EMBO reports

Mitochondrial tyrosyl-DNA phosphodiesterase 2 and its TDP2^{S} short isoform

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Review timeline:

Submission date:	1 February 2016
Editorial Decision:	17 February 2016
Resubmission:	8 June 2017
Editorial Decision:	18 July 2017
Revision received:	20 October 2017
Editorial Decision:	22 November 2017
Revision received:	4 December 2017
Accepted:	5 January 2018

Editor: Esther Schnapp

Transaction Report: This manuscript was transferred to *EMBO reports* following review at *The EMBO Journal*
(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 17 February 2016

Thank you for the transfer of your manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

I am sorry to say that the evaluation of your manusript is not a positive one. As you will see, while the referees agree that the data support a mitochondrial localization of TDP2, they also all point out that role of the short TDP2 isoform compared with the long one remains unclear and insufficiently investigated. The referees further raise technical concerns and indicate contradictory data.

Given these concerns, the amount of work required to address them, and the fact that EMBO reports can only invite revision of papers that receive enthusiastic support from a majority of referees, I am sorry to say that we cannot offer to publish your manuscript.

I am sorry to have to disappoint you this time. I nevertheless hope, that the referee comments will be helpful in your continued work in this area, and I thank you once more for your interest in our journal.

REFEREE REPORTS

Referee #1:

Overall, the manuscript convincingly demonstrates that TDP2 is present in the mitochondria. However, the data addressing the short isoform and its relevance to mitochondria are not

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convincing, despite this being a major concession of the manuscript. Specific issues are below,

1. In Fig.1a and Supplementary Fig.1, the co-localisation between TDP2 and mitochondria is not very convincing, since the oxphos and mitotracker stain is much broader than the TDP2 stain. Colocalisation of a known mitochondrial protein is needed as an extra control along with better imaging. Also, the IF experiments might be better conducted on A549 cells, which appear to show higher levels of the short isoform.

2. In Fig.2C and S2B both normal TDP2 and TDP2(S) are enriched in the cytoplasm and mitochondria, yet in the IF experiments in Fig.S1A the enzyme localizes to the nucleus. The authors need to explain this discrepancy.

3. The prediction that TDP2S is mitochondrial is not convincing - do any other software packages predict this? TDP2S looks cytosolic but does not convincingly look mitochondrial in any of the fluorescence images.

4. The authors' data show that the standard isoform of TDP2 is also present in the mitochondria, and indeed they employ the standard isoform to complement the sensitivity of TDP2 KO DT40 cells to a mitochondrial specific form of doxorubicin. It is thus not at all clear that the short isoform is important, either for the mitochondrial phenotype or for TDP2 function at all.

5. For the mitochondrial phenotype experiments, the authors show that the standard isoform complements the hypersensitivity phenotype to the mitochondrial specific doxorubicin. However, the conclusion would be more convincing if they compared in these experiments TDP2 lacking the N-terminl domain but containing an artificial NLS (e.g. SV40) or an artificial mitochondrial targeting sequence.

6. The data showing a reduction in mitochondrial copy number are not very convincing. Only one concentration of mtDox shows a difference in wild type versus TDP2 KO cells and, despite the apparent statistical difference, the error bars on the data look large and overlapping.

Referee #2:

This is a short report which aims to show that the DNA repair enzyme TDP2 (and a short form) can co-localise and function in mitochondria. The first part of the paper focusses on localisation with immunofluorescence microscopy and subfractionations. Although a little unclear, the data does seem to support the presence of TDP2 and a shorter isoform in the mitochondria of some cell lines. The authors claim that after protease K shaving of their mitochondrial extracts, the remaining intact proteins must be in the mitochondrial matrix. Although the authors use a 0.1% treatment of their mito extracts with digitonin, to be sure that the OM and IMS proteins have been removed, they have to show this by using an OM or IMS marker. At the moment, their controls show only that the TDP2/TDP2s are either in the IMS or the matrix. I was not convinced by Fig 3B, particularly with the apparent colocalisation of the GFP-tagged MTS-TDP2s. Indeed, the whole story of the TDP2s is a little unclear. The data in Fig5 is much more compelling. I had to go back to previous papers until I found that clonogenic assays are left for 14 days before quantification. This is important to include, as I was confused by how dramatic the effect of the mtDOX was. I could not explain the clonogenic data other than by the interpretation the authors use.

Referee #3:

Huang et al. first described identification of a short isoform of tyrosyl-DNA phosphodiesterase (TDP2s) by immunofluorescence microscopy and western blotting analysis with combination of RNA silencing and subcellular fractionation. In the human genome databank, they found a cDNA sequence with an alternative transcription start site coding a TDP2 isoform, which may correspond to TDP2s detected by anti-TDP2, containing a different N-terminal leading sequence with un altered catalytic domain (85 to 362aa in TDP2), which is replacing the nuclear localization signal in the N-terminal domain of TDP2 with a putative mitochondrial targeting sequence. They showed that GFP-tagged TDP2 localizes in nucleus and TDP2s localizes to human mitochondria.

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Next they confirmed that the nuclear and mitochondrial extracts of wild-type but not tdp2-knockout DT40 cells contain specific 5'-tyrosyl-DNA phosphodiesterase activity, although they could not identify authentic TDP2 polypeptide in DT40 cells. They showed that tdp2-knockout DT40 cells are hypersensitive to the mitochondrial-specific Top2 poison, mitochondrial-targeted doxorubicin (mtDox). Introduction of human TDP2 (hTDP2) expressing plasmid restored resistance to mtDox. Furthermore, mtDox selectively depleted mitochondrial DNA (mtDNA) in tdp2-knockout DT40 cells, but not in wild-type or hTDP2-expressing cells, indicating that hTDP2 protects against mtDNA damage.
Based on these observations, the authors concluded that this study adds TDP2 to the mtDNA repair enzymes, with the short isoform TDP2S predominantly localizing to mitochondria of human cells.

