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A new pathway that regulates 53BP1 stability implicates Cathepsin L and Vitamin D in DNA repair

Ignacio Gonzalez-Suarez, Abena Redwood, David Grotsky, Martin Neumann, Emily Cheng, Colin Stewart, Adriana Dusso, Susana Gonzalo

Corresponding author: Susana Gonzalo, Washington University

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

1st Editorial Decision 01 February 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Please accept my apologies for the fact that it has taken significantly longer than usual to get back to you with the outcome of its evaluation. The manuscript was sent to three referees before the christmas holiday break, however one of them informed us only in mid-January to not be able to complete the review assignment, while another one was delayed until now with sending his/her report. With two reports at hand, I am now finally able to get back to you with an editorial decision, given that these two reports are in fair overall agreement. Both referees find your results linking lamin A loss and genome stability defects via cathepsin L (CTSL) and 53BP1 degradation interesting and potentially important. Nevertheless they also agree that a number of concerns need to addressed before publication, to make the results more conclusive and insightful.

In light of these evaluations, I would like to invite you to prepare a revised version of the manuscript. Pending satisfactory addressing of the major issues, we should then be able to consider such a revised manuscript for publication. Among these issues raised by both referees are microscopy experiments to further study the subcellular localization alterations of 53BP1, and looking further at the downstream effects of Vitamin D-mediated CTSL inhibition beyond Comet assays and 53BP1 levels, i.e. at correction of nuclear abnormalities typically associated with lamin A loss such as gamma-H2AX foci and telomere dysfunction. In addition, both referees ask for some better understanding of mechanistic links, such as whether CTSL directly or indirectly targets 53BP1, or how Vitamin D may inhibit CTSL in murine cells. On the other hand, I would not insist on referee 2's request for further validation of the in vivo relevance in Lmna -/- or Cathepsin -/- animals as long as you are able to decisively address the issues detailed above.

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When preparing your revision, please also make sure to take care of editorial and presentational issues as indicated also by the referees. I should further point out that it is EMBO Journal policy to allow a single round of major revision only, making it important to diligently answer to all the various points raised at this stage. Finally, please bear in mind that your letter of response will form part of the Peer Review Process File, available online to the community in the case of publication (please visit http://www.nature.com/emboj/about/process.html for more details). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This work is the follow-up of a previous work of the S. Gonzalo team on the effects of A-type lamin dysfunction on 53BP1 stability. They identified here a mechanism linking the loss of A-type function, cathepsin L overexpression and 53BP1 down-expression. Moreover, they provided evidence that vitamin D can be a mean to overcome the 53BP1 down expression and thus to restore genome stability in A-type Lamin-compromised cells. Overall, these results are important for our understanding of the pathways controlling genome stability but also for the mechanisms of progeria and cancer, where cathepsin L is found overexpressed.

The presented experiments are well done and presented. However, this reviewer thinks that some conclusions might be reinforced by the following approaches :
- It is not clear from the data whether 53BP1 is directly the target of capthepsin or whether cathepsin degrades a regulator of 53BP1. Could it be envisaged that cathepsin L processes a protein involved in 53BP1 nuclear import and that the degradation of this protein results from its cytoplasmic localization ? One way to address this point would be to set up an in vitro assay of 53BP1 degradation in the presence of purified cathepsin L. At least, this should be discussed.
- The delocalization of part of 53BP1 into the cytoplasm upon cathepsin overexpression is strking but the mechanism is unclear. This conclusion is based solely on cell fractionation. I would suggest to confirm these results by microscopy. The precise localization of 53BP1 within the nucleus and within the cytoplasm should shed some light on the underlying mechanism.
- They used as readout of DNA damage the Comet assay, which is fine. Nevertheless, if their conclusion is correct, one would expect that an inhibition of cathepsin L would restore the fusion of deprotected telomeres. This experiment would be of great help to link their new data to their previous work and to better understand the nature of telomere dysfunction in progeria cells.
- - the lysosomal system is increased at senescence. Does replicative senescence lead to cathepsin L overexpression and 53BP1 destabilization ?
- Does expression of progerin mirror the effects of Lamin-A down expression for cathepsin L overexpression and 53BP1 destabilization ?

