THE EMBO JOURNAL

Manuscript EMBO-2015-91998

TDP-43 loss of function increases TFEB activity and blocks autophagosome-lysosome fusion

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Review timeline:

Submission date:	09 May 2015
Editorial Decision:	08 June 2015
Revision received:	12 October 2015
Editorial Decision:	26 October 2015
Revision received:	12 November 2015
Accepted:	16 November 2015

Editor: Andrea Leibfried

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 08 June 2015

Thank you for submitting your manuscript entitled 'mTORC1-TFEB-linked autophagy and lysosomal biogenesis contribute to TDP-43-mediated neurotoxicity'. I have now received reports from all referees, which are enclosed below.

As you will see, the referees appreciate your findings. However, they think that your analyses do not offer sufficient insight and that the data do not fully support your conclusions. They therefore think that the current manuscript is not suitable for publication in The EMBO Journal.

However, given the interest into the topic and the constructive comments provided by the referees, I could offer to consider a revised version should you be able to address all concerns raised and to substantiate your manuscript along the lines suggested by the referees.
Importantly,
- more insight into why and how autophagolysosome formation is impaired upon TDP-43 KD is needed (see especially referee #1, points 1-2 and proposed RIP-seq experiment)
- a formal demonstration that the reduction in autolysosome formation causes the phenotype is needed (see especially referee #1, point 3)
- further controls and statistical analysis of the results are needed (see especially comments by referee #2)
- the role of the RRM domains of TDP-43 and the mechanism of Raptor downregulation by TDP-43 needs to be further analyzed (see referee #2, point 11; and referee #3, point 2)
- overall mTORC1 levels need to be analyzed and alternative conclusions need to be discussed (see referee #3, point 1)

- mTORC1-independent effects need to be discussed in the context of your results (see referee #1 and #3).

Addressing all concerns raised by the referees clearly demands a lot of work and time, as many of the experiments would have to be repeated and refined, and additional ones would have to be performed as well, with uncertain outcome. I can extend the revision time to 6 months, should that be helpful.
Please note that a revised version will be sent back to all referees, and that I would need strong support from them in order to move forward with the paper here. Please contact me in case of other questions regarding the revision of your manuscript.

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

REFEREE REPORTS

Referee #1:

In this work Xia et al. studied the regulation of the mTORC1 pathway by TDP-43, a protein found in the ubiquitin-positive inclusions in both ALS and FTLD.
They convincingly show that loss of TDP-43 leads to the reduction in Raptor mRNA levels. Consequently, mTORC1 signaling is inhibited and the transcription factor TFEB is in the nucleus where it activates the transcription of lysosomal and autophagic target genes. As a result, autophagy and lysosome biogenesis is induced in TDP-43 -/- cells. In addition, the authors propose that the increase in AV biogenesis upon TDP-43 KD is not efficiently coupled to autophagolysosome formation, resulting in a progressive accumulation of undigested autophagosomes and of P62 substrate. Lastly they show that loss of TDP-43 in drosophila reduces mTORC1 activity and increases the expression levels of autophagy and lysosomal genes and that Rapamycin treatment worsens whereas PA treatment (mTORC1 activator) slightly improves the phenotype of TDP-43-/- flies. Based on these observations the authors concluded that abnormal regulation of autophagy and lysosomal biogenesis by mTORC1 and TFEB contributes to TDP-43-mediated neurodegeneration.

I have three major criticisms:
1) It is totally unclear why the later steps of autophagy are impaired upon mTORC1 inhibition and TFEB activation. These data are completely unexpected according to the current literature; it is well known that Rapamycin, Starvation or silencing any member of the mTORC1 complex induces functional autophagy flux, which is characterized by enhanced AV biogenesis, AV-lys fusion and AV substrate degradation, such as P62 and Htt-150q. Why does the TDP-43-mediated mTORC1 inhibition have a completely different outcome? Furthermore, if the author's model is correct and the toxic effect of TDP-43 silencing is mediated by mTORC1 inhibition, why doesn't Rapamycin treatment induce a TDP-43 like phenotype in WT flies?

2) The data in fig 7b do not demonstrate that autophagy is impaired. In addition the observation that starvation (which inhibits mTORC1) does not normalize the behavior of SiTDP-43 cells strongly suggests that other, mTORc1 independent mechanisms are accounting for the observed phenotype.

3) There is no formal demonstration that the slight reduction in autolysosome formation in TDP 43 -/- flies causes the phenotype.

In principle TDP-43 could bind many other targets that can contribute to the phenotype. Since the authors can perform RIP analysis I strongly encourage them to perform RIP-seq in order to characterize all of the RNAs targeted by TDP-43. For example, it could be possible that TDP43 binds to RNAs encoding for proteins that are important for lysosomal function or for AV-LYS fusion (E.g. SNARE or Motor proteins).

Other points:
- In cellular (or fly) models of ALS or FTLD, where TDP-43 is found in ubiquitin positive aggregates, is the mRNA level of Raptor reduced and mTORC1 activity decreased?
- The rescue experiment in fig2I is minimal. Western blotting of mTORC1 substrates should be

shown. In addition, the expression levels of all known members of the mTORC1 machinery on the lysosome should be tested in TDP-43 depleted cells. (In fig. 2f only a few of them were tested).
- Transcriptional levels of P62 should be checked. In Fig 7E the increase in P62 could be the consequence of increased transcription.

Referee #2:

This manuscript by Qin Xia et al. aimed to investigate the effect of the TDP-43 depletion on mTORC1 components and transcription factor EB (TFEB) as well as on global gene expression of the autophagy-lysosome pathway (ALP). The authors report that depletion of TDP 43 both in cells and in a Drosophila model down-regulates the mTORC1 component raptor and up-regulates the genes involved in ALP. They report that nuclear translocation of TFEB is a consequence of TDP-43 knock down and causes the global ALP expression activation. In addition they show an enhanced autophagosome synthesis but an impaired autophagic flux in TDP-43 depleted cells. Finally the authors report that mTORC1 inhibition by rapamycin worsened the neurodegenerative phenotype in TBPH-/- flies, while a mTORC1 agonist like phosphatidic acid (PA) ameliorated it.

The data and mechanism outlined in the manuscript are novel and of interest. However, the manuscript is not easy to follow. The language is occasionally imprecise and while the authors present an abundance of experimental data the conclusions are not always drawn clearly from the data presented, particularly because the data presented often comes from experiments that lack appropriate controls, evidence of reproducibility, statistical analysis.
The authors need to provide the data before drawing the conclusion and the text should be written in more precise way with the clear conclusions given at the end of each paragraph.

The Materials and Methods section should be expanded with more experimental details. For example a description of the methodology used for the quantification of the co-localizations. Many Figure legends contain mistakes, wrong indications making it difficult to follow.. Authors should keep in mind that "figure legends should contain sufficient information to allow the reader to follow the data presented without referring back to the text".

Specific points:

1- Figure 1C: A western blot control of TDP 43 depletion is needed.

