Redox signaling directly regulates TDP-43 via cysteine oxidation and disulfide cross-linking

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1st Editorial Decision 02 March 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see, the referees do not offer strong support for publication of the paper in The EMBO Journal - at least at this stage of analysis. I will not repeat all their individual points of criticism here, but while the referees consider the study as interesting in principle they feel strongly that the conclusiveness and the completeness of the experiments is insufficient at this point to justify the conclusions drawn and to make a strong case for the functional and (patho)physiological significance of your findings. Clearly, the referees point to major shortcomings in key aspects of the experimental evidence provided, and an extensive amount of further experimentation which includes crucial controls would be required to address these issues. Furthermore, the outcome of such experiments cannot be predicted at this point. I therefore see little choice but to come to the conclusion that we cannot offer to publish the manuscript.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS

Referee #1

In this work, Cohen et al., investigate the effects of disulfide formation on TDP-43 aggregation proteins when subjected to oxidative stress. Overall this is a very interesting work on the factors that may regulate TDP-43 aggregation properties during the development of pathology. In particular, the authors have studied the role of some cysteine residues from TDP-43 protein (173, 175, 198, 244) in aggregation, protein stability, and activity under stress conditions. Several experimental issues, however, still need some refinement in order to be more convincing.

First of all, according to Figure 1 arsenite treatment is more efficient at obtaining the most rapid change in TDP-43 solubility. Therefore, why did the authors use only H2O2 in the experiments performed for Fig.2? What if the experiments shown in this figure were repeated using arsenite? Aside from this, in Fig.2B the authors show the formation of high molecular weight TDP-43 species following H2O2 treatment in non-reducing immunoblot conditions. Although the appearance of these bands following H2O2 treatment is clear, it is somewhat doubtful whether it may be as “dramatic” as suggested by the authors. The reason for this is that the amounts of normal TDP-43 protein shown at the bottom of the gel is clearly not affected in either the - and + lanes for all the Abs tested. This fact suggests that only a very minor amount of the cellular TDP-43 gets converted to HMW TDP-43. Ideally, some way of measuring the amount of HMW TDP-43 bands with respect to the input should be used to provide a more accurate quantification of this phenomenon. Such a quantification procedure would also be particularly useful for the experiments shown in Fig.3D, where the difference between the two cysteine carrying mutants and the other mutants tested is not very clear, especially for the G348C mutant (as a side note, only the lower band indicated by the asterisk can be ascribed to the presence of an “extra” cysteine in TDP-43 as the upper band is really present in all samples).

In my opinion this is probably the most critical issue to be addressed in this work because if only a minor fraction of cellular TDP-43 is converted to HMW TDP-43 through the action of the cysteine residues then the clear loss in solubility observed following H2O2 and arsenite treatment (Fig.1) must be ascribed largely or significantly to some other factor other than disulfide bond formation.

Another issue that needs some clarification are the results using the 4CS mutants reported in Fig.4C where the total amounts of TDP-43 monomer (soluble) in the + and - lanes for the CS is much lower than WT expression levels. In these conditions, it is a little difficult to understand whether no HMW TDP-43 formation is due to a specific effect of these mutations or simply to low expression levels of this mutant. In this case, the authors should repeat the experiment trying to achieve equal WT and mutant expression levels in order to be able to really compare their respective solubilities/HMW TDP-43 formation.

Finally, in Fig.6 the authors show that arsenite treatment can functionally affect the splicing properties of TDP-43 using a CFTR exon 9 minigene reporter system. Although the effects shown in Figs.6A and B are rather convincing it is not clear why the C137S and C175S mutants have an effect on splicing with respect to WT. In fact, these mutants are mentioned for the first time in this figure and have not been previously analyzed with respect to HMW TDP-43 formation. Do they behave as the C137S/C175S and 4CS mutants shown in Fig.5C? In addition, for this experiment have the authors verified by Western-blot the amount of soluble TDP-43 variants following transfection in order to better correlate the overexpression of TDP-43 cys mutants and CFTR exon 9 splicing after arsenite treatment?

Minor comments

1) in Fig. 1C the authors have only measured the amount of TDP-43 mRNA levels. Is this observed
increment specific for TDP or also other related proteins mRNA levels are elevated? (ie. FUS/TLS etc.).

2) in Fig.3B, R and U lanes for untreated COS7 cells should be added to the figure.

3) please make the labelling of Figs. 5B and 5C consistent: in 5B the two cysteine mutant 2CS apparently corresponds to C137S/C175S in 5C. The name of this mutant is also spelled wrongly in page 12, line 6 from the top (C173/175S)

4) in Fig. 6 the representation of relative values of CFTR transcripts as a log ratio is a little confusing. Why not substituting it by reporting the direct ratio of spliced/unspliced products?

5) please modify the conclusion of the sentence on page 14 "...while TDP-43 over-expression led to enhanced accumulation of the exon 9 excluded product (Figure 6A, compare lanes 2 and 3), confirming that TDP-43 promotes CFTR splicing". In reality, TDP-43 is really an inhibitor of CFTR exon 9 inclusion (ie. splicing). It might thus be better if the authors concluded sayin "... confirming that TDP-43 inhibits CFTR exon 9 splicing").

Referee #2

Review EMBOJ-2011-77273

This manuscript contains several remarkable pieces of information how the pathogenic protein TDP-43 may become altered in common neurodegenerative diseases. First, a noticeable selectivity of TDP-43 incorporation into stress granules induced by several agents is described. Cellular stresses, particularly oxidizing, cause a dramatic loss of TDP-43 solubility in cell culture. This is claimed to involve cysteine disulfide bond formations, which are further studied with regard to biochemistry and molecular cell biology. Finally, effects on TDP-43 function are measured using an established splice reporter assay. Although potentially very interesting, the story is very bumpy at present and lacks rigorous advancement of knowledge.

Major Concerns:

1. By far the greatest effects are seen for insoluble monomers, the relative amounts of oxidized HMW TDP-43 species are comparably low. What is not easily understandable, if such an oxidative mechanism accounts for TDP-43 insolubility, why does boiling in reducing loading buffer not resolubilize the monomeric TDP-43?

2. It is a great pity that the present MS data fail to cover parts of TDP-43, which could be pivotal. Moreover, it is not clearly specified if additional oxidative modifications are absent, e.g. oxidation of specific cysteine or methionine side chains, dityrosine crosslinks, etc.

3. Considering points (1) and (2), it seems important to separate at least the in vitro oxidized TDP-43 species by size exclusion chromatography and subject to meticulous biochemical and biophysical characterizations. If the authors wish to uphold the exciting indication of distinct crosslinkings in the disease-associated G348C and S379C mutants, in which cysteine residues are introduced in the glycine-rich domain, where there are normally no cysteines, the same comprehensive characterizations should be included as well. In the present manuscript, no further analyses are done with these mutants, regrettably.

