments:
Overall the findings in the paper are very interesting and important to the pathogenic mechanisms of FXTAS. However, a significant concern about the results is that the experiments are carried out in a kidney cell line (COS7) instead of neuronal cell line. When the author is trying to answer key questions about the pathogenic mechanisms of a neurodegenerative disease, it would have been ideal to execute the experiments in a neuronal cell line rather than any other cell line; i.e. not to ignore cell type specific expression of proteins which may be important in contributing to the disease state or the biologic phenomenon being studied. In FXTAS research people have used neuronal cell lines (Ref. Induction of inclusion formation and disruption of lamin A/C structure by premutation CGG-repeat RNA in human cultured neural cells. Hum Mol Genet. 2005 1;14(23):3661-71). Similarly the authors reference the work of Chawla, et. al. in MCB, Jan 2009, where most of the work on Sam68 regulation of alternative splicing was done using neuronal cell lines (N2A and NPC cells). The author provides images from the RNA FISH experiments to show the intranuclear CGG aggregate in mouse neuron primary culture and the PC12 neuronal cell line, suggesting that experiments could have been carried out in these cell lines.

Also, the other thing I don't understand is the concept of the RNA foci getting larger and the sequential recruitment of proteins to the RNA aggregates over a 72 hour period. These experiments were done using COS7 cells and transient transfection assays. COS7 cells are not terminally differentiated. They continue to divide with a doubling time of approximately 24-28 hours. Wouldn't the aggregates have to dissolve and reform in the daughter cells with each cell division?

Chawla et. al. reported about 30 transcripts that are affected by Sam68 knockdown. About half of these alternative splicing events involved exon skipping while the other half involved inclusion of an exon. It would have been interesting to see if the FXTAS patients showed more than the ATP11B change. The authors do mention changes in Bin1, Dncic2, and Clasp2 alternative splicing though the changes look rather modest. The data could be included in the supplement. At the bottom of page 11, the authors state "These observed changes in alternative splicing are weak but similar to those

observed after Sam68 depletion by shRNA in neuronal cell cultures (Chawla et al., 2009)". In fact, if one were to look at the data it is quite different. In the current study the authors report Bin1-exon 7 splicing decreases from 34% to 26% in FXTAS. However, according to Chawla, when Sam68 is knocked down in N2A cells, the splicing increases from 30% to 43%. Similarly, the authors report that Clasp2 changes from 54% in unaffected tissues to 44% in FXTAS, while Chawla reports it increases from 25% to 48%. The only similar one is Dncic2 where there is a modest increase in both papers.

It would seem obvious and relevant to test out the splicing defects in the tissues from the knock-in mice that were used in Figure 5. However, no data is provided. Was this not done, or was there a discrepancy between the data generated from human tissues and those from mouse models? Sam68 knockout mice are also available; the hypothesis would predict that these mice should have the splicing defects as well. Was this tested, as this would obviously strengthen the arguments made in this paper?

Lastly, the sequential aggregation model predicts that eventually a number of RNA binding proteins including Sam68, but also MBNL1, hnRNP-A would be depleted in neuronal cells of FXTAS patients. Thus, splicing defects seen in the post-mitotic tissues of the brain where these large aggregates have formed over a long time, would presumably reflect the cumulative deficits of a number of RNA-binding proteins (including MBNL1, hnRNP-A and Sam68, and perhaps others). So, how can one attribute the observed splicing defects to only Sam68? Also, have splicing defects associated with MBNL1 depletion been assessed in FXTAS tissues, mouse models and the 72 hour cell extracts?

In addition it is not clear if there is a pattern to the affected alternative splicing events; i.e. is it exon inclusion or exon exclusion that is affected or both. Since Sam68 has either one of these effects depending on the target, it would have been interesting to note a trend if any. If the author had done some of their experiments in primary neuronal cell lines, they could have compared the changes found in patient tissues with those observed in the cell culture experiments.