Referee #2 (Remarks to the Author):

In this manuscript, Gonzalez-Suarez et al. describe a novel pathway involving the cysteine protease cathepsin L as a regulator of the levels and localization of 53BP1 protein, a DNA damage response mediator. Through the study of Lmna knockout cells, the authors establish an inverse relationship between cathepsin L and 53BP1 levels. Lmna-/- mouse embryo fibroblasts (MEFs) show low levels of 53BP1 protein as a consequence of the increased levels of the active form of cathepsin L. The

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specific knockdown of this protease increases the levels of 53BP1, supporting its involvement in the degradation of 53BP1. Besides, cathepsin L interference is able to correct the defective DNA double-strand break repair shown by Lmna-/- cells through the increase of 53BP1 levels. Conversely, cathepsin L overexpression in wild-type MEFs reduces 53BP1 levels, reproducing the situation of Lmna-/- MEFs. The administration of vitamin D is able to correct these alterations in Lmna-/- cells through the inhibition of cathepsin L. Thus, vitamin D treated Lmna-/- MEFs show increased levels of 53BP1 and a normal response to DNA damage induced by ionizing radiation. This manuscript deals with a very relevant topic, with important implications not only for the study of progeroid laminopathies but also for the molecular study of DNA damage response. The signaling pathway proposed by the authors is novel and generally well supported by experimental work. The possible pharmacological intervention on the pathway through vitamin D administration could have important clinical applications. The manuscript is well written, with the results presented in a very straightforward fashion. The main weakness of the work is the lack of in vivo data, which would reinforce decisively the biological significance of the findings reported by the authors.

Specific points:
1- The authors should provide some in vivo data to support the physiological relevance of their findings. To test in vivo their findings, the authors could investigate 53BP1 levels and DSB repair in cathepsin L-deficient mice.

2- The alterations in subcellular distribution of 53BP1 and cathepsin L could be supported with immunofluorescent analysis of these cells. This information would be complementary to the cellular fractionation experiments shown.

3- Although the relationship between cathepsin L and 53BP1 is well supported with these experiments, in this reviewer's opinion the specific effect of vitamin D in this context should be experimentally demonstrated. Thus, cathepsin L enzymatic activity assays should be performed in Lmna-/- cells treated with vitamin D to demonstrate a specific inhibition of this protease in comparison with non-treated Lmna-/- cells.

4- The authors speculate that vitamin D treatment leads to inhibition of cathepsin L activity via upregulation of cystatin gene expression, an explanation which is supported by the previously reported upregulation of cystatin D in human colon cancer cells in response to this vitamin. However, as far as I know, the murine genome does not encode a cystatin D orthologue. Consequently, it would be interesting to investigate if this role is played in mouse cells by a different cysteine protease inhibitor.

5- Since this manuscript suggests that vitamin D administration might revert some of the alterations present in Lmna-/- cells, an experimental approach that could confirm the biological effect of this substance would improve the manuscript. Thus, the reduction of the number of nuclear abnormalities or the -H2AX foci in vitamin D-treated Lmna-/- cells could be an important proof of the potential effect of vitamin D in these pathologies. Furthermore, the authors could consider investigating the in vivo effect of vitamin D on the phenotype of Lmna-/- mice.

Minor points
Some references are incomplete: the year is missing in some of them (Bouwman et al; Bunting et al, page 4; Lankelma et al, page 12, several in page 14, the same occurs in the References section; it seems to affect to all references for year 2010).
Some Figure legends should include a better explanation of the abbreviations used (for example in Supplementary Figure 1). In the legend for Fig. 4 there is an extra (C).
Apparently, Westerns for 53bp1 are not completely homogenous. Thus, bands shown in Fig 1A, 1B and 1C are quite different in aspect. Likewise, those shown in the central panel of Fig.1F are also dissimilar from those of other panels in the same Figure. Is there any explanation for these discrepancies??

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1st Revision - authors' response 15 May 2011

Enclosed please find a new version of the manuscript entitled "Novel pathway regulating 53BP1 stability implicates Cathepsin L and Vitamin D in DNA repair", for your consideration.