4. Please explain why the disease-associated A90V and R361S mutants form less HMW species that wild-type TDP-43 (Fig. 3D). And the G348C lane is useless, practically only the endogenous TDP-43 can be seen in the middle panel. Please provide anti-Myc probings to visualize transfected TDP-43 (also in Fig. 5C). Overall, in Fig. 3D much less loss of soluble TDP-43 proteins is seen +arsenite compared to the previous figures. In fact, in Fig. 5C there is no loss of soluble TDP-43 +arsenite at all. Does this indicate a problem in reproducibility?
5. Another heavy problem is the difficult, if not impossible separation of effects mediated by specific cysteine residues and global structural perturbations that are hampering firm conclusions especially for the 4CS mutant. First of all, the 4CS mutant expression appears lower in Fig. 5 (parallel control TDP-43 protein probings are absent for Fig. 6). This vitiates the negative conclusions for this mutant, where all effect reductions could simply reflect lower protein expression. In addition, the 4CS mutant appears to be misfolded/aggregating even under basal conditions (Fig. 5D), so no clear conclusions can be drawn for the specific cysteine residues investigated here. If understood correctly, a clean cysteine mutant confirming the authors’ hypothesis should behave exactly like wild-type but must be refractory to the stresses applied here, and remain (splicing) functional under stress (?) This is not the case for the present cysteine mutants.

6. As for the apparent disulfide bond formation between the adjacent C173 and C175, the experiments shown in Fig. 5B,C disprove an involvement in HMW crosslinking. In fact, HMW species can be seen for 2CS even in the absence of arsenite, which is opposite to the expectation and authors’ conclusions. And why are the single mutants dysfunctional in the CFTR reporter assay? Is it not more likely that these residues are important for the normal structure of TDP-43, potential hnRNP interactions, and/or RNA binding?

7. In Fig. 5E the endogenous mouse TDP-43 appears not only cleared from the nucleus, but actually gone altogether in the 2 strange-looking (morphologically altered?) 4CS-transfected cells shown. Does this reflect some regulation of the endogenous TDP-43 expression? Please address this point more comprehensively.

Additional Comments:

8. Although the immunostainings and solubility assays are very convincing, it is surprising that (nuclear) TDP-43 looks the same in control cells as after arsenite treatment. Could this impression be due to overexposure of panels (a) and (g)? Please provide in Fig. 1A the entire set of data including lower magnification photomicrographs and higher magnification inserts for all conditions. And did heat shock not induce stress granules?

9. The authors discuss that the resolubilization of TDP-43 after arsenite treatment is not because of de novo synthesis based on a turnover rate mentioned elsewhere. However, under these conditions TDP-43 mRNA is up-regulated, which should increase new protein synthesis. It would be desirable to determine protein levels, solubility and turnover rate as well as mRNA levels both at the short and prolonged time points.

10. Include total protein stains (e.g. Ponceau) for normalization of the solubility assays.

11. Why is there HMW TDP-43 in the control lane 1 of Fig. 2C?

12. Are the cysteine mutants recruited into stress granules or not?

13. Do the stress conditions used in Fig. 6 induce stress granules? If yes, a failure of CFTR reporter splicing might result from a sequestration of other important factors into stress granules, such as e.g. hnRNPA1. The authors may use a splice assay independent of TDP-43 to prove that the arsenite effects are specifically mediated via TDP-43 and not due to general alteration/sequestration of the cellular splicing machinery.

14. How many replicates were measured in Fig. 6? Add statistics. And include parallel quantifications of TDP-43 proteins.

15. Please provide a model scheme with as much atomic resolution as possible. The molecular model mentioned on p. 16 as "personal observations" should be shown.

16. The first sentences in the abstract and introduction are formulated in a circular manner ...TDP-43 is the major disease protein in [diseases with] TDP-43 pathology... this could be phrased more
elegantly.

Referee #3

Cohen et al describe a biochemical analysis of the TDP-43 protein in the context of various cellular stressors, and report that disulfide cross linking is observed in this context correlating with a shift from soluble to insoluble TDP-43. They also show which cysteine residues appear to mediate this via mass spec analysis using purified protein and cell lysates, as well as via mutagenesis studies, and then show that cellular stress alters a CFTR splicing assay which involves TDP-43 function. The work is overall sound, and the focus of connecting TDP-43 function and stress is important to advancing the field. As currently presented, there are some gaps in their ability to make this connection.

Major points:

1) A key finding that would bring relevance to their findings would be confirming disulphide crosslinked TDP-43 species in human tissues from ALS, FTLD patients but not normal tissues or disease controls. Such a finding would strengthen the paper tremendously. The absence of this is concerning, given that the authors have ready access such tissues, and this could be easily done.

2) The interpretation of the CFTR splicing assay should be taken with caution, as it is not a selective measure of TDP-43 function. This assay is well characterized to be influenced by numerous other splicing factors including hnRNPs (REF). Furthermore, other hnRNPs are well known to respond to various stressors including oxidation by altering their localization and function. The fundamental problem is that even though the splicing assay changes with stress and this correlates with TDP-43 solubility change, this does not prove that the splicing change observed is due to altered TDP-43 function. Possible solutions to this problem would include: i) rescuing the altered splicing pattern induced by Ars by overexpressing TDP-43. It is unclear why they chose to do most of their stress treatments in the context of TDP-43 overexpression, as it precludes this experiment from being done. ii) examining other assays of TDP-43 function (transcriptional suppression) which though similarly nonspecific likely do not depend on the same adjunctive factors as the CFTR splicing assay.

3) In Fig 1A, they show that TDP-43 does not enter stress granules on Ars treatment in COS7 cells. This of course is in distinction to some recent papers. One of the challenges in comparing the different papers is that quantitation (i.e. reporting the percentage of cells with stress granules that are TDP-43 positive) is rarely reported. The authors should report in both the H2O2 and Ars treated conditions what percentage of cells have stress granules that are positive for both TDP-43 and TIA-1.

4) The cysteine mutagenesis data is very difficult to interpret. Since the mutants clearly are not properly folded, one cannot interpret that these particular cysteines are involved disulfide bonding, rather they likely disrupt all aspects of TDP-43 function (such as RNA binding which is supported by their nuclear aggregation), including disulfide bond formation.

5) Along these lines, they show that the 4CS mutant depletes endogenous TDP-43 via immuno (Fig 5E), and later show that CFTR splicing is altered in these cells (Fig 6C), suggesting that this alteration is via TDP-43 depletion. Again to prove this is related to TDP-43 depletion, they should overexpress TDP-43 together with the 4CS and show that it rescues the altered splicing, if they wish to claim that the altered splicing activity is via secondary loss of TDP-43.

Minor points:

1) Figure 1 B (middle panel) shows ARS treatment in COS7 cells, with complete loss of TDP-43 in the RIPA soluble fraction. Then in Fig 1D, apparently the same experiment shows a different result, with residual TDP-43 in the RIPA soluble material. Is there a difference in the Ars concentration or treatment time? If so this is not stated.

2) Fig 1. It is curious that in the condition where TDP-43 becomes the most insoluble, (Ars treatment), there is no detectable TDP-43 aggregation on immuno. What is the author's explanation
for this apparent contradiction?

Additional correspondence (author) 03 March 2011

Thank you for the rapid turnaround in the review of our manuscript and we thank the 3 reviewers for their comments. Although all three reviewers found our study interesting and the overall work sound, they also raised a number of concerns. While we believe that we can address their concerns by conducting additional experiments as they recommended and clarify other issues that they have raised, we like to know that EMBO J will entertain a revised manuscript since as you know, it will take major efforts from us to conduct these additional experiments and address the issues raised by the reviewers.

