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Inhibition of Mitochondrial Fusion by α-Synuclein is rescued by PINK1, Parkin, and DJ-1

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1st Editorial Decision 25 March 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the delay in getting back to you with a decision. However, two of the referees were not able to return their reports as quickly as initially expected.

Your manuscript has now finally been seen by three referees whose comments to the authors are shown below. As you will see while all three referees consider the study as highly interesting in principle they are not convinced that the conclusiveness and completeness of the experimental evidence provided is sufficient to justify the conclusions drawn; and they therefore do not offer strong support for publication of the study here at this stage of analysis. I will not repeat all their individual points, but the major criticism is that in their view considerably stronger evidence is needed for an inhibitory effect of alpha-synuclein on mitochondrial fusion in cells. Another issue raised is that the functional interplay between alpha-synuclein and the other genes involved in familial Parkinson's disease remains at a level of limited understanding. Clearly, all in all the referees point to major shortcomings of key aspects of the experimental evidence provided which precludes publication of the study here at this stage of analysis.

Still, given the interest expressed by the referees in principle we could give you the chance to address the referees' concerns by major revision. In particular, it will be indispensable to provide considerably stronger evidence for an inhibitory effect of alpha-synuclein on mitochondrial fusion and to rule out any indirect effects along the lines put forward by and to the full satisfaction of

referees 1 and 2. While it would certainly be highly desirable to provide at least some deeper mechanistic understanding of the functional link between alpha-synuclein, Pink1, Parkin and DJ-1 we would not necessarily ask for an in-depth analysis of this aspect of the study as long as the main aspect of the study - a direct inhibitory effect of alpha-synuclein on mitochondrial fusion - is sufficiently developed. Furthermore, while the referees do not ask for it, it would certainly strengthen the study if you included the in vivo data in the nervous system in C. elegans that you referred to in your letter. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees.

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

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In this manuscript, Authors describe the effect of a-synuclein, aggregated in Parkinson's disease, in fusion of membrane and in mitochondrial fusion in particular. A nice in vitro assay shows the effect of a-synuclein inhibits fusion of liposomes. They then shift to the analysis of the effect of a-synuclein on mitochondrial morphology, given its localization on mitochondrial membranes, as shown by nice immuno-EM experiments. Expression of a-synuclein leads to mitochondrial fragmentation which was not rescued by expression of Mfn2 and silencing of Drp1. Accordingly, reduction in the levels of a-synuclein cause mitochondrial elongation and potentiate the effect of the pro-fusion Mfn2. Surprisingly, Pink1, parkin and DJ-1 all rescue the morphological defect by a-synuclein. Authors suggest that a-synuclein might act as a stabilizer of the lipid defects required for mitochondrial membrane fusion.

In general this manuscript could be of great interest, showing a specific mitochondrial fusion defect triggered by a-synuclein. However at this stage the model of the authors seems to be mostly based on extrapolation of the solid in vitro data and is not well developed especially for the part of the interaction with the other PD genes. In particular

1. it is very much surprising that all the genes that are involved in the familial PD revert the effect of a-synuclein, especially since the authors propose that the effect on mitochondrial morphology are independent of the fusion machinery (or upstream of it). It is unclear why Pink1 and parkin, whose function is very much different, should be reverting the fragmentation by a-synuclein. In particular, parkin is expected to targed mitochondria to autophagosomes (Narendra et al 2009). Moreover, evidence exists from the same lab that the effect of Pink1 on mitochondrial morphology depends on

Drp1 (Lutz et al., 2009). It is very difficult to reconcile the Drp1 dependency of the Pink1 effect with the Pink1 effects on a-synuclein, which is conversely independent of Drp1

2. a number of specific issues should be addressed.