2- From the data in Figure 2A the authors conclude that "TDP-43-depleted cells showed a dramatic reduction of mTOR cytoplasmic puncta, which reflects lysosomal-enriched mTOR". This sentence is imprecise. What can be seen in the figure 2A is the reduction of the punctate mTOR distribution in si-TDP-43 cells (mTOR dispersion) that could indicate its lower lysosomal localisation. This conclusion can only be made after showing lower mTOR co-localization with the late endosomes/lysosomes marker LAMP1 in si-TDP-43 cells in comparison to the control (figure 2C and 2H).

3-Figure 2B shows the lower phosphorylation of mTORC1 substrates p70S6K and 4E-BP1 in si-TDP-43 cells. The immunoblot with antibody for total 4E-BP1 is not informative.

4-The authors claim that "mTOR was colocalized with a lysosomal specific marker LAMP1, indicating that mTOR is localized to the lysosomal surface" and that "mTOR failed to localize to lysosomes in TDP-43-depleted cells regardless of the presence or absence of amino acids (Figure 2C, lower panel)". Colocalization of mTOR and LAMP1 in the figure 3C should be demonstrated in the merged images and not leave the reader to imagine their colocalization.

5-The authors claimed, "re-treatment of amino acids failed to restore S6K1 phosphorylation in TDP-43-depleted cells, whereas it significantly induced S6K1 phosphorylation in control cells (Figure 2D)". In order to be able to establish a significant phosphorylation induction, quantitative analysis of the immunoblots from the three independent experiments needs to be provided together with an immunoblot analysis with antibody against total p70S6K1.

6- Figure 2G is redundant as it completely overlaps with the figure 2H, five upper panels.

7- In figure 3A the authors show that TDP-43 silencing causes higher TFEB and mTOR co-localization in the cytoplasmic lysosome-like puncta that have been quantified in the figure 3B as lysosomes. These vesicles should be positive for LAMP1 suggesting that they correspond to late endosomes/lysosomes. As it has been shown that mTOR colocalizes with lysosomes in around 20% of si-TDP43 cells (figure 2I) it is not clear how the authors conclude that the presented colocalization of TFEB and mTOR is actually in the lysosome and how they have performed the quantification analysis. The merged enlarged sections are not even mentioned in the text, nor is any reference made to figures 3B and 3C.

8- Immunofluorescence analysis of the EGFP-TFEB cellular localization upon TDP-43 silencing, raptor silencing, TDP-43 and raptor silencing and si-TDP-43+HA-raptor shown in figure 3A should be confirmed by immunoblots assays after subcellular fractionation analysis.

9- Figure 3E would be easier to follow if the respective controls (si-control figures) are included and if the section results and the figure legend contain a more precise explanation.

10- In order to confirm Rag GTPases independent TDP-43 regulation of the TFEB localization the authors should perform silencing experiments with si-RagB together with the adding back of the RagB and follow the subcellular distribution of TFEB under the microscope, as well as in the immunoblot assay after subcellular fractionation.

11- On the base of the experiments presented in the figure 4 the authors concluded "that RRM domains of TDP-43 specifically bind to raptor mRNA and mediate the regulation of raptor expression". In the same figure 4C they present the adding back experiment with glycine-rich domain (GRD) deletion mutant of TDP-43 where it is clear that this mutant, as well as RRM mutants, failed to restore the raptor level in si-TDP-43 cells. Keeping in mind that ΔGRD TDP-43 mutant contain intact and functional RRM domains it would be expected to be able to restore mRNA and protein levels of raptor in TDP-43 silenced cells. The use of ΔGRD TDP-43 mutant is not mentioned in the results section.

Fluorescence intensity of Lysotracker (Figure 5A) could be assessed with the flow cytometry in order to confirm an increased volume of acidic compartments upon TDP-43 silencing.

Minor points:

1- In the figure 1B what the white arrows are indicating is not explained

2- Supplementary figure 1 should include the quantification data of TFEB localization.

3- When the authors state "TDP-43-depleted cells showed a decrease of raptor, but not mTOR, p18 and RagB, compared with control cells" they refer just to the figure 2E. As the figure 2B includes mTOR expression level in si-TDP-43 cells compared to control this should be included in the text.

4-Figure legend 2 contains an error: "The quantification of mTOR on lysosomes is shown in Figure 2H". The right panel is 2I.

5- Supplementary figure 2B legend contains an error: "anti-mTOR is (red)". The mTOR signal is green in the supplementary figure 2B. Figures 2G and supplementary figure 2B are the same ones but the text in these figure legends is different.

6- Title of the supplementary figure 2 is too broad and vague.

7- In the paragraph "we observed that TFEB showed a diffuse cytoplasmic distribution in normal control cells (Figure 3A)", normal refers to si-control cells therefore it should be corrected.

8- Figures 3B, 3C and 4C are not mentioned and explained in the section results.

9- Based on the figure legend 3D the authors explained the use of Rag GTPase mutants. The corresponding figure and the text in the section results are not in agreement with this.

10- In the section results there is an error: "similar results were obtained in various cell lines, such as SH-SY5Y (Figure 5D and E)". Mentioned results are on the figure 5E and F.

11- When explaining that "the protein level of p62, the autophagic substrate, was up-regulated in TDP-43 deficient cells under basal and starvation conditions" for the easier reading the authors should indicate in which figure it has been shown (figure 7E).

12. Figure 8: A and D show western blots apparently coming from larval tissue. I imagine this is a typo as the figure is divided in head and whole body. Probably these two images are referred to the adult fly, and F to the larvae. Instead E shows the protein levels in each larval stage; in this case the larval western blot (A, D or F?) would not be necessary.

13. In the discussion it is suggested that a possible strategy to treat the TDP-43 mediated cytotoxicity, which is due to autophagosome accumulation, could be the triggering of the fusion between autophagosome and lysosomes. The authors could try to treat the TDP-43 silenced cells with an mTOR-independent autophagy inducer, as for example Trehalose, in order to see if an increase in the autophagy flux ameliorates the phenotype.

14. Several typos needed to be addressed. The manuscript should be reread thoroughly and corrected. Below are just a few examples
- In several places the authors use the word "base on" instead of "based on".
- In the Discussion: "Although the current view of autophagy in neuron..." should read "Although the current view of autophagy in neurons
- "In consistent with these studies..." should read. In accordance with these studies....
- Flies should not be capital,
- etc, etc

Referee #3:

Xia et. al. perform a number of in vitro and in vivo experiments showing that TDP43 knockdown affects autophagy and lysosomal biogenesis. They show that TDP43 knockdown results in down regulation of RAPTOR which in turn leads to an increase in TFEB activity and thereby an increase in autophagy and lysosomes. Specific comments on the manuscript are as follows:

1. The authors determine that knock-down of TDP43 drastically reduces RAPTOR levels, as well as mTORC1 signaling. However throughout the paper the authors suggest that effects of TDP43 on TFEB are modulated by effects on mTORC1 localization. While less mTORC1 is at the lysosome upon TDP43 knock-down, this is most probably due to less mTORC1 in the cell since there is less RAPTOR. The authors should rephrase their findings accordingly and acknowledge that overall levels of mTORC1, not its localization per se, are important in the TDP43 knock-down phenotype.