Additional correspondence (editor) 04 March 2011

Thank you for your message asking us whether we would be prepared to consider a revised version of your manuscript. I have now had a chance to consider the case once more. It becomes clear from all three referees’ reports that there are major issues with the conclusiveness of the data and with the strength of the evidence provided for making a case for the functional and (patho)physiological significance of your findings. Now, in such a situation, when the outcome of an extensive amount of additional experiments including controls cannot be predicted, we cannot consider a formal revision. Still, I would like to point to the possibility to submit a new, considerably strengthened version of the manuscript as a new submission (rather than a revision) at a later time in case you will be able to strengthen the study - in particular those aspects that affect the functional and physiological significance of your findings - sufficiently along the lines put forward by the referees. To be completely clear, however, I would like to stress that if you wish to send a new manuscript this will be treated as a new submission rather than a revision. It will be evaluated again at the editorial level and reviewed afresh involving our original referees if available at the time of resubmission, also with respect to the literature and the novelty of your findings at the time of resubmission.

I hope that these thoughts will help you in your decision how to proceed.

Yours sincerely,

Editor
The EMBO Journal

Additional correspondence (author) 04 March 2011

Thank you for your recommendation. The reviewers comments have been very helpful in pointing out some of the deficits in our submission. We are currently conducting additional experiments along the line recommended by the reviewers and by your email below. I will let you know after conducting additional experiments if we decide to submit our revised manuscript to EMBO J.

Resubmission 22 August 2011

Major Revisions and Additions:

• One major concern raised by the reviewers was that only a minor fraction of TDP-43 becomes
oxidized to form high molecular weight (HMW) cross-linked species in response to oxidative stress, thereby questioning the importance of this modification. However, we like to emphasize that in addition to formation of HMW species, monomeric TDP-43 also forms intra-molecular disulfide cross-links in response to oxidative stress. These new data demonstrating robust formation of both intra- and inter-molecular disulfide cross-links are included in our revised manuscript. To briefly summarize our new findings: 1) both intra- and inter-molecular disulfide cross-link species are readily observed in vitro and in cultured cells by non-reducing immunoblotting (Figure 2-3), and mass spectrometry of TDP-43 protein bands confirmed these observations (Figure 4); 2) cysteine oxidation of monomeric TDP-43 was further confirmed by 14C-iodoacetate labeling (Figure 2); 3) we have quantified the oxidized TDP-43 species in arsenite-treated cells and estimated that a significant percentage of total TDP-43 forms HMW species (~13%), providing additional evidence that TDP-43 is highly susceptible to cysteine oxidation. Thus, our additional findings using several different methods support that it is unlikely that only a minor fraction of total TDP-43 protein becomes oxidized during stress.

• Although we demonstrated disulfide cross-linking in vitro and in cultured cells, the reviewers questioned the patho-physiological relevance of disulfide cross-linking in the human brain. We now provide new data supporting that TDP-43 forms detectable disulfide bonds in normal brain tissue, and importantly, these species are dramatically increased in pathological FTLD-TDP brain containing insoluble TDP-43 aggregates (Figure 6). These new findings provide evidence to support a novel pathological link between disulfide cross-links and TDP-43 aggregation observed in diseased FTLD-TDP brain.

• We demonstrated in cultured cells that specific ALS-associated TARDBP genetic mutations that introduce cysteine residues (i.e. G348C and S379C) in TDP-43 could increase distinct disulfide cross-linked species thereby providing evidence for a potentially novel pathogenic mechanism for these mutations. However, the reviewers questioned the validity and reproducibility of the distinct cross-linked species generated by G348C and S379C mutations. We have now performed a more comprehensive disulfide analysis using a panel of ALS mutations both in vitro and in cultured cells. Our data demonstrate that the cysteine-generating G348C and S379C mutants introduce a distinct ~90kD disulfide bonded TDP-43 species due to the additional cysteine residue, and this species is absent in WT or non-cysteine generating ALS mutations. Moreover, we quantified the production of the ~90kD oxidized TDP-43 species, which is now estimated as a percentage of the total TDP-43 pool (see Figure 7).

• We demonstrated in cultured cells that TDP-43 shows stress-type specific localization patterns, with more prominent localization to hydrogen peroxide-induced cytoplasmic stress granules (SGs), and a predominantly nuclear localization in response to arsenite stress. However, given the robust decrease in TDP-43 solubility observed after all stress treatments, the reviewers questioned whether TDP-43 forms aggregates in response to stress. Thus, a more extensive immunofluorescence analysis was performed to examine TDP-43 localization. We confirmed and quantified the peroxide-induced re-localization to cytoplasmic SGs, and secondly, we observed a profound nuclear aggregation of insoluble TDP-43 that occurs in response to arsenite, cadmium, and heat shock, thus providing evidence for distinct cytoplasmic and nuclear TDP-43 aggregation in response to different stressors. However, we emphasize that despite different subcellular localizations of TDP-43 in response to different stressors, disulfide cross-linking appears to be a common and more general mechanism mediating redox regulation of TDP-43.

Below, we respond to each reviewer’s comments and describe additional experiments we have done to address their concerns.

Referee #1

1) In this work, Cohen et al., investigate the effects of disulfide formation on TDP-43 aggregation proteins when subjected to oxidative stress. Overall this is a very interesting work on the factors that may regulate TDP-43 aggregation properties during the development of pathology. In particular, the authors have studied the role of some cysteine residues from TDP-43 protein (173, 175, 198, 244) in aggregation, protein stability, and activity under stress conditions. Several experimental issues, however, still need some refinement in order to be more convincing. First of all, according to Figure
1 arsenite treatment is more efficient at obtaining the most rapid change in TDP-43 solubility. Therefore, why did the authors use only H2O2 in the experiments performed for Fig.2? What if the experiments shown in this figure were repeated using arsenite?

The data presented in Figure 2 are all in vitro oxidation experiments using recombinant TDP-43 and require the use of H2O2 as a reactive oxygen species (ROS) to directly mediate cysteine oxidation. In contrast, arsenite does not directly oxidize cysteines, but instead mediates oxidative stress via several intracellular mechanisms, as cited throughout the manuscript. Thus, H2O2 is preferentially used for in vitro oxidation experiments (Figure 2), while both stressors are used in cell-based experiments (Figure 3-4). To further clarify, we have updated the references in the results section to better reflect the nature of these stressors.

2) Aside from this, in Fig.2B the authors show the formation of high molecular weight TDP-43 species following H2O2 treatment in non-reducing immunoblot conditions. Although the appearance of these bands following H2O2 treatment is clear, it is somewhat doubtful whether it may be as “dramatic” as suggested by the authors. The reason for this is that the amounts of normal TDP-43 protein shown at the bottom of the gel is clearly not affected in either the - and + lanes for all the Abs tested. This fact suggests that only a very minor amount of the cellular TDP-43 gets converted to HMW TDP-43. Ideally, some way of measuring the amount of HMW TDP-43 bands with respect to the input should be used to provide a more accurate quantification of this phenomenon. Such a quantification procedure would also be particularly useful for the experiments shown in Fig.3D, where the difference between the two cysteine carrying mutants and the other mutants tested is not very clear, especially for the G348C mutant (as a side note, only the lower band indicated by the asterisk can be ascribed to the presence of an "extra" cysteine in TDP-43 as the upper band is really present in all samples). In my opinion this is probably the most critical issue to be addressed in this work because if only a minor fraction of cellular TDP-43 is converted to HMW TDP-43 through the action of the cysteine residues then the clear loss in solubility observed following H2O2 and arsenite treatment (Fig.1) must be ascribed largely or significantly to some other factor other than disulfide bond formation.