Main comments
This is the first paper describing mitochondrial form of TDP2, and showing its functional importance to maintain mitochondrial DNA in vertebrate cells. The found smaller mitochondrial polypeptide reactive to anti-TDP2 and confirmed that RNA silencing of TDP2 expression abolished its expression in human cells, providing an evidence for TDP2 gene encodes the smaller polypeptide. They described in Supplementary Figure S2, "The band at 37 kDa was excised and subjected to tandem mass spectrometry analysis, performed using an Orbitrap Fusion tribrid mass spectrometer (Thermo Scientific), which confirmed that the protein was TDP2 with 15 unique peptide sequences and 60% sequence coverage.", however they did not show which peptides were identified. In order to convince readers, it is better to present the results obtained from the mass spectrometry analysis. Moreover, they found and expressed cDNA which may encode the TDP2s polypeptide, however, there is no evidence indicating the molecule identity of this cDNA encoding polypeptide with the TDP2s detected by anti-TDP2. The data obtained from the mass spectrometry analysis provide strong evidence for the TDP2s identity.

The authentic TDP2s polypeptide detected by anti-TDP2, expressed in high level in some human cancer cell lines, however, the authors claimed that recombinant human TDP2s expression in chicken DT40 cell is very low, and they could not present any data regarding its function. Instead, the authors presented data with recombinant human TDP2 protein functions which is localized both nucleus and mitochondria in DT 40 cells with mitochondria-targeted doxorubicin (mtDox), in order to demonstrate its function as mitochondrial DNA repair enzyme. These day, it is very simple to obtain gemome-edited human cancer derived cell lines, so the reviewer request to establish TDP2-disrupted human cells, and then the authors should characterize functions of the two isoforms of TDP2 separately, because DT40 cells may not behave as do human cells.

Minor comments
In figure 3(B), they examined TDP-2-GFP fusion or other fusion proteins to confirm intracellular localization of the two TDP2 isoforms. First, the authors should present GFP itself to make sure the effect of each fused protein, NLS or MTS. Secondly, they concluded "Fluorescence confocal microscopy imaging (Fig. 3B) showed that TDP2S colocalized with mitochondria, whereas the TDP2 was located in the nucleus." This result is contradictory to the data obtained by Western blotting from various cell lines. The authors should pay attention this contradictory data.

Resubmission 8 June 2017

We are grateful for the reviewers' insightful comments from our initial submission, which have helped us enhance our report following revision. We have provided further evidence to definitively assign the new TDP2 transcript TDP2^{S} (short TDP2 isoform) to mitochondria by using isoform-specific siRNA in addition to biochemical cellular fractionation and fluorescence microscopy constructs. We have specifically investigated the role of the short TDP2 isoform with a human cell line expressing only the short isoform of TDP2 using CRISPR gene editing to disrupt the full-length TDP2 isoform without disturbing the expression of the TDP2 short isoform. Thus, our data now provide solid genetic, biochemical and cellular evidence for the existence of TDP2 in vertebrate mitochondria.

We have also reorganized the manuscript (including the new data) to improve the logical flow and better highlight the novel role of TDP2 in mitochondria. Our point-by-point response to the reviewers' comments is appended in the following pages.

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Specifically, our revised study reveals that:

- A previously unidentified short isoform of TDP2 is the product of an alternative transcript arising from a different transcription start site; we also show that both human cells and murine tissues express the short TDP2 isoform;
- TDP2 is a targeted mitochondrial enzyme, as the short isoform of human TDP2 bears a functional mitochondrial targeting sequence (MTS), while the full-length TDP2 localizes to both the nucleus and mitochondria of all cell lines examined;
- Both TDP2 isoforms (the short new isoform TDP2S and the previously identified full-length isoform TDP2) protect against DNA damage caused by the mitochondrial-specific TOP2 poison, mtDox (mitochondrial-targeted doxorubicin).

Our study is also the first to show that alternative transcription start sites of the *TDP2* gene enable TDP2 to function in different cellular compartments (mitochondria and nucleus for full-length TDP2, and mitochondria for the new short isoform). It also reveals the role of TDP2 in DNA repair caused by topoisomerases in mitochondria.

Referee #1:
"Overall, the manuscript convincingly demonstrates that TDP2 is present in the mitochondria. However, the data addressing the short isoform and its relevance to mitochondria are not convincing, despite this being a major concession of the manuscript."

Response: We thank the reviewer for finding that our manuscript convincingly demonstrates that TDP2 is present in the mitochondria. The relevance of the short isoform of TDP2 to mitochondria is implied by the facts this widely expressed and previously unidentified isoform bears a functional mitochondrial targeting sequence (MTS) that replaces both the nuclear localization (NLS) and UBA (Ubiquitin association) domains present in the canonical TDP2 isoform. This point has been clarified in the revised manuscript (Fig 1F and Fig S2). In addition, we have constructed human cell lines expressing only the short isoform of mitochondrial TDP2 using CRISPR gene editing technology and shown their differential response to the mitochondrial-targeted TOP2cc poison mtDox (see Fig 4 in the revised manuscript).

"Specific issues are below.
1. In Fig.1a and Supplementary Fig.1, the co-localisation between TDP2 and mitochondria is not very convincing, since the oxphos and mitotracker stain is much broader than the TDP2 stain. Colocalisation of a known mitochondrial protein is needed as an extra control along with better imaging. Also, the IF experiments might be better conducted on A549 cells, which appear to show higher levels of the short isoform".

Response: We agree with the reviewer that the previously presented immunofluorescence images were not, on their own fully convincing, which is related to the low sensitivity of the available TDP2 antibodies against native TDP2. To address this point, we have performed additional and more direct experiments (Figure 2 in the revised manuscript) with tagged constructs and mitochondrial fractionation with mitochondrial- and nuclear-specific markers. We have retained the immunofluorescence data and decided to present them in the supplementary information section (Fig S3 in the revised manuscript). Experiments in A549 cells were similar from those in HCT116. We feel that although the immunofluorescence data are consistent with our findings reported in this study, the presence and activity of TDP2 in the mitochondria are most definitively demonstrated by the biochemical cellular fractionation followed by immunoblotting or activity assays, which is now combined with genetic studies to form the main thrust of our study. Moreover, the natural selection of the TDP2S isoform with the MTS provides converging evidence for the importance of TDP2 in mitochondria.

"2. In Fig.2C and S2B both normal TDP2 and TDP2(S) are enriched in the cytoplasm and mitochondria, yet in the IF experiments in Fig.S1A the enzyme localizes to the nucleus. The authors need to explain this discrepancy."

