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Knockdown of transactive response DNA-binding protein (TDP-43) downregulates histone deacetylase 6

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(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 06 May 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. I have now had a chance to read it carefully and to discuss it with my colleagues and I am sorry to say that we cannot offer to publish it.

We appreciate that you have identified HDAC6 as being regulated by TDP-43. Your results demonstrate that TDP-43 knockdown leads to decreased levels of HDAC6, with a consequent increase in levels of acetylated tubulin. Given that processed TDP-43 aggregates have been strongly implicated in a number of neurodegenerative disorders, and that HDAC6 is known to regulate aggresome formation and autophagy-mediated aggregate degradation, this is an intriguing link. However, we find that, currently, your analysis does not go far enough - either towards determining the mechanism by which TDP-43 regulates HDAC6 expression, or towards showing that loss of HDAC6 contributes to the development of TDPopathies. Given these limitations, I am afraid we do not feel that we can offer to consider your manuscript further at this stage. However, we can see the potential interest and importance of your findings. Therefore, if you are able to provide significant additional insight along the directions outlined above - particularly in terms of demonstrating that decreased HDAC6 expression is a critical event in the pathological context - we would certainly be willing to consider your manuscript again, with a view to sending it out for formal peer review.

Please note that we publish only a small percentage of the many manuscripts that we receive at the EMBO Journal, and that the editors have been instructed to subject only those manuscripts to external review which are likely to receive enthusiastic responses from our reviewers and readers. I am sorry to have to disappoint you at this stage, but I hope that this negative decision will not prevent you from considering the EMBO Journal for publication of future studies.

Yours sincerely,

Editor
The EMBO Journal

1st Resubmission – Authors' Response 28 May 2009

Please find attached an enhanced version of manuscript EMBOJ-2009-71258 entitled "Knockdown of transactive response DNA-binding protein TDP-43 downregulates histone deacetylase 6" by Fiesel et al. We would like to submit this manuscript for re-consideration as original article in the EMBO Journal.
The first version fell short in providing (1) mechanistic insight how TDP-43 regulates HDAC6 expression and (2) how HDAC6 reduction contributes to TDPopathies. We have performed additional experiments addressing these issues.
(1) We propose that TDP-43 influences HDAC6 levels by direct binding to the HDAC6 RNA, as shown in Figure 5. To strengthen this point, we have performed additional rescue experiments with structural TDP-43 mutants. We generated deletion constructs lacking the RNA recognition motifs, and used a nuclear localization signal mutant TDP-43. Transfection of these mutants failed to rescue HDAC6 mRNA and protein expression both in stably silenced shRNA^{TDP-43} HEK293E cells and in siRNA $^{TDP-43}$treated HEK293E cells (new Figure 4C-E). Thus, the HDAC6 regulating activity of TDP-43 depends on RNA binding and proper nuclear localization, consistent with the suggestion that TDP-43 influences HDAC6 mRNA processing.
(2) HDAC6 is known to mediate aggresome formation and autophagy of expanded polyQ proteins and thereby reduce polyQ proteotoxicity. TDP-43 knockdown mediated HDAC6 downregulation would be expected to reduce aggregate formation and enhance proteotoxicity. To test this hypothesis, we have employed as a model protein eGFP-tagged ataxin-3 (new Figure 7). When containing a very long (148 a.a.) polyQ expansion, but not a normal short (15 a.a.) glutamine stretch, Atx148Q-eGFP formed visible aggregates in 30% of the transiently transfected HEK293E cells. The percentage of aggregate-bearing cells was significantly reduced to 15% in stably silenced shRNATDP-43 cells. Consistently, Atx148Q-eGFP transfection caused a three-fold increase in cytotoxicity, whereas Atx15Q-eGFP or eGFP alone showed much less toxicity.
Importantly, both the reduced aggregate formation and the enhanced cytotoxicity of Atx148QeGFP were restored by co-transfection of HDAC6. These results are entirely consistent with the proposed model that TDP-43 deficiency down-regulates HDAC6 and thereby impairs the cellular defense against aggregating protein toxicity.

We hope very much that these major improvements of the manuscript render it suitable for consideration by the EMBO Journal.

2nd Editorial Decision 18 June 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, referees 2 and 3 are overall more positive, although both still raise a number of serious concerns with your manuscript, while referee 1 is generally negative - finding that your study does not provide a sufficient advance in our understanding of TDP-43 function. Given the positive recommendations of the majority of the referees, we are prepared to overlook the negative opinion of referee 1, and invite you to submit a revised version of your manuscript. However, it is clear that the study will require substantial

revision before we are able to consider publication.

