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Loss of Fused in sarcoma (FUS) promotes pathological Tau splicing

Denise Orozco, Sabina Tahirovic, Kristin Rentzsch, Benjamin M. Schwenk, Christian Haass and Dieter Edbauer

Corresponding author: Dieter Edbauer, German Center for Neurodegenerative Diseases DZNE

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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 07 February 2012

Thank you for the submission of your manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. Referee 1's report is in a somewhat different format, as this referee participated in an ongoing structured referee report trial. As you will see, referees 1 and 3 find the topic of general interest, although referee 2 is less supportive of the study in this regard. All of them raise a number of issues and of technical improvements of the data that would need to be addressed during revision.

Given that all referees provide constructive suggestions on how to strengthen the work, and the majority support publication here, I would like to give you the opportunity to revise your manuscript. If all the referee concerns can be adequately addressed (including a the demonstration of direct FUS binding to Tau RNA, the use of shRNA#2 in the toxicity assays and further phenotypic characterization of shFUS-transfected neurons) we would be happy to accept your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

1. Do the contents of this manuscript report a single key finding? YES

2. Is the main message supported by compelling experimental evidence? YES

Overall, the experimental evidences presented by the authors are convincing and very well performed.

There is however one area of vagueness that should be clarified, and this regards the putative binding site for FUS/TLS in the vicinity of exons 3 and 10. First of all, it is not clear what are the Tau pre-mRNA and mRNA regions amplified by the primers for the experiment reported in Fig.3. Did the pre-mRNA amplified fragment contain the intron 3 or 10 motifs that resembled the FUS binding motif proposed by Hoell et al.?. If this was the case the authors should state this clearly. If not, there is always the possibility that FUS may be interacting with Tau RNA through some other sequences.

In general, therefore, this issue of the putative binding sites needs to be addressed in a little more detail. For example, what exactly do they mean by "resemble" (page 6, lines 1 to 3)?. An exact sequence comparison of the putative binding motifs near exons 3 and 10 with the established consensus should be shown, perhaps through a supplementary figure. How well are these motifs conserved in humans?.

Secondly, and at least for exon 10, it would be very important if the authors were to perform at least some kind of preliminary direct binding assay (EMSA with recombinant protein or UV-crosslinking of endogenous FUS to a labelled RNA followed by IP) to definitively identify the stem-loop sequence as a direct FUS binding site. In fact, although the knockdown, add-back, and minigene experiments all agree with a direct role played by FUS in exon 10 recognition they would have yielded pretty much the same results also if FUS was acting with the Tau RNA through a secondary protein-protein interaction. Alternatively, the authors could add back in Fig.2 a shRNA resistant HA-FUS protein uncapable of binding to RNA and checking whether this mutant was less capable of restoring the suppression of the 2N and 4R isoforms than the wild-type HA-FUS.

Finally, regarding exon 3, why did the authors not try to perform a minigene analysis similar to the one reported in Fig. 3C for exon 10?.

3. Have similar findings been reported elsewhere (e.g. on a closely related protein; in another organism or context)? NO

4. Is the main finding of general interest to molecular biologists? YES

5. After appropriate revision, would a resubmitted manuscript be most suited for publication:

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6. Please add any further comments you consider relevant:

Minor issues:

1) according to the text in Fig.2 the authors have used shFUS#2 to KD the protein. However, in the figure body the shRNA is indicated as just shFUS, that could possibly lead to some confusion. Please correct.

2) In the Western blot shown in Fig.2A does the overexpression of human HA-FUS downregulate endogenous expression?. Due to the intensity of the bands it is difficult to tell but if true it might be an interesting side observation.

3) why was the exon 10 minigene obtained from a mouse BAC library rather than rat (that is the main organism studied in this work)?. Are the putative FUS binding motifs also conserved in this species?.

Referee #2:

The authors identified the mRNA of microtubule associated protein Tau as physiological splicing target of FUS. Tau pre-mRNA is associated with FUS in mouse brain and knockdown of FUS in hippocampal neurons leads to preferential inclusion of exons 3 and 10. Moreover, FUS knockdown causes significant growth cone enlargement and disorganization reminiscent of Tau loss-of-function, suggesting that disturbed cytoskeletal function and enhanced expression of the neurodegeneration-associated exon 10 may contribute to FTLD/ALS with FUS inclusions. While the results are clear and convincing, their impact is somewhat mitigated. I believe these results could easily be published in a shorter format in journal with a more specialized focus.

comments:
In Figure 1a two different FUS shRNAs were used and the knockdowns were efficient. This enabled the authors to observe an upregulation of Tau 4R at the protein level. In parallel to this Tau 4R change, the authors observed the Tau 2N and 4R but at the mRNA level. The authors measured the viability of the cells using an XTT-based assay but only made such testing for the hsRNA#1. Given the shRNA#2 is the one used in the following experiments; it is unclear why the toxicity assay was not made using this same shRNA. This should be corrected in a revised manuscript. Furthermore in n Figure S2b it was the same shRNA#1 that was used. Why not the shRNA#2?