Response: Immunofluorescence signals are reflective of the relative TDP2 concentration in the whole cell and accessibility to the antibody, which shows that TDP2 is mainly a nuclear protein with

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minor cytoplasmic distribution. By contrast, in Western blotting, the signals are reflective of the relative TDP2 concentration in the nuclear and mitochondrial compartments, since we loaded equal amount of total proteins from those cellular fractions in each lane. Mitochondrial protein contents represent only a small percentage of total cellular protein mass. While the low levels of mitochondrial TDP2 might not be easily detected by immunofluorescence microscopy, by greatly enriching mitochondrial protein concentration after a mito-prep, mitochondrial TDP2 can be detected readily via immunoblotting analysis. Our results show that, although (the canonical) TDP2 with its NLS and UBA (see Fig. S2) is abundant in the nucleus, it is also in the mitochondria of all cell lines examined (see Figs 2-4 and S1). This is now addressed in the Discussion section. Thank you.

"3. The prediction that TDP2S is mitochondrial is not convincing - do any other software packages predict this? TDP2S looks cytosolic but does not convincingly look mitochondrial in any of the fluorescence images."

Response: The manuscript has been revised to provide converging evidence that $TDP2^S$ is mitochondrial. We agree that the software calculating the tendency of protein localizing to mitochondria is only a prediction and does not constitute a proof. We demonstrated experimentally that $TDP2^S$-GFP and the MTS portion of $TDP2^S$ tagged with GFP both have excellent colocalization with MitoTracker, as opposed to the control full-length TDP2-GFP (Fig. 2A in the revised manuscript). The lack of NLS and UBA in the $TDP2^S$ together with the presence of a functional MTS (see Fig. 2A in the revised manuscript) and the immunoblotting of mitochondrial fractions coupled with proteinase K digestion demonstrate the presence of $TDP2^S$ in mitochondria. Thank you.

"4. The authors' data show that the standard isoform of TDP2 is also present in the mitochondria, and indeed they employ the standard isoform to complement the sensitivity of TDP2 KO DT40 cells to a mitochondrial specific form of doxorubicin. It is thus not at all clear that the short isoform is important, either for the mitochondrial phenotype or for TDP2 function at all".

Response: We thank the reviewer for this insightful comment. We note that the regulation of the $TDP2^S$ are different from cell line to cell line. Some cell lines express high levels of $TDP2^S$ and would presumably rely on $TDP2^S$ more in the mitochondria, while the cell lines that express low levels of $TDP2^S$ tend to rely on the full-length TDP2 in the mitochondria. We have discussed this point in the revised manuscript. As suggested, we have performed additional experiments and generated cells that only have the short isoform of TDP2 by knocking out the standard isoform of TDP2. The mitochondrial fraction of these cells contains the $TDP2^S$ and lacks the standard isoform of TDP2. The presence of short isoform of TDP2 rescued the hypersensitivity to mtDox exhibited by the tdp2-KO cells, demonstrating that both short and standard isoforms of TDP2 in the mitochondria contribute to mitochondria maintenance.

"5. For the mitochondrial phenotype experiments, the authors show that the standard isoform complements the hypersensitivity phenotype to the mitochondrial specific doxorubicin. However, the conclusion would be more convincing if they compared in these experiments TDP2 lacking the N-terminl domain but containing an artificial NLS (e.g. SV40) or an artificial mitochondrial targeting sequence."

Response: We thank the reviewer for raising this important question, which is now addressed in the new Figure 4 of the manuscript and discussed in the previous comments. As suggested, we have repeatedly attempted to complement the *tdp2*-knockout cells (both DT40 and human cancer cells) with the short isoform of TDP2 or TDP2 containing an artificial mitochondrial targeting sequence but failed. It might be that the exogenous expression of these TDP2 protein products is toxic. To overcome this problem, we have generated human cells that only express the short isoform of TDP2 by knocking out the standard isoform of TDP2 by CRIPR-Cas9 (new Fig. 4). The full-length TDP2 is depleted in these cells, and the mitochondrial fraction of these cells contain the short isoform of TDP2. The fact that in these cells, $TDP2^S$ rescues the sensitivity to mtDox is functional evidence that $TDP2^S$ plays a role in maintenance of mitochondria.

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"6. The data showing a reduction in mitochondrial copy number are not very convincing. Only one concentration of mtDox shows a difference in wild type versus TDP2 KO cells and, despite the apparent statistical difference, the error bars on the data look large and overlapping"

Response: We appreciate the reviewer comment. Higher concentration of mtDox treatment did not yield consistent results and therefore were excluded from our report. The data with 2 uM mtDox-treatment were the average of 8 individual experiments. The plot showed the standard deviation (±SD), and not the standard error of the mean (±SEM). The resulting P-values calculated by two-tailed t-test indicated that there is a significant difference between the WT and tdp2 KO, and again between the tdp2 KO and hTDP2 cells. The relatively limited difference in mtDNA copy number could be due to the rapid degradation of damaged mtDNA. This point has been clarified in the revised manuscript.

Referee #2:

"This is a short report which aims to show that the DNA repair enzyme TDP2 (and a short form) can co-localise and function in mitochondria. The first part of the paper focusses on localisation with immunofluorescence microscopy and subfractionations. Although a little unclear, the data does seem to support the presence of TDP2 and a shorter isoform in the mitochondria of some cell lines. The authors claim that after protease K shaving of their mitochondrial extracts, the remaining intact proteins must be in the mitochondrial matrix. Although the authors use a 0.1% treatment of their mito extracts with digitonin, to be sure that the OM and IMS proteins have been removed, they have to show this by using an OM or IMS marker. At the moment, their controls show only that the TDP2/TDP2s are either in the IMS or the matrix. I was not convinced by Fig 3B, particularly with the apparent colocalisation of the GFP-tagged MTS-TDP2s. Indeed, the whole story of the TDP2s is a little unclear.
The data in Fig5 is much more compelling. I had to go back to previous papers until I found that clonogenic assays are left for 14 days before quantification. This is important to include, as I was confused by how dramatic the effect of the mtDox was. I could not explain the clonogenic data other than by the interpretation the authors use."