The authors write about routes of treatment for FXTAS and emphasize the importance and specificity of tautomycin as it abolishes the Sam68 co-localization and CGG aggregate formation. This seems to be specific to CGG aggregates. The implication is that this might be a therapeutic approach that could rescue the splicing defects caused by Sam68 sequestration. Therefore, it would certainly strengthen the paper if the splicing data of ATPIIB after treatment with tautomycin is also provided. Is there a rescue of the splicing defect?

Overall, the paper is lengthy and can be made concise. Only specific details can be given and reiterations can be avoided.

This paper is primarily based on imaging data but no where in the paper or in "Materials and Methods" are microscopy details given. It will be good if the author provides details like whether these images are confocal or taken with a CCD camera, what the magnification was and where ever possible scale bars should be given.

Only for FRAP measurements the author mentions using a confocal microscope. Since, FRAP measurements are dynamic and very sensitive it will be good if the author also provides information on the details of whether a life-time imaging system, CO2 and humidified 37∫C chamber or a microscope stage warmer was used FRAP analysis as it may help readers to devise their FRAP experiments.

Also, when describing the FRAP experiment the author should be careful in how it is described. For example, on apge 10, the author writes "FRAP of transfected GFP-Sam68 was measured in nuclear regions containing CGG aggregates and compared to nuclear regions containing no CGG aggregates...". FRAP analysis is done on live cells so how does the author make sure that these nuclear regions contained the CGG aggregates without doing RNA FISH? The nuclear regions taken for FRAP analysis in this study only tells us about the aggregates of GFP-Sam68 and not about the CGG aggregates. It is presumed that the RNA aggregates are there, but not demonstrated in this specific experiment.

Minor comments:
1.Since, the co-localization data or the images for hnRNP A1 and hnRNP G-T with the CGG repeat have not been provided in the paper it is not required to mention about these protein throughout the paper. Readers can refer to the supplementary tables.

2.Details of alternative splicing in Results and Discussion can be made concise (pages 10-11 and 16-18).

3.Page 14, end of 1st paragraph- reference for the observations in CUG knock-in mouse models should be provided.

4.Page 16, author writes "FRAP analysis demonstrated that a significant fraction of nuclear Sam68 protein is immobilized within CGG RNA aggregates" whereas, FRAP analysis only demonstrates that after photobleaching the recovery is only ~60% because of Sam68 aggregation as there is less of mobile fraction of GFP-Sam68.

5.The results of RNAse and DNAse treatment are provided in the supplementary data. Therefore experimental details should be given in the figure legend of the same and removed from Materials and Methods.

6.There are some spelling mistakes. Page 15 1st paragraph "cytoplasmic" ; Supplementary Figure S3, "combined"; Supplementary Figure S4, "protein interactions" have been mis-spelled.

7.Cos 7 should be written instead Cos as on pages 7 and 22.

1st Revision - authors' response 02 November 2009

REFEREE #1.

1 -Endogenous proteins vs. over-expressed proteins**.**

Endogenous proteins were detected by immunofluorescence, and replaced over-expression in Fig. 2, 3, 4a, 9 and 10. Quantifications are in the supplementary table 3, and the open field images (X20) of Fig. 2 are in the referee-supplementary material.

Endogenous or transfected GFP-MBNL1 and GFP-hnRNP G gave similar results. However and thanks to referee's suggestions, we removed data that were non-physiological: over-expression of GFP-Sam68 leads to larger CGG aggregates (compare endogenous in Fig 2 to overexpressed WT and mutants in sup. Fig. 3). Similarly, over-expression of GFP-hnRNP A1 or A2 results in aggregation of these proteins 72 hours after transfection, while endogenous hnRNP A1 and A2 are recruited later (96 hours after transfection), and only within the giant aggregates that form in dying cells. In conclusion, over-expression of GFP-tagged proteins were removed and replaced by endogenous MBNL1, hnRNP G, Sam68 and hnRNP A proteins in Fig. 2, 3, 4a, 9, 10 and referee-supplementary material.