We believe that the majority of TDP-43 proteins are modified by disulfide-cross-linking following oxidative stress treatments. In addition to the formation of HMW species, we now demonstrate that monomeric TDP-43 also forms intra-molecular disulfide cross-links in response to oxidative stress (Figures 2-4). Briefly, our new findings can be summarized as follows: 1) both intra- and inter-molecular disulfide cross-linked species are readily observed in vitro and in cultured cells using non-reducing immunoblotting methods (Figures 2-3), and mass spectrometry of these TDP-43 protein bands confirmed the presence of an intra-molecular C173-C175 disulfide bond (Figure 4), 2) cysteine oxidation of monomeric TDP-43 was further confirmed using [14C]-iodoacetate labeling (Figure 2), 3) we have quantified the HMW oxidized TDP-43 species in arsenite-treated cells and estimated that a significant percentage (~13%) of total TDP-43 forms HMW species, providing additional evidence that TDP-43 is highly susceptible to cysteine oxidation. Thus, our additional findings using several different methods support that a significant fraction of total TDP-43 protein becomes oxidized to form both intra- and inter-molecular disulfide bonds.

3) Another issue that needs some clarification are the results using the 4CS mutants reported in Fig.4C where the total amounts of TDP-43 monomer (soluble) in the + and - lanes for the CS is much lower than WT expression levels. In these conditions, it is a little difficult to understand whether no HMW TDP-43 formation is due to a specific effect of these mutations or simply to low expression levels of this mutant. In this case, the authors should repeat the experiment trying to achieve equal WT and mutant expression levels in order to be able to really compare their respective solubilities/HMW TDP-43 formation.

We now have repeated the experiment as recommended by this reviewer to achieve equal expression levels of monomeric WT and mutant TDP-43 in cultured cells (compare lanes 2, 4, 6, and 8 in Fig. 5D). Our new data show abrogation of HMW cross-linked bands (see darker exposure in Figure 5D) only in cells expressing the 4CS but not the WT and 2CS proteins. Thus, our results confirm that these cysteine residues mediate disulfide bond formation.

4) Finally, in Fig.6 the authors show that arsenite treatment can functionally affect the splicing properties of TDP-43 using a CFTR exon 9 minigene reporter system. Although the effects shown in
Figs. 6A and B are rather convincing it is not clear why the C137S and C175S mutants have an effect on splicing with respect to WT. In fact, these mutants are mentioned for the first time in this figure and have not been previously analyzed with respect to HMW TDP-43 formation. Do they behave as the C137S/C175S and 4CS mutants shown in Fig. 5C? In addition, for this experiment have the authors verified by Western-blot the amount of soluble TDP-43 variants following transfection in order to better correlate the overexpression of TDP-43 cyst mutants and CFTR exon 9 splicing after arsenite treatment?

Our results in Figure 5, both in vitro (Fig. 5B) and in cultured cells (Fig. 5D), demonstrate that Cys 173, 175, 198 and 244 are critical in the formation of TDP-43 disulfide cross-links. However, in agreement with the reviewer’s observations, we found that several of the cysteine mutants analyzed (including 4CS mutant) were partially insoluble in the absence of stress and remained non-responsive to stress treatments (i.e. they are redox-resistant), thus compromising our splicing analysis. Although we do not fully understand the unique solubility properties associated with the cysteine mutants, this altered baseline solubility prevented a reliable characterization of any purely redox-resistant mutants. Therefore the functional analysis of these mutants was omitted from this revised manuscript, but could form the basis for a future study on TDP-43 conformation. We would like to emphasize that the exquisite sensitivity of native TDP-43 conformation to cysteine modifications suggests that disulfide bond formation at these particular residues could have a major structural impact on TDP-43 function.

Minor comments 5) in Fig. 1C the authors have only measured the amount of TDP-43 mRNA levels. Is this observed increment specific for TDP or also other related proteins mRNA levels are elevated? (i.e. FUS/TLS etc.).

In revising and restructuring our manuscript, these data have been omitted from the revised manuscript and are no longer applicable.

6) in Fig. 3B, R and U lanes for untreated COS7 cells should be added to the figure.

In untreated COS-7 cells, TDP-43 is consistently observed in the soluble fraction, which is shown in triplicate in Figure 1C.

7) please make the labelling of Figs. 5B and 5C consistent: in 5B the two cysteine mutant 2CS apparently corresponds to C137S/C175S in 5C. The name of this mutant is also spelled wrongly in page 12, line 6 from the top (C173/175S)

The labeling and spelling issues have been corrected in the revised manuscript.

8) in Fig. 6 the representation of relative values of CFTR transcripts as a log ratio is a little confusing. Why not substituting it by reporting the direct ratio of spliced/unspliced products?

The CFTR splicing data is now shown in Fig. 1G (instead of Fig. 6) in the revised manuscript. We have represented CFTR splicing as log ratio to provide a standardized baseline as a control reference to compare all other samples. Any condition that resulted in increased exon 9 exclusion (i.e. increased splicing) has a positive value, while any condition that reduced exon 9 exclusion (i.e. reduced splicing) has a negative value. Thus, for clarity purposes, we believe that this is best way to display our data.

9) please modify the conclusion of the sentence on page 14 "...while TDP-43 over-expression led to enhanced accumulation of the exon 9 excluded product (Figure 6A, compare lanes 2 and 3), confirming that TDP-43 promotes CFTR splicing". In reality, TDP-43 is really an inhibitor of CFTR exon 9 inclusion (i.e. splicing). It might thus be better if the authors concluded saying "... confirming that TDP-43 inhibits CFTR exon 9 splicing”.

Based on the reviewer’s comment, we have re-worded the main text associated with Fig. 1G to better reflect that TDP-43 inhibits CFTR exon 9 inclusion rather than promotes exon 9 exclusion (paragraph 4 starting on bottom of page 6 to page 7).

Referee #2
This manuscript contains several remarkable pieces of information on how the pathogenic protein TDP-43 may become altered in common neurodegenerative diseases. First, a noticeable selectivity of TDP-43 incorporation into stress granules induced by several agents is described. Cellular stresses, particularly oxidizing, cause a dramatic loss of TDP-43 solubility in cell culture. This is claimed to involve cysteine disulfide bond formations, which are further studied with regard to biochemistry and molecular cell biology. Finally, effects on TDP-43 function are measured using an established splice reporter assay. Although potentially very interesting, the story is very bumpy at present and lacks rigorous advancement of knowledge. Major Concerns: 1. By far the greatest effects are seen for insoluble monomers, the relative amounts of oxidized HMW TDP-43 species are comparably low. What is not easily understandable, if such an oxidative mechanism accounts for TDP-43 insolubility, why does boiling in reducing loading buffer not resolubilize the monomeric TDP-43? We now demonstrate that in addition to the formation of oxidized HMW species, insoluble monomeric TDP-43 proteins are also oxidized and form intra-molecular disulfide cross-linked species in response to oxidative stress (see response to Reviewer #1, comment #2). Our solubility assays are performed by sequential extraction with buffers of increasing strength (i.e. RIPA buffer extraction, followed by UREA extraction of the insoluble pellet). Our data show that TDP-43 is soluble and readily extractable by RIPA buffer in untreated cells, however, cross-linked TDP-43 species generated in response to stress cannot be extracted by RIPA buffer, but are readily extractable in urea buffer. Thus, these distinct fractions provide a reliable readout for determining TDP-43 solubility.