Response: We thank the reviewer for raising several important points that needed clarification. We agree with the point on the protease K test of the whole mitochondria and we have thus repeated the experiments and included additional markers. The OM marker was indeed removed by proteinase K, while the IMS marker was not. Therefore, we corrected our conclusion to the localization of TDP2 to the IMS or the matrix of mitochondria in the revised manuscript. To further establish and clarify the role of the new TDP2 mitochondrial isoform, $TDP2^S$, we have performed additional experiments by generating human cells that only have the $TDP2^S$ by knocking out the standard isoform of TDP2 using CRISPR gene editing. TDP2 was depleted in these cells and, consistent with our prior conclusion, the mitochondrial fraction of these cells contains the short isoform $TDP2^S$. The fact that $TDP2^S$ rescues the sensitivity of *tdp2* knockout cells to mtDox supports the functional role of $TDP2^S$ in the maintenance of mitochondria. As suggested, we have included the details on the clonogenic assays (Fig 3D and S5) in the method section. Thank you.

Referee #3:

"Huang et al. first described identification of a short isoform of tyrosyl-DNA phosphodiesterase (TDP2s) by immunofluorescence microscopy and Western blotting analysis with combination of RNA silencing and subcellular fractionation. In the human genome databank, they found a cDNA sequence with an alternative transcription start site coding a TDP2 isoform, which may correspond to TDP2s detected by anti-TDP2, containing a different N-terminal leading sequence with un altered catalytic domain (85 to 362aa in TDP2), which is replacing the nuclear localization signal in the N-terminal domain of TDP2 with a putative mitochondrial targeting sequence. They showed that GFP-tagged TDP2 localizes in nucleus and TDP2s localizes to human mitochondria.
Next, they confirmed that the nuclear and mitochondrial extracts of wild-type but not tdp2-knockout DT40 cells contain specific 5'-tyrosyl-DNA phosphodiesterase activity, although they could not identify authentic TDP2 polypeptide in DT40 cells. They showed that tdp2-knockout DT40 cells are hypersensitive to the mitochondrial-specific Top2 poison, mitochondrial-targeted doxorubicin

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(mtDox). Introduction of human TDP2 (hTDP2) expressing plasmid restored resistance to mtDox. Furthermore, mtDox selectively depleted mitochondrial DNA (mtDNA) in tdp2-knockout DT40 cells, but not in wild-type or hTDP2-expressing cells, indicating that hTDP2 protects against mtDNA damage. Based on these observations, the authors concluded that this study adds TDP2 to the mtDNA repair enzymes, with the short isoform TDP2S predominantly localizing to mitochondria of human cells.

Main comments
This is the first paper describing mitochondrial form of TDP2, and showing its functional importance to maintain mitochondrial DNA in vertebrate cells. The found smaller mitochondrial polypeptide reactive to anti-TDP2 and confirmed that RNA silencing of TDP2 expression abolished its expression in human cells, providing an evidence for TDP2 gene encodes the smaller polypeptide. They described in Supplementary Figure S2, "The band at 37 kDa was excised and subjected to tandem mass spectrometry analysis, performed using an Orbitrap Fusion tribrid mass spectrometer (Thermo Scientific), which confirmed that the protein was TDP2 with 15 unique peptide sequences and 60% sequence coverage.", however they did not show which peptides were identified. In order to convince readers, it is better to present the results obtained from the mass spectrometry analysis. Moreover, they found and expressed cDNA which may encode the TDP2s polypeptide, however, there is no evidence indicating the molecule identity of this cDNA encoding polypeptide with the TDP2s detected by anti-TDP2. The data obtained from the mass spectrometry analysis provide strong evidence for the TDP2s identity."

Response: We thank the reviewer for stating that the mass spectrometry data provide strong evidence for TDP2 identity. As suggested, we have included the identified unique peptide sequences in Figure S2 (highlighted in red in panel A). We have also included in the same figure the sequence of the canonical (full-length) TDP2 with its NLS and UBA domains, which are replaced by the MTS in $TDP2^S$, as well as data showing the robust phosphodiesterase activity of the recombinant $TDP2^S$ polypeptide. To directly confirm the $TDP2^S$ polypeptide detected by anti-TDP2 antibody as a previously unidentified TDP2 transcript (ENST00000341060) encoded by the *TDP2* gene, we have included new experiments with isoform-specific silencing RNA targeting transcript for either the TDP2-362 aa or the TDP2-304 aa. The fact that the $TDP2^S$ band is selectively depleted by TDP2-304 aa siRNA (Figs. 1D-E) unequivocally show that the lower band in the immunoblot is the protein product of alternative transcript of TDP2 (ENST00000341060). These results are now included and discussed in the revised manuscript to strengthen our conclusion that TDP2 (both the canonical isoform and $TDP2^S$) are mitochondrial enzymes.

"The authentic TDP2s polypeptide detected by anti-TDP2, expressed in high level in some human cancer cell lines, however, the authors claimed that recombinant human TDP2s expression in chicken DT40 cell is very low, and they could not present any data regarding its function. Instead, the authors presented data with recombinant human TDP2 protein functions which is localized both nucleus and mitochondria in DT 40 cells with mitochondria-targeted doxorubicin (mtDox), in order to demonstrate its function as mitochondrial DNA repair enzyme. These day, it is very simple to obtain genome-edited human cancer derived cell lines, so the reviewer request to establish TDP2-disrupted human cells, and then the authors should characterize functions of the two isoforms of TDP2 separately, because DT40 cells may not behave as do human cells."

Response: We agree with the reviewer's point. Yet, further attempts to overexpress $TDP2^S$ in DT40 and human cells have failed to generate viable clones, possibly because overexpression of $TDP2^S$ is toxic. As suggested, we have generated human cells that only have the $TDP2^S$ by knocking out the standard isoform of TDP2 using CRISPR editing. We now present evidence that TDP2 is depleted in the nuclear fraction of these cells, and that the mitochondrial fraction of these cells contains the short TDP2 isoform, $TDP2^S$ but not the standard isoform of TDP2. These cells that only express $TDP2^S$ regain resistance to mtDox, which is consistent with $TDP2^S$ playing a role in the maintenance of the mitogenome. Our data support that both isoforms of TDP2 function in the mitochondria. The reliance on the full-length TDP2 vs. $TDP2^S$ in the mitochondria depends on the regulation of expression for each isoform in each cell line. These points have been included in the revised manuscript. Thank you.