Although differing in their overall enthusiasm for the study, all three referees highlight the same broad concerns (which are the same as those we initially raised). Firstly, they find that the mechanistic link between TDP-43 and HDAC6 levels needs to be strengthened - particularly in terms of mapping the protein-RNA interaction more precisely. Secondly, and perhaps more critically, the link to the pathological situation still remains somewhat weak, and it would be important to conduct additional experiments, particularly using constructs representing the human mutations, to address these major concerns. In addition, both referees 1 and 2 feel that it would be valuable to include a more complete description of the initial microarray data. A final specific comment: referee 2, in point 4, suggests that further analysis of the function of HDAC6 in this context would be valuable; clearly this is an important question, but we recognise that it is not the main topic of this study, and therefore we would not insist on experiments addressing this.

I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript.

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

Yours sincerely,
Editor
The EMBO Journal

REFEREE Reports

Referee #1 (Remarks to the Author):

The manuscript by Fiesel et al. (EMBOJ-2009-71484) uses RNAi knockdown in tissue culture cells to examine the molecular function of TDP-43, a protein found in cytoplasmic aggregates in the neurodegenerative diseases FTLD and ALS. The manuscript includes very strong data (Figures 2-4 and 6) supporting a role for TDP-43 in positively regulating the expression of HDAC6 (i.e, loss of TDP-43 causes reduced expression of HDAC6 and increased levels of acetylated tubulin). However, the rest of the data are not as strong and the importance of TDP-43-dependent regulation of HDAC6 in FTLD and ALS is not addressed. Therefore, the manuscript does not significantly advance our understanding of the molecular function of TDP-43.

Specific comments:
1. TDP-43 has been shown by others to predominantly reside in the nucleus, so this is not new information (Figure S1). Moreover, the studies presented in Figures 1 and S2 are difficult to interpret. There is some co-localization with markers of nuclear bodies, but this could simply be due to the fact that TDP-43 is spread throughout the nucleoplasm. Without functional studies, these data are not useful.
2. Since only selected genes from the microarray analysis of TDP-43 knockdown cells are discussed, it is impossible to effectively evaluate the importance of HDAC6 downregulation. A more complete presentation of the microarray data, including all of the genes that changed expression, needs to be provided in the manuscript. Also, this study should be repeated in a physiologically relevant cell type (i.e., neurons).
3. In Figure 4, it would be interesting to determine the extent to which TDP-43 mutant proteins identified in FTLD or ALS individuals are able to rescue HDAC6 expression in TDP-43 knockdown cells. This would begin to address the physiological importance of TDP-43-dependent regulation of HDAC6 expression.
4. The only data supporting a direct role for TDP-43 in regulating HDAC6 expression is the *in vitro* co-immunoprecipitation data shown in Figure 5. However, these data do not address the issue of specificity. TDP-43 is an RNA binding protein and thus may bind all RNAs to some extent in vitro. To address this possibility other mRNAs need to be examined. Furthermore, TDP-43 may be expected to preferentially interact with the intron-containing HDAC6 pre-mRNA. This should be examined.

5. The physiological relevance of the data presented in Figure 7 is unclear. Has TDP-43 been linked to polyQ-expansion diseases? Furthermore, the data are not convincing. Are the observed changes in toxicity statistically significant? Is RNAi knockdown of HDAC6 sufficient to increase toxicity? These experiments should be conducted in a physiologically relevant cell type (i.e., neurons).

Minor comments:
1. In the manuscript title, TDP-43 should be in parentheses. This will make it clear that TDP-43 is an acronym for transactive response DNA-binding protein.
2. There are spelling and grammatical errors throughout the manuscript.

In summary, the observation that HDAC6 levels are reduced in TDP-43 knockdown cells is interesting but, in the absence of experiments that probe the relevance of lower HDAC6 levels to neurodegeneration in FTLD and ALS, is insufficient for publication in EMBO J.

Referee #2 (Remarks to the Author):

In this work, Fiesel et al. describe the reduction in HDAC6 expression following TDP-43 depletion in a variety of cell lines and its effects in an experimental model of protein aggregation based on the polyQ-expanded Atx3 protein. HDAC6 is a peculiar deacetylase that localizes in the cytoplasm and harbors two copies of the catalytic domain. Among its substrates are tubulin and the heat shock protein 90. HDAC6 is thought to mediate cytotoxic responses. The results suggest that loss-of-function effects following TDP-43 removal from the nucleus might contribute to the pathological phenotype observed in patients affected by TDP-43 proteinopathies.
In general, the results are described clearly and the experiments are well performed.

Major point:

1- The authors should provide additional information regarding the differences observed in their microarray experiments. Considering that the levels of HDAC6 expression only drop to approximately 50-60% of the control (as shown in Fig.2C) it would be interesting to see the wider picture of the transcripts that are either up- or down-regulated in their experiment. As it stands, it is impossible to place the HDAC6 expression data in the wider perspective of potential pathogenic mechanisms. Having a more complete picture would explain why the authors focused on HDAC6 excluding other possible variations besides the analysis of ALS-associated genes reported in Table 1. In any case, I would expect them to report at least what was the effect of TDP-43 depletion on other HDAC family members.