First of all, I would like to thank you and the reviewers for the suggestions, since they have allowed us to improve significantly the characterization of the pathway impacting on the stability of 53BP1. The concerns of the reviewers have been addressed experimentally, and we now include the following new data:
(1) In vitro degradation assay showing that incubation of nuclear extracts with recombinant Cathepsin L leads to degradation of 53BP1 in a time-dependent manner. This indicates that Cathepsin L can degrade 53BP1 when the two proteins are in contact; such is the case of Lmna-/- MEFs, which exhibit increased levels of Cathepsin L in the nucleus.
(2) Immunofluorescence studies confirming the decrease in 53BP1 levels in the nucleus upon depletion of A-type lamins and rescue of the nuclear levels of 53BP1 upon treatment with vitamin D. However, we were unable to detect cytoplasmic 53BP1 in any of the conditions, probably due to the fact that immunofluorescence is not sensitive enough to detect limited amounts of 53BP1 dispersed throughout the cytoplasm.
(3) A variety of assays showing that treatment with vitamin D rescues phenotypes of lamins-deficient cells other than defects in DNA DSBs by comet assays. In particular, immunofluorescence studies show that vitamin D reduces the percentage of lamins-deficient cells with _H2AX foci indicative of basal DNA damage and with aberrant nuclei. Furthermore, vitamin D treatment rescues the ability of lamins-deficient cells to process dysfunctional telomeres by NHEJ.

All together, the new studies stress the notion that Cathepsin L-mediated degradation of 53BP1 could contribute to the genomic instability characteristic of cancer and progeria cells. Most importantly, we now demonstrate that treatment with vitamin D can rescue many of the phenotypes of lamins-deficient cells in culture. These results represent a proof-of-concept for the use of vitamin D treatment in vivo for those laminsrelated diseases that exhibit aberrant loss of 53BP1. Furthermore, regulation of 53BP1 protein by vitamin D could serve as a therapeutic strategy in BRCA1-deficient and triplenegative breast tumors, which have recently been shown to rely on 53BP1 loss for viability.

You will find attached, the detailed answers to the reviewers questions, as well as a file with "referee-only" supplementary material.

Answers to Referee #1:

This work is the follow-up of a previous work of the S. Gonzalo team on the effects of A-type lamin dysfunction on 53BP1 stability. They identified here a mechanism linking the loss of A-type function, cathepsin L overexpression and 53BP1 down-expression. Moreover, they provided evidence that vitamin D can be a mean to overcome the 53BP1 down expression and thus to restore genome stability in A-type Lamin-compromised cells. Overall, these results are important for our understanding of the pathways controlling genome stability but also for the mechanisms of progeria and cancer, where cathepsin L is found overexpressed.
The presented experiments are well done and presented. However, this reviewer thinks that some conclusions might be reinforced by the following approaches:
- It is not clear from the data whether 53BP1 is directly the target of capthepsin or whether cathepsin degrades a regulator of 53BP1. Could it be envisaged that cathepsin L processes a protein involved in 53BP1 nuclear import and that the degradation of this protein results from its cytoplasmic localization? One way to address this point would be to set up an in vitro assay of 53BP1 degradation in the presence of purified cathepsin L. At least, this should be discussed.

We thank the reviewer for this suggestion. As advised, we have performed an in vitro assay to determine if 53BP1 is a direct target of Cathepsin L. Nuclei from wild-type cells were isolated and subjected to mild solubilization, followed by incubation with recombinant Cathepsin L. As shown in new Figure 4D, incubation with Cathepsin L leads to degradation of 53BP1 in a time-dependent manner, suggesting that CTSL can degrade 53BP1 when the proteins are in contact, such is the case of lamins-deficient cells. In addition, we agree with the reviewer in that the increase in 53BP1 in the

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cytoplasm upon depletion of A-type lamins or overexpression of CTSL could be the result of either decreased entry or decreased retention of 53BP1 inside the nucleus. Thus, we have modified the text to discuss these two possibilities.

The delocalization of part of 53BP1 into the cytoplasm upon cathepsin overexpression is strking but the mechanism is unclear. This conclusion is based solely on cell fractionation. I would suggest to confirm these results by microscopy. The precise localization of 53BP1 within the nucleus and within the cytoplasm should shed some light on the underlying mechanism.