"Minor comments

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In figure 3(B), they examined TDP-2-GFP fusion or other fusion proteins to confirm intracellular localization of the two TDP2 isoforms. First, the authors should present GFP itself to make sure the effect of each fused protein, NLS or MTS. Secondly, they concluded "Fluorescence confocal microscopy imaging (Fig. 3B) showed that TDP2S colocalized with mitochondria, whereas the TDP2 was located in the nucleus." This result is contradictory to the data obtained by Western blotting from various cell lines. The authors should pay attention this contradictory data".

Response: As suggested, we have included the images showing that GFP by itself has no specific localization in the revised manuscript (Fig 2A, bottom row in the revised manuscript). We have also clarified the point on the localization of the standard isoform of TDP2 in the Discussion section: it is mainly a nuclear protein with minor mitochondrial distribution. The immunofluorescence approach reflects the *in-situ* concentration, whereas Western blotting shows the amount of TDP2 present in the cellular compartment relative to total protein content of the particular cellular compartment. Combined, our results show that the standard isoform of TDP2 (which contains the NLS and UBA; see new Fig S2) is mainly nuclear. A fraction of the standard isoform of TDP2 is also present in mitochondria, which remains detectable by Western blotting of purified and concentrated mitochondrial fraction. We have clarified these points in the revised manuscript.

2nd Editorial Decision 18 July 2017

Thank you for the submission of your revised manuscript to our journal. We have now received the reports from the referees that are pasted below.

As you will see, while the referees acknowledge that the study has been strengthened, they still raise several and important concerns that would need to be satisfactorily addressed for publication of the manuscript here. The main concerns regard the unconvincing mitochondrial localization and unclear function of the short TDP2 isoform, TDP2s.

I would like to give you the opportunity to address the remaining points, but please note that we can only offer to publish your study if the data will be considerably strengthened and the majority of the referees convinced. Please also submit a detailed point-by-point response with the revised version, and let me know if you have any questions.

I look forward to seeing a newly revised manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

This is an interesting paper in which the authors describe a new isoform of TDP2, and argue that this isoform is present in the mitochondria. There are some problems with this manuscript, however as it stands. Currently, it is a paper that draws two separate conclusions. The first is the existence of a short isoform of TDP2. This is convincingly demonstrated, and includes CRISPR data that greatly enhance this conclusion. The problem though is that no function for the short isoform is demonstrated. It is argued that it is a mitochondrial form, but actually the full-length isoform is also mitochondrial in the data presented. Indeed, in the DT40 experiments, the only apparent isoform in nuclei and mitochondria is the full-length one. Thus, no specific role for the short isoform is identified. The second conclusion is that TDP2 is present in the mitochondria. This looks convincing from the data, though a few more controls are needed.

1. Does the mitochondrial specific doxorubicin also damage nuclear genome? The authors should rule this out by looking at gH2AX formation under the conditions employed, including standard doxorubicin as a positive control. I appreciate the supplementary images suggest that the mt Dox is properly localised to mitochondria but a clear and convincing test would be to look directly for (absence of) damage in the nucleus.
2. In the IF images in Fig.2, there is still a problem that the GFP tagged TDP2 short isoform is not convincingly mitochondrial. It is certainly cytoplasmic, but the colocalisation with mitotracker Is not very clear. This is also true for the images in Fig.S2 with oxphos markers. I agree it is clear that

the putative mt localisation peptide targets GFP to mitochondria, but it's not clear that it does so in TDP2.

Referee #2:

Thank you very much for sending me this updated version by Huang and colleagues. It is certainly an improved manuscript and the authors should be complemented. It is often difficult to convince people of dual localised proteins to mitochondria, due to a variety to reasons. Often the possible 'mitochondrial' form is present at low level and then there is the difficulty of producing subfractions that are absolutely clean. In this case, a second, short isoform of TDP2 is believed to localised specifically to mitochondria and the authors provide a substantial amt of data to support their claim. On the whole, the story holds together but there are a few things I am still surprised about that should be added. It is a surprise to me that the steady state level of this short isoform is present at such relatively high levels, often close to the level of the full length protein. Why has this isoform not been noted before ? I would like to see a simple northern blot to show the relative steady state levels of the alternatively spliced mRNA. Second, in Fig 2 the MTS TDP2S GFP fusion protein is not shown to localise convincingly to mitochondria. Why? Third, in Fig S3 why is the TDP2 staining within mitochondria so punctate ? COuld it be localising to mtDNA ? If so, it needs to be shown, if not the authors need to comment. Overall, I'm not sure whether the focus was on the characterisation of the short potentially mitochondrial isoform or on the observation that a subset of the complete TDP2 apparently localises to the mitochondrial matrix. If the latter, why is it necessary to express the short mitochondrial isoform ? If the major TDP2 isoform is indeed inside the mitochondrion, how does it get there ? Overall, there is lots of interesting data here and although to my mind this is a little surprising, I think it should be published to allow others to make up their own minds.

Referee #3:

In this revised manuscript, the authors provided more convincing data for identity of mitochondrial TDP2s form, and its biological function in human cancer derived cell lines using gene editing technology. This is much better than the first version, however, some concerns are raised as shown below.

Major comments

1. Results page 3, line 32: The authors performed isoform specific RT-PCR to demonstrate the existence of TDP2s, however, no data was presented. Those data should be presented at least in the supplementary information
2. The authors used His-tagged recombinant TDP2 as a control for SDS-PAGE, in Figure 1A and Figure 3A. Migration of the His-tagged recombinant TDP2 in the Figure 1A is slower than that of endogenous human TDP2, while exogenously expressed human TDP2 in DT40 cells exhibits similar migration as did the recombinant the His-tagged recombinant TDP2. The authors need to explain this inconsistency.
3. In Figure 3A and B, migration of the exogenously expressed human TDP2 in DT40 cells looks different; 55-kDa in the former and smaller than 55-kDa in the latter.
4. It is better to show whether the MTS in TDP2s protein is processed in mitochondria or not.
5. In Figure 4D, please provide TDP2 biochemical activities in both nuclei and mitochondria prepared from the three cell lines (WT, TDP2S, tdp2-/-) in order to ensure the biochemical activity of endogenous TDP2S in human cells.
6. In Figure 4D, please compare sensitivities of the three cell lines to classical doxorubicin and mtDox.
7. In Figure 4D, mitochondrial DNA copy should be examined.