2- In Fig.4D an experiment should be added to test the effect of over expression of TDP 43 lacking the C-terminal domain. This experiment will show if the recovery of HDAC6 regulated expression is also mediated by interactions of the C-terminal region of the protein where most of the patient's mutations are localized

3- In Fig.5, the authors demonstrate that TDP-43 can specifically interact with the 3'UTR sequence of HDAC6. At least a rough mapping analysis of the sequence bound by this protein should have been attempted (are there consensus binding sequences for TDP 43 in this sequence?).

4- The evidence for the connection between HDAC6 downregulation and cellular viability is weak and models concerning the role for this novel target in disease would require further studies. The authors should provide more information on the processes downstream of HDAC6 decrease that lead to cell death. HDAC6 plays a role in various cellular processes that involve microtubule/cytoskeletal integrity and cellular response to stress stimuli. Therefore, experiments that touch upon these processes in response to TDP-43 depletion should be addressed.

4- The experiment with the *Drosophila* model that shows reduction of HDAC6 levels following TBPH knock-down (Fig.6), supports the author's findings. However this experiment is rather preliminary. It is essential that the authors show directly, for example by immunoblotting, that the decrease in TABPH levels in transgenic flies is followed by the decrease in HDAC6 protein levels. Furthermore there is no attempt to analyze if Human and *Drosophila* HDAC6 share extensive

homology in their 3'UTR region that would justify an eventual interaction of TBPH with these sequences. Some experiments to prove at least this point should definitively be in order. As in the human cellular experiments, attempts should be done to analyze what happens to the other HDACs in *Drosophila* and establish if the drop is exclusive for HDAC6 or is also extended to the other family members.

Minor points

1- The studies of TDP-43 nuclear localization and colocalization with major nuclear structures do not provide novel information and should be removed or be presented as supplementary material. The authors assertion that there is lack of colocalization between TDP-43 and hnRNP A, hnRNP A2/B1 in HEK293 cells is not accurate. This work and previous studies show that, in addition to being localized in discrete foci, much of TDP-43 is found diffuse in the nucleus. The hnRNP A proteins are present in a diffuse nuclear pattern, therefore precluding the possibility to asses TDP-43 and hnRNP A complex formation through immunofluorescence.

2- In the last paragraph of the discussion section, the sentence: "Necessary for the elimination of aggregated protein is thereby a fine-tuned balance between HDAC6 and VCP (Boyault et al., 2006) that is genetically associated with IBMPFD (Watts et al., 2004b)." should be rewritten.

Referee #3 (Remarks to the Author):

TDP-43 defects have been linked to the development to FTD and ALS. The mechanism by which TDP-43 affects neurodegeneration remains uncertain. In this manuscript, the authors presented evidence that TDP-43 silencing by siRNA led to down regulation of HDAC6 mRNA and protein. This reduction of HDAC6 could be rescued by wild-type but not mutant forms of TDP-43. They also showed that TDP-43 bound to HDAC6 mRNA, which is consistent with the known RNA binding activity of TDP-43. Using expanded polyQ ataxin-3(Atx3) as a model proteotoxic protein, they showed that knockdown of TDP-43 reduced formation of large aggregates and increased cell death due to HDAC6 deficiency.

This report identifies HDAC6 as a target of TDP-43. The regulation of HDAC6 expression level by TDP-43 is interesting of potential significance. Overall this is an interesting report that provides a potential mechanism connecting TDP-43 to HDAC6-dependent protein aggregate clearance and neurodegeneration. However, the patho-physiological importance of this observation in TDP-43-related diseases was not sufficiently explored. For example, whether TDP-43 mutations found in human FTD would affect HDAC6 expression and aggregates formation should be examined. The documentation of a change in HDAC6 levels in a few FTD patients with TDP43 mutations/aggregates would further strengthen the importance of this report. The better establishment of the patho-physiological relevance of this finding would be needed to justify its publication in EMBO.

Other specific comments:
1) Figure 5. The binding of TDP43 to *in vitro* transcribed HDAC6 is not as convincing. At the minimum, the inclusion of an RNA binding-deficient TDP43 mutant should be included as control for this assay. If TDP43 regulates HDAC6 mRNA stability independent of HDAC6 UTR, as proposed by the author, it would be helpful to demonstrate that TDP43-siRNA should also reduce the levels of ectopically expressed HDAC6 lacking the UTR.
2) Figure 6B. The effect of fly TDP-43 (dmTBPH) mutation on HDAC6 mRNA is less obvious and could be strengthened by quantification of mRNA from a few flies using real time PCR.
3) Fig.7 representative and quality confocal images of inclusion bodies should be shown to document the effect of TDP-43 on mutant at3-induced inclusion bodies formation. A knockdown of HDAC6 should also be included as a control.
4) p12; {Bichelmeier, 2007#286} should be formatted. p13; Reference to Figure 7B should be included.

1st Revision - Authors' Response 14 September 2009

Please find attached a revised version of manuscript EMBOJ-2009-71484 entitled "Knockdown of transactive response DNA-binding protein (TDP-43) downregulates histone deacetylase 6" by Fiesel et al.