2. As the main effect of TDP43 knock-down on mTOR activity is down regulation of RAPTOR, the authors need to dwell more on the mechanism of how TDP43 reduces RAPTOR levels. TDP43 is known to have transcriptional and post transcriptional effects. The authors need to determine if TDP43 directly controls RAPTOR mRNA expression at the level of transcription. The authors show that TDP43 can bind RAPTOR mRNA; they need to further investigate the cause of this interaction to determine if TDP43 is involved in splicing or stability of RAPTOR mRNA.

3. In figure 2C, the authors need to include a "Merge" panel as it is not very clear if mTOR and LAMP1-RFP are co-localized throughout the conditions tested. In figure 2, panel G also appears in supplemental figure 2, the authors need to address this discrepancy. Throughout the paper authors measure mTORC1 activity by measuring phosphorylation of mTORC1 targets; they need to show levels of total protein together with the phosphorylated form of the substrate protein.

4. Commenting on the results shown in figure 3, the authors describe a model where TDP43 effects

on TFEB localization are dependent on mTORC1. The authors need to substantiate this claim by performing an experiment using a phosphomimetic mutant of TFEB for serine 211; serine phosphorylated by mTORC1. The authors need to determine if the localization of this phosphomimetic mutant changes upon TDP43 knockdown.

5. In figure 7, the authors show that knock-down of TDP43 has effects on autophagosome and autolysosome formation in starved cells. During starvation mTORC1 signaling is turned off, as such most probably the effects of TDP43 knock-down during starvation are mTORC1 independent. This will mean that knock-down of TDP43 has mTORC1 dependent and independent effects. The authors need to acknowledge and explain this point and possibly delineate mTORC1 dependent and independent effects.

6. In figure 9, the authors show that in TBPH -/- larvae, treating with rapamycin exacerbates and treating with PA ameliorates their locomotion. However rapamycin or PA treatment does not affect WT larvae. The authors need to measure mTORC1 activity throughout these treatments in WT as well as TBPH -/- larvae to account for these differences.

1st Revision - authors' response 12 October 2015

We thank you for encouraging us to revise the manuscript and thank Reviewers for their precious comments and suggestions on our manuscript. We modified our manuscript as suggested. The followings are our point-to-point responses to the issues raised by the three referees.

Referee #1:

In this work Xia et al. studied the regulation of the mTORC1 pathway by TDP-43, a protein found in the ubiquitin-positive inclusions in both ALS and FTLD. They convincingly show that ....

General response: We thank Reviewer 1 for the positive comments.

Point 1
1) It is totally unclear why the later steps of autophagy are impaired upon mTORC1 inhibition and TFEB activation. These data are completely unexpected according to the current literature; it is well known that Rapamycin, Starvation or silencing any member of the mTORC1 complex induces functional autophagy flux, which is characterized by enhanced AV biogenesis, AV-lys fusion and AV substrate degradation, such as P62 and Htt-150q. Why does the TDP-43-mediated mTORC1 inhibition have a completely different outcome? Furthermore, if the author's model is correct and the toxic effect of TDP-43 silencing is mediated by mTORC1 inhibition, why doesn't Rapamycin treatment induce a TDP-43 like phenotype in WT flies?

Response
We gratefully thank Reviewer 1 for the critical comments and questions, which are clearly helpful to improve the quality of our paper. In fact the later steps of autophagy process are impaired in an mTORC1-independent manner. We find that depletion of TDP43 has both mTORC1 dependent and independent effects on autophagy-lysosome pathway, as pointed out by Reviewer 1 (point 2) and Reviewer 3 (point 5). Importantly, our data indicate the autophagosome and lysosome biogenesis were enhanced through mTORC1 inhibition and TFEB activation in TDP-43-depleted cells, whereas the autophagosome-lysosome fusion (later steps of autophagy) are impaired through a mTORC1-independent manner (a Dynactin 1 (DCTN1)-associated manner, will be described in response to point 4) in those cells . Regarding this mTORC1 independent mechanism, we initially thought that it was beyond the scope of this study, therefore we did not focus on this part and did not aim to include other TDP-43 cellular functions in this paper. Nevertheless, we agree with Reviewer 1 that it's important to clarify the different contributions of mTORC1 dependent and independent mechanisms to autophagy-lysosome pathway in our models. Now we are pleased to include these additional data of mTORC1 independent mechanism and modify the text in our revised manuscript (also, will be described in response to point 4). Hence, now we can understand "why does the TDP-43 depletion have a completely different outcome from Rapamycin, Starvation or silencing any member of the mTORC1 complex (mTORC1 inhibition alone), since TDP-43 depletion does not only affect mTORC1, but also affect the AV-LYS fusion. Also, Rapamycin treatment differs from

TDP-43 depletion, since it will not impair autophagosome-lysosome fusion and will not induce a TDP-43 like phenotype in flies.

Point 2
2) The data in fig 7b do not demonstrate that autophagy is impaired. In addition the observation that starvation (which inhibits mTORC1) does not normalize the behavior of SiTDP-43 cells strongly suggests that other, mTORc1 independent mechanisms are accounting for the observed phenotype.

Response
We appreciate Reviewer 1 for raising this excellent point (which is also raised by Reviewer 3, point 5), and this point is relevant to Point 1.Yes, there is indeed mTORC1 independent mechanism that accounts for the observed phenotype (as response to point 1), and we show the detailed mechanism in response to point 4 and in our revised manuscript. Regarding "The data in fig 7b do not demonstrate that autophagy is impaired", we apologize for this carelessness since fig. 7b alone would not strongly suggest an impairment of autophagy flux. We should have described and discussed the data in fig. 7e regarding autophagic substrate degradation (as also suggested by Reviewer 2, minor point 11). In our revised manuscript, we combine the data in Figure 7b, 7e, Supplementary Figure 11 and 12 to demonstrate that functional autophagy flux is impaired.

Point 3
3) There is no formal demonstration that the slight reduction in autolysosome formation in TDP 43 -/- flies causes the phenotype.

Response
This is obviously a very good point. Autophagy has been well studied in the starvation-induced fruit fly larval fat body, a tissue comparable to human liver and fat (Scott et al Dev Cell. 2004 Aug;7(2):167-78). We performed this assay according to the previous studies. Our results from electron microscopy (EM) showed that the autolysosome formation was indeed impaired in starvation-induced larval fat bodies of TDP 43 -/- flies, compared with wild type flies (Supplementary Figure 13 in our revised manuscript).

Point 4
In principle TDP-43 could bind many other targets that can contribute to the phenotype. Since the authors can perform RIP analysis I strongly encourage them to perform RIP-seq in order to characterize all of the RNAs targeted by TDP-43. For example, it could be possible that TDP43 binds to RNAs encoding for proteins that are important for lysosomal function or for AV-LYS fusion (E.g. SNARE or Motor proteins).