1st Revision - authors' response 20 October 2017

Referee #1

This is an interesting paper in which the authors describe a new isoform of TDP2, and argue that this isoform is present in the mitochondria. There are some problems with this manuscript, however as it stands. Currently, it is a paper that draws two separate conclusions. The first is the existence of a short isoform of TDP2. This is convincingly demonstrated, and includes CRISPR data that greatly enhance this conclusion. The problem though is that no function for the short isoform is demonstrated. It is argued that it is a mitochondrial form, but actually the full-length isoform is also mitochondrial in the data presented. Indeed, in the DT40 experiments, the only apparent isoform in nuclei and mitochondria is the full-length one. Thus, no specific role for the short isoform is identified. The second conclusion is that TDP2 is present in the mitochondria. This looks convincing from the data, though a few more controls are needed.

Response: Thank you for finding our report interesting, and for construtive comments and specific recommendations to further improve our potential publication. We agree with the reviewer that our study draws two conclusions and we are grateful that the reviewer acknowledges that both conclusions are convincing from the data. The two conclusions (existence of a short isoform of TDP2 and presence of TDP2 in mitochondria) are actually complementary and provide the first evidence for mitochondrial localization of TDP2, and therefore mitochondrial function of TDP2. Our study demonstrates that full-length TDP2 is present and plays a role in the mitochondria in addition to its nuclear localization and functions. Furthermore, our study provides evidence that the new short TDP2 isoform ($TDP2^S$) contains a mitochondrial targeting sequence (MTS), which replaces the nuclear localization sequence (NLS present only in the long TDP2 isoform). The existence of the mitochondrial short isoform emphasizes the importance of TDP2 in mitochondria. There might be additional cellular functions for the novel isoform. Indeed, in the revised manuscript, we note that the short TDP2 isoform ($TDP2^S$) is also abundant in the cytosolic fraction, potentially reflecting additional roles of TDP2 beyond its repair function for abortive topoisomerase II cleavage complexes (consistent with its identification as the cytosolic VpG unlinkase and as TTRAP). The question raised by the reviewer as to the potential functional differences between the TDP2 isoforms is interesting. Our study shows that both are in the mitochondria but that only the long isoform is in the nucleus. With respect to mitochondria, we note that this type of redundancy has been observed, where more than one protein isoform is present in the mitochondria while only one isoform specifically bears a MTS (e.g. FEN1 and FENMIT). The conclusion that TDP2 functions in mitochondria is supported by the existence of the mitochondrial-specific TDP2 short isoform and is consistent with the existence of topoisomerases II alpha and beta in mitochondria. Using mitochondria-specific doxorubicin, we provide evidence that TDP2 repairs TOP2cc in mitochondria. Moreover, we have included in the revised manuscript new data showing that TDP2-deficient cells show reduced mitochondrial transcripts, indicative of a role for TDP2 (and TOP2) in mitochondrial transcription. We have included the requested controls listed below, and revised our manuscript to address your constructive suggestions.

1. Does the mitochondrial specific doxorubicin also damage nuclear genome? The authors should rule this out by looking at gH2AX formation under the conditions employed, including standard doxorubicin as a positive control. I appreciate the supplementary images suggesting that the mt Dox is properly localised to mitochondria but a clear and convincing test would be to look directly for (absence of) damage in the nucleus.

Response: As suggested, we have examined gH2AX formation using standard doxorubicin as control. We have compared mtDox-treated cells vs. doxorubicin-treated cells using immunofluorescence imaging and find that mtDox does not induce DNA damage in the nucleus under the condition under which it induces mitochondrial DNA damage and under which standard doxorubicin induces nuclear gH2AX foci. These data are now included in the supplementary information (Fig S7C). Thank you.

2. In the IF images in Fig.2, there is still a problem that the GFP tagged TDP2 short isoform is not convincingly mitochondrial. It is certainly cytoplasmic, but the colocalisation with mitotracker is not very clear. This is also true for the images in Fig.S2 with oxphos markers. I agree it is clear that the putative mt localisation peptide targets GFP to mitochondria, but it's not clear that it does so in TDP2.

Response: We appreciate the reviewer's attention and precise analysis of the images. We agree that $TDP2^S$ is found in the cytosol in addition to mitochondria, a point which is further clarified in the

revised manuscript by including cytosolic fractions in our immunoblotting analyses. We agree that the fluorescent microscopy images reflect that fact. We have also included additional newly-acquired images of TDP2^S-GFP in Fig 2A to better demonstrate the localization of the TDP2 short isoform colocalizes with Mitotracker and is also present in the cytosol. There are likely other translocation and interaction signals in the remaining portion of TDP2^S as the MTS of TDP2^S shows an exclusive mitochondria distribution. This point has been included and addressed in the Result and Discussion sections. Thank you.

Referee # 2

Thank you very much for sending me this updated version by Huang and colleagues. It is certainly an improved manuscript and the authors should be complimented. It is often difficult to convince people of dual localised proteins to mitochondria, due to a variety to reasons. Often the possible 'mitochondrial' form is present at low level and then there is the difficulty of producing subfractions that are absolutely clean. In this case, a second, short isoform of TDP2 is believed to localised specifically to mitochondria and the authors provide a substantial amount of data to support their claim.

Response: Thank you for the compliment and for finding that our revised manuscript is improved and provides a substantial amount of data to support our claims. Thank you also for the suggestions listed below, which have been addressed point-by-point in our revision. We believe the manuscript has been further improved thanks to your suggestions.

On the whole, the story holds together but there are a few things I am still surprised about that should be added. It is a surprise to me that the steady state level of this short isoform is present at such relatively high levels, often close to the level of the full length protein. Why has this isoform not been noted before ? I would like to see a simple northern blot to show the relative steady state levels of the alternatively spliced mRNA.