The general needs for improvement shared by the referees and editors were (1) to provide stronger evidence for the link between TDP-43 protein and HDAC6 mRNA, including specificity controls and some mapping of the TDP-43 binding site on HDAC6 mRNA, (2) assessment of effects of clinical TDP-43 mutations on HDAC6 expression and (3) more complete description of the microarray data. We have performed more experiments to address these issues and provide the requested information. Moreover, we have made several improvements in compliance to additional and minor comments by all 3 referees. All these substantial additions increased the character count. To keep the limits, a few established methods were moved to the Supplementary Information.

(1) As a specificity control we have employed HDAC1 in key experiments. In contrast to HDAC6, *in vitro* biotinylated HDAC1 RNA did not specifically pull down TDP-43. Moreover, we obtained antibodies against *Drosophila* HDACs, and confirmed the specific reduction of dmHDAC6 protein but not of dmHDAC1 in $TBPH^{+/-}$ fly heads.
Following the advice of referee #2.4 we have aligned the human and *Drosophila* HDAC6 mRNA sequences and found considerable homology only in the coding region. The 3'-UTR sequences are very divergent. Together with our observations that the HDAC6 3'-UTR does not increase TDP-43 binding compared to the open reading frame alone would indicate binding in this region. To roughly map the TDP-43 binding region, we generated HDAC6 RNA fragments comprising the coding sequence with progressive 3'-truncations for new pull-down experiments. These suggest that the nucleotides ~1000-1500 of the coding sequence are important for efficient interaction of TDP-43 with the HDAC6 RNA. Although we cannot exclude the existence of other interaction sites our data strongly suggest that TDP-43 binds specifically to the coding region within HDAC6 mRNA.

(2) We have investigated the effects of a number of TDP-43 missense mutations on HDAC6 expression. In our new retransfection experiments most clinical TDP-43 mutants acted like wild-type TDP-43. This seems surprising at first glance and given the published claims by Johnson et al. (2009) that C-terminal mutations should enhance aggregation of purified TDP-43 protein and cytotoxic inclusion formation in yeast. In sharp contrast, Nonaka et al. (2009) found no effects of several clinical mutations in the context of full-length TDP-43 with regard to aggregate formation, pathological phosphorylation, and CFTR splice activity in mammalian cells culture even for the most effective mutations in the Johnson study. Our results are in general accord with the latter findings. Nevertheless, we noted consistently reduced HDAC6 mRNA rescue potential for one clinical TDP-43 mutation (D169G) and an abolishment by the R361S mutation originally identified in a French ALS patient with no reported family history. Interestingly, the non-conservative R361S mutation resides within the minimal hnRNP interaction domain of TDP-43, suggesting a functionally important localization.

(3) To illustrate better the choice of HDAC6 for extensive validation, we added the complete list of genes altered above fold-change 2. Moreover, we have provided RT-PCR analyses for 15 prominently altered genes identified in the original microarray. Although some were altered with much greater fold-changes than HDAC6, many were not consistently regulated with all 4 validating siRNAs and thus considered as off-target effects. We mention in the revised supplementary information that an additional gene, annexin A1 was consistently upregulated after TDP-43 knockdown at the mRNA (supplementary Fig. 4) and protein (not shown) level. Another gene, KIAA1324/EIG121 was strongly and consistently upregulated after TDP-43 knockdown, but we could not detect the corresponding increase at the protein level. These additional informations may clarify our reasons to focus on HDAC6.

Responses to specific referee comments:

Referee #1

1. Although our (sub)nuclear localization results for TDP-43 may contribute some

information for the exact characterization of T-bodies, these figures are not essential for this report on HDAC6 as novel TDP-43 target gene. Thus, we moved Fig. 1 into the supplement and omitted Fig. S2 altogether. The criticism pertaining to the general diffuse co-localization of nuclear TDP-43 and hnRNPs is correct (hence, Fig. S2 is omitted), although enrichment of TDP-43 in gems and Cajal bodies can be clearly seen in the green channel of the high resolution images (note also the supplementary movie showing the localization of a T body within the coilin-positive subnuclear body).

2. We realize that the choice of HDAC6 for comprehensive validation cannot be appreciated without the context of the whole microarray data. All this information is now provided. A new supplementary table lists all genes differentially expressed >2-fold upon $siRNA^{TDP-43}$ treatment. Moreover, we have added a new supplementary figure showing RT-PCR validations of 15 selected genes. Many interesting genes (for example a cluster in the secretory pathway) were not further evaluated because the effects were not consistently seen with all 4 siRNAs and were thus considered as off-target effects. However, we did identify additional genes that were consistently upregulated upon TDP-43 knockdown. Of these, annexin A1 was confirmed at the mRNA and protein level. In addition, the KIAA1324 gene was strongly upregulated by all 4 TDP-43 siRNAs in HEK293E cells, but we could not confirm the corresponding effect for the EIG121 protein. All this information is now disclosed and discussed in the supplementary information. We hope in this context the rationale becomes plausible why we focused initially on HDAC6, which is implicated in the cellular management of aggregating toxic proteins involved in neurodegenerative diseases.