Two exceptions required expression of tagged proteins: the in cellulo FRAP analysis (Fig. 6) which necessitated a GFP-tagged protein, and the analysis of hnRNP G in Sam68 depleted cells (Fig. 4b), which necessitated a GFP-hnRNP G due to the poor quality of the hnRNP G antibodies (see below).

hnRNP G : please, note that all the antibodies against hnRNP G that we tested failed to resist the FISH conditions. Therefore we developed a plasmid expressing 60 CGG repeats fused to 3 MS2 RNA tags. MS2 tags are small RNA hairpins that are recognized with very high affinity and specificity by the MS2 coat protein. Thus, in figure 2, we were successful in detecting endogenous hnRNP G by immunofluorescence, and the CGG-MS2 RNA by localization of a transfected GFP-MS2 coat protein. Furthermore, still due to the poor quality of the hnRNP G antibodies, we were not able to detect both endogenous hnRNP G and Sam68 in Fig. 4b (both antibodies are rabbit and tested monoclonal antibodies did not work), so we used in that experiment a transfected GFP-hnRNP G .

2 - Mechanism by which Sam68 is recruited.

We put much effort into identifying the protein which bridge the CGG RNA repeats to Sam68, and found one candidate. This protein binds to CGG RNA and robustly co-immunoprecipitated with Sam68 (they are still associated in 0.4 M salt + RNase). Furthermore depletion of that protein by siRNA abolishes recruitment of Sam68 within CGG aggregates. This confirms that Sam68 is associated to a protein partner, which binds to CGG repeats. However, we need to study further that candidate and its function before considering publishing. Furthermore, description of that candidate is beyond the scope of the present article.

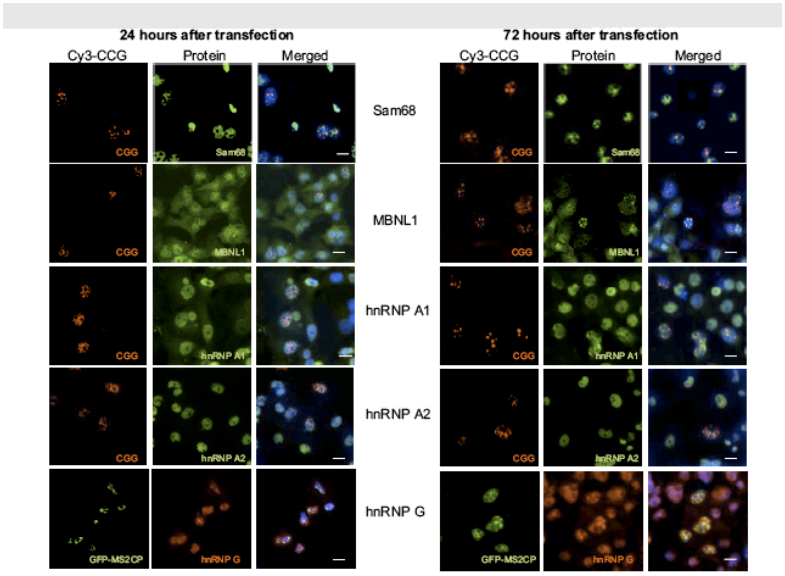
We confirmed hnRNP G-Sam68 interaction by yeast 2-hybrid and co-immunoprecipitation (Sup. Fig. 3A), but found no robust interactions between MBNL1 and Sam68 or hnRNP G. Therefore, recruitment of hnRNP G is explained by protein-protein interaction with Sam68, while the recruitment of MBNL1 must involves other intermediary proteins, which are currently not yet identified.

3 - Size of the CGG aggregates.