Response
We highly appreciate Reviewer 1 for these constructive comments and great suggestions, which are helpful to identify the other targets that may contribute to the phenotype. It can help us to elucidate the mechanism underlying the impaired AV-LYS fusion in this study, and it will certainly improve the quality of our paper. Since genome-wide analysis using RIP-seq or CLIP-seq may take too much time at this moment, and given that this suggestion is relevant to AV-LYS fusion, we performed comprehensively analysis of gene expression level and function of genes/proteins involved in AV-LYS fusion in TDP-43-depleted cells instead. We tested nearly 20 genes (including SNARE and Motor proteins as suggested) that have been implicated in autophagosome-endosome/lysosome fusion process according to the recent studies. Interestingly and importantly, in this screening we found the gene expression level of Dctn1 (gene encoding Dynactin 1) was reduced. This observation was consistent with a previous study (Nat Neurosci. 2011 Apr;14(4):459-68) in which Polymenidou *et al* convincingly identified RNA targets of TDP-43 using CLIP-seq. We further confirmed the protein level of Dynactin 1 was also reduced in TDP-43-depleted cells. Dynactin 1 is a motor component of Dynein-Dynactin complex that mediates the retrograde transport of lysosomes in cells, and dysfunction of Dynein/Dynactin results in abnormality of lysosomal positioning and AV-LYS fusion as reported by Jahreiss *et al* Traffic. 2008 Apr;9(4):574-87, Korolchuk *et al* Nat Cell Biol. 2011 Apr;13(4):453-60, Nixon RA. Nat Med. 2013 Aug;19(8):983-97. We show that knockdown of TDP-43, similarly as knockdown of Dynactin 1, could affect lysosomal positioning to the periphery area (away from MTOC), and thereby block the AV-LYS fusion, indicating Dynactin 1 contributes to the phenotype observed in TDP-43-depleted cells. Again, we thank Reviewer 1 for

the helpful suggestion to figure out the mechanism in the impaired AV-LYS fusion in TDP-43-depleted cells. In our revised manuscript, we include these data in Supplementary Figure 9, 10 and 11 and modify the text as response to point 1.

Other points:
1- In cellular (or fly) models of ALS or FTLD, where TDP-43 is found in ubiquitin positive aggregates, is the mRNA level of Raptor reduced and mTORC1 activity decreased?

Response
We thank Reviewer 1 for this good question. In models and patients of ALS or FTLD, ubiquitin positive TDP-43 aggregates always accompany with the loss of nuclear TDP-43. To better take the reviewer's advice, we performed the raptor and mTORC1 activity restoration experiments using TDP-43 mutants as in Figure 4. Similar to many published papers, we failed to see TDP-43 cytoplasmic aggregation using TDP-43 constructs with pathogenic mutations. Alternatively, we expressed a TDP-43 C-terminal fragments (25 kD, named "TDP-25") in cells, and then we can observe the cytoplasmic aggregates as reported (Supplementary Figure 6A in our revised manuscript). In TDP-43-depleted cells re-expressed with TDP-25, we did not observe any restoration of raptor and mTORC1 activity (Supplementary Figure 6B in our revised manuscript). Thus, we conclude that cytoplasmic TDP-43 with aggregates would not affect raptor or mTORC1 activity.

2- The rescue experiment in fig2I is minimal. Western blotting of mTORC1 substrates should be shown. In addition, the expression levels of all known members of the mTORC1 machinery on the lysosome should be tested in TDP-43 depleted cells. (In fig. 2f only a few of them were tested).

Response
In our revised manuscript, we performed the experiments as suggested. Western blotting of mTORC1 substrates is shown in Supplementary Figure 2E, and the expression levels of other known members of the mTORC1 machinery on the lysosome are shown in Supplementary Figure 2C.

3- Transcriptional levels of P62 should be checked. In Fig 7E the increase in P62 could be the consequence of increased transcription.

Response
We checked the transcriptional levels of p62 relevant to Fig. 7E as suggested, and our results indicate that transcriptional levels of p62 were not affected in those cells. The data were added in the Supplementary Figure 12 of our revised manuscript

Referee #2:
.....
The data and mechanism outlined in the manuscript are novel and of interest. However, the manuscript is not easy to follow. The language is occasionally imprecise and while the authors present an abundance of experimental data the conclusions are not always drawn clearly from the data presented, particularly because the data presented often comes from experiments that lack appropriate controls, evidence of reproducibility, statistical analysis.
The authors need to provide the data before drawing the conclusion and the text should be written in more precise way with the clear conclusions given at the end of each paragraph.
The Materials and Methods section should be expanded with more experimental details. For example a description of the methodology used for the quantification of the co-localizations. Many Figure legends contain mistakes, wrong indications making it difficult to follow.. Authors should keep in mind that "figure legends should contain sufficient information to allow the reader to follow the data presented without referring back to the text".

General response: We are pleased that the reviewer considered our manuscript to be “novel and of interest”. Also, we gratefully thank the reviewer for the detailed valuable comments and suggestion on the clarity of text and data presentation, which are helpful to improve the quality of our paper. We apologize for the mistakes in the text and the confusions, and we have addressed all the comments below as suggested. We have also corrected other mistakes that were not mentioned by the reviewer, such as missing descriptions and wrong names of Figure 6C and E, now corrected as

Figure 6E and C in our revised manuscript). The revised manuscript has been modified throughout, and now we believe that it should read better.

Point 1
Figure 1C: A western blot control of TDP 43 depletion is needed.

Response
We appreciate Reviewer 2 for this suggestion. In Figure 1C of our revised manuscript, we have provided the western blot control of TDP 43 depletion as suggested.

Point 2
From the data in Figure 2A the authors conclude that "TDP-43-depleted cells showed a dramatic reduction of mTOR cytoplasmic puncta, which reflects lysosomal-enriched mTOR". This sentence is imprecise. What can be seen in the figure 2A is the reduction of the punctate mTOR distribution in si-TDP-43 cells (mTOR dispersion) that could indicate its lower lysosomal localisation. This conclusion, can only be made after showing lower mTOR co-localization with the late endosomes/lysosomes marker LAMP1 in si-TDP-43 cells in comparison to the control (figure 2C and 2H).

Response
We deeply thank Reviewer 2 for pointing this out. We agree with Reviewer 2 that although many reports have shown that mTOR cytoplasmic puncta reflects its lysosomal localization, the conclusion "reduction of lysosomal-enriched mTOR "can only be made after showing lower mTOR co-localization with the late endosomes/lysosomes marker LAMP1 in si-TDP-43 cells in comparison to the control (figure 2C). Therefore, we have modify the text in our revised manuscript as suggested.

Point 3
Figure 2B shows the lower phosphorylation of mTORC1 substrates p70S6K and 4E-BP1 in si-TDP-43 cells. The immunoblot with antibody for total 4E-BP1 is not informative.