It should be noted that TDP-43 pathology is not restricted to CNS neurons, but also occurs for example in muscle from patients with sporadic inclusion body myositis and IBMPFD due to VCP mutations (cf added reference of Weihl et al., 2008). Moreover, the pathological nuclear depletion of TDP-43 is not only well documented for neurons, but also for muscle fibers by the recent works of OlivÈ et al. (2009) and Salajegheh et al. (2009), which is emphasized in the revised Introduction (p. 4). Nevertheless, we agree that performing microarray analyses with neurons or brain cells is eventually desirable. In the present study, we focused on the novel TDP-43 target HDAC6 and validated it not only in non-neuronal HEK293E cells, but also in neuroblastoma cells. Moreover, we have extended the validation of specific HDAC6 downregulation in adult TBPH+/- fly heads to the protein level, and we established qRT-PCR measurements on brains dissected from TBPH-/- larvae, demonstrating dramatic reduction of HDAC6 mRNA in TDP-43 knockout brain. These efforts prove the HDAC6 downregulation by TDP-43 deficiency in neural cell types.

3. Determination of the rescue capacities of clinical TDP-43 mutations is putatively a very relevant experiment, which we did (see new Fig. 4I). Surprisingly, our experiments revealed wild-type properties of disease-associated TDP-43 mutants. We detected a consistent reduction of HDAC6 mRNA rescue in transiently silenced HEK293E cells only for 1 ALS-associated mutation, R361S. Interestingly, this mutation resides in the minimal hnRNP interaction domain, suggesting a functionally important localization. While we observed a trend of reduced HDAC6 rescue activity for D169G, most clinical TDP-43 mutants were not impaired. These data are in general accord with a very recent report by Nonaka et al. (2009), who showed no effects of clinical mutations on solubility, fragmentation, phosphorylation and CFTR reporter splicing in the context of the full-length TDP-43 protein. Thus, assessment of pathological influences by introduction of disease-associated mutations into the TDP-43 sequence is not as straightforward as expected.

4. To address the important issue of specificity we have cloned as control HDAC1, and we performed parallel RNA crosslink pull-down experiments with HDAC6 (new Fig. 5D, E). Unlike *in vitro* biotinylated HDAC6 RNA, HDAC1 pulled down neither endogenous nor Flag-tagged TDP-43 specifically.

We did RNA-IP experiments to show an interaction between TDP-43 and endogenous HDAC6 mRNA, despite difficulties with non-specific binding. From these experiments we could not conclude that TDP-43 binds preferentially to the HDAC6 pre-mRNA, which might be expected if TDP-43 were a strict splice factor for HDAC6. However, we do not claim that. TDP-43 knockdown reduced HDAC6 expression, which could be mediated by mRNA processing, transport or stability. These general functions might be exerted by TDP-43 binding to the HDAC6 pre-mRNA or the mature mRNA. The *in vitro* experiments were performed with the cloned HDAC6 coding region; addition of the 3'-UTR did not increase the interaction with TDP-43. Moreover, our new mapping data indicate that TDP-43 can directly bind to coding sequences within the HDAC6 open reading frame. Thus, the TDP-43 effects involve, but may not be limited to binding of coding sequences within the HDAC6 mRNA.

5. We used Atx3-Q148 as a model protein whose turnover is affected by HDAC6. This set of experiments provides a general validation of impaired turnover of an aggregating, toxic protein upon

TDP-43 deficiency in an HDAC6-dependent manner. Tan et al. (2009) described TDP-43 neuropathology in the lower spinal cord of Machado-Joseph disease patients, however TDP-43 inclusions were distinct from ataxin-3 deposits. We agree that TDP-43 is not commonly and directly linked to polyQ-expansion diseases, but the used polyQ construct shows the impairment of proteotoxic turnover by TDP-43 deficiency.
The significance values for the aggregate formations are provided in the revised figure, and more LDH release assays were done to reach statistical power to draw significant conclusions.
Importantly, we performed all requested experiments establishing HDAC6 RNAi. As expected, HDAC6-specific siRNA treatment perfectly phenocopied the observed siRNATDP-43ñmediated reductions of aggregate formation as well as increases of cytotoxicity after Atx3-Q148 transfection (new Fig. 6C, E). Thus, we further prove that the effects on toxic protein turnover in TDP-43 silenced cells critically involve HDAC6. The literature reports of HDAC6 RNAi effects on proteotoxicity (also in neuronal cells) are more distinctly pointed out in the revised text.

Minor 1: In the title the acronym TDP-43 was put in parenthesis, as requested.
Minor 2: The text document was run through with spell and grammar checker.