a. there is the likely possibility that changes in mitochondrial morphology are caused by a secondary mitochondrial dysfunction. Authors should consider the possibility supported by recent findings that Ca2+ levels are higher in the neurons expressing a-synuclein, causing mitochondrial dysfunction (Su et al, 2009, for example). Mitochondrial function, levels of fusion/fission proteins, their subcellular localization, their post-transcriptional modifications should be all tested here

b. the use of DioC6 is worrisome, since this compound is very much toxic for mitochondria (see the works by Hagai Rottenmberg for example, and Bernardi et al 1999 for a review). All the experiments on mitochondrial morphology must be repeated with a mitochondrially targeted fluorescent protein

c. there is no formal proof anywhere in the paper that expression of a-synuclein reduces the rate of mitochondrial fusion. this should be measured for both inner and outer membrane fusion, using for example PEG fusion assays like the ones developed by Rojo, or even better in vitro using the ones developed by Nunnari.

d. authors limit the modulation of fusion proteins to Mfn2: why not trying Mfn1, Opa1? What are the levels of Drp1 following siRNA? what happens if they use a dominant negative Drp1?

e. the effects of siRNA of a-synuclein on mitochondrial length seem marginal. Maybe if they follow re-elongation of mitochondria following mitochondrial fission by FCCP the effect is going to be clearer.

Referee #2 (Remarks to the Author):

Kamp et al. propose that alpha-synuclein, a key factor of Parkinson's disease, inhibits mitochondrial fusion. This is based on two lines of evidence: (i) alpha-synuclein inhibits fusion of artificial vesicles in vitro, (ii) overexpression of alpha-synuclein induces mitochondrial fragmentation in cultured cells and C. elegans while its depletion leads to the formation of large interconnected networks. Intriguingly, alpha-synuclein dependent mitochondrial fragmentation is rescued by PINK1, parkin and DJ-1, three factors known to be involved in Parkinson's disease. The manuscript contains a number of interesting observations. However, the following points should be considered by the authors.

Major points

1. I am not convinced that the in vitro fusion assays presented in Fig. 1 faithfully reflect the physiological function of alpha-synuclein. Spontaneous fusion of protein-free SUVs that consist of only one type of phospholipids is a highly artificial situation that never exists in the cell. Furthermore I assume that the concentration of alpha-synuclein in these reactions was much higher than in the cell. In my eyes, these experiments might have some value for the study of the behavior of artificial vesicles, but their relevance for fusion of cellular membranes is only very limited.

2. The authors observed that overexpression of alpha-synuclein induces mitochondrial fragmentation, while its depletion generates large interconnected mitochondria. The authors conclude that alpha-synuclein inhibits mitochondrial fusion. However, their data are fully compatible with the alternative view that alpha-synuclein stimulates mitochondrial division. They should try to discriminate between these possibilities (for example, they could use an established in vivo fusion assay that monitors the mixing of fluorescent mitochondrial markers in fused cells).

Further points

3. Fluorescent micrographs in Figs. 2A and 6A should be in black and white for better visibility of mitochondria.

4. Error bars should be explained in the Figure legends.

5. Figure 4 shows immuno EM of alpha-synuclein on mitochondria. I wonder whether the authors were able to detect signals also on other organelles, as alpha-synuclein seems to interfere with membrane dynamics in the secretory pathway.

6. Page 9, second row: 'cytoplasm' should read 'cytosol'.

Referee #3 (Remarks to the Author):

This manuscript "Inhibition of Mitochondrial Fusion by Synuclein is rescued by PINK1, Parkin and DJ-1" Frits Kamp et.al. demonstrated that synuclein has an inhibitory function in the membrane in vitro and synuclein binds to mitochondria which leads to mitochondrial fragmentation in vivo. On the other hand, down regulation of synuclein results in elongated mitochondria. Furthermore mitochondrial fragmentation is rescued by PINK1, Parkin and DJ-1, but not in these mutants. Finally the authors conclude that synuclein functionally interacts with disease associated genes, however, the conclusion is very preliminary and requires further experimentation. For example, one might suggest that the authors show the mechanism why PINK1, Parkin and DJ-1 would rescue fragmented mitochondria. Furthermore the authors used SH-SY5Y cells in this experiment. But to demonstrate the effect of synuclein, the authors should use another cell line which does not have synuclein. The mitochondria change their morphology by continuous fission and fusion physiologically, so we do not understand why deficiency of synuclein alone induces elongation of mitochondria.