Response
Our antibody for total 4E-BP1 was not good enough for immunoblot and we apologize for this low quality. We do not have other available 4E-BP1 antibodies at this time during revision. Since our antibodies of total and phosphorylation level of mTORC1 substrates p70S6K are informative enough to indicate the mTORC1 activity, we have removed the immunoblot data of total and phosphorylated 4E-BP1 in Figure 2B of our revised manuscript.

Point 4
The authors claim that "mTOR was colocalized with a lysosomal specific marker LAMP1, indicating that mTOR is localized to the lysosomal surface" and that "mTOR failed to localize to lysosomes in TDP-43-depleted cells regardless of the presence or absence of amino acids (Figure 2C, lower panel)". Colocalization of mTOR and LAMP1 in the figure 3C should be demonstrated in the merged images and not leave the reader to imagine their colocalization.

Response
In our revised manuscript, the picture for the colocalization of mTOR and LAMP1 in the figure 2C has been added as merged images.

Point 5
The authors claimed, "re-treatment of amino acids failed to restore S6K1 phosphorylation in TDP-43-depleted cells, whereas it significantly induced S6K1 phosphorylation in control cells (Figure 2D)". In order to be able to establish a significant phosphorylation induction, quantitative analysis of the immunoblots from the three independent experiments needs to be provided together with an immunoblot analysis with antibody against total p70S6K1.

Response
In our revised manuscript, we have included the quantitative data of Figure 2D, and provided quantitative analysis together with immunoblot of total p70S6K1 as suggested.

Point 6
Figure 2G is redundant as it completely overlaps with the figure 2H, five upper panels.

Response
Thank you for this point, we have removed Figure 2G in our revised manuscript.

Point 7
In figure 3A the authors show that TDP-43 silencing causes higher TFEB and mTOR co-localization in the cytoplasmic lysosome-like puncta that have been quantified in the figure 3B as lysosomes. These vesicles should be positive for LAMP1 suggesting that they correspond to late endosomes/lysosomes. As it has been shown that mTOR colocalizes with lysosomes in around 20% of si-TDP43 cells (figure 2I) it is not clear how the authors conclude that the presented colocalization of TFEB and mTOR is actually in the lysosome and how they have performed the quantification analysis. The merged enlarged sections are not even mentioned in the text, nor is any reference made to figures 3B and 3C.

Response
We appreciate Reviewer 2 for these detailed comments and suggestions. In Supplementary Figure 3B of our manuscript, we demonstrate that the TFEB puncta are indeed lysosomal localized TFEB, suggesting the quantification analysis we performed in figure 3B indicate the TFEB puncta in the lysosomes. In fact it is hard to see the "colocalization of TFEB and mTOR" in the lysosomes in si-TDP-43 cells, since in this case TFEB can form lysosomal puncta but the mTOR lysosomal puncta disappear (Figure 3A). Thus, the quantification analysis in figure 2I (now figure 2H in the revised manuscript) reflects the disappeared mTOR lysosomal puncta in si-TPD-43 cells, while the quantification analysis in figure 3B reflects enhanced TFEB lysosomal puncta in si-TPD-43 cells. Note that we did not attempt to make the quantification of the co-localizations, since we only counted the numbers of cells with "TFEB puncta" and figure 3B showed quantification data of TFEB puncta. Also, we have modified the figure legends to describe the merged enlarged sections in figure 3 and other figures.

Point 8
Immunofluorescence analysis of the EGFP-TFEB cellular localization upon TDP-43 silencing, raptor silencing, TDP-43 and raptor silencing and si-TDP-43+HA-raptor shown in figure 3A should be confirmed by immunoblots assays after subcellular fractionation analysis.

Response
Immunoblots assays after subcellular fractionation analysis of the EGFP-TFEB cellular localization in Figure 3A have been performed as suggested, and the data have been provided in Supplementary Figure 3C of our revised manuscript.

Point 9
Figure 3E would be easier to follow if the respective controls (si-control figures) are included and if the section results and the figure legend contain a more precise explanation.

Response
We highly appreciate Reviewer 2 for raising this issue, which is helpful for the improvement of the quality of our manuscript. In Supplementary Figure 5 of our revised manuscript, we have provided the respective controls (si-control figures) of Figure 3E.

Point 10
In order to confirm Rag GTPases independent TDP-43 regulation of the TFEB localization the authors should perform silencing experiments with si-RagB together with the adding back of the RagB and follow the subcellular distribution of TFEB under the microscope, as well as in the immunoblot assay after subcellular fractionation.

Response
In our revised manuscript, we have provided the immunofluorescence and immunoblot assays as suggested. Our results indeed confirmed a Rag GTPases independent TDP-43 regulation of the TFEB localization, as shown in Supplementary Figure 4.

Point 11
On the base of the experiments presented in the figure 4 the authors concluded "that RRM domains of TDP-43 specifically bind to raptor mRNA and mediate the regulation of raptor expression". In the same figure 4C they present the adding back experiment with glycine-rich domain (GRD) deletion mutant of TDP-43 where it is clear that this mutant, as well as RRM mutants, failed to restore the raptor level in si-TDP-43 cells. Keeping in mind that ΔGRD TDP-43 mutant contain intact and functional RRM domains it would be expected to be able to restore mRNA and protein levels of raptor in TDP-43 silenced cells. The use of ΔGRD TDP-43 mutant is not mentioned in the results section.

Response
We thank Reviewer 2 for this good point regarding the role of the RRM domains of TDP-43 and the mechanism of Raptor downregulation by TDP-43, which was also raised by Reviewer 3, point 2. The GRD domain in TDP-43 has been shown to be involved in the interaction with other key regulators (such as hnRNPs) that are critical for the TDP-43 function, and these factors could cooperate together with TDP-43 to regulate TDP-43 RNA targets, indicating GRD domain deletion/mutation will affect TDP-43 overall function. We found that GRD deletion failed to restore the TDP-43-targeting RNA level in si-TDP-43 cells, as reported by Fiesel *et al* EMBO J. 2010 Jan 6;29(1):209-21. In the revised manuscript, we carefully discussed these in the results section and moved the figure 4C/D to Supplementary Figure 6D and E. Also, we added the use of GRD mutant as suggested.

Point 12
Fluorescence intensity of Lysotracker (Figure 5A) could be assessed with the flow cytometry in order to confirm an increased volume of acidic compartments upon TDP-43 silencing.

Response
In our revised manuscript, we performed the flow cytometry to show the fluorescence intensity of Lysotracker as suggested (Figure 5A), and we moved the original Figure 5A/B to Supplementary Figure 8.

Minor points:
Point 1
In the figure 1B what the white arrows are indicating is not explained

Response
We seriously apologize for this carelessness and thank Reviewer 2 so much for pointing out this issue. In the figure legends section of our revised manuscript, we have provided appropriate information about the white arrows.

Point 2
Supplementary figure 1 should include the quantification data of TFEB localization.

Response
In our revised manuscript, we added the quantitative data of supplementary figure 1 as suggested.