Referee #2:

1. To show the wider picture of TDP-43 RNAi-responsive transcripts, we provide a new supplementary table listing all genes differentially expressed >2-fold upon siRNATDP-43 treatment. Moreover, we have added a new supplementary figure showing RT-PCR validations of 15 genes with high fold changes. Many interesting genes (for example a cluster in the secretory pathway) were not further evaluated because the effects were not consistently seen with all 4 siRNAs and were thus considered as off-target effects. However, we did identify additional genes that were consistently upregulated upon TDP-43 knockdown. Of these, annexin A1 was confirmed at the mRNA (new supplementary Fig. S4) and protein level (data not shown). In addition, the KIAA1324 gene was strongly upregulated by all 4 TDP-43 siRNAs in HEK293E cells, but we could not confirm the corresponding effect for the EIG121 protein. All this information is now disclosed and discussed in the supplementary information. We hope in this context the rationale becomes plausible why we focused initially on HDAC6, which is implicated in the cellular management of aggregating toxic proteins involved in neurodegenerative diseases.
Other HDAC family members were not altered in the microarrays (supplementary table I). In fact, we employed HDAC1 as a specificity control in new RNA binding experiments and in a new Western blot experiment demonstrating the specific reduction of HDAC6 but not HDAC1 protein in TBPH+/- fly heads.
2. This is an excellent suggestion. To test the relevance of the C-terminal glycine-rich domain (GRD) for HDAC6 regulation, we have cloned TDP-43 GRD and used it for retransfection experiments. Indeed, TDP-43 lacking GRD was not able to restore HDAC6 mRNA. This is consistent with the hypothesized functional importance of the TDP-43 GRD. Remarkably, the only HDAC6 recovery impaired missense mutation, [R361S]TDP-43 (new Fig. 4I) lies within the previously reported minimal hnRNP interaction domain within the GRD (D'Ambrogio et al., 2009).
3. It might have been formulated not clear enough in the previous manuscript version that we found evidence for binding of TDP-43 to the open reading frame, not the 3'-UTR. Adding the 3'-UTR of HDAC6 RNA did not increase TDP-43 binding in *in vitro* crosslinking experiments when compared to coding sequence alone. We have done more efforts to map the TDP-43 binding to HDAC6. As suggested, we checked if predicted TDP-43 binding sites are present within the HDAC6 gene. TAR DNA consensus sequences (Ou et al., 1995) were not present. The only appreciable GU-rich region (several (GU)5 repeats) lies within intron 22 of the HDAC6 pre-mRNA. Thus, we performed RNA-IP experiments to determine TDP-43 interaction with endogenous HDAC6 (pre-)mRNA. Despite technical difficulties with non-specific binding we could co-immunoprecipitate HDAC6 mRNA along with TDP-43. Judging from the length of the amplificates, the TDP-43 co-immunoprecipitated HDAC6 was mature mRNA. We never observed pre-HDAC6 mRNA in the immunoprecipitates. Given our discovery that *Drosophila* HDAC6 is also regulated by TDP-43 (TBPH) we aligned human and fly HDAC6 sequences and found homology only in the coding region (see also point 5). Most importantly, we directly mapped the binding region within the HDAC6 CDS by RNA-IP experiments with a newly generated series of deletion mutants, pointing to an involvement of nucleotides ~1000-1500 of the human HDAC6 coding sequence.

4. The cellular processes by which HDAC6 regulates responses towards misfolded (polyQ) proteins is demonstrated by a good body of literature, which we have cited and discussed. HDAC6 is necessary for aggresome formation probably by coupling the misfolded ubiquitinated cargo to the retrograde transport machinery. Moreover, HDAC6 is thought to recruit the autophagic machinery and thus help to eliminate aggregates via autophagy. In addition, it was reported that HDAC6 expression protected against cell death due to ubiquitin-proteasome-system impairment in a fly model of spinal muscular atrophy and from poly-glutamine induced cell death in a VCP mutant cell culture model. Further investigation of the mechanisms of aggregate formation and cytotoxicity by HDAC6 downregulation most interesting but beyond the scope of this report. Nevertheless, we did strengthen the demonstration of HDAC6-dependence of impaired toxic protein turnover in our experimental system by the use of HDAC6-specific siRNA (new. Fig. 6C, E).
5. As requested, we obtained antibodies against *Drosophila* HDAC proteins and prove selective reduction of dmHDAC6 protein but not dmHDAC1 on Western blots prepared from TBPH+/- fly head lysates.
Alignment of human and *Drosophila* HDAC6 sequences revealed homology only in the coding region, not in the 3'-UTR. Since the addition of the 3'-UTR did not increase the interaction of HDAC6 RNA with TDP-43, the TDP-43 effects involve binding of conserved coding sequences within the HDAC6 mRNA (see point 3).