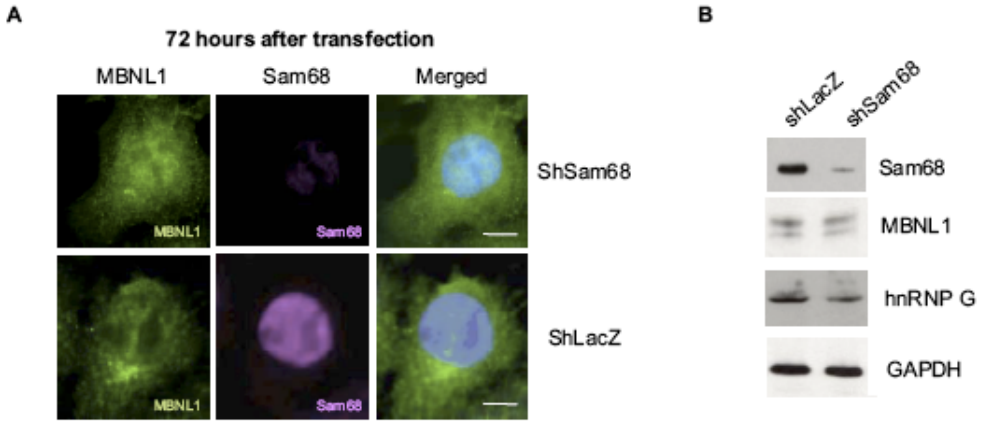
Sup. Fig. 3B: the CGG aggregates are larger when GFP-Sam68 is co-expressed. As suspected by the referees (see point #1), this is due to over-expression of GFP-Sam68, of which recruitment leads to bigger CGG aggregates. In contrast, the Sam68 N-terminal mutant, which does not co-localize with CGG repeats, does not induce larger aggregates.

4 - Sam68 shRNA.

Western-blotting showing decrease of endogenous Sam68 upon expression of an shRNA directed against Sam68 is presented in Fig. 2C. Both immunofluorescence and western blotting demonstrate that shRNA against Sam68 does not alter the localization of MBNL1 or the quantities of MBNL1 and hnRNP-G are presented in the referee-supplementary material.



COS7 cells were transfected with a plasmid expressing 60 CGG repeats and analyzed by RNA FISH combined to immunofluorescence using an antibody against MBNL1, Sam68, hnRNP A1 and hnRNP A2 24 or 72 hours after transfection. Endogenous hnRNP G was analyzed by co-transfection of COS7 cells with a plasmid expressing 60 CGG repeats fused to MS2 tags and a plasmid expressing the GFP-MS2 Coat Protein. Magnification 20x. Scale bar, 10 μm.



(**A**) COS7 cells were transfected with a plasmid expressing control or Sam68 shRNA and analyzed by immunofluorescence using antibodies against MBNL1 and Sam68, 72 hours after transfection. Magnification 63x. Scale bar, 10 μm. (Note: due to the poor quality of the hnRNP G antibodies, we were not able to detect both endogenous hnRNP G and Sam68).
(**B**) COS7 cells were transfected with a plasmid expressing control or Sam68 shRNA and analyzed by western blotting using antibodies against MBNL1, Sam68, hnRNP G and GAPDH 72 hours after transfection.

5 - Sam68 IHC.

We repeated the IHC but with longer incubation times with DAB (8 min instead of 3 min previously). Labeling of Sam68 is now evident in control and in FXTAS patient and presented in Fig. 5C. Sam68 is diffuse within the nucleoplasm in control, but localized in aggregates in FXTAS.

6 - Bcl-x splicing.

Analyzes of Bcl-x minigene is now included as Fig. 7A. Our data are identical to the one previously published (Paronetto et al., 2007), which demonstrated that Sam68 depletion by shRNA promotes the expression of the Bcl-xL isoform.

7 - ATP11B.