Point 3
When the authors state "TDP-43-depleted cells showed a decrease of raptor, but not mTOR, p18 and RagB, compared with control cells" they refer just to the figure 2E. As the figure 2B includes mTOR expression level in si-TDP-43 cells compared to control this should be included in the text.

Response
In our revised manuscript, we have moved the mTOR immunoblots from figure 2B to figure 2E. Now figure 2E contains the expression levels and quantitative data of raptor, RagB, p18, TDP-43 and mTOR. Also, we have modified the text. Now our revised manuscript should read much better.

Point 4
Figure legend 2 contains an error: "The quantification of mTOR on lysosomes is shown in Figure 2H". The right panel is 2I.

Response
We deeply thank Reviewer 2 for pointing this out. Now the quantification of mTOR on lysosomes is shown in Figure 2H in the revised manuscript.

Point 5
Supplementary figure 2B legend contains an error: "anti-mTOR is (red)". The mTOR signal is green in the supplementary figure 2B. Figures 2G and supplementary figure 2B are the same ones but the text in these figure legends is different.

Response
We apologize for this and thank Reviewer 2 so much for pointing out this issue. In our revised manuscript, we have corrected the error in Supplementary figure 2B legend. As response to point 6, we have already removed the original Figure 2G. Now we have provided appropriate information on the figure legends of Figure 2 and Supplementary figure 2B.

Point 6
Title of the supplementary figure 2 is too broad and vague.

Response
We thank Reviewer 2 for pointing out this. In the supplementary figure 2 of our revised manuscript, we have added appropriate modifications. Now it should be much better.

Point 7
In the paragraph "we observed that TFEB showed a diffuse cytoplasmic distribution in normal control cells (Figure 3A)", normal refers to si-control cells therefore it should be corrected.

Response
Thank you again! We have corrected the description to "si-control cells" in our revised manuscript.

Point 8
Figures 3B, 3C and 4C are not mentioned and explained in the section results.

Response
Figures 3B and C are the quantification data of Figures 3A, and those figures have been mentioned in the results section of our revised manuscript. In addition, original Figure 4C and D has been moved to Supplementary Figure 6D and E, and they have also been mentioned in the revised manuscript.

Point 9
Based on the figure legend 3D the authors explained the use of Rag GTPase mutants. The corresponding figure and the text in the section results are not in agreement with this.

Response
We have modified the text in the section results and figure legends so that the readers can understand why and how we checked activated Rags in cells using these mutants.

Point 10
In the section results there is an error: "similar results were obtained in various cell lines, such as SH-SY5Y (Figure 5D and E)". Mentioned results are on the figure 5E and F.

Response
In our revised manuscript, (original) Figure 5E and F have been named as Figure 5D and E.

Point 11
When explaining that "the protein level of p62, the autophagic substrate, was up-regulated in TDP-43 deficient cells under basal and starvation conditions" for the easier reading the authors should indicate in which figure it has been shown (figure 7E).

Response

Again, we are so sorry for this carelessness. In the results section of our revised manuscript, we have indicated figure 7E as suggested.

Point 12
Figure 8: A and D show western blots apparently coming from larval tissue. I imagine this is a typo as the figure is divided in head and whole body. Probably these two images are referred to the adult fly, and F to the larvae. Instead E shows the protein levels in each larval stage; in this case the larval western blot (A, D or F?) would not be necessary.

Response
Figures A and D indeed indicate the head and whole body of larval tissue, but not the adult fly (they are still necessary since E indicates the whole body of larval tissue). Meanwhile, F indicates the whole body of adult fly. In our revised manuscript, we have modified the figure legends to avoid the confusion and to make it easier to understand.

Point 13
In the discussion it is suggested that a possible strategy to treat the TDP-43 mediated cytotoxicity, which is due to autophagosome accumulation, could be the triggering of the fusion between autophagosome and lysosomes. The authors could try to treat the TDP-43 silenced cells with an mTOR-independent autophagy inducer, as for example Trehalose, in order to see if an increase in the autophagy flux ameliorates the phenotype.

Response
This is obviously a very good question and suggestion. Unfortunately, we failed to see an increase in the autophagy flux after Trehalose treatment, therefore we did not include these data in our paper. Trehalose can function upstream of the fusion between autophagosome and lysosomes (possibly acts through AMPK and/or other targets), and we observed an impairment of the autophagosome-lysosome fusion in TDP-43-depleted cells (as response to reviewer 1 and 3). In this case, we need to find a "fusion inducer" to make the rescue. However, currently there is no such available compounds and we hope that we can use such compounds that benefits from the research progress of autophagy field in the future.

Point 14
Several typos needed to be addressed. The manuscript should be reread thoroughly and corrected. Below are just a few examples
- In several places the authors use the word "base on" instead of "based on".
- In the Discussion: "Although the current view of autophagy in neuron..." should read "Although the current view of autophagy in neurons
- "In consistent with these studies..." should read. In accordance with these studies....
- Flies should not be capital,
- etc, etc

Response
We deeply thank Reviewer 2 for pointing out this. In our revised manuscript, we have addressed the typos as suggested. Also, we have reread and corrected the paper thoroughly, and it should read much better now.

Referee #3:

Xia et. al. perform a number of in vitro and in vivo experiments showing that TDP43 knockdown affects autophagy and lysosomal biogenesis. They show that TDP43 knockdown results in down regulation of RAPTOR which in turn leads to an increase in TFEB activity and thereby an increase in autophagy and lysosomes.

General response: Thank you for pointing these out.

Point 1
The authors determine that knock-down of TDP43 drastically reduces RAPTOR levels, as well as mTORC1 signaling. However throughout the paper the authors suggest that effects of TDP43 on

TFEB are modulated by effects on mTORC1 localization. While less mTORC1 is at the lysosome upon TDP43 knock-down, this is most probably due to less mTORC1 in the cell since there is less RAPTOR. The authors should rephrase their findings accordingly and acknowledge that overall levels of mTORC1, not its localization per se, are important in the TDP43 knock-down phenotype.

Response
The reviewer is right. We should have stated that effects of TDP43 on TFEB are modulated by effects on mTORC1 levels (activity), but not mTORC1 localization. In fact we should have stated mTOR localization instead of mTORC1 localization. We rephrase our findings accordingly, and our revised manuscript should read much better.

Point 2
As the main effect of TDP43 knock-down on mTOR activity is down regulation of RAPTOR, the authors need to dwell more on the mechanism of how TDP43 reduces RAPTOR levels. TDP43 is known to have transcriptional and post transcriptional effects. The authors need to determine if TDP43 directly controls RAPTOR mRNA expression at the level of transcription. The authors show that TDP43 can bind RAPTOR mRNA; they need to further investigate the cause of this interaction to determine if TDP43 is involved in splicing or stability of RAPTOR mRNA.