Which shRNA is is used in figure 3?

At the beginning of the second paragraph of the result section, the authors refer to figure S2b, it should be S1b.

Referee #3:

In the report by Orozco, FUS knockdown, but not TDP-43 knockdown, caused an increase in 4-repeat tau isoform protein and mRNA in cultured rat hippocampal neurons. Expression of HA-tagged WT human FUS could rescue the effect and suppressed the 4-repeat tau level below that of controls. FUS was found to associate with tau pre-mRNA and mRNA in mouse brain, and FUS knockdown favored tau exon 10 inclusion in a exon 9-11 minigene assay. Primary neurons in which FUS was knocked down showed greatly increased spreading at axonal growth cone tips, which was associated with the appearance of decreased microtubule bundling.

This is a very interesting study that links FUS expression to preferential tau isoform expression that

may be related to tau pre-mRNA as a splicing target of FUS in neurons. Some of the effects of FUS knockdown on axonal cytoskeletal dynamics may be relevant to the mechanisms of neurodegeneration in FTLD or other conditions.

Were there any differences in the number of neurites or branching patterns in hippocampal neurons transfected with shFUS? After longer time in culture, did FUS knockdown affect spine morphology or neuronal viability?

1st Revision - authors' response 22 May 2012

General response to the referees

The revised version of the manuscript contains the following new data:
-direct interaction of FUS and Tau RNA through UV-crosslinking experiments (Figure 3C)
-replication of data in Supplemental Figure S1A and S1B with the second shRNA targeting FUS
-extended analysis of neuronal morphology upon FUS knockdown (Supplemental Figure S4B)
-prolonged FUS knockdown is not toxic to neurons in vitro (Supplemental Figure S4C)

Point-by-point response to the referees

Referee #1:

1. Do the contents of this manuscript report a single key finding? YES

2. Is the main message supported by compelling experimental evidence? YES

Overall, the experimental evidences presented by the authors are convincing and very well performed.

There is however one area of vagueness that should be clarified, and this regards the putative binding site for FUS/TLS in the vicinity of exons 3 and 10.

We agree with the reviewer that this is an important point. We now appropriately addressed it following the helpful suggestions and show direct interaction of FUS and Tau pre-mRNA close to exon 3 and 10 in crosslinking experiments (new Figure 3C). For details see below.

First of all, it is not clear what are the Tau pre-mRNA and mRNA regions amplified by the primers for the experiment reported in Fig.3. Did the pre-mRNA amplified fragment contain the intron 3 or 10 motifs that resembled the FUS binding motif proposed by Hoell et al.? If this was the case the authors should state this clearly. If not, there is always the possibility that FUS may be interacting with Tau RNA through some other sequences.

We now explain the location of the PCR-amplicons more clearly in the legend (compare also Supplemental Figure 1C for a diagram). The PCR-amplicons from Figure 3A/B for Tau pre-mRNA (covering exon 10) and Tau mRNA (exon 12 to 13) do not contain stem-loop motifs that completely match the Hoell criteria (N_{4sense}-TA-N_{3-17}-Y-$N_{4antisense}$). The reviewer is correct that we cannot conclude from this experiment that FUS directly interacts with Tau transcripts at exon 10. However, we added UV-crosslinking data supporting direct interaction of FUS and Tau RNA (new Figure 3C and see below).

In general, therefore, this issue of the putative binding sites needs to be addressed in a little more detail. For example, what exactly do they mean by "resemble" (page 6, lines 1 to 3)? An exact sequence comparison of the putative binding motifs near exons 3 and 10 with the established consensus should be shown, perhaps through a supplementary figure. How well are these motifs conserved in humans?