2. It is a great pity that the present MS data fail to cover parts of TDP-43, which could be pivotal. Moreover, it is not clearly specified if additional oxidative modifications are absent, e.g. oxidation of specific cysteine or methionine side chains, dityrosine crosslinks, etc.

Mass spectrometry is most efficient at identifying intra-molecular disulfide bonds in short peptides (~5-15 amino acids) and this was substantiated by our ability to identify the disulfide bond between C173 and C175 (Figure 4). If the intra-molecular disulfide bonds on cysteine residues are further apart or if inter-molecular disulfide cross-links are formed, mass spectrometry is less effective. However, we confirmed the identity of the additional cysteine residues by mutagenesis (Figure 5). Other oxidative modifications of TDP-43 could be present, but studies to analyze and characterize them are outside the scope of the present study.

3. Considering points (1) and (2), it seems important to separate at least the in vitro oxidized TDP-43 species by size exclusion chromatography and subject to meticulous biochemical and biophysical characterizations. If the authors wish to uphold the exciting indication of distinct crosslinkings in the disease-associated G348C and S379C mutants, in which cysteine residues are introduced in the glycine-rich domain, where there are normally no cysteines, the same comprehensive characterizations should be included as well. In the present manuscript, no further analyses are done with these mutants, regrettably.

We agree with the reviewer that a detailed examination of oxidized TDP-43 is desirable. However, stress-induced disulfide cross-linking generates a heterogeneous mix of HMW species that are increasingly insoluble (Figure 2-3), a property that is not amenable to detailed biochemical and biophysical characterizations. Thus, in response to the reviewer’s comments, we have generated a panel of purified recombinant proteins containing ALS-linked mutations and analyzed their stress-induced disulfide banding patterns (Figure 7). We now provide evidence in vitro (Figure 7A, 7B) and in cultured cells (Figure 7C) that ALS-linked mutations that introduce an additional cysteine residue (G348C and S379C) generate a distinct disulfide signature characterized by a ~90kD species that is absent in WT or non-cysteine generating mutants. Hence, we raise the exciting possibility that abnormal TDP-43 cross-linking could represent a pathogenic mechanism associated with these mutations.

4. Please explain why the disease-associated A90V and R361S mutants form less HMW species that wild-type TDP-43 (Fig. 3D). And the G348C lane is useless, practically only the endogenous TDP-43 can be seen in the middle panel. Please provide anti-Myc probeds to visualize transfected TDP-43 (also in Fig. 5C). Overall, in Fig. 3D much less loss of soluble TDP-43 proteins is seen +arsenite.
compared to the previous figures. In fact, in Fig. 5C there is no loss of soluble TDP-43 +arsenite at all. Does this indicate a problem in reproducibility?

We agree with the reviewer that there is some variability in the formation of HMW species among the different ALS mutations. However, we attribute this variability to the transient and dynamic nature of the TDP-43 disulfide cross-linking that occurs in cells treated with arsenite, as well as the accumulation of insoluble and fibrillar HMW TDP-43 species (Figure 2-3 in the revised manuscript), since these experiments have been repeated > 6 times with similar results. Using both in vitro (revised Figure 7A, 7B) and cell-based (revised Figure 7C) assays, we demonstrate that only G348C and S379C results in the formation of a distinct ~90kD HMW species, while WT and other ALS mutants lead to the formation of the expected ~120-250kD species. We have now visualized transfected TDP-43 using anti-myc 9E10 antibodies in the revised Figure 5D and 7C.

5. Another heavy problem is the difficult, if not impossible separation of effects mediated by specific cysteine residues and global structural perturbations that are hampering firm conclusions especially for the 4CS mutant. First of all, the 4CS mutant expression appears lower in Fig. 5 (parallel control TDP-43 protein probeds are absent for Fig. 6). This vitiates the negative conclusions for this mutant, where all effect reductions could simply reflect lower protein expression. In addition, the 4CS mutant appears to be misfolded/aggregating even under basal conditions (Fig. 5D), so no clear conclusions can be drawn for the specific cysteine residues investigated here. If understood correctly, a clean cysteine mutant confirming the authors' hypothesis should behave exactly like wild-type but must be refractory to the stresses applied here, and remain (splicing) functional under stress (?) This is not the case for the present cysteine mutants.

We now provide new data in cultured cells showing that total monomeric proteins are comparable among these samples (new Figure 5D, compare lanes 2, 4, 6, 8), but only the 4CS mutant abrogated formation of HMW cross-linked TDP-43 bands (see darker exposure in Figure 5D), thereby confirming that these cysteines mediate disulfide bonding. These results are supported by our in vitro mutagenesis showing a similar requirement for these cysteines (Figure 5B, 5C). We agree with the reviewer that several of the cysteine mutants analyzed (including 4CS mutant) were partially insoluble in the absence of stress, precluding a functional characterization of these mutants. See response to Reviewer #1, comment #4.

6. As for the apparent disulfide bond formation between the adjacent C173 and C175, the experiments shown in Fig. 5B,C disprove an involvement in HMW crosslinking. In fact, HMW species can be seen for 2CS even in the absence of arsenite, which is opposite to the expectation and authors' conclusions. And why are the single mutants dysfunctional in the CFTR reporter assay? Is it not more likely that these residues are important for the normal structure of TDP-43, potential hnRNP interactions, and/or RNA binding?

We agree with the reviewer that C173 and C175 are likely not involved in forming HMW cross-linked species of TDP-43. In fact, we showed that C173 and C175 form an intra-molecular disulfide bond, which is detected in both monomeric TDP-43 and HMW TDP-43 protein bands (Figure 4). We favor the idea that all four cysteines engage in a complex network of both intra- and inter-molecular interactions, and this is supported by the lack of HMW species in the 4CS mutant lacking all four cysteine residues. This model is now more elaborately stated in the discussion at the bottom of page 15-16. Functional characterization of the cysteine mutants was omitted from the revised manuscript as per the response to Reviewer #1, comment #4.

7. In Fig. 5E the endogenous mouse TDP-43 appears not only cleared from the nucleus, but actually gone altogether in the 2 strange-looking (morphologically altered?) 4CS-transfected cells shown. Does this reflect some regulation of the endogenous TDP-43 expression? Please address this point more comprehensively.

These data were omitted from the revised manuscript as per the response to Reviewer #1, comment #4.

Additional Comments: 8. Although the immunostainings and solubility assays are very convincing, it
is surprising that (nuclear) TDP-43 looks the same in control cells as after arsenite treatment. Could this impression be due to overexposure of panels (a) and (g)? Please provide in Fig. 1A the entire set of data including lower magnification photomicrographs and higher magnification inserts for all conditions. And did heat shock not induce stress granules?

We now provide new data showing that arsenite treatment caused accumulation of insoluble TDP-43 within nuclear aggregates, similar to heat shock and cadmium treatment (Supplementary Figures 1-2). Thus, to summarize briefly, peroxide treatment led to robust re-localization to stress granules, while arsenite caused a distinctly different TDP-43 aggregation phenotype within nuclei. However, we would like to emphasize that redox signaling via cysteine residues occurred under both conditions, supporting our view that redox regulation is a general response to oxidant stress, while additional mechanisms likely exist to control TDP-43 localization in response to different stressors.