Response
We thank Reviewer 3 for this point and appreciate a lot for the detailed suggestions to address the mechanism of how TDP43 reduces raptor RNA levels. To test whether TDP43 regulates raptor RNA at the transcriptional level, we used Click-iT technology (Invitrogen) to label the newly transcribed RNA in TDP-43-depleted cells and control cells, and then the labeled RNA was selected and subjected to qRT-PCR. We found the level of newly transcribed raptor RNA was not changed by TDP-43 depletion (figure 4F in the revised manuscript). Furthermore, we analyzed the half-lives (stability) of raptor RNA by inhibiting transcription using Actinomycin D (ActD). Our results showed that the stability raptor mRNA was significantly altered by TDP-43 depletion (figure 4G and Supplementary figure S7 in the revised manuscript). Therefore, we conclude that TDP-43 can bind to raptor mRNA and regulate the stability of raptor mRNA at the post-transcriptional level, but not at the transcription level. Again, we thank the reviewer for the helpful suggestion to explore the mechanism underlying TDP-43-mediated raptor regulation and we modified the text in the revised manuscript.

Point 3
In figure 2C, the authors need to include a "Merge" panel as it is not very clear if mTOR and LAMP1-RFP are co-localized throughout the conditions tested. In figure 2, panel G also appears in supplemental figure 2, the authors need to address this discrepancy. Throughout the paper authors measure mTORC1 activity by measuring phosphorylation of mTORC1 targets; they need to show levels of total protein together with the phosphorylated form of the substrate protein.

Response
Thank you for these points, which have been also raised by Reviewer 2. In our revised manuscript, the colocalization of mTOR and LAMP1 in the figure 2C has been added as merged images. Figure 2G has been removed. Currently we do not have good antibodies to detect total protein levels of p70S6K1 and 4E-BP1 in flies, therefore we show gene expression level of fly p70S6K1 (dS6K) using qPCR assay (Supplementary figure 12A), and we also show total protein levels of p70S6K1 together with phosphorylated p70S6K1 in cells (Figure 2). We have also included the quantitative analysis of phosphorylated/total p70S6K1 in Figure 2B and D in the revised manuscript.

Point 4
Commenting on the results shown in figure 3, the authors describe a model where TDP43 effects on TFEB localization are dependent on mTORC1. The authors need to substantiate this claim by performing an experiment using a phosphomimetic mutant of TFEB for serine 211; serine phosphorylated by mTORC1. The authors need to determine if the localization of this phosphomimetic mutant changes upon TDP43 knockdown.

Response
This is obviously a very good suggestion. Theoretically, phosphomimetic mutant TFEB should interact with cytosolic 14-3-3 and stay in the cytosol as speculated from the results in recent reports.

We have made the phosphomimetic mutant TFEB constructs (S211D and S211E) and performed the experiments as suggested. Surprisingly, we found that TFEB S211D and S211E localized in the nucleus, but not in the cytosol. We reason that hyper-phosphorylated may interact with other factors that associate with TFEB nuclear-cytoplasmic translocation, or these mutations may affect TFEB protein conformation. It is also possible that these mutations may affect the phosphorylation on other phosphorylation sites of TFEB. Overall, we cannot explain this unexpected observation at this moment and exploration of the mechanism undying this phenomenon is beyond the scope of this study. Hence, we did not include these data in the present paper.

Point 5
In figure 7, the authors show that knock-down of TDP43 has effects on autophagosome and autolysosome formation in starved cells. During starvation mTORC1 signaling is turned off, as such most probably the effects of TDP43 knock-down during starvation are mTORC1 independent. This will mean that knock-down of TDP43 has mTORC1 dependent and independent effects. The authors need to acknowledge and explain this point and possibly delineate mTORC1 dependent and independent effects.

Response
We thank Reviewer 3 for raising this excellent point, which is also raised by Reviewer 1 (point 2). There is indeed mTORC1 independent mechanism that accounts for the observed phenotype. Our data indicate autophagosome-lysosome fusion is impaired through an mTORC1-independent manner (a Dynactin 1 associated manner) in TDP-43 depleted cells, and we include the detailed mechanism (as suggested by Reviewer 1) and delineate this mTORC1 independent effects in our revised manuscript.

Point 6
In figure 9, the authors show that in TBPH -/- larvae, treating with rapamycin exacerbates and treating with PA ameliorates their locomotion. However rapamycin or PA treatment does not affect WT larvae. The authors need to measure mTORC1 activity throughout these treatments in WT as well as TBPH -/- larvae to account for these differences.

Response
We have measured mTORC1 activity (phosphorylated p70S6K1) throughout the treatments on WT as well as TBPH -/- larvae in Figure 9. The results are shown in Supplementary figure 12B and C in our revised manuscript.

2nd Editorial Decision 26 October 2015

Thank you for submitting your revised manuscript for our consideration. Your manuscript has now been seen once more by the original referees (see comments below), and I am happy to inform you that they are broadly in favor of publication, pending satisfactory minor revision.

I would therefore like to ask you to address the remaining points and to provide a final version of your manuscript. Referee #1 and #3 note that the data on dynactin need to be incorporated into the main part of your manuscript. Furthermore, point #3 of referee #3 needs to be addressed in the discussion. Importantly, both referee #1 and #2 request a few additional experiments that will better support your conclusions. They all seem straight forward, and I am certain that addressing the remaining issues as outlined by these referees will make your manuscript an outstanding one.

I am therefore formally returning the manuscript to you for a final round of minor revision. Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

I am overall satisfied with the revision. I think that the dynactin part is very relevant for the paper and must be included in a main figure, mentioned in the abstract and included in the model of last figure.

In addition a missing experiment (crucial in my opinion) is the rescue of AV-Lys fusion upon dynactin overexpression in TDP-43 null cells. In principle this experiment should rescue most of the phenotypes observed.

Referee #2:

The revised manuscript is easier to follow and contains more careful interpretation of the results observed. Experiments contain more controls and the conclusions are more accurate. The authors addressed all of the critical points arisen during the revision process. Above all, the quality of the revised manuscript improved in terms of the deeper dissection of the mTORC1-independent mechanism underlying TDP-43 depletion, namely AV-LYS fusion impairment in mTORc1-independent way.

Regarding the experiment with overexpression of TDP-25 (Supplementary Figure 6A) the more interesting experiment would be to check the level of mRNA level of raptor and mTORC1 activity in these cells with the cytoplasmic aggregates. In other words to investigate if the aggregates are able to sequester TDP-43 wt and cause the same effect as TDP-43 silencing such as raptor and mTORC1 inhibition. Alternatively some other model of TDP-43 aggregation with TDP-43 wt sequestration in the aggregates would be more suitable to answer this question.

Referee #3:

To the Authors:
Xia et. al. have tried to answer almost all the comments from the first revision. The authors' responses to the comments are satisfactory. Specific comments on the revised manuscript are as follows:

1. In the revised manuscript the authors describe an mTOR independent way of action for TDP-43. They argue that changes in Dynactin 1 can modulate the effects on autophagy, seen upon TDP43 depletion. The authors should include this mTOR independent branch of TDP-43 action in their model in Figure 9D.