We removed the sentence concerning the putative stem-loop in exon 10, as it does not match the Hoell criteria exactly. Moreover, only one of the FUS-binding probes (i9-2) contained a stem-loop according to Hoell et al., while one non-binding probe (e9) also contained such a stem loop. Data from Hoell et al suggests that FUS binds promiscuously to its target RNA in vivo with a preference for AU-rich intronic sites, as documented by several observed crosslinking-sites. Indeed, using UV-crosslinking of RNA probes with FUS from mouse brain, we could identify several ~300 nucleotide regions in the Tau RNA (exon 10 and 11 and within intron 2 and 9) that directly bind FUS. Several of these binding regions are well conserved in humans and thus provide a starting point for future analysis (new Supplemental Figure S5).

Secondly, and at least for exon 10, it would be very important if the authors were to perform at least some kind of preliminary direct binding assay (EMSA with recombinant protein or UV-crosslinking of endogenous FUS to a labeled RNA followed by IP) to definitively identify the stem-loop sequence as a direct FUS binding site. In fact, although the knockdown, add-back, and minigene experiments all agree with a direct role played by FUS in exon 10 recognition they would have yielded pretty much the same results also if FUS was acting with the Tau RNA through a secondary protein-protein interaction. Alternatively, the authors could add back in Fig.2 a shRNA resistant HA-FUS protein uncapable of binding to RNA and checking whether this mutant was less capable of restoring the suppression of the 2N and 4R isoforms than the wild-type HA-FUS.

We agree with the reviewers concerns and are grateful for the technical suggestions. Using UV-crosslinking we now could show a direct interaction of endogenous FUS with Tau RNA probes at several regions within intron 2 and 9 and to a lesser extent in exon 10 and 11. This is in agreement with recent data from Hoell et al, showing that FUS crosslinks predominantly to intronic RNA with a preference for AU-rich sequences.

Finally, regarding exon 3, why did the authors not try to perform a minigene analysis similar to the one reported in Fig. 3C for exon 10?

We focused our efforts on exon 10 splicing, because this splice event is critical for many neurodegenerative diseases. We now indicated this in the text. Additional, we now show direct binding of FUS to region in Tau intron 2 close to exon 3 (new Figure 3C), suggesting FUS-dependent splicing of exon 3 might be a direct effect, too.

3. Have similar findings been reported elsewhere (e.g. on a closely related protein; in another organism or context)? NO

4. Is the main finding of general interest to molecular biologists? YES

5. After appropriate revision, would a resubmitted manuscript be most suited for publication:

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Minor issues:

1) according to the text in Fig.2 the authors have used shFUS#2 to KD the protein. However, in the figure body the shRNA is indicated as just shFUS, that could possibly lead to some confusion. Please correct.

We added the exact name/number of all shRNA in Figure 2 in the figure body as requested and corrected this in all other figures where applicable.

2) In the Western blot shown in Fig.2A does the overexpression of human HA-FUS downregulate endogenous expression? Due to the intensity of the bands it is difficult to tell but if true it might be an interesting side observation.

We agree with the reviewer that this may be an interesting possibility. However, to reliably distinguish transfected and endogenous FUS a larger tag would be necessary. Without additional

data, we would prefer not to discuss a possible autoregulation of FUS. However we added new data (Figure 3B) showing that FUS is associated with its own RNA (in agreement with Hoell et al), supporting the idea of putative FUS autoregulation.

3) why was the exon 10 minigene obtained from a mouse BAC library rather than rat (that is the main organism studied in this work)? Are the putative FUS binding motifs also conserved in this species?

We used the mouse BAC, because a suitable BAC from rat was not commercially available. Moreover, we think that having independent data from two different species even strengthens our finding. We have evidence for a FUS/Tau interaction in two species: rat (FUS knockdown), mouse (RNA binding and crosslinking). Additionally, human HA-FUS rescues knockdown of rat FUS and knockdown of human FUS in HEK293 cells enhances exon 10 inclusion in a minigene assay with mouse Tau. For a conservation of intronic FUS-binding regions in Tau compare the new Supplemental Figure S5.

Referee #2:

The authors identified the mRNA of microtubule associated protein Tau as physiological splicing target of FUS. Tau pre-mRNA is associated with FUS in mouse brain and knockdown of FUS in hippocampal neurons leads to preferential inclusion of exons 3 and 10. Moreover, FUS knockdown causes significant growth cone enlargement and disorganization reminiscent of Tau loss-of-function, suggesting that disturbed cytoskeletal function and enhanced expression of the neurodegeneration-associated exon 10 may contribute to FTLD/ALS with FUS inclusions. While the results are clear and convincing, their impact is somewhat mitigated. I believe these results could easily be published in a shorter format in journal with a more specialized focus.