9. The authors discuss that the resolubilization of TDP-43 after arsenite treatment is not because of de novo synthesis based on a turnover rate mentioned elsewhere. However, under these conditions TDP-43 mRNA is up-regulated, which should increase new protein synthesis. It would be desirable to determine protein levels, solubility and turnover rate as well as mRNA levels both at the short and prolonged time points.

We now provide new data demonstrating that TDP-43 redox regulation occurs at the protein level and does not require new protein synthesis (Figure 1E). Briefly, we demonstrate that in the presence of cycloheximide, which inhibits new protein synthesis, cells treated with arsenite show the expected stress-induced accumulation of insoluble TDP-43. Upon subsequent arsenite removal (also in the presence of cycloheximide), TDP-43 transitioned from an insoluble fraction back to a soluble fraction. These data confirm that TDP-43 solubility is determined independently of new protein synthesis (Figure 1E), and firmly support our hypothesis of redox-regulation of TDP-43.

10. Include total protein stains (e.g. Ponceau) for normalization of the solubility assays.

Based on the reviewer’s comment, GAPDH is now included as protein loading controls in our solubility assays to ensure proper protein extraction and also equal loading.

11. Why is there HMW TDP-43 in the control lane 1 of Fig. 2C?

In this experiment shown in Figure 2C, EGS [ethylene glycol bis(succinimidyl succinate)], a sensitive protein cross-linking agent, induces a low basal level of TDP-43 aggregation (lane 1), which is not surprising given that TDP-43 is an aggregation-prone protein (J Biol Chem. 2009 Jul 24;284(30):20329-39). However, acute treatment with H2O2 caused a robust accumulation of HMW cross-linked species that were reduced by subsequent treatment with dithiothreitol (DTT) (Figure 2C, lane 4), confirming the formation of HMW disulfide bonds.

12. Are the cysteine mutants recruited into stress granules or not?

The cysteine mutants are not recruited into SGs. However, we have focused our manuscript on a general TDP-43 redox mechanism that is independent of TDP-43 localization, and therefore these data have not been included in this revised manuscript.

13. Do the stress conditions used in Fig. 6 induce stress granules? If yes, a failure of CFTR reporter splicing might result from a sequestration of other important factors into stress granules, such as e.g. hnRNPA1. The authors may use a splice assay independent of TDP-43 to prove that the arsenite effects are specifically mediated via TDP-43 and not due to general alteration/sequestration of the cellular splicing machinery.

QBI-293 cells show limited formation of stress granules, and we have confirmed that the stress conditions used in the splicing assay do not induce formation of stress granules in this cell type.

14. How many replicates were measured in Fig. 6? Add statistics. And include parallel quantifications of TDP-43 proteins.

Splicing assays were done in triplicate using three independent experiments, and statistical analysis
has been added to this experiment (revised Figure 1G). Comparable TDP-43 protein solubility analysis in response to stress is shown in Figure 1C and Figure 3A.

15. Please provide a model scheme with as much atomic resolution as possible. The molecular model mentioned on p. 16 as "personal observations" should be shown.

A detailed atomic model incorporating the cysteine residues can only be estimated with current structural approaches. Our major conclusion that TDP-43 is a redox regulated target does not require a structural analysis, but could be addressed in a future study on this topic.

16. The first sentences in the abstract and introduction are formulated in a circular manner ...TDP-43 is the major disease protein in [diseases with] TDP-43 pathology... this could be phrased more elegantly.

The abstract and introduction have been re-worded based on the reviewer’s comment.

Referee #3

Cohen et al describe a biochemical analysis of the TDP-43 protein in the context of various cellular stressors, and report that disulfide cross linking is observed in this context correlating with a shift from soluble to insoluble TDP-43. They also show which cysteine residues appear to mediate this via mass spec analysis using purified protein and cell lysates, as well as via mutagenesis studies, and then show that cellular stress alters a CFTR splicing assay which involves TDP-43 function. The work is overall sound, and the focus of connecting TDP-43 function and stress is important to advancing the field. As currently presented, there are some gaps in their ability to make this connection. Major points: i) A key finding that would bring relevance to their findings would be confirming disulphide crosslinked TDP-43 species in human tissues from ALS, FTLD patients but not normal tissues or disease controls. Such a finding would strengthen the paper tremendously. The absence of this is concerning, given that the authors have ready access such tissues, and this could be easily done.

We now provide critical new data demonstrating that TDP-43 forms cross-linked species in normal human brain but these species are much more prominent in pathological FTLD-TDP brain (Figure 6). This experiment was accomplished using a sequential extraction of human brain tissue followed by immunoblotting with several different TDP-43 antibodies (Figure 6). These findings support our view that aberrant TDP-43 redox regulation is a pathological feature associated with disease, and they also provide patho-physiological relevance to our study by showing that 1) there is TDP-43 redox signaling in the brain and 2) TDP-43 disulfide cross-linking appears to be augmented in FTLD-TDP brains.

2) The interpretation of the CFTR splicing assay should be taken with caution, as it is not a selective measure of TDP-43 function. This assay is well characterized to be influenced by numerous other splicing factors including hnRNPs (REF). Furthermore, other hnRNPs are well known to respond to various stressors including oxidation by altering their localization and function. The fundamental problem is that even though the splicing assay changes with stress and this correlates with TDP-43 solubility change, this does not prove that the splicing change observed is due to altered TDP-43 function. Possible solutions to this problem would include: i) rescuing the altered splicing pattern induced by Ars by overexpressing TDP-43. It is unclear why they chose to do most of their stress treatments in the context of TDP-43 overexpression, as it precludes this experiment from being done. ii) examining other assays of TDP-43 function (transcriptional suppression) which though similarly nonspecific likely do not depend on the same adjunctive factors as the CFTR splicing assay.

We agree with the reviewer about the limitations of this functional assay, however, it is the most widely used and accepted functional assay of this type to determine TDP-43 activity. Other attempts to assess TDP-43 function using transcriptional reporter assays were not as robust or reliable. Our data clearly show that arsenite impairs TDP-43-mediated exon 9 exclusion (Figure 1G), and we attribute this to inactivation of TDP-43 via cysteine oxidation and disulfide bond formation followed by TDP-43 aggregation. Indeed, TDP-43 is an aggregation-prone protein, and this is supported by the nuclear aggregation of TDP-43 observed in response to arsenite, which would likely inhibit any
effects on CFTR splicing (Supplementary Figure 1). Although other mechanisms could alter TDP-43 solubility and function, we believe that disulfide cross-linking is upstream from other changes in TDP-43 conformation.

3) In Fig 1A, they show that TDP-43 does not enter stress granules on Ars treatment in COS7 cells. This of course is in distinction to some recent papers. One of the challenges in comparing the different papers is that quantitation (i.e. reporting the percentage of cells with stress granules that are TDP-43 positive) is rarely reported. The authors should report in both the H2O2 and Ars treated conditions what percentage of cells have stress granules that are positive for both TDP-43 and TIA-1.

Based on the reviewer’s comments, we have now performed detailed quantification of SG formation in COS cells treated with either arsenite or peroxide. We show that although the SG marker TIAR localizes to both types of SGs, TDP-43 shows preferential recruitment to peroxide-induced SGs (Figure 1A and 1B), indicating stress-type specific regulation.