As suggested by the reviewer, we performed immunofluorescence studies to monitor the localization of 53BP1 upon depletion of A-type lamins and treatment with vitamin D. The results are shown in new Supplementary Figure 5. We found a marked decrease in nuclear levels of 53BP1 upon depletion of A-type lamins by IF. In addition, we find that treatment with vitamin D restores the intensity of labeling of nuclear 53BP1. However, we were unable to find an increase in 53BP1 in the cytoplasm in any of the conditions using two different antibodies. This is probably due to the fact that most of the 53BP1 protein is found in the nucleus in both lamins-proficient and ñdeficient cells. And thus, IF is not sensitive enough to detect the limited amounts of 53BP1 dispersed throughout the cytoplasm in lamins-deficient cells. Cytoplasmic 53BP1 is detected however by western blot upon subcellular fractionation. In these assays the amount of nuclear extract loaded into the gel is half that of the cytoplasmic extract, in order to detect cytoplasmic and nuclear 53BP1 within the same gel. We have now made this clear in the figure legends.

They used as readout of DNA damage the Comet assay, which is fine. Nevertheless, if their conclusion is correct, one would expect that an inhibition of cathepsin L would restore the fusion of deprotected telomeres. This experiment would be of great help to link their new data to their previous work and to better understand the nature of telomere dysfunction in progeria cells.

As suggested by the reviewer, we have tested whether treatment with vitamin D rescues NHEJ of dysfunctional telomeres in lamins-deficient cells. We find that in fact, Lmna-deficient cells treated with vitamin D restore the ability of dysfunctional telomeres to be processed by NHEJ, as shown in new Figure 8.

- the lysosomal system is increased at senescence. Does replicative senescence lead to cathepsin L overexpression and 53BP1 destabilization ?
As suggested by the reviewer we have monitored the levels of 53BP1 and CTSL in senescent wild-type MEFs and did not find any obvious differences. However, we would like to investigate this question further both in human and mouse cells that are induced to undergo senescence by different mechanisms (replicative senescence, oncogene- or damage-induced), before making any conclusion.

Does expression of progerin mirror the effects of Lamin-A down expression for cathepsin L overexpression and 53BP1 destabilization?

We have addressed this question and obtained inconclusive data, which we have included in Figure 1 of the "referee-only" supplementary material.

Answers to Referee #2:

In this manuscript, Gonzalez-Suarez et al. describe a novel pathway involving the cysteine protease cathepsin L as a regulator of the levels and localization of 53BP1 protein, a DNA damage response mediator. Through the study of Lmna knockout cells, the authors establish an inverse relationship between cathepsin L and 53BP1 levels. Lmna-/- mouse embryo fibroblasts (MEFs) show low levels of 53BP1 protein as a consequence of the increased levels of the active form of cathepsin L. The specific knockdown of this protease increases the levels of 53BP1, supporting its involvement in the degradation of 53BP1. Besides, cathepsin L interference is able to correct the defective DNA

double-strand break repair shown by Lmna-/- cells through the increase of 53BP1 levels. Conversely, cathepsin L overexpression in wild-type MEFs reduces 53BP1 levels, reproducing the situation of Lmna-/- MEFs. The administration of vitamin D is able to correct these alterations in Lmna-/- cells through the inhibition of cathepsin L. Thus, vitamin D treated Lmna-/- MEFs show increased levels of 53BP1 and a normal response to DNA damage induced by ionizing radiation. This manuscript deals with a very relevant topic, with important implications not only for the study of progeroid laminopathies but also for the molecular study of DNA damage response. The signaling pathway proposed by the authors is novel and generally well supported by experimental work. The possible pharmacological intervention on the pathway through vitamin D administration could have important clinical applications. The manuscript is well written, with the results presented in a very straightforward fashion. The main weakness of the work is the lack of in vivo data, which would reinforce decisively the biological significance of the findings reported by the authors.
Specific points:
1- The authors should provide some in vivo data to support the physiological relevance of their findings. To test in vivo their findings, the authors could investigate 53BP1 levels and DSB repair in cathepsin L-deficient mice.