The concept of this manuscript is interesting, however, it would be better if the authors confirmed that this phenomenon applied to other mutations as well, in order to confirm the true ortholog of synuclein. Every experiment needs more controls and there are several points that should be addressed.

In Fig. 1, the authors should monitor fusion by the increase in static light scatting upon the addition of aliquot of C12E8 including synuclein, PINK1, Parkin, DJ1 and each mutants.

In Fig.1D, the authors should add other synuclen deletion mutants including N-terminal and both deletion.

In Fig. 2B, we do not understand how synuclein mutants differ from WT synuclein. This phenomenon does not explain why these mutants are pathogenic to PD. The authors should explain the reason.

1st Revision - authors' response 06 July 2010

All points raised by the reviewers were addressed in detail as follows:

Referee #1 (Remarks to the Author):

This reviewer points out that " In general this manuscript could be of great interest, showing a specific mitochondrial fusion defect triggered by αS".

The following critical points were raised:

1. "it is very much surprising that all the genes that are involved in the familial PD revert the effect of αS, especially since the authors propose that the effect on mitochondrial morphology are independent of the fusion machinery (or upstream of it). It is unclear why Pink1 and

parkin, whose function is very much different, should be reverting the fragmentation by αS. In particular, parkin is expected to target mitochondria to autophagosomes (Narendra et al., 2008)

The reviewer raised an interesting point, but in our view the effects of parkin, PINK1 or DJ-1 on synuclein-induced mitochondrial morphology can be explained based on the following facts: (i) Evidence from different model systems is accumulating that PINK1 and parkin can act in the same pathway, in particular, parkin can revert the PINK1-deficient phenotype (Clark et al., 2006; Exner et al., 2007; Lutz et al., 2009; Park et al., 2006; Yang et al., 2006);. (ii) Parkin, PINK1 and DJ-1 have been shown to protect cells from stress-induced cell death in cell culture and animal models (iii) The acute knockdown of parkin, PINK1 or DJ-1 has the same effect on mitochondrial morphology, and unpublished data from our group (and other groups) indicate that DJ-1 acts not in the same pathway as parkin and PINK1, but in parallel to maintain mitochondrial integrity. This observation strongly argues for an at least partial functional convergence of these three PD-associated genes on stress protection and prevention of mitochondrial damage.

We discussed these points on pages 18 and 19 of the Discussion.

Moreover, evidence exists from the same lab that the effect of Pink1 on mitochondrial morphology depends on Drp1 (Lutz et al., 2009). It is very difficult to reconcile the Drp1 dependency of the Pink1 effect with the Pink1 effects on αS, which is conversely independent of Drp1)"

We did not mean to claim that the PINK1 rescue is independent of Drp1. We only say that αS binds to mitochondria independently of the fusion/fission machinery. Thus, the reviewer is right and the rescuing activity of PINK1 may indeed work via interacting with the fusion/fission machinery as described before. We greatly apologize for this misunderstanding and explained this in the discussion on page 19-20.

2. specific comments:

2a." there is the likely possibility that changes in mitochondrial morphology are caused by a secondary mitochondrial dysfunction. Mitochondrial function, levels of fusion/fission proteins, their subcellular localization, their post-transcriptional modifications should be all tested here"

This is indeed an important consideration. In the new Figure 4 we now demonstrate that mitochondrial membrane potential and ATP production are not changed in the SH-SY5Y cells which overexpress αS. Furthermore, we also did not observe alterations in the expression, subcellular localization or modification of mitochondrial fusion/fission proteins. These data are now shown in the new Figure S7. In the Discussion we added: "Another alternative explanation for the observed effects of αS on mitochondrial dynamics would be that αS expression alters the levels of expression of fission or fusion proteins, their subcellular localizations and/or posttranslational modifications. However, no such effects were observed (Figure S7)."