The method and rational behind the bioinformatics search are now referenced ("The Germ Cell Nuclear Proteins hnRNP G-T and RBMY Activate a Testis-Specific Exon" Liu et al., PLOS Genet. In press). Briefly, ATP11B exon 28b was first identified as it was adjacent to a testis-enriched exon, but proved to be both expressed in the human CNS and to be regulated by SAM68 and its paralog Slm2 (T-STAR). We can add this data to the supplementary material if the referees would like.

8 - FXTAS patients.

Number and of FXTAS patients are now included and referenced (cases 6, 7 , 9 and 10 in Greco et al., 2006).

9 - Phosphorylation of Sam68.

Sam68 tyrosine-phosphorylations after Sik transfection or AG490 or Dephostatin treatments are now presented as Fig. 9B. Overall our results are similar to previous finding (Lukong et al., 2005).

However, we were unable to definitively confirm Sam68 serine or threonine phosphorylation. Despite much effort, the anti-phospho serine or anti-phospho threonine antibodies that we tested were of poor quality to draw any conclusions. Therefore we moved TPA, PD98059 and AO treatments in the supplementary Fig. 6, and mention that Sam68 serine or threonine phosphorylation was not confirmed.

10 - Minor points.

The probe used is a Cy3-labelled (CCG)8x oligonucleotide as it should hybridize to CGG repeats. This is now clarified in supplementary Fig. 1A. Magnifications in all cell experiments are now identical and indicated (63x). Scale bars are now indicated.

REFEREE #2.

1 - Quantification of the splicing defects.

Alteration of alternative splicing of the ATP11B pre-mRNA was confirmed in FXTAS patients by real time RT-PCR, and is now included in Fig. 8A.

However and thanks to referee's suggestions, we found no significant changes of the splicing of BIN1, DNCIC2 and CLASP2 by qRT-PCR. Thus, the weak splicing changes that were previously found by classic RT-PCR were not significant and thus have been removed.

2 - The comment that "Sam68 is the only protein ..." was indeed not justified, and has been removed. Data on the proteomic analysis are now included and referenced.

3 - Sam68 shRNA.

Western-blotting showing decrease expression of Sam68 protein in cells transfected by Sam68 shRNA is now included (Fig. 4C).

Referee #3.

1 - Neuronal cells.

Endogenous localization of Sam68 within CGG aggregates in differentiated neuronal PC12 cells is now included (Fig. 2C).
However, PC12 cells are more sensitive than COS7 cells to CGG expression, and die in less than 48 hours after CGG transfection. Thus, while we observed at 48 hours giant CGG aggregates and late recruitment of hnRNP G and MBNL1, the toxicity of CGG repeats in PC12 cells impairs kinetic studies. We also tested other "neuronal" cell lines such as NG-108-15, IMR-32, Neuro-2a, SH-SY5Y, SK-N-MC, and SK-N-SH but no CGG aggregates were observed, confirming a previous report (Arocena et al., 2005) that not all cell lines can support CGG repeat aggregate formation. This has been addressed in the text.

2 - cell divisions.

As mentioned by the referee, no aggregate accumulation (CUG, AUUCU or CGG) can be observed in dividing cells. Therefore, all experiments were performed in 0.1% serum to inhibit cell divisions. This is now clarified in the manuscript.

3- Quantification of the splicing defects.

We confirmed by qRT-PCR the splicing alteration of ATP11B exon 28B (Figure 8, $p<0,005$ t-test). This is now included in Fig. 8.

However, and as noted by referees, the splicing changes in BIN1, CLASP2 and DNCIC2 found by classic RT-PCR were modest and after quantification by qRT-PCR proved to be not statistically significant, and therefore have been removed from the manuscript.

4 - Splicing in Knock-in mice.