We agree with the reviewer in that the in vivo data would improve the significance of our study. Some of these studies have been initiated. However, due to time limitations on the characterization of this pathway in vivo, we are unable to include the results in the present manuscript. However, in the current version we include new in vitro data that shows that vitamin D can rescue some of the phenotypes of lamins-deficient cells, such is the case of the degree of unrepaired DNA damage, nuclear morphological abnormalities, and NHEJ of dysfunctional telomeres (see below).

2- The alterations in subcellular distribution of 53BP1 and cathepsin L could be supported with immunofluorescent analysis of these cells. This information would be complementary to the cellular fractionation experiments shown.

As suggested by the reviewer, we performed immunofluorescence studies to monitor the localization of 53BP1 upon depletion of A-type lamins and treatment with vitamin D. The results are shown in Supplementary Figure 5. We found a marked decrease in nuclear levels of 53BP1 upon depletion of A-type lamins by IF. In addition, we find that treatment with vitamin D restores the intensity of labeling of nuclear 53BP1. However, we were unable to find an increase in 53BP1 in the cytoplasm in any of the conditions using two different antibodies. This is probably due to the fact that most of the 53BP1 protein is found in the nucleus in both lamins-proficient and ñdeficient cells. And thus, IF is not sensitive enough to detect the limited amounts of 53BP1 dispersed throughout the cytoplasm in lamins-deficient cells. Cytoplasmic 53BP1 is detected however by western blot upon subcellular fractionation. In these assays the amount of nuclear extract loaded into the gel is half that of the cytoplasmic extract, in order to detect cytoplasmic and nuclear 53BP1 within the same gel. We have now made this clear in the figure legends.

3- Although the relationship between cathepsin L and 53BP1 is well supported with these experiments, in this reviewer's opinion the specific effect of vitamin D in this context should be experimentally demonstrated. Thus, cathepsin L enzymatic activity assays should be performed in Lmna-/- cells treated with vitamin D to demonstrate a specific inhibition of this protease in comparison with non-treated Lmna-/- cells.

We agree with the reviewer that this control is very important. Thus, we have performed Cathepsin L activity assays in Lmna-proficient and ñdeficient cells that are treated with vitamin D or vehicle control. As shown in new Figure 5H, depletion of A-type lamins leads to increased Cathepsin L activity which is partially inhibited by treatment with vitamin D.

4- The authors speculate that vitamin D treatment leads to inhibition of cathepsin L activity via upregulation of cystatin gene expression, an explanation which is supported by the previously reported upregulation of cystatin D in human colon cancer cells in response to this vitamin. However, as far as I know, the murine genome does not encode a cystatin D orthologue.

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Consequently, it would be interesting to investigate if this role is played in mouse cells by a different cysteine protease inhibitor.

The reviewer is absolutely right. Driven by the reviewer comments we performed Cystatins promoter analysis for vitamin D responding elements. We analyzed the promoters of 13 mouse cystatin genes with the Genomatix software using ElDorado mouse genome database. From all the analyzed genes, we selected four whose promoter contained at least two RXR/VDR heterodimer binding sites. In those selected genes, we evaluated the changes in gene expression in response to treatment with vitamin D, both in wild-type and Lmna-/- MEFs. In the case of Cystatin B, both cell types exhibited an increase in gene expression upon vitamin D treatment. Thus, to determine if Cystatin B is mediating the effect of vitamin D on 53BP1, we assayed five different shRNAs specific for the mouse cstb gene. We identified three shRNAs (sh1, sh2 and sh5) which resulted in over 90% knock-down of cstb protein. Unfortunately, cystatin B depletion seemed to have harmful effects in our infected cells. After selection, cells infected with one of the shRNAs died and in other two cases cells showed a dramatic decrease in cell proliferation. Proliferation rates were recovered after a few days in culture, however we observed that cells had also recovered most of the cystatin B expression. As a consequence, and despite we tried to evaluate the response to vitamin D, the results we obtained were inconclusive. We have included these data in Figure 2 of the "referee-only" supplementary material

5- Since this manuscript suggests that vitamin D administration might revert some of the alterations present in Lmna-/- cells, an experimental approach that could confirm the biological effect of this substance would improve the manuscript. Thus, the reduction of the number of nuclear abnormalities or the γ-H2AX foci in vitamin D-treated Lmna-/- cells could be an important proof of the potential effect of vitamin D in these pathologies. Furthermore, the authors could consider investigating the in vivo effect of vitamin D on the phenotype of Lmna-/- mice.