Minor 1: Although our (sub)nuclear localization results for TDP-43 may contribute some information for the exact characterization of T-bodies, these figures are not essential for this report on HDAC6 as novel TDP-43 target gene. Thus, we moved Fig. 1 into the supplement and omitted Fig. S2 altogether. The criticism pertaining to the general diffuse co-localization of nuclear TDP-43 and hnRNPs is correct (hence, Fig. S2 is omitted).
Minor 2: The sentence about the interplay between HDAC6 and VCP, and the genetic link of VCP to FTD is rewritten more clearly. Moreover, the neuropathological link between VCP and TDP-43 is emphasized by the citation of Neumann et al. (2007) and Weihl et al. (2008) who described TDP-43 neuropathology in patients with VCP mutations.

Referee #3

To provide insight into the pathophysiological relevance of HDAC6 downregulation by TDP-43 deficiency, we determined the rescue capacities of clinical TDP-43 mutations (see new Fig. 4I). Surprisingly, our retransfection experiments revealed wild-type properties of disease-associated TDP-43 mutants. We detected a consistent impairment of HDAC6 mRNA rescue in transiently silenced HEK293E cells only for one ALS-associated mutation, R361S. Interestingly, this mutation resides in the minimal hnRNP interaction domain, suggesting a functionally important localization. Furthermore, we observed a trend of reduced HDAC6 rescue activity for D169G. However, genetic TDP-43 mutants mostly showed wild-type HDAC6 rescue activity. These data are in general accord with a very recent report by Nonaka et al. (2009), who showed no effects of clinical mutations on solubility, fragmentation, phosphorylation and CFTR reporter splicing in the context of the full-length TDP-43 protein. Thus, assessment of pathological influences by introduction of disease-associated mutations into the TDP-43 sequence is not as straightforward as expected.
We did attempts to determine alterations of HDAC6 expression in post-mortem frontal cortex tissue from human FTD patients and controls. However, such measurements were extremely variable and obscured by the high general expression levels of HDAC6, including the many non-affected cells present in a tissue block.

1. As requested, we have included our synthetic RNA-binding deficient TDP-43 (RRM) mutants in the *in vitro* crosslinking experiments (new Fig. 4E). We found that RRM1 is essential for HDAC6 RNA binding, whereas RRM2 seems dispensable. This interesting finding is consistent with TDP-43 binding studies to CFTR (UG) repeats (Buratti et al., 2001). While the RRM1 appears to confer direct specific RNA binding of TDP-43, the RRM2 may be important for correct complex formation and activity of TDP-43. This would also explain the failure of TDP-43 RRM2 to restore HDAC6 levels.
We have addressed the question whether TDP-43 can regulate HDAC6 expression from the cDNA plasmid that entirely lacks all non-coding sequences. We depleted cells of TDP-43 by shRNA as well as siRNA and as expected the transfected HDAC6 but not HDAC1 RNA levels were decreased in TDP-43 silenced cells. Still, we prefer to exclude the results from these rather artificial

experiments. However, if desired we are willing to send the figure for referees disclosure.
2. We greatly expanded the measurements in TBPH knockout flies. To obtain results from complete TBPH null animals, we established the requested qRT-PCR for larval fly brains. Since TBPH-/- flies die during development (which is why the adult fly measurements are restricted to heterozygous knockout fly heads), we have developed a procedure to measure mRNA levels of individual first instar larvae brains (new Fig. 5D). As expected, TBPH mRNA levels were not detectable in TBPH-/- larval brains, and importantly HDAC6 mRNA levels were dramatically reduced. Moreover, we successfully confirmed the specific HDAC6 but not HDAC1 downregulation in TBPH+/- fly head at the protein level by Western Blot analysis (new Fig. 5C). This complete set of experiments demonstrates in vivo that TDP-43 (TBPH) controls HDAC6 expression.
3. We have expanded the supplementary picture and included representative images of inclusions and control transfections (eGFP, Atx3-Q15-eGFP and Atx-Q148-eGFP in parental and shRNATDP-43 treated HEK293E cells) and moved these images into the main manuscript (new Fig. 6A) for better visibility.
The requested HDAC6 RNAi experiments were successfully performed. As expected, HDAC6-specific siRNA treatment perfectly phenocopied the observed siRNATDP-43ñmediated reductions of aggregate formation as well as increases of cytotoxicity after Atx3-Q148 transfection.
4. Reference Bichelmeier et al. (2007) was formatted on p. 12.
Reference to Fig. 7B was added on p. 13.

We hope very much that these major improvements of the manuscript render it suitable for consideration by the EMBO Journal.

3rd Editorial Decision 05 October 2009

Many thanks for submitting the revised version of your manuscript EMBOJ-2009-71484R. It has now been seen again by the original referees 2 and 3, whose comments are enclosed below. As you will see, referee 2 finds that you have satisfactorily addressed the concerns raised in the previous round of review and supports publication without further revision. Referee 3 is also supportive of publication but does raise a couple of concerns, as outlined below.