2. The authors do not mention the TDP-43 effects on Dynactin 1 in their Discussion section. They should include a part describing these findings and contrasting them with the mTOR dependent branch of TDP-43 action.

3. In experiments shown in figure 9 treatment of WT larvae with rapamycin does not have any effect, whereas TBPH null larvae on their own have a drastic effect. However levels of mTORC1 activity are similar in TBPH null larvae and WT larvae treated with rapamycin (supplemental figure 14), suggesting that the effects are probably not due to mTORC1 downregulation. The author should address this in the Discussion session.

2nd Revision - authors' response 12 November 2015

Referee #1:

Point 1
I am overall satisfied with the revision. I think that the dynactin part is very relevant for the paper

and must be included in a main figure, mentioned in the abstract and included in the model of last figure.

Response
We thank Reviewer 1 for this point, which is also raised by Reviewer 3. In our revised manuscript, we have moved the Dynactin 1 part to the main figure (figure 7), combined the original figure 7 with figure 6 and moved some redundant parts of figure 6/7 to Expanded View 4 (Figure EV4) as suggested. Also, we have mentioned and included the Dynactin 1 part in the abstract and the model of last figure (figure 9D) as suggested.

Point 2
In addition a missing experiment (crucial in my opinion) is the rescue of AV-Lys fusion upon dynactin overexpression in TDP-43 null cells. In principle this experiment should rescue most of the phenotypes observed.

Response
We appreciate Reviewer 1 for this good suggestion, which is of help for us to improve the quality of our paper. We performed the rescue experiments as suggested, and we found that re-transfection of Dynactin 1 could restore the AV-Lys fusion in TDP-43-depleted cells as speculated by Reviewer 1. We include these data in Figure 7F and G and modify the text in our revised manuscript.

Referee #2:

The revised manuscript is easier to follow and contains more careful interpretation of the results observed. Experiments contain more controls and the conclusions are more accurate. The authors addressed all of the critical points arisen during the revision process. Above all, the quality of the revised manuscript improved in terms of the deeper dissection of the mTORC1-independent mechanism underlying TDP-43 depletion, namely AV-LYS fusion impairment in mTORc1-independent way.

Response
We thank Reviewer 2 for the positive comment.

Regarding the experiment with overexpression of TDP-25 (Supplementary Figure 6A) the more interesting experiment would be to check the level of mRNA level of raptor and mTORC1 activity in these cells with the cytoplasmic aggregates. In other words to investigate if the aggregates are able to sequester TDP-43 wt and cause the same effect as TDP-43 silencing such as raptor and mTORC1 inhibition. Alternatively some other model of TDP-43 aggregation with TDP-43 wt sequestration in the aggregates would be more suitable to answer this question.

Response
We are grateful to Reviewer 2 for this very interesting question and suggestion. We found that the aggregates in "TDP-25" cells were not able to sequester endogenous TDP-43. We reasoned that the endogenous wild type TDP-43 level in cytosol is very low and endogenous TDP-43 is not easy to be translocated into the cytosol from the nucleus under normal conditions in our cellular model, or perhaps the C-terminal fragment of TDP-43 that we used is not long enough to recruit endogenous TDP-43, since previous studies showed that the RRM2 and the N-terminus of TDP-43 are important for its self-association and oligomerization. Similar to several published papers, we failed to see TDP-43 cytoplasmic aggregation using the C-terminal fragment of TDP-43 longer than 25 kD and TDP-43 constructs with pathogenic mutations in our cultured cellular model, as responded to Reviewer 1, minor point 1 in the 1st revision. Thus, at this stage, we can only present the data that the raptor level and mTORC1 activity restoration experiments using aggregate-prone "TDP-25" in the experiment in Supplementary Figure 6A (now Appendix Figure S2), showing that cytoplasmic TDP-43 aggregates themselves would not affect raptor or mTORC1 activity. We discussed this issue in the result section of our revised manuscript, and we think that it is an interesting issue. Since in disease conditions, the formation of TDP-43 aggregates are always accompanied by a loss of nuclear TDP-43, which may reflect the loss of TDP-43 function in our study. We thank Reviewer 2 for this suggestion, which is much of help for us to think about preparing models for our future work.

Referee #3:

To the Authors:
Xia et. al. have tried to answer almost all the comments from the first revision. The authors' responses to the comments are satisfactory. Specific comments on the revised manuscript are as follows:

Point 1
In the revised manuscript the authors describe an mTOR independent way of action for TDP-43. They argue that changes in Dynactin 1 can modulate the effects on autophagy, seen upon TDP43 depletion. The authors should include this mTOR independent branch of TDP-43 action in their model in Figure 9D.

Response
We thank Reviewer 3 for this point, which is also raised by Reviewer 1. In our revised manuscript, we have included this part in the model in Figure 9D and modified our manuscript as suggested.

Point 2
The authors do not mention the TDP-43 effects on Dynactin 1 in their Discussion section. They should include a part describing these findings and contrasting them with the mTOR dependent branch of TDP-43 action.

Response
In our revised manuscript, we have included this mTOR independent branch of TDP-43 action (Dynactin 1 part) and contrasting this with the mTOR dependent branch of TDP-43 action in discussion section as suggested.

Point 3
In experiments shown in figure 9 treatment of WT larvae with rapamycin does not have any effect, whereas TBPH null larvae on their own have a drastic effect. However levels of mTORC1 activity are similar in TBPH null larvae and WT larvae treated with rapamycin (supplemental figure 14), suggesting that the effects are probably not due to mTORC1 downregulation. The author should address this in the Discussion session.

Response
This is a very good question, which has been pointed out by Reviewer 1 in the 1st revision. The reviewers are right, since the effect in TBPH null larvae was not only due to mTORC1 downregulation, but also due to the impairment of autophagosome-lysosome fusion. We find that depletion of TDP43 has both mTORC1 dependent and independent effects on ALP. Rapamycin treatment differs from TDP-43 depletion, since it will not impair autophagosome-lysosome fusion and will not induce a TDP-43 like phenotype in flies. In detail, our data indicate that the autophagosome and lysosome biogenesis were enhanced through mTORC1 inhibition and TFEB activation in TDP-43-depleted cells (similar effect as rapamycin treatment), whereas the autophagosome-lysosome fusion are impaired through a mTORC1-independent manner (a Dynactin 1 (DCTN1)-associated manner) in our TDP-43 loss of function model, which does not happen in Rapamycin treatment. Hence, now we can understand why "WT larvae with rapamycin does not have any effect, whereas TBPH null larvae on their own have a drastic effect", since the toxic outcome in TBPH null larvae might result from the combination of both mTORC1 dependent and independent effects, as described in the discussion section and new Figure 9D in our revised manuscript. Now our revised manuscript should read much better and the authors are very grateful for the suggestions made by the Reviewers.

3rd Editorial Decision 16 November 2015

Thank you for sending the revised version of your manuscript to us. I appreciate the introduced changes and I am happy to accept your manuscript for publication in The EMBO Journal.