We thank the reviewer for the positive feedback, but disagree on the impact of our study. The role of Tau in neuronal cell biology and common neurodegenerative diseases has been of continuous interest to many labs for the last decades. In our study we discovered an unexpected link between two distinct disease groups of FTLD, FTLD-FUS and FTLD-Tau, because we identified exons 3 and 10 of Tau mRNA as physiological splicing targets of FUS. Importantly, cytoplasmic redistribution of FUS strongly suggests a loss-of-function component in the disease and increased expression of 4R Tau isoforms has been linked to several neurodegenerative diseases in the presence or absence of overt Tau aggregates. Therefore we strongly believe that our study will attract broad interest.

comments:
In Figure 1a two different FUS shRNAs were used and the knockdowns were efficient. This enabled the authors to observe an upregulation of Tau 4R at the protein level. In parallel to this Tau 4R change, the authors observed the Tau 2N and 4R but at the mRNA level. The authors measured the viability of the cells using an XTT-based assay but only made such testing for the shRNA#1. Given the shRNA#2 is the one used in the following experiments; it is unclear why the toxicity assay was not made using this same shRNA. This should be corrected in a revised manuscript. Furthermore in Figure S2b it was the same shRNA#1 that was used. Why not the shRNA#2?

We agree that is important to confirm these results with the second FUS shRNA. As shown in the extended Supplemental Figure S1A both shRNAs targeting FUS are not toxic to neurons. Additionally, we repeated the western blot from Supplemental Figure S1B with both shRNA constructs and show this data in the new Supplemental Figure S1B.

Which shRNA is used in figure 3?

We apologize for the confusion. Because neither shFUS#1 nor #2 target human FUS in HEK293 cells, we used a third shRNA targeting human FUS. We indicated this now clearly in the figure (shFUS-hu). The target sequences of all shRNAs are listed in the methods and the Supplemental Table S1.

At the beginning of the second paragraph of the result section, the authors refer to figure S2b, it should be S1b.

We corrected this mistake.

Referee #3:

In the report by Orozco, FUS knockdown, but not TDP-43 knockdown, caused an increase in 4-repeat tau isoform protein and mRNA in cultured rat hippocampal neurons. Expression of HA-tagged WT human FUS could rescue the effect and suppressed the 4-repeat tau level below that of controls. FUS was found to associate with tau pre-mRNA and mRNA in mouse brain, and FUS knockdown favored tau exon 10 inclusion in a exon 9-11 minigene assay. Primary neurons in which FUS was knocked down showed greatly increased spreading at axonal growth cone tips, which was associated with the appearance of decreased microtubule bundling.

This is a very interesting study that links FUS expression to preferential tau isoform expression that may be related to tau pre-mRNA as a splicing target of FUS in neurons. Some of the effects of FUS knockdown on axonal cytoskeletal dynamics may be relevant to the mechanisms of neurodegeneration in FTLD or other conditions.

We thank the reviewer for the supportive and helpful comments.

Were there any differences in the number of neurites or branching patterns in hippocampal neurons transfected with shFUS? After longer time in culture, did FUS knockdown affect spine morphology or neuronal viability?

We extended our phenotypic characterization of shFUS-transfected neurons by analyzing the requested parameters of neurite morphology. Overall, we found that the neurite number is not affected by FUS knockdown. Additionally, we analyzed neurite branching pattern. Because the minor neurites do not show significant branching at the developmental stage analyzed (Figure 4A and Dotti et al, J Neuroscience 1988), we quantified axonal branching and found no differences between FUS knockdown and control-transfected neurons. The new data is included in Supplemental Figure S4B. Regarding spine morphology, we had cited the work of Fujii et al. (Current Biology 2005) showing spine changes in the absence of FUS. They showed strongly reduced number of mushroom spines and increased number of filopodia-like protrusions in FUS/TLS knockout neurons. We now mention these data in greater detail in the revised version of our manuscript (page 9).

To test the effects of long-term FUS knockdown on cell viability, we analyzed neurons 14 days after nucleofection. Interestingly, even such a prolonged depletion of FUS does not cause overt neurotoxicity in vitro, suggesting that the neurodegeneration seen in vivo either requires a much longer incubation time or possibly other (environmental) factors. The results are shown in the new Supplemental Figure S4C.

2nd Editorial Decision 01 June 2012

Thank you for the submission of your revised study to EMBO reports. It has now been seen by referees 1 and 3, who recommend its publication and have no further comments. I am thus very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Many thanks for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports

