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TDP-43 regulates its mRNA levels through a negative feedback loop

Youhna M. Ayala, Laura De Conti, S. Eréndira Avendaño Vázquez, Ashish Dhir, Maurizio Romano, Andrea D'Ambrogio, James Tollervey, Jernej Ule, Marco Baralle, Emanuele Buratti and Francisco E. Baralle

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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 28 May 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. Your manuscript has been evaluated by three referees and I enclose their reports below. As you will see from their comments the majority of referees find the study to be potentially interesting but require some further experimental analysis before it can be further considered for the EMBO Journal. The issues that need to be addressed focus on two aspects, one being the link to disease and secondly the mechanism of autoregulation, both are central to the study.

Given the demonstration that multiple splicing factors and RNA binding proteins can autoregulate their levels, it is important to more directly link TDP-43 autoregulation to the TDP-43 proteinopathies, such as testing disease mutations like the RRM1 mutant in ALS patiants mentioned by referee #2. This is required for further consideration at the EMBO Journal. Secondly, both referees #1 and #3 require that some further insight into the mechanism of autoregulation be provided. Given the interest in the study and its potential implication for the TDP-43 proteinopathies, should you be able to address these issues, we would be wiling to 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. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments.

Please note that when preparing your letter of response to the referees' comments 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):

This is an interesting manuscript from the Baralle lab demonstrating that the RNA binding protein TDP-43 autoregulates its own expression by down-regulating the stability of its own mRNA. TDP-43 is of particularl interest because misregulation of its expression, localization and post-translational modifications are associated with various neurodegenerative conditions.

This paper shows that: i) overexpression of TDP-43 downregulates endogenous TDP-43 protein and mRNA, ii) mutations that impair the first RRM domain, or deletion of a C-terminal region known to interact with hnRNP A and C proteins, both impair the autoregulation, iii) pure TDP43 binds to an element within its own 3'UTR. iv) NMD is ruled out as a possible autoregulatory mechanism because no alteration in TDP43 mRNA levels were observed by northern blot after cycloheximide treatment. v) a morpholino antisense molecule targeting the 3'UTR element leads to increased TDP43 protein, vi) the stability of TDP43 mRNA is reduced by overexpression of exogenous TDP43, vii) knockdown of exosome components (Rrp6 and 44) increases the levels of TDP43 mRNA.

To my mind the authors have clearly established that TDP43 regulates the stability of its own mRNA and that it can bind to a 3'UTR element. However, what follows is weaker. The only data attempting to link the 3'UTR element to the autoregulatory loop is the morpholino experiment. But the data shown is not quantitated and only shows protein levels (rather than mRNA levels or stability). It also lacks controls to show, for example, that morpholino binding in other 3'UTR locations is without effect. There are numerous experimental approaches that are in routine use for analysis of regulation of mRNA stability via elements in 3'UTRs and binding of cognate factors. For example, the authors could use reporters containing the TDP43 3'UTR to test the necessity and sufficiency of TDPBE, test whether tethering of TDP43 affects stability etc. Combined with their existing range of reagents it should be possible to rapidly reach a deeper level of mechanistic understanding.

Specific comments

p7 "The greatest structural difference... is the lack of the 3'UTR". What about the complete lack of introns and the different promoter? These seem to be comparable differences.

Figure 5 is a negative result and not essential as a separate Figure. The two lanes showing that CHX has no observable effects could be included in Fig 2.

Figure 4. The data should be quantitated. Having already established that regulation is at the level of mRNA the effects on mRNA levels (and ideally stability - as in Fig 6A - should be shown). Additional negative control morpholinos that bind elsewhere to the TDP43 UTR would be good to demonstrate the specificity.

Figure 6B. While the data show that TDP43 levels are upregulated upon knockdown of exosome

components it is not clear that this is related to the autoregulatory loop. In knockdown conditions there is still a > 2-fold reduction in endogenous TDP43 in response to exogenous overexpression. It might be better to measure TDP43 mRNA levels in these experiments.

As well as references to studies of individual RNA processing factors regulated by NMD (Sun, Sureau, Wollerton, Rossbach) the authors should cite the more systematic analyses of Ni et al Genes Dev 21, 708-718, Lareau et al. Nature 446, 926-929, Salzmann et al. Mol Cell Biol. 13, 4320-30.

Minor typos etc.

p5 "TARDBP" - use TDP43 consistently
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Supp Fig 2B. Given the indicated position of PCR primers the upper band can only be V1pA4 but not V1pA1?
Supp Fig 2D. Annotation is wrong - I think Tet should go - + - + (left to right)?
P14 line 9. "Autoregulatory" should be "regulatory" when applied to other transcripts.

Referee #2 (Remarks to the Author):

Overview: The authors generated human cell lines expressing tagged forms of wild-type and mutant TDP-43 and observed that TDP-43 controls its own expression through a negative feedback loop. They demonstrated that the RNA binding properties of TDP-43 involved in autoregulatory are in its 3' UTR sequence. Tis autoregulation does not change splicing, but promotes RNA instability. They conclude that it is likely that disease-associated TDP-43 aggregates disrupt TDP-43 self-regulation, thus contributing to pathogenesis.

Comments: I believe the present study was very well conducted and the results are convincing. My only concern is that the authors could have chose to use the RRM1 mutant identified in ALS patients in parallel to the mutations they chose to introduce in the RRM1 domain.

Referee #3 (Remarks to the Author):

The manuscript by Ayala et al. describes what might be a new method of autoregulation by an RNA-binding protein. The authors expressed an exogenous version of TDP-43 in HEK293 cells and found that levels of the endogenous protein and mRNA declined relative to tubulin (Figures 1 and 2). Using CLIP (data not shown), the authors had shown that TDP-43 bound to its own 3'UTR. This observation may have triggered the investigation. They showed in the manuscript that a short portion of the 3'UTR was able to bind to recombinant TDP-43 with an affinity apparently greater than that of a known substrate (GU6; Figure 3) and that transfecting with an oligonucleotide complementary to this portion increased the level of expression (Figure 4), suggesting that the binding of TDP-43 to this sequence causes degradation. Degradation was not prevented by cycloheximide, suggesting that NMD was not involved (Figure 5). The authors analysed the stability of the endogenous mRNA after inhibiting transcription and demonstrated that the lifetime of a portion of the mRNA was reduced when exogenous TDP-43 was expressed (Figure 6). The levels of two proteins known to be components of the exosome were reduced by transfection with siRNA, and it was concluded that the preferential degradation of the endogenous mRNA was mediated by the exosome.

This is a straightforward and well-planned set of experiments that form a simple and interesting story. Moreover, the mechanism suggested would have implications for other examples of autoregulation and even, the authors suggest, for the development of proteinopathies.

There are a few points at which the evidence might need to be reviewed a bit.

1. In Figure 4, the complementary oligo produces an increase in the level of endogenous mRNA. However, the biological system is different from the one used throughout the paper, in that there is no exogenous protein. If the argument is that TDP-43-driven decreases in endogenous mRNA are mediated via this element, then the authors need to show that the oligonucleotide prevents endogenous mRNA levels falling after expression of exogenous TDP-43. Moreover, this woud provide an important control, currently missing, showing that TDP-43 mRNA lacking the 3'UTR element is unaffected by the presence of the oligonucleotide.

2. The important comparisons in Figure 6, as described on page 10, concern the ratios of levels of protein +/- Tet. In other words, the +/- ratio for control samples is 0.3, while for siRNA-treated samples it is 0.5. These are the values that should be plotted, and the error bars should be given for these ratios (adding the variances) so that we can judge whether 0.3 is significantly different from 0.5. I am sceptical of this, from the data shown. If the results are not significant then the conclusion that the process is exosome-mediated would not be tenable.

1st Revision - authors' response 28 September 2010

Referee #1

Comment:

This is an interesting manuscript from the Baralle lab demonstrating that the RNA binding protein TDP-43 autoregulates its own expression by downregulating the stability of its own mRNA. TDP-43 is of particularl interest because misregulation of its expression, localization and post-translational modifications are associated with various neurodegenerative conditions. This paper shows that: i) overexpression of TDP-43 downregulates endogenous TDP-43 protein and mRNA, ii) mutations that impair the first RRM domain, or deletion of a C-terminal region known to interact with hnRNP A and C proteins, both impair the autoregulation, iii) pure TDP43 binds to an element within its own 3'UTR. iv) NMD is ruled out as a possible autoregulatory mechanism because no alteration in TDP43 mRNA levels were observed by northern blot after cycloheximide treatment. v) a morpholino antisense molecule targeting the 3'UTR element leads to increased TDP43 protein, vi) the stability of TDP43 mRNA is reduced by overexpression of exogenous TDP43, vii) knockdown of exosome components (Rrp6 and 44) increases the levels of TDP43 mRNA. To my mind the authors have clearly established that TDP43 regulates the stability of its own mRNA and that it can bind to a 3'UTR element. However, what follows is weaker. The only data attempting to link the 3'UTR element to the autoregulatory loop is the morpholino experiment. But the data shown is not quantitated and only shows protein levels (rather than mRNA levels or stability). It also lacks controls to show, for example, that morpholino binding in other 3'UTR locations is without effect. There are numerous experimental approaches that are in routine use for analysis of regulation of mRNA stability via elements in 3'UTRs and binding of cognate factors. For example, the authors could use reporters containing the TDP43 3'UTR to test the necessity and sufficiency of TDPBE, test whether tethering of TDP43 affects stability etc. Combined with their existing range of reagents it should be possible to rapidly reach a deeper level of mechanistic understanding.

Response to the referee's comment:

We agree with this reviewer that in this work the mechanistic link between this element and the autoregulation was based on the CLIP result, its validation in vitro and the morpholino experiments (Figs 3 and 4). As requested, therefore, we have tried to expand and confirm these results both in regards to the

quantification of protein intensities and the presentation of additional recovery and up regulation data at the mRNA level. However, after repeating the experiments many times, we realized that that cells suffer considerably following morpholino transfection. We have synthesized new morpholinos with different fluorescent markers and can see clearly that these oligo nucleotides distribute in all the cell compartments and in good proportion within the cytoplasm, making it difficult to interpret the results (i.e.: is it a translation or processing effect?, etc.). Please, refer to the figure included in the referee-only supplementary file.

As a consequence, the results were highly variable per se and we could no longer feel comfortable with the conclusions that originated using this technique. To obviate these difficulties, we decided to use an alternative approach to block the binding of TDP-43 to the TDPBE 3' UTR region in a more nuclear specific fashion. We then centered on the 34 bp. TDPBE region to produce multiple U1snRNP molecules that targeted the 5' end, central region and 3' end of the TDPBE plus a control region. The advantage of using this approach was threefold: first of all, in our hands it never caused any kind of cellular stress, it is nuclear specific, and due to the greater size of the U1snRNP molecules with respect to MPOs they were expected to be more efficient in inhibiting binding of TDP-43. The results of these analyses, however, did not match our expectations either as there was no clear increase of endogenous TDP-43 protein synthesis in both control and Tet induction conditions following U1snRNP transfection.

In parallel with these experiments, however, we also performed a re-evaluation of the CLIP data applying new bioinformatics approaches. This analysis highlighted some additional minor CLIP hits for TDP-43 in the RNA region nearby the TDPBE (see Suppl. Fig.4). We therefore suspected that the previous difficulties using the morpholino and U1snRNPs were also due to the fact that we had focused on the TDPBE sequence alone rather than in a wider segment of the 3’ UTR.

This was also in keeping with additional results generated in our lab. In fact, while fulfilling this reviewer request of further validating our conclusions by analyzing TDP-43 3’ UTR function in an heterologous context we observed that hybrid GFP-TDP 3' UTR constructs which carry the full exons 5, 6 and 3'UTR sequences of the TDP-43 gene are subject to autoregulation just as the endogenous TDP-43 gene (see revised Fig.4). However, when we deleted a sensibly larger area than just the 34 nt. TDPBE sequence the regulation was progressively lost depending on the amount of TDP-43 binding sequence removed. Since these experiments have given more reliable and accurate results we have now replaced the morpholino approach for the heterologous functional one.

Specific comment #1

p7 "The greatest structural difference... is the lack of the 3'UTR". What about the complete lack of introns and the different promoter? These seem to be comparable differences.

Response to specific comment #1

The reviewer is correct in pointing out that this issue must be discussed in greater depth. These additional differences were also considered by us, but we focused mainly in the 3' UTR region for the following reasons: First of all, regarding the absence of introns, it must be taken into account that the 3' UTR region of TDP-43 is highly conserved among different species whilst none of the intronic regions share an even similar degree of conservation (see Suppl. Fig.1). We also know that evolutionary conservation is probably very important

for autoregulation as three heterozygous transgenic mouse models of TDP-43 overexpression have shown that +/- mice were observed to produce the same amount of protein and mRNA as the wild-type +/+ mice (Kraemer et al, 2010; Sephton et al, 2010; and Wu et al., 2010). Secondly, with regards to the use of different promoters, it should be considered that autoregulation can still be observed in the GFP heterologous constructs where promoter usage is different from the endogenous situation. Finally, and as a sidenote, the use of two different systems to produce stable cell lines also rules out an artefactual effect mediated by the protein tag sequences attached to the transgenic TDP-43 as these differed from each other in either system: FLAG and HA-strep tags. All these data suggest that autoregulatory properties are not unique to human TDP-43 and that they are probably shared among different species, at least vertebrate ones. Taken together, therefore, we consider that the major difference between our transgenes and the endogenous TDP-43 pre-mRNA is represented by the 3' UTR and not by the absence of introns or different promoters. This result is also supported by the GFP-3'UTR constructs (see revised Fig.4) where the promoter, most exons and introns of the TARDBP gene are missing.

Following the reviewer's comment, however, we now realize that this point should be better discussed in the revised version. Therefore, we have now added these considerations to the revised version of the manuscript.

Specific comment #2

Figure 5 is a negative result and not essential as a separate Figure. The two lanes showing that CHX has no observable effects could be included in Fig 2.

Response to specific comment #2

We have tried to implement these suggested changes in the revised version of the manuscript. However, the figure thus revised became very complex and although we agree with the referee that figure 5 is a negative result, the mechanism being investigated is NMD, which normally acts as a major player in many other autoregulatory mechanisms (as discussed in our work). For this reason, it is our feeling that although negative the information contained in Figure 5 represents a major result deserving a figure of its own.

Specific comment #3

Figure 4. The data should be quantitated. Having already established that regulation is at the level of mRNA the effects on mRNA levels (and ideally stability - as in Fig 6A - should be shown). Additional negative control morpholinos that bind elsewhere to the TDP43 UTR would be good to demonstrate the specificity.

Response to specific comment #3

As explained above, this issue has been specifically addressed in a new revised Fig.4 using a heterologous construct based on a GFP-expression vector that contains part of human TDP-43 exons 5 and 6, including all the 3'UTR region and deletions therein (especially in the light of recent data that show TDP-43 binding to additional elements beside the TDPBE reported in Suppl. Fig.4).

Specific comment #4

Figure 6B. While the data show that TDP43 levels are upregulated upon knockdown of exosome components it is not clear that this is related to the

autoregulatory loop. In knockdown conditions there is still a > 2-fold reduction in endogenous TDP43 in response to exogenous overexpression. It might be better to measure TDP43 mRNA levels in these experiments.

Response to specific comment #4

We have now performed quantitative PCR to quantify mRNA levels of the endogenous TDP-43 following control and exosome knock-down. The results are now included in the revised Fig.6.

Specific comment #6

As well as references to studies of individual RNA processing factors regulated by NMD (Sun, Sureau, Wollerton, Rossbach) the authors should cite the more systematic analyses of Ni et al Genes Dev 21, 708-718, Lareau et al. Nature 446, 926-929, Salzmann et al. Mol Cell Biol. 13, 4320-30.

Response to specific comment #6

As suggested by the reviewer, the references are now included in the revised version.

Minor typos etc.

p5 "TARDBP" - use TDP43 consistently

done

p5 last line "mutations in RRM1" should be "RRM1 mutants"

done

Supp Fig 2B. Given the indicated position of PCR primers the upper band can only be V1pA4 but not V1pA1?

The label of the isoform in Supp Fig2B was corrected to indicate V1pA1 only

Supp Fig 2D. Annotation is wrong - I think Tet should go - + - + (left to right)?

The annotation in the figure is correct.

P14 line 9. "Autoregulatory" should be "regulatory" when applied to other transcripts.

done

Referee #2 (Remarks to the Author):

Overview: The authors generated human cell lines expressing tagged forms of wild-type and mutant TDP-43 and observed that TDP-43 controls its own expression through a negative feedback loop. They demonstrated that the RNA binding properties of TDP-43 involved in autoregulatory are in its 3' UTR sequence. Tis autoregulation does not change splicing, but promotes RNA instability. They conclude that it is likely that disease-associated TDP-43 aggregates disrupt TDP-43 self-regulation, thus contributing to pathogenesis. Comments: I believe the present study was very well conducted and the results are convincing. My only concern is that the authors could have chose to use the RRM1 mutant identified in ALS patients in parallel to the mutations they chose to introduce in the RRM1 domain.

Response Referee #2's comment

Here, the referee is referring to the D169G mutation that has been previously described in one patient affected by a familial form of ALS (Kabashi et al, 2008) and that may have affected the RNA binding properties of TDP-43 thus presumably interfering with the autoregulatory process. This is indeed a fascinating possibility (although it should be considered that TDP-43 inclusions are universal in all patients, TDP-43 mutations are found in less than 5% of ALS/FTD patients. The D169G has been described only once in all the studies performed so far. In addition, this mutation, although falling within the RRM1 sequence, is very near to the C-terminal edge of this region (that spans 106-183) and is not predicted to affect the RNA binding abilities of TDP-43 but to affect its aggregation properties (Nonaka et al., 2009) and interaction with the ubiquilin factor (Kim et al., 2009). In keeping with this, we have now expressed this mutant as a GST-TDP43 fusion protein and our experiments show that its RNA and hnRNP-binding abilities (also important for autoregulation) do not seem to differ appreciably from those of the wild-type protein. Nonetheless, we agree with this reviewer that this issue should be discussed in the revised manuscript and therefore these results are now also included as Supplementary Fig.4 and briefly mentioned in the revised Discussion.

Referee #3 (Remarks to the Author):

The manuscript by Ayala et al. describes what might be a new method of autoregulation by an RNA-binding protein. The authors expressed an exogenous version of TDP-43 in HEK293 cells and found that levels of the endogenous protein and mRNA declined relative to tubulin (Figures 1 and 2). Using CLIP (data not shown), the authors had shown that TDP-43 bound to its own 3'UTR. This observation may have triggered the investigation. They showed in the manuscript that a short portion of the 3'UTR was able to bind to recombinant TDP-43 with an affinity apparently greater than that of a known substrate (GU6; Figure 3) and that transfecting with an oligonucleotide complementary to this portion increased the level of expression (Figure 4), suggesting that the binding of TDP-43 to this sequence causes degradation. Degradation was not prevented by cycloheximide, suggesting that NMD was not involved (Figure 5). The authors analysed the stability of the endogenous mRNA after inhibiting transcription and demonstrated that the lifetime of a portion of the mRNA was reduced when exogenous TDP-43 was expressed (Figure 6). The levels of two proteins known to be components of the exosome were reduced by transfection with siRNA, and it was concluded that the preferential degradation of the endogenous mRNA was mediated by the exosome.
This is a straightforward and well-planned set of experiments that form a simple and interesting story. Moreover, the mechanism suggested would have implications for other examples of autoregulation and even, the authors suggest, for the development of proteinopathies.

There are a few points at which the evidence might need to be reviewed a bit.

Response to referee #3's comments

1. In Figure 4, the complementary oligo produces an increase in the level of endogenous mRNA. However, the biological system is different from the one used throughout the paper, in that there is no exogenous protein. If the argument is that TDP-43-driven decreases in endogenous mRNA are mediated via this element, then the authors need to show that the oligonucleotide prevents endogenous mRNA levels falling after expression of exogenous TDP-43. Moreover, this woud provide an important control, currently missing, showing that TDP-43 mRNA lacking the 3'UTR element is unaffected by the presence of the oligonucleotide.

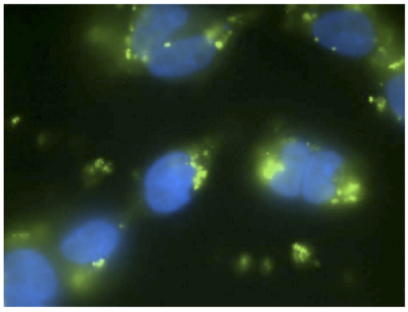
We have performed several controls with regards to the morpholino experiment (as detailed in the answer to Reviewer #1). As a result, using more recent CLIP and binding data that extends the region of 3'UTR bound by TDP-43 and analyzing the functionality of hybrid GFP-TDP 43 3'UTR constructs (Suppl. Fig.4 and revised Fig.4) we have considerably modified this approach in the revised version of the manuscript.

2. The important comparisons in Figure 6, as described on page 10, concern the ratios of levels of protein +/- Tet. In other words, the +/- ratio for control samples is 0.3, while for siRNA-treated samples it is 0.5. These are the values that should be plotted, and the error bars should be given for these ratios (adding the variances) so that we can judge whether 0.3 is significantly different from 0.5. I am sceptical of this, from the data shown. If the results are not significant then the conclusion that the process is exosome-mediated would not be tenable.

We have now performed additional analysis of the results and confirmed that these differences are statistically significant, albeit not in a major way. It should be noted, however, that the possible involvement of the exosome complex in TDP-43 autoregulation is also supported by the connection between TDP-43 and some components of this complex, as reported in the screening study on human mRNA degradation performed by Lehner and Sanderson (2004). Using a yeast two hybrid screening approach these researchers have reported TDP-43 as capable of interacting with the HRDC domain of PM/Scl-100 and Xrn2/Rat1. All this has now been better highlighted in the revised version. In a more general way, however, we are also fully aware that in other circumstances the mechanisms is probably more complex than presented in this work, and that mRNA stability and exosome processing probably do not tell the whole story, as insightfully pointed out by this reviewer. For example, the exact role played by NMD in the V2 or other TDP-43 isoforms, which are underor not expressed in our experimental system (but which may be predominant in other still uncharacterized circumstances), will certainly require more extensive studies. It should be considered, moreover, that the amount of binding of TDP-43 to its 3'UTR is almost certainly not the only check-point that acts within cells to keep the levels of this protein constant. In fact, considering the very high degree of conservation of the >3,000 long 3'UTR of TDP-43 (Suppl. Fig.1) it is very likely that several other interactions will participate in the fine-tuning of this process. Moreover, considerable changes in the protein half-life have been recently demonstrated to occur in different cell lines, suggesting that regulation could also occur at a post-translational level (Ling et al., 2010). Furthermore an additional control point at the level of transcription cannot be ruled out. Therefore, we expect that additional studies will be required to better unravel this issue. All these considerations have now been better discussed in the revised version to provide what we hope is a balanced view of this subject.

Response to referee #1 comment

Immunofluorescence following fluorescently tagged morpholino (blue -nuclei, green - MPO green) showing cytoplasmic accumulation.



2nd Editorial Decision 25 October 2010

Your revised manuscript manuscript has been reviewed once more by the original three referees. From their comments a number of issues need to be addressed prior to publication. This includes changes to the presentation of the manuscript, including the full description of the CLIP data. Both referee #1 and #3 also have remaining concerns about the exosome mediated destabilisation data in Fig 6 this needs to be addressed. If you already have the data describing the contribution of other mRNA decay pathways this should be added, even if it is negative data. Pending satisfactory minor revision, we would be willing to publish your manuscript in the EMBO Journal.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review. 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 reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have addressed most of my minor concerns. They have also replaced the previous morpholino experiments that subsequently proved to be problematic. Instead they have included a more convincing GFP-minigene approach that establishes that the TDP43 3'UTR is sufficient for down-regulation by exogenously expressed TDP43 protein. Using this vector they show that a 3'UTR region of 369-669 nt encompassing several CLIP tags is necessary for mediating down-regulation. However, deletion of the 34 nt region identified by the first CLIP tag had no effect.

One problem with the revised manuscript is that the authors still present the story based on the first CLIP tag they identified. They still refer to this as the TDP binding element (TDPBE), even though it is only one of a number of TDP binding elements in the 3'UTR (supplementary Figure 3), and it is not even a necessary element for autoregulation (data not shown). This is highlighted on p9: "A recent reassessment of the CLIP data...". Given that this is the first paper to use the CLIP data it would be appropriate to start with the full assessment of the CLIP data and develop the story from there.

The other problem - from this referee's perspective - is that the revised manuscript does not go any further than the original submission towards understanding the mechanism of TDP43 induced mRNA degradation. We know that TDP43 protein needs to be able to bind RNA via RRM1 and that it also needs the C-terminal domain, and we know that a region in the 3' UTR containing TDP43 binding sites is required, and that together these lead to degradation of TDP43 mRNA. However, numerous RNA binding proteins are known to autoregulate in various ways by binding to their own mRNA/pre-mRNA. So the interesting questions concern the mechanism by which TDP43 binding in the 3' UTR leads to mRNA destabilization.
The only data that starts to address mechanism is Figure 6, which includes new data panel 6D. Previously, knockdown of exosome components was shown to reduce the effect of exogenously expressed TDP43 upon endogenous TDP43 protein from a 70% decrease to a 50% decrease (6C).

This was a marginal effect, although statistically significant. The new panel 6D shows that the {plus minus} Tet ratio for endogenous TDP43 mRNA is 0.4 under control conditions, but 1.0 under exosome siRNA conditions. Are the authors really saying that exogenous TDP43 expression has no effect upon endogenous TDP43 mRNA when DIS-3 and PM/Scl-100 are knocked down (as implied by the ratio of 1.0)? Taken together with the 50% reduction in protein this would imply an effect of TDP43 on its own translation (or protein turnover), which would surely be worth pursuing. Or has the data somehow been normalized? Either way, it would be best to show the mRNA (6D) data in the same way as the protein data of panel 6C (i.e. show the four values for {plus minus} Tet, control vs exosome siRNA). The broader critique of Figure 6, is why the authors have focused only on one component of one mRNA decay pathway. Why not test other obvious factors e.g. decapping enzymes, deadenylases?

In summary, I think the authors provide a clear and compelling demonstration that TDP43 expression is autoregulated at the level of mRNA destabilization via binding to its own 3'UTR. This is certainly publishable. Given the lack of mechanistic insights, the broader interest of the manuscript derives from the connection of TDP43 misregulation with neurodegenerative conditions. In this respect, the demonstration of the feedback loop, with the potential for disruption of TDP43 expression, raises the general level of interest.

Referee #2 (Remarks to the Author):

The revised version of the manuscript rightly adressed this reviewer's concerns and those of the other two reviewers. I believe the results presented in this manuscript are convincing and believe the authors were quick to adress this question that is a hot topic in the field of ALS. In my view the evidence was already very convincing in the first version. My recommendation is therefore to publish it given its clean data and the question is burning the mind of several investigators.

Referee #3 (Remarks to the Author):

I had two concerns previously. The first was that Figure 4 lacked important controls. The material here has been replaced completely and the new version seems to me to be fine.

My second concern was that Figure 6 had not been analysed properly and it was not clear whether the absence of exosome components really did affect protein levels as would be predicted. The authors reply to this point does not address this rigorously. They say that they have analysed the results further and confirm that the 'differences are statistically significant, albeit not in a major way.' I would like to be helpful, but this is too vague.

The problem in Figure 6 is that the analysis of protein in 6C is incomplete. The important question is whether the reduction in endogenous TDP43 protein upon Tet addition is less after RNAi of exosome components. Figure 6D shows the results for mRNA levels, which are persuasive, but I would just like to see the corresponding ratios for protein. It looks, from Figure 6C, that this may not be the case. This is not fatal for the claims of the paper, but we need to know how strong or weak the data are. As it is, the authors avoided presenting the data in 6C as I had requested (in the same form as 6D) and this could easily have been done. Why not? It would make it easier for the reader to see the important comparisons.

2nd Revision - authors' response 29 October 2010

Referee #1

The authors have addressed most of my minor concerns. They have also replaced the previous morpholino experiments that subsequently proved to be problematic. Instead they have included a more convincing GFP-minigene approach that establishes that the TDP43 3'UTR is sufficient for down-regulation by exogenously expressed TDP43 protein. Using this vector they show that a

3'UTR region of 369-669 nt encompassing several CLIP tags is necessary for mediating down-regulation. However, deletion of the 34 nt region identified by the first CLIP tag had no effect.

One problem with the revised manuscript is that the authors still present the story based on the first CLIP tag they identified. They still refer to this as the TDP binding element (TDPBE), even though it is only one of a number of TDP binding elements in the 3'UTR (supplementary Figure 3), and it is not even a necessary element for autoregulation (data not shown). This is highlighted on p9: "A recent reassessment of the CLIP data...". Given that this is the first paper to use the CLIP data it would be appropriate to start with the full assessment of the CLIP data and develop the story from there.

The referee is absolutely correct in pointing out this need for a better presentation of the CLIP data. We did not reorganize the paper along these lines before to avoid excessive changes but we are happy to do it now. Therefore, in this revised version we have moved the CLIP and EMSA analyses originally displayed in Supplementary Fig.3 to revised Fig.3 in the manuscript. In addition, we have provided a more complete assessment of the CLIP data in the Results section (revised manuscript page 8, line 9 to bottom; page 9, line 1 to 10). A wider more general CLIP analysis is beyond the scope of this paper that focuses on the 3'UTR regulatory function. From Fig.3 we have focused the ms. on the more extended region TDPBR and not in the shorter TDPBE. The sequence of the latter was used only for the validation of binding of the non canonical sequence and for comparative affinity experiments with the canonical $(UG)_6$ (all in revised Fig.3).

The other problem - from this referee's perspective - is that the revised manuscript does not go any further than the original submission towards understanding the mechanism of TDP43 induced mRNA degradation. We know that TDP43 protein needs to be able to bind RNA via RRM1 and that it also needs the C-terminal domain, and we know that a region in the 3' UTR containing TDP43 binding sites is required, and that together these lead to degradation of TDP43 mRNA. However, numerous RNA binding proteins are known to autoregulate in various ways by binding to their own mRNA/pre-mRNA. So the interesting questions concern the mechanism by which TDP43 binding in the 3' UTR leads to mRNA destabilization.

The only data that starts to address mechanism is Figure 6, which includes new data panel 6D. Previously, knockdown of exosome components was shown to reduce the effect of exogenously expressed TDP43 upon endogenous TDP43 protein from a 70% decrease to a 50% decrease (6C). This was a marginal effect, although statistically significant. The new panel 6D shows that the {plus minus} Tet ratio for endogenous TDP43 mRNA is 0.4 under control conditions, but 1.0 under exosome siRNA conditions. Are the authors really saying that exogenous TDP43 expression has no effect upon endogenous TDP43 mRNA when DIS-3 and PM/Scl-100 are knocked down (as implied by the ratio of 1.0)? Taken together with the 50% reduction in protein this would imply an effect of TDP43 on its own translation (or protein turnover), which would surely be worth pursuing. Or has the data somehow been normalized? Either way, it would be best to show the mRNA (6D) data in the same way as the protein data of panel 6C (i.e. show the four values for {plus minus} Tet, control vs exosome siRNA). The broader critique of Figure 6, is why the authors have focused only on one component of one mRNA decay pathway. Why not test other obvious factors e.g. decapping enzymes, deadenylases?

As suggested by the reviewer we have shown the data in the same format as figure 6C. Using this format, it should now be more clear that from our quantitative PCRs (and notwithstanding the significant change observed in the ratio values, see old figure) the increase in TDP-43 endogenous levels following knockout of the exosome components is significant only in the +Tet samples. These results suggests that exosome degradation of the endogenous TDP-43 mRNA plays a predominant role when there are high amounts of total TDP-43 protein expressed in the cell. At the moment, we cannot explain why the fact that endogenous TDP-43 protein levels are also raised in -Tet conditions (Fig.6C) is not reflected at the mRNA level (Fig.6D, -Tet graphs). As the reviewer suggests, other factors, like translation modulation/mRNA compartments, may be playing a role in regulating TDP 43 protein levels. We are currently testing several hypotheses that hopefully will be a natural follow up to this work.

Nonetheless, we think that this reviewer is correct in pointing out the need to further clarify this issue and therefore several considerations to this effect have been added to the revised Results

(revised manuscript, page 13, lines 9-15) and Discussion (revised manuscript, page 15, lines 24-25; page 16, lines 1-4) section of the manuscript.

In summary, I think the authors provide a clear and compelling demonstration that TDP43 expression is autoregulated at the level of mRNA destabilization via binding to its own 3'UTR. This is certainly publishable. Given the lack of mechanistic insights, the broader interest of the manuscript derives from the connection of TDP43 misregulation with neurodegenerative conditions. In this respect, the demonstration of the feedback loop, with the potential for disruption of TDP43 expression, raises the general level of interest.

Referee #2 (Remarks to the Author):

No issues to be addressed.

Referee #3 (Remarks to the Author):

I had two concerns previously. The first was that Figure 4 lacked important controls. The material here has been replaced completely and the new version seems to me to be fine.

We thank the reviewer for appreciating all the effort that has gone in strengthening this part of the manuscript.

My second concern was that Figure 6 had not been analysed properly and it was not clear whether the absence of exosome components really did affect protein levels as would be predicted. The authors reply to this point does not address this rigorously. They say that they have analysed the results further and confirm that the 'differences are statistically significant, albeit not in a major way.' I would like to be helpful, but this is too vague.

The problem in Figure 6 is that the analysis of protein in 6C is incomplete. The important question is whether the reduction in endogenous TDP43 protein upon Tet addition is less after RNAi of exosome components. Figure 6D shows the results for mRNA levels, which are persuasive, but I would just like to see the corresponding ratios for protein. It looks, from Figure 6C, that this may not be the case. This is not fatal for the claims of the paper, but we need to know how strong or weak the data are. As it is, the authors avoided presenting the data in 6C as I had requested (in the same form as 6D) and this could easily have been done. Why not? It would make it easier for the reader to see the important comparisons.

As suggested also by this reviewer we have shown the data in the same format as figure 6C (see reply to referee 1 for additional considerations with regards to these changes).

3rd Editorial Decision 02 November 2010

I have looked through your newly revised version of the manuscript and find that you have addressed the concerns raised by the referees. I am happy to accept the manuscript fro publication in The EMBO Journal. One issue that needs to be resolved is the labels in Fig 3c. I would be grateful if you could send this as soon as possible. You should receive the official acceptance letter in the next day or so.

Yours sincerely,

Editor
EMBO Journal