2b. "*the use of DioC6 is worrisome, since this compound is very much toxic for mitochondria (see the works by Hagai Rottenmberg for example, and Bernardi et al 1999 for a review). All the experiments on mitochondrial morphology must be repeated with a mitochondrially targeted fluorescent protein)"*

We repeated all our experiments with SH-SY5Y cells in which mito-GFP was expressed and imaged the mitochondria from wt-cells and cells with elevated and reduced αS levels (new Figures 2 and 8). There were no apparent differences between this assay and the assay provided previously where the mitochondria were imaged with DiOC6.
We provide additional data as information for Referee #1 where the two methods to label mitochondria (DiOC6 and mito-GFP) were directly compared in a set of experiments that were performed in parallel. There was no difference, especially no increase, in the number of cells with fragmented mitochondria when DiOC6 was used compared to mito-GFP

(Additional Information 1, see below).

2c. there is no formal proof anywhere in the paper that expression of αS reduces the rate of mitochondrial fusion. This should be measured for both inner and outer membrane fusion, using for example PEG fusion assays like the ones developed by Rojo, or even better in vitro using the ones developed by Nunnari)

We carried out the requested PEG fusion assay as suggested. We fused cells with redfluorescent mitochondria with cells containing green-fluorescent mitochondria. Compared with the empty vector control, the fused cells displayed a significantly reduced mitochondrial fusion rate upon overexpression of αS. These new findings are now shown in Figure 3A and B.

2d. authors limit the modulation of fusion proteins to Mfn2: why not trying Mfn1, Opa1? What are the levels of Drp1 following siRNA? what happens if they use a dominant negative Drp1?)

We included Mfn1 and Opa1 in our new Figure 9 and show that their expression also increases the fraction of cells with elongated mitochondria as expected. However, coexpression of αS reduced this effect consistent with our previous findings (see new Fig. 9). Expression levels of Drp1 following downregulation by siRNA were significantly reduced as shown in Figure S6C. As suggested, we performed the same experiment with a dominant negative mutant Drp1 K38E and came to the consistent result that fusion is reduced in αS overexpresing cells (new Figure 9).

2e. the effects of siRNA of αS on mitochondrial length seem marginal. Maybe if they follow re-elongation of mitochondria following mitochondrial fission by FCCP the effect is going to be clearer.

We included the experiment suggested by the reviewer. Upon CCCP induced mitochondrial fragmentation the subsequent re-elongation of mitochondria was indeed accelerated in the absence of αS as compared to control cells (new Figure 8). Vice versa, upon reduction of αS the number of fragmented mitochondria decreased more rapidly (new Fig. 8)

Referee #2 (Remarks to the Author):

This reviewer also finds that our manuscript "contains a number of interesting observations. However, the following points should be considered by the authors".

Major points

1. "I am not convinced that the in vitro fusion assays presented in Fig. 1 faithfully reflect the physiological function of αS. Spontaneous fusion of protein-free SUVs that consist of only one type of phospholipids is a highly artificial situation that never exists in the cell".

We included a fusion experiment with vesicles comprised of a lipid mixture that has been described to be particularly fusogenic (Haque et al., 2001; Lentz, 2007). This is now shown in the new Figure 1G. We also included an experiment of spontaneous rapid fusion of vesicles with lipids of opposite charges (new Figure 1H). In both experiments αS inhibited fusion. In the discussion we added the following line: "Although we recognize that the in vitro fusion assays do not fully represent completely in vivo membrane fusion events, our biophysical experiments provided the basis for the in vivo experiments".

"Furthermore I assume that the concentration of αS in these reactions was much higher than in the cell"

In the brain wt- αS is extremely abundant. In fact αS is one of the most abundant neuronal proteins. In brain homogenates 0.5-1% of the total cytosolic protein is made up by αS. Cytosolic concentrations of αS between 30-60 μM have been reported (Bodner et al., 2009; Iwai et al., 1995). We mention this now in the Introduction. In our vitro experiments we never

used αS concentrations exceeding 30 μM, thus we are within the physiological concentrations of αS.