4) The cysteine mutagenesis data is very difficult to interpret. Since the mutants clearly are not properly folded, one cannot interpret that these particular cysteines are involved disulfide bonding, rather they likely disrupt all aspects of TDP-43 function (such as RNA binding which is supported by their nuclear aggregation), including disulfide bond formation.

We have now shown that all four cysteines are critical for disulfide bond formation using in vitro purified proteins to support our cell-based experiments (Figure 5A, 5B). See response to Reviewer #1, comment #4.

5) Along these lines, they show that the 4CS mutant depletes endogenous TDP-43 via immuno (Fig 5E), and later show that CFTR splicing is altered in these cells (Fig 6C), suggesting that this alteration is via TDP-43 depletion. Again to prove this is related to TDP-43 depletion, they should overexpress TDP-43 together with the 4CS and show that it rescues the altered splicing, if they wish to claim that the altered splicing activity is via secondary loss of TDP-43.

The functional characterization and immunostaining analysis using the cysteine mutants has been omitted from the revised manuscript for reasons stated in response to Reviewer #1, comment #4.

Minor points:

6) Figure 1 B (middle panel) shows ARS treatment in COS7 cells, with complete loss of TDP-43 in the RIPA soluble fraction. Then in Fig 1D, apparently the same experiment shows a different result, with residual TDP-43 in the RIPA soluble material. Is there a difference in the Ars concentration or treatment time? If so this is not stated.

The effects of arsenite on TDP-43 solubility were dependent on additional factors including cell confluency and cell plating conditions. However, we consistently observed 50-100% of total TDP-43 protein transitioning from a soluble to insoluble fraction upon 1 hr arsenite treatment in several cell types including QBI-293, COS-7, and Neuro2A cells.

7) Fig 1. It is curious that in the condition where TDP-43 becomes the most insoluble, (Ars treatment), there is no detectable TDP-43 aggregation on immuno. What is the author's explanation for this apparent contradiction?

We now provide immunofluorescence data using a Triton extraction method demonstrating that arsenite treatment caused accumulation of insoluble TDP-43 within nuclear aggregates, similar to heat shock and cadmium treatment (Supplementary Figure 1, please see response to Reviewer #2, comment #8).
Thank you for sending us a new version of your manuscript (previously EMBOJ-2011-77273) as a new submission. In the meantime, referee 1 and 2 have seen it again. Referee 3 was not available at the time of resubmission. As you will see, while both referees acknowledge the effort you have made to address their concerns, they are not convinced that the new version makes a strong enough case for the functional significance of oxidative-stress induced TDP-43 HMW formation, a specific function or an unspecific loss-of-function. Referee 2 is not in favour of publication of the study here. Referee 1 makes a number of suggestions how this key aspect of the study could be addressed by additional experimentation. Now, given that this is a resubmission, there is still the option for one round of revision. On balance, and given the interest expressed by the referees in principle, we would thus be willing to consider a revised version of the manuscript if you can address the points raised with respect to the functional role of TDP-43 oxidation by substantial further experimentation and beyond any doubt to the full satisfaction of the referees.

I should add that it is EMBO Journal policy to allow only a single round of revision and that acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version as well as on the final assessment by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

We do realise that addressing all the referees' criticisms may require quite some additional time and effort and be technically challenging. I would therefore understand if you wish to publish the manuscript rapidly and without any significant changes elsewhere, in which case please let us know, so we can withdraw it from our system.

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

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS

Referee #1

In this work, Cohen et al. resubmit an earlier work focused on highlighting the importance of redox signalling on TDP-43 regulation. The link through which redox signalling can directly act on TDP-43 solubility and hence, functions, is four cystein residues localized within and surrounding the RRM-2 domain.

First of all, the authors should be commended for having experimentally answered many queries that were originally made by this and other reviewers. In addition, in this new version they provide additional data coming from patient's brains that show an increase in HMW TDP-43 with respect to controls. In this respect, therefore, the paper has been very much improved.

However, still persists the concern regarding the real functional effects of oxidative stress leading to TDP-43 HMW formation and hence loss of normal functions. Is it present to a detectable degree or
not?. At present, the only data regarding this issue still remains the minigene analysis now reported in Fig.1G. In their rebuttal to reviewer #3, point 2 the authors quite correctly point out that it is the most widely used and accepted functional assay. However, there are now many additional functional consequences of TDP-43 removal/inactivation that could have been tested in parallel by the authors. For example, recent reports (Polymenidou et al, Ayala et al) have shown that an increase in TDP-43 mRNA level production should be expected following its depletion/inactivation. Has any increase in TDP-43 mRNA levels been observed by the authors following oxidative stress treatment with any of their reagents?. In addition, Polymenidou et al and Tollervey et al. also published prominent alterations in the splicing process of the POLDIP3 and BIM genes following TDP-43 removal. Do cells treated with H2O2 and/or Ars display similar changes in the splicing process of these genes?. Alternatively, do the levels of HDAC6 mRNA change as previously reported by Fiesel et al.?.

Minor points.

-In Fig.7B, panels 1 mM H2O2, it seems that the number of HMW species formed for the R361S mutant following H2O2 treatment is greater than the neighbouring S379C mutant. The reason may be that looking at the levels of TDP-43 monomer these are also much lower than those of R361S. A better quality picture should therefore be provided for this inset.

Referee #2

The authors have streamlined the revised manuscript. Most of the minor suggestions were successfully dealt with and many experiments were qualitatively improved. Importantly, the evidence for intramolecular oxidation-dependent band shift is pointed out, and oxidized TDP-43 HMW species are shown for human disease brain tissue.

What remains weak is the identification of specific cysteine residues that become oxidized (original point #5). The 4CS mutant indeed shows no HMW shifted material, but it is also underexpressed. Thus, it remains a serious difficulty to distinguish between specific loss of oxidation and general loss of protein. Moreover, the new Fig. 5C is unclear. Should there not be a loss of monomer TDP-43ox band shift in the 2CS mutant when concluding that the electrophoretic motility shift is due to C173-C175 disulfide bonding?

Minor correction: Fig. 7C legend refers to "soluble/insoluble fractions" and "reducing or non-reducing immunoblotting" but only 1 condition is shown (?)

Below, we respond to each reviewer’s comments in a point by point manner and describe additional experiments that we have conducted to address their concerns.

Referee #1

In this work, Cohen et al. resubmit an earlier work focused on highlighting the importance of redox signalling on TDP-43 regulation. The link through which redox signalling can directly act on TDP-43 solubility and hence, functions, is four cystein residues localized within and surrounding the RRM-2 domain.