We found that the Sam68 and CGG aggregates were smaller, dispersed and less frequent in knock-in mice, than in patients, which is consistent with the milder neuromotor and behavioral disturbances observed in mice compared to FXTAS patients. We estimated than in knock-in mice less than 5-10% of the cells contain Sam68 aggregates, while depending of the FXTAS patient, we found

Sam68-positive inclusions in 10 to 20% (up to 30% in hippocampal area of one patient) of the cells. Thus, we did not tested ATP11B splicing in CGG-knock in mice as, with less than 5-10% of affected cells, very little modifications were expected.

5 - Sam68 Knock-out.

In contrast, we tested by qRT-PCR ATP11B, BIN1, CLASP2 and DNCIC2 splicing in Sam68 KO mice, but found no statistical differences between brain samples of control and KO mice. This is most probably due to the compensatory effects of Sam68 paralogs, Slm1 and Slm2, both of which are highly expressed in brain. Consistent with this, ATP11B exon 28b minigene is also regulated at least by Slm2 (data not shown, although this data can be included if requested).The CGG depletion would sequester each of these proteins within nuclei, rather than just Sam68 in the KO mouse. These experiments are shortly discussed in the revised manuscript.

6 - MBNL1 depletion.

As noted by referees, a potential depletion of MBNL1 is a crucial question, specially considering Myotonic Dystrophies. We found that MBNL1 is present within CGG aggregates at late time point after CGG transfection and in FXTAS patients. However, we found that alternative splicing events known to be regulated by MBNL1 were not altered in CGG expressing cells nor in FXTAS patients. These results suggest that, while MBNL1 does co-localize with CGG repeats, it is not sequestered and does not lose its splicing function. This novel results are presented in supplementary Fig. 4.

Similarly, a splicing event (APP exon 7) known to be regulated by hnRNP A1 is not altered in FXTAS, suggesting that hnRNP A1 is also not sufficiently sequestered within CGG aggregates. This is now discussed in the revised manuscript.

7 - Tautomycin and splicing defects.

In the presence of Tautomycin we observed no deleterious effects of CGG repeats on splicing of the ATP11B minigene. This is now included in Fig. 10D. We obtained similar results in differentiated PC12 cells. Furthermore, splicing analysis in presence of Sik, AG490 and Dephostatin were performed and are now included in Fig. 9D.

8 - FRAP and microscopy.

Indeed and thanks to referee comments, in our FRAP experiment we did not detect CGG aggregates but Sam68 aggregates. FRAP experiments were performed using a confocal microscope combined to a heated stage (37ºc), but with no CO2 or humidifier as the experiments last 15 minutes. The manuscript was modified accordingly. Microscopy, FRAP details, magnification and scale bars are now included. Results and discussion sections are now shorter.

Overall, we believe to have answered all referees questions and comments. Furthermore, thanks to there suggestions, previous imprecisions due to over-expression of tagged-proteins and non-quantitative RT-PCR have been removed, and important issues have been clarified.

In conclusion, we believe that our manuscript demonstrates for the first time that an RNA binding protein (Sam68) is recruited by CGG repeats in transfected cells, neurons from CGG knock-in mice and FXTAS patients and, consequently, partially loses its normal splicing functions, as demonstrated by FRAP and splicing analysis in cells and in patients. Furthermore, Tautomycin treatment proves that CGG aggregates can be disrupted and are "druggable" targets for FXTAS patients. To my knowledge, both finding are new, and hopefully suitable for publication in EMBO Journal.

2nd Editorial Decision 25 November 2009

Thank you for submitting your revised manuscript for consideration by the EMBO Journal. It has now been seen by two of the original referees whose comments are shown below. While both agree that the current manuscript is improved, referee #1 finds a couple of issues are outstanding and the issue regarding the effect of Sam68 phosphorylation needs to be resolved.

Should you be able to address these criticisms, we would be happy to consider a revised manuscript. While it is EMBO Journal policy to only allow a single round of revision we can extend this to give you an opportunity to address these issues and acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. 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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The revised manuscript from Sellier et al., is now improved by the addition of analyses on endogenous proteins and controls missing in the first version of the manuscript.
However, there are still some points that need to be addressed or clarified before the publication in the EMBO journal.