As suggested by the reviewers, we analyzed the ability of vitamin D to rescue a variety of phenotypes of lamins-deficient cells. In new Figure 7D&E we show that depletion of A-type lamins leads to an increase in the percentage of cells with H2AX, indicative of basal DNA damage, and an increase in the percentage of cells with nuclear morphological abnormalities. Interestingly, we found that treatment with vitamin D reduces both phenotypes. Furthermore, vitamin D treatment rescues the ability of lamins-deficient cells to process dysfunctional telomeres by NHEJ (New Figure 8).

Minor points:
Some references are incomplete: the year is missing in some of them (Bouwman et al; Bunting et al, page 4; Lankelma et al, page 12, several in page 14, the same occurs in the References section; it seems to affect to all references for year 2010). Some Figure legends should include a better explanation of the abbreviations used (for example in Supplementary Figure 1). In the legend for Fig. 4 there is an extra (C). Apparently, Westerns for 53bp1 are not completely homogenous. Thus, bands shown in Fig 1A, 1B and 1C are quite different in aspect. Likewise, those shown in the central panel of Fig.1F are also dissimilar from those of other panels in the same Figure. Is there any explanation for these discrepancies??

We are thankful to the reviewer for pointing out these errors, which have been corrected in the new version. With respect to the westerns of 53BP1, we agree with the reviewer in that the appearance of the protein varies among westerns. This is a consistent finding for which we donít have a clear explanation. 53BP1 has a MW of 250 Kd, however it has a Mr of 400 Kd. Depending upon how far the gel is run, we either see one band or two bands, and sometimes even three bands. In control experiments performed in our laboratory in which 53BP1 is depleted by shRNA, we found disappearance of all the different bands, therefore we concluded that all these different forms correspond to 53BP1 protein. It is likely that post-translational modifications of 53BP1 contribute to the different mobility of the protein. Future studies will need to determine if the pathway that we describe here impacts on post-translational modifications of 53BP1 which in turn could correspond to differential mobility in SDS-PAGE.

2nd Editorial Decision 06 June 2011

Thank you for submitting your revised manuscript for our consideration. Both of the original referees have now assessed it once more, and I am happy to inform you that there are no further concerns raised from their side against publication in The EMBO Journal.

Before formal acceptance, we will nevertheless still require addressing of a few editorial issues, which I noted during my pre-acceptance checks:

- please provide us with higher quality original figure files. On close inspection, I noted that especially the blots in the current PDFs show signs of compression/conversion artifacts (e.g. shadows around bands) that could obscure the representation of the original data, and which are usually alleviated by source files of larger size and with less compression.

- two issues regarding quantitation and statistical analysis (please see also our Guide to Authors for further reference):
* for graphs, individual data points should be connected by straight direct lines (e.g. as in Fig 7D), not by smoothened curves (e.g. as in Fig 6 and several other panels)
* for all bar diagrams with N=2, calculation of a SEM is not statistically meaningful. In those cases, we would require either an increase in the number of experimental replicates (N{greater than or equal to}3) to allow for proper statistical analysis (e.g. SEM calculation), or a re-plotting of these data to show individual data points in vertical arrangement instead of a column plot. This applies to Figures 1, 4, 5, 6, S1, S4.

- Finally, I would like to suggest a slightly modified title: "A new pathway that regulates 53BP1 stability implicates Cathepsin L and Vitamin D in DNA repair"

I am therefore returning the manuscript once more to make these final changes and to upload the modified files through the link below. Once we will have received the properly modified final version, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Best regards,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The revised version adequately addressed my comments.

Referee #2 (Remarks to the Author):

The authors have made an effort to address most of my previous comments and criticisms. I am still missing some in vivo studies to support the physiological relevance of their in vitro findings. Nevertheless, I understand the time limitations argued by the authors.

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