I can give you a firm commitment that we will be able to accept your manuscript for publication in the EMBO Journal, but before we are able to do so, I would like to ask you to address two specific points raised by referee 3:
1) He/she states that the images presented in Figure 6A do not show identifiable inclusion bodies. I realise that this is not correct, since the brightly staining GFP/Atx3 positive punctae presumably represent inclusion bodies. However, if you do have better quality, and perhaps more representative, images, I would encourage you to include these in place of the current images.
2) He/she comments on the poor efficiency of HDAC6 knockdown in the HEK293 cells, and questions the data presented in Figure 6E. Since it appears that you have only achieved about 30% knockdown, I tend to agree with these concerns. I would therefore strongly encourage you to repeat these experiments in order to show a more convincing knockdown. While I realise that, since you do already see a significant phenotype with the low-efficiency knockdown, the results of the experiment should not change (or should only be improved) but given the concern of the referee, and the fact that this is a critical experiment, I think it would be important to show more convincing data on this point.

Please let me know if you have any further questions, and I look forward to receiving your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #2 (Remarks to the Author):

The authors have added a considerable amount of new experiments that satisfactorily clarifies several points raised by this reviewer. As a result, the manuscript has been very improved.

Referee #3 (Remarks to the Author):

The revision by Fiesel et al. has addressed many of the concerns raised by this reviewer although the analysis of TDP43 mutants did not yield results that would have supported the importance of TDP43-HDAC6 connection in FTD. Accordingly, data on Figure 6 would be the only evidence that TDP43 is important for HDAC6-dependent activity.
However, there are no identifiable AT3 inclusion bodies from the images presented (Fig 6A and 6B). It is not clear how authors can quantify inclusion bodies based on those images. As commented in the original review, better and high quality images are needed to show that TDP43 affects the formation of AT3 or misfolded protein-induced inclusion bodies. Further, the efficiency of HDAC6 knockdown is also quite low but an effect on inclusion body formation was nonetheless reported. These data are not convincing. These concerns should be addressed before its publication.

2nd Revision – Authors' Response 09 October 2009

In compliance with the requests of referee #3 we have added more information to Figure 6.

1) no identifiable AT3 inclusion bodies
Maybe this referee is diverted because of the many negative control pictures showing no inclusions. To make things clearer, we have added arrows pointing to the Atx3- Q148 / eGFP positive inclusions in the bottom panels of Fig. 6A.
Moreover, we have added a higher magnification of the sh(TDP-43) cell with Atx3-Q148-eGFP inclusions.
We have also done just yesterday co-stainings with p62/ sequestosome-1, which indicate that some Atx3-Q148-eGFP inclusions in the cytosol are positive for the ubiquitin-binding p62, which delivers ubiquitinated inclusion proteins to the autophagic machinery. Perfectioning the stainings and repeating all conditions would take quite some time, to prove something that is actually known. We take this preliminary data as reassurance that we are dealing with Atx3-Q148-eGFP inclusions that are turned over in cells via the known HDAC6-p62-autophagy pathway.

2) the efficiency of HDAC6 knockdown is also quite low I see the issue. The reason is that we are transiently silencing HDAC6 (for 24h) and then transfect the various cDNA constructs. Aggregates are allowed to form, and counted/viability determined several days later (120h). At this point in time, cell aliquots were taken for the protein control Western blots. After this period, we still see HDAC6 knockdown, but the siRNA effect vanishes a bit. In another experiment of the original dataset, we had stronger knockdown, which is added to the revised Fig. 6. More importantly, we have added the Western analysis of our first siRNA validations, demonstrating the enormous knockdown efficiency after 48h, as expected for these commercial, validated-quality siRNAs. Thus, during the critical period of the Atx3 aggregation experiment, we have very strong HDAC6 knockdown, and even at the end of the observation period, HDAC6 protein levels remain low, but not as much, because the siRNA begins to be used up after longer time points.
I think the extended Western analysis in the re-revised Fig. 6 should resolve the valid concern of referee #3.

Could you please have a look at the re-revised Figure 6 and let me know if this is satisfactory? Then I could go ahead and make the matching additions to the text. Or do you need further amendments to the manuscript?
Finally, I will have the entire microarray dataset uploaded these days, I have not forgotten it.

Thank you very much for your continued consideration and helpful suggestions.

2nd Additional Correspondence 09 October 2009

Many thanks for sending your proposed revised figure 6. The Western blot data is convincing, and if you can incorporate text along the lines of what you write in your message, that should satisfy the concern of the referee.

In terms of the IF data in 6A, I have to say that I find the blow-up you include in the new version somewhat distracting; the arrows highlighting the inclusion bodies make things very clear, and I would suggest you leave out the additional panel.
Otherwise, it looks fine.

So if you could go ahead and make the necessary changes in the text and then submit your revision, we should then be able to accept the manuscript without further delay. I know you mention it here, but please don't forget to include the accession number(s) for your microarray data in this final version.

I look forward to receiving your revised version.

Yours sincerely,

Editor
The EMBO Journal