1. It is well known that Sam68 loses its affinity for RNA upon tyrosine phosphorylation. For most of the splicing events tested (Bcl-x for Sam68 and SMN2 for the Slm1 paralog), tyrosine phosphorylation reverts the effect of Sam68 or Slm1. Thus, it is unexpected that treatments that increase Sam68 tyrosine phosphorylation lead to a recovery of its splicing activity. If the authors want to claim this, it should be directly tested on the ATP11B minigene in transfection experiments using Sam68 with or without Syk. However, the expected result is that Syk counteracts the effect of Sam68 on the splicing event and, indeed, this is what the figures 9 and 10 show: Syk and tautomycin induce splicing changes in the minigene like those caused by the CGG repeats. The fact that CGG repeats lose their effect could be due to saturation of the splicing regulation in the system (i.e. Sam68 is already excluded from the reaction because phosphorylated in tyrosine residues and released from target RNAs). This aspect should be reconsidered in the interpretation of the data.

2. The effect of tautomycin on CGG repeats is clear, but, as mentioned above, the alternative splicing of the ATP11B minigene goes in the same direction of the deleterious CGG repeats. Moreover, it is also evident the general effect of this drug on alternative splicing (Mermoud, et al., 1992). If Sam68 is the nucleation factor that drives the recruitment of other RNA binding proteins on CGG repeats, it would be better to act directly on it, impairing its localization into these CGG-foci, instead of using drugs that have a general and clear effect on the alternative splicing response of the cells. For instance, the authors should transfect the Sam68DNter to rescue the splicing events impaired in the disease or upon transfection of CGG repeats.

3. The authors claim that Sam68 acts as a nucleation site for the accumulation of additional RNA binding proteins in the CGG foci, but the RNA binding activity of Sam68 is dispensable whereas the N-terminus is required. The authors put forward the hypothesis that Sam68 is recruited to the

aggregates by protein-protein interactions. The authors should clarify in more detail the mechanism by which Sam68 is recruited to these foci. For example, the authors could use the N-terminus domain of Sam68 as bait to identify interacting proteins, and test them for the CGG-binding affinity.

Referee #3 (Remarks to the Author):

The authors have adequately addressed the concerns of the reviewers.

2nd Revision - authors' response 12 January 2010

REFEREE #1.

1 - Tyrosine phosphorylation of Sam68 inhibits its splicing activity.

As noted by the referee, the absence of cumulative effects on splicing of ATP11B observed in presence of SIK and CGG repeats could be also due to saturation of the splicing regulation. This is now clarified in the manuscript.

2 - Sam68ΔNter rescues the CGG-induced splicing changes.

Thanks to referee's suggestion, we tested a mutant of Sam68 (Sam68ΔNter), which is not sequestered by CGG repeats, and found that it rescued the splicing changes induced by expression of CGG repeats. These data suggest that CGG repeats affect splicing mainly through sequestration of Sam68. These results have been included as Supplementary Figure S4.

3 - Mechanism by which Sam68 is recruited.

We identified the complex which bridge the CGG RNA repeats to Sam68. This protein complex binds to CGG RNA and co-immunoprecipitates with Sam68 but not with Sam68ΔNter. Furthermore depletion of a protein of that complex by siRNA abolishes recruitment of Sam68 within CGG aggregates. However, our work requires more studies before considering publication. Notably, we need to characterize the activity of this complex in presence of CGG repeats and in FXTAS patients. Furthermore, description of this complex is way beyond the scope of the present article.

In conclusion, we believe to have answered all referee questions and comments. Furthermore, thanks to his/her suggestions, we added "rescue" experiments, which suggest that CGG repeats affect splicing mainly through sequestration of Sam68. We hope that our manuscript will be now suitable for publication in the EMBO Journal.