First of all, the authors should be commended for having experimentally answered many queries that were originally made by this and other reviewers. In addition, in this new version they provide additional data coming from patient's brains that show an increase in HMW TDP-43 with respect to controls. In this respect, therefore, the paper has been very much improved.
1. However, still persists the concern regarding the real functional effects of oxidative stress leading to TDP-43 HMW formation and hence loss of normal functions. Is it present to a detectable degree or not? At present, the only data regarding this issue still remains the minigene analysis now reported in Fig.1G. In their rebuttal to reviewer #3, point 2 the authors quite correctly point out that it is the most widely used and accepted functional assay. However, there are now many additional functional consequences of TDP-43 removal/inactivation that could have been tested in parallel by the authors. For example, recent reports (Polymenidou et al, Ayala et al) have shown that an increase in TDP-43 mRNA level production should be expected following its depletion/inactivation. Has any increase in TDP-43 mRNA levels been observed by the authors following oxidative stress treatment with any of their reagents? In addition, Polymenidou et al and Tollervey et al. also published prominent alterations in the splicing process of the POLDIP3 and BIM genes following TDP-43 removal. Do cells treated with H2O2 and/or Ars display similar changes in the splicing process of these genes? Alternatively, do the levels of HDAC6 mRNA change as previously reported by Fiesel et al.? It may well be that, although significant, a 13% "loss" in active TDP-43 to HMW species may not be enough to cause detectable changes in all or some of these events. However, this is something that is very easy to test for and represents an information which should be provided by the authors.

We agree with the reviewer that downstream effects of oxidative stress on TDP-43 targets should be further evaluated and documented in our paper. Based on the reviewers’ helpful suggestions, we have performed quantitative RT-PCR to determine whether arsenite treatment causes an increase in TARDBP mRNA and a decrease in HDAC6 mRNA levels, as would be expected by TDP-43 inactivation during stress. The inclusion of these new functional data required a reorganization of the figures with a new Figure 2 demonstrating the effects of oxidative stress on TDP-43-mediated RNA functions. The new Figure 2A is the splicing data which were included as Figure 1G in the last submission. The new data in Figure 2B now show that arsenite caused a ~2-fold increase in TARDBP mRNA levels. Our analysis of TARDBP mRNA levels was specific, as a TDP-43 siRNA used as a control showed the expected reduction in TDP-43 mRNA levels. Furthermore, we observed a significant stress-induced decrease in HDAC6 mRNA levels (~3-fold), further confirming TDP-43 inactivation during stress. Given the stress-induced depletion of HDAC6, we asked if arsenite treatment caused the accumulation of the major HDAC6 substrate, acetylated-tubulin (Figure 2C). As expected, immunoblot analyses showed a dramatic increase in acetylated-tubulin levels concomitant with a reduction in HDAC6 levels in response to stress treatment. Taken together, we have used several different approaches in Figure 2 to support stress-induced inactivation of TDP-43 function and, consequently, alterations in downstream RNA targets including the HDAC6 transcript and the auto-regulated TARDBP transcript itself.

Minor points.

2. In Fig.7B, panels 1 mM H2O2, it seems that the number of HMW species formed for the R361S mutant following H2O2 treatment is greater than the neighbouring S379C mutant. The reason may be that looking at the levels of TDP-43 monomer these are also much lower than those of R361S. A better quality picture should therefore be provided for this inset.

We have repeated this experiment to achieve comparable levels of TDP-43 monomer among the WT and ALS mutants analyzed. The revised data shown in Figure 8B (formerly Figure 7B) demonstrate similar monomeric protein levels, with stress-induced accumulation of > 120kD HMW bands present in all TDP-43 proteins analyzed, however, a distinct ~90kD protein signature is present only in G348C and S379C mutants after exposure to 1 or 10 mM peroxide. These revised data firmly establish the unique disulfide properties of cysteine-generating ALS mutants (G348C and S379C), which are not observed in WT or non-cysteine generating mutants (G294A, R361S).

Referee #2

The authors have streamlined the revised manuscript. Most of the minor suggestions were successfully dealt with and many experiments were qualitatively improved. Importantly, the evidence for intramolecular oxidation-dependent band shift is pointed out, and oxidized TDP-43 HMW species are shown for human disease brain tissue.

3. What remains weak is the identification of specific cysteine residues that become oxidized
The 4CS mutant indeed shows no HMW shifted material, but it is also underexpressed. Thus, it remains a serious difficulty to distinguish between specific loss of oxidation and general loss of protein.

We agree with the reviewer that the 4CS mutant protein is slightly under-represented compared to WT protein extracts. To more accurately compare HMW species between WT and 4CS mutant proteins, we have performed immunoblotting analysis using 2-fold more 4CS protein (2.5 - 10% total insoluble protein input) compared to WT protein (1.2 - 5% total insoluble protein input). Using this method, we have more accurately normalized the amount of monomeric protein input, thus allowing a better determination of the formation of HMW species. As shown in the revised Figure 6E, HMW disulfide bonds are significantly reduced, but not completely abrogated, in the 4CS mutant compared to WT. Thus, these new data provide additional evidence to support our view that these four residues are the major redox-regulated cysteines present in TDP-43. However, we cannot rule out the possible involvement of additional cysteine residues, which is a point highlighted in the discussion at the top of page 16.

4. Moreover, the new Fig. 5C is unclear. Should there not be a loss of monomer TDP-43ox band shift in the 2CS mutant when concluding that the electrophoretic motility shift is due to C173-C175 disulfide bonding?

Our data suggest that C173-C175 intra-molecular cross-linking represents one of many possible disulfide configurations that occur in vitro and in cells exposed to oxidative stress. Although we were able to detect this particular cross-link (C173-C175) by mass spectrometry (Figure 5), it is equally plausible that other intra-molecular interactions occur, which are not readily identified by mass spectrometry due to the limitations of this approach. Therefore, we agree with the reviewer’s comment that intra-molecular interactions are not completely abrogated in the 2CS mutant. In contrast, we favor the idea that a complex, heterogeneous mixture of both intra- and inter-molecular interactions likely occur in response to stress-induced cysteine oxidation. Supporting this possibility, our in vitro and cell-based mutagenesis data in Figure 6 clearly show a substantial reduction in both intra- and intermolecular disulfide bonding in the absence of all four cysteine residues, suggesting these are in fact the major redox-regulated cysteines in TDP-43. Thus, we have further elaborated our current model involving these cysteine residues as a discussion point at the top of page 16.

5. Minor correction: Fig. 7C legend refers to "soluble/insoluble fractions" and "reducing or non-reducing immunoblotting" but only 1 condition is shown (?)

We have updated the legend in the revised Figure 8C (formerly Figure 7C) to better reflect the details of the experiment shown.

3rd Editorial Decision 28 November 2011

Thank you for sending us your (re-)revised manuscript. After some difficulties with their availability at the time of resubmission, our original referees have now seen it again, and you will be pleased to learn that in their view you have addressed their criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal.

Still, referee 2 puts forward one minor point that should be addressed (see below). Furthermore, there are two editorial issues that need further attention.

First, please add scale bars and explanations to figures 7A and S1.

Second, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers;
further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Please let us have a suitably amended manuscript as soon as possible. I will then formally accept the manuscript.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1
The authors have adequately answered all the questions that were raised by this reviewer.

Referee #2
The authors have provided more functional data and protein normalizations. In my opinion, the manuscript is now acceptable for publication.

Just one last correction (missed in the previous revision):
Page 11, line 10: "...the monomeric 4CS was less (*not more*) compact as the electrophoretic motility was slightly slower (*not faster*) compared to the more diffuse WT..." (?)

2nd Revision - authors' response 30 November 2011

Enclosed please find the final amendments to our accepted manuscript entitled “Redox signaling directly regulates TDP-43 via cysteine oxidation and disulfide cross-linking” by T.J. Cohen et al. We have addressed the one final concern from Reviewer #2 below, and have provided a final amended manuscript, figure files, and source data accompanying this submission.