Response: Indeed, a shorter isoform of TDP2 has been reported in prior publications as a TDP2 variant by Li et al. [2011 Oncogene], Thompson et al. [2013 Anal Biochem] and by Virgen-Slane et al. [2012 PNAS]. Those independent studies did not address the origin of the shorter isoform and focused on the biological functions of TDP2 both as a nuclear repair protein and cytoplasmic protein. This point has now been included in our revision along with the corresponding references. Our study carried out a systematic characterization of the shorter isoform (TDP2^S) and defines its genetic origin arising from an alternative transcription start site. We also provide evidence for the functional importance of TDP2^S as a mitochondrial form of TDP2. Consistent with the report of Li et al. who also studied a broad range of cancer cell lines, we find that the steady state levels of TDP2^S varies across cell lines with some having high levels of TDP2^S. As suggested, we have included in the revised manuscript additional data to further ascertain the existence of the alternatively spliced mRNA for TDP2^S. First, we have performed isoform-specific qPCR showing the mRNA levels for both isoforms are reduced after si*TDP2* treatment; these data are now included in the supplementary section (Fig S3A). Second, we have analyzed RNA-seq data for several cell lines examined in this study, which detect the isoform-specific transcripts for both *TDP2* and *TDP2^S*. We also show the relative steady state levels of the alternatively spliced mRNA counts determined from RNA-seq data. These results have been included in our revision in the supplementary section (Fig S4A).

Second, in Fig 2 the MTS TDP2S GFP fusion protein is not shown to localise convincingly to mitochondria. Why?

Response: We have included newly-acquired images of TDP2^S-GFP in the results section (revised Fig 2A) to better show the colocalization of TDP2S with Mitotracker. We agree that TDP2^S fusion protein is found in the cytosol in addition to mitochondria while being excluded from the nucleus. The immunoblotting analysis in the revised manuscript now includes the cytosolic fractions to demonstrate the presence of the short TDP2^S isoform in the cytosol. This raises the interesting possibility that TDP2^S is also functioning in the cytosol, which is consistent with the identification of TDP2 as the VpG unlinkase. These points have been discussed in the Result and Discussion sections. Thank you.

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Third, in Fig S3 why is the TDP2 staining within mitochondria so punctate ? Could it be localising to mtDNA ? If so, it needs to be shown, if not the authors need to comment.

Response: We thank the reviewer for asking us to clarify this point. We note that the images reported by Virgen-Slane and colleagues using the same antibody showed very similar patterns as ours. We agree it is plausible that TDP2 is localized to mtDNA as a repair enzyme for abortive topoisomerase II cleavage complexes in the mitochondrial nucleoids. Unfortunately, we do not feel that the specificity of the available antibodies is high enough to show colocalization of TDP2 with mtDNA conclusively. As suggested, we commented on this point in the revised manuscript.

Overall, I'm not sure whether the focus was on the characterisation of the short potentially mitochondrial isoform or on the observation that a subset of the complete TDP2 apparently localises to the mitochondrial matrix. If the latter, why is it necessary to express the short mitochondrial isoform ? If the major TDP2 isoform is indeed inside the mitochondrion, how does it get there ? Overall, there is lots of interesting data here and although to my mind this is a little surprising, I think it should be published to allow others to make up their own minds.

Response: We thank the reviewer for finding our data interesting and noting they should be published and made available to the scientific community. What necessitates the expression of a short mitochondrial isoform of TDP2 is an interesting question. Indeed our data show that the two isoforms (TDP2 and TDP2^S) have different expression levels across a broad range of cell lines. The fact that both isoforms function in mitochondria and that TDP2^S tends to be excluded from the nucleus suggests the importance of TDP2 activity in mitochondria and in the cytosol. We note that the levels of the two isoforms are not closely correlated in different cell lines and murine tissue (see Fig 1 and new Fig S4A). This type of redundancy of multiple isoforms of an enzyme in the mitochondria has been reported before (e.g. FEN1 and FENMIT). We show that the full-length TDP2 is also present in the mitochondria, demonstrated by the protease K digestion assay of purified, intact mitochondria. It is indeed notable that the full-length TDP2 and many other proteins functioning in mitochondria (including TOP2a and TOP2b, as well as TDP1) lack a canonical MTS. These point has been further discussed in our revised manuscript.

Referee #3

In this revised manuscript, the authors provided more convincing data for identity of mitochondrial TDP2s form, and its biological function in human cancer derived cell lines using gene editing technology. This is much better than the first version, however, some concerns are raised as shown below.

Response: We thank the reviewer for the overall positive and constructive comments, which have been addressed point-by-point below.

Comments

1. Results page 3, line 32: The authors performed isoform specific RT-PCR to demonstrate the existence of TDP2s, however, no data was presented. Those data should be presented at least in the supplementary information

Response: As requested, the isoform-specific qPCR data are now included in the supplementary section (Fig S3A in the revised manuscript).

2. The authors used His-tagged recombinant TDP2 as a control for SDS-PAGE, in Figure 1A and Figure 3A. Migration of the His-tagged recombinant TDP2 in the Figure 1A is slower than that of endogenous human TDP2, while exogenously expressed human TDP2 in DT40 cells exhibits similar migration as did the recombinant the His-tagged recombinant TDP2. The authors need to explain this inconsistency.

Response: In Figure 1A, the recombinant protein is tagged with a His-tag through a linker; therefore larger by ~ 4 kDa than the endogenous TDP2. In Figure 3A, the exogenously expressed human

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TDP2 in DT40 cells actually bears a FLAG tag, making its size comparable to the His-tagged recombinant TDP2. We have now included this information in the Materials and Methods section and clarified the Results. Thank you.

3. In Figure 3A and B, migration of the exogenously expressed human TDP2 in DT40 cells looks different; 55-kDa in the former and smaller than 55-kDa in the latter.

Response: We thank the reviewer for noting this discrepancy in the labeling of the bands. We apologize for the mistake. The bands in Figure 3B are now correctly labeled.

4. It is better to show whether the MTS in TDP2s protein is processed in mitochondria or not.

Response: Cleavage of MTS in $TDP2^S$ would give rise to difference in molecular weight of only 2 kD. After trying several types of eletrophoresis methods, we are not able to confidently resolve the size difference to that precise degree. However, our data (Fig 2) show that the MTS in $TDP2^S$ drives its cellular localization to the mitochondria. We also show that recombinant $TDP2^S$ bearing the MTS is fully catalytically active (Fig. S4B), as is the N-terminally-truncated TDP2. Hence, cleavage of the MTS does not impact $TDP2^S$ function. We have now clarified this point in the revised manuscript. Thank you.

5. In Figure 4D, please provide TDP2 biochemical activities in both nuclei and mitochondria prepared from the three cell lines (WT, TDP2S, tdp2-/-) in order to ensure the biochemical activity of endogenous TDP2S in human cells.