"In my eyes, these experiments might have some value for the study of the behaviour of artificial vesicles, but their relevance for fusion of cellular membranes is only very limited".

The in vitro experiments provided a *working hypothesis* for the in vivo function of αS (as mentioned in the Discussion). These findings were then confirmed in vivo in cultured cells and even in a living animal model (*C. elegans*; see Figures 2, 3, 5 and 8).

2. "The authors observed that overexpression of αS induces mitochondrial fragmentation, while its depletion generates large interconnected mitochondria. The authors conclude that αS inhibits mitochondrial fusion. However, their data are fully compatible with the alternative view that αS stimulates mitochondrial division. They should try to discriminate between these possibilities (for example, they could use an established in vivo fusion assay that monitors the mixing of fluorescent mitochondrial markers in fused cells)."

This point was also raised by reviewer #1 (see above). As described above we performed the requested fusion assay. We fused cells with red-fluorescent mitochondria with cells containing green-fluorescent mitochondria. Compared with the empty vector control, the fused cells displayed a significantly reduced mitochondrial fusion rate upon overexpression of αS. These new findings are now shown in Figure 3A and B. Moreover, upon CCCP induced mitochondrial fragmentation the subsequent re-elongation of the mitochondria was accelerated in the absence of αS as compared to control cells (new Figure 8). *Vice versa*, upon reduction of αS the number of fragmented mitochondria decreased more rapidly (new Fig. 8)

These new findings are now addressed in the Discussion (p.16): "An alternative explanation for the observed effects of αS on mitochondrial dynamics would be that αS enhances mitochondrial fission. This is unlikely as the free-energy change involved with the structural switch of αS upon membrane binding (Nuscher et al., 2004) is not enough to cause fission, and mitochondrial fission is an GTP requiring event (Westermann, 2008). The experiment of Figure 3, in which fusion of red and green labeled mitochondria in fused cells was slower when αS was overexpressed, can only be explained by an inhibitory effect of αS on fusion. Furthermore, the experiment from Figure 8E showed that re-elongation of mitochondria upon CCCP induced fragmentation was faster when cytosolic αS levels were suppressed by αS siRNA. Since during the re-elongation phase hardly any mitochondrial fission occurs, the slower re-elongation in the presence of αS can only be explained by a specific inhibitory effect of αS on the fusion of mitochondrial membranes".

3. "Fluorescent micrographs in Figs. 2A and 6A should be in black and white for better visibility of mitochondria)"

The new Figures 2 and 8 are in black and white as requested.

4. "Error bars should be explained in the Figure legends"

We now describe the error bars in the legends.

5. "Figure 4 shows immuno EM of αS on mitochondria. I wonder whether the authors were able to detect signals also on other organelles, as αS seems to interfere with membrane dynamics in the secretory pathway.)"

There was only sparse labelling detectable throughout the cytosol which may have coincided in some cases with the ER. We did not see, however, an accumulation of synuclein on any other organellar membrane than the mitochondrial outer membrane. We specifically compared mitochondrial and Golgi membranes.

6. "Page 9, second row: 'cytoplasm' should read 'cytosol'"

This has been corrected accordingly

Referee #3 (Remarks to the Author):

One might suggest that the authors show the mechanism why PINK1, Parkin and DJ-1 would rescue fragmented mitochondria.

In line with the editor's notion in the decision letter, we did not include a detailed analysis on the mechanism of Pink1, Parkin and DJ-1 mediated rescue, since so far nobody succeeded in the recombinant expression of functional and soluble Pink-1 and Parkin, which therefore makes such experiments currently impossible. Nevertheless, we have produced sufficient amounts of soluble DJ-1 for in vitro experiments. First experiments indicate that DJ-1