Response: As requested, we have now performed the TDP2 biochemical assay with the mitochondrial extracts of the three cell lines (*WT*, $TDP2^S$, $TDP2^{-/-}$), which show that $TDP2^S$ in the mitochondrial extracts are biochemically competent (Fig. 4D in the revised manuscript). Thank you.

6. In Figure 4D, please compare sensitivities of the three cell lines to classical doxorubicin and mtDox.

Response: As suggested, the sensitivities of three TK6 cell lines (*WT*, $TDP2^S$, $TDP2^{-/-}$) to doxorubicin have been tested and the results are now included in the supplementary data (Fig S9). $TDP2^S$ cells show similar sensitivity as the $TDP2^{-/-}$ cells to doxorubicin, as expected.

7. In Figure 4D, mitochondrial DNA copy should be examined.

Response: Under the conditions we have examined (short treatment of mtDox to avoid potential cell death induced by longer treatments), we have not found significant changes in mtDNA copy numbers between the three cell lines. This point has been included in our revised manuscript. Thank you.

3rd Editorial Decision 22 November 2017

Thank you for the submission of your newly revised manuscript. We have now received the enclosed referee reports as well as referee cross-comments. As you will see, while referee 2 does not seem to be convinced by the data, referees 1 and 3 think that their concerns have been adequately addressed. Both referees still have suggestions that I would like you to address and incorporate before we can proceed with the official acceptance of your manuscript.

As referee 1 notes, better confocal images of the TDP2/mitoch colocalisation for Fig. 2A need to be provided.

A few other changes are also required:

The manuscript has 5 main figures and should thus be classified as a short report. However, the results and discussion sections are separate. They either need to be combined, or one more main figure needs to be added to change the paper into a full article.

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Please add scale bars to fig 2A, Appendix fig S5A (both for the origin images and the zoom), S5B, S7B (both for the origin images and the zoom) and S7C.

Figs 3D,E, 4D,E, 5A,B, S6, S8, S9, S10 currently do not and need to specify "n" as the number of independently performed experiments (n=number of cells is not sufficient!). If the data are based on less than 3 independently performed experiments no error bars can be shown or calculated. Please also note that no error bars should be calculated for technical replicates.

Please add a callout for fig 5B in the main manuscript text.

Please update the callouts and legends for Appendix figs from Fig S[n] to Appendix Fig S[n].

Please add a table of content to the Appendix.

Please rewrite the abstract and your findings in present tense.

Several blots seem to be overexposed or have a bad resolution, e.g. especially in fig 4C. Please provide better blots.

I look forward to seeing a new final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

the concerns from the last review seem to have been addressed

Referee #2:

I'm afraid I'm just not convinced about this paper. In my opinion there is not sufficient evidence to unequivocally believe that the full length form of TDP2 is really being taken in to the mitochondrial matrix (I don't understand the mechanism of how this would happen) or that there is a clear role for the short form of TDP2. A great deal of this work in my opinion could be explained by artefact. My feeling with the last iteration was that we should just let other scientists in the field judge the work, but now I feel that it is just not credible enough.

Referee #3:

Now, the authors appropriately responded to most of all three reviewers' comments, however, I found that the authors' response to my comment #3 was not appeared in the revised Figure 3B, yet. In Figure 3A and B, migration of the exogenously expressed human TDP2 in DT40 cells looks different; 55-kDa in the former and smaller than 55-kDa in the latter.

Cross-comments from referee 1:

Overall they have added the controls we asked for. The critical conclusion is whether TDP2 is mitochondrial. Their biochemical data looks robust and it's hard to argue against that. Their imaging is not great still, though, and I would still like higher resolution/magnification confocal images of the isoform (in Fig.2A) showing mitochondrial co-localisation, but that's the only technical improvement I think I would like to see.

Cross-comments from referee 3:

I understund the the reviewer #2's concern, especially how full-length TOP2 localizes in mitochondria. When I examined possible cellular localization of human TOP2 sequence using

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PSORT II software, more than 10% possibility for mitochondrial localization was predicted, even without apparent MTS, and similar prediction was also made for hTOP2-short form.

Experimental data provided by the authors are sufficient to support the contribution of both hTOP2 and its short form in mitochondria, and I can not express any doubt at this moment.

2nd Revision - authors' response 4 December 2017

We thank the reviewers for their construtive comments and for concluding that our revised manuscript has addressed the comments of the Referees adequately.

We also thank you and the reviewers for the additional suggestions, which we have addressed in our revision. To comply with the format as a research article, the current manuscript now includes six main figures. This improves the presentation, the logical flow and the ease of reading.

The following specific changes have been made:

1. We reprocessed the fluorescence confocal microscopy raw images (now in Fig 3) to allow higher resolutions. We modified the color scheme and included zoomed-in areas to better demonstrate the colocalizations.
2. Scale bars are now included in every microscope images throughout the manuscript.
3. The number of independently performed experiments is stated for every data set, and error bars are shown only for data set with more than 3 independently performed experiments (not including technical replicates).
4. We corrected the labeling of bands in Figure 4B.
5. Several blots were rescanned at maximum resolution to provide better image qualities.

We are confident that these modifications further clarify and strengthen our study, and we appreciate the opportunity to have our manuscript published in EMBO Reports.

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Journal Submitted to: EMBO Report
Manuscript Number: EMBOR-2016-42139V2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	All immunoblotting analyses were carried out in at least 2 independent experiment with similar results. All biochemical assays were carried out at least 2 independent times. Clongenic and viability assays were carried out at least 3 times, each time with technical duplicates or triplicates. Quantitative real-time qPCR were carried out for at least 6 independent experiments, each with its technical replicates.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	None of the samples were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Cell lines that are treated or not treated with a particular chemical reagents are taken from the same culture vials, which were kept to the similar growth conditions between cell lines.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	N/A
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5. For every figure, are statistical tests justified as appropriate?	Yes, please see relevant figure legends for description of every statistical test performed.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We mainly used non-parametric tests, which did not assume normal distribution.

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Is there an estimate of variation within each group of data?	Yes, either SD or SEM is presented.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	See relevant section in Materials and Methods and Figure Legends.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines used in this study is free of mycoplasma. HCT116 has been authenticated by STR profiling.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
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10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

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11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
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21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

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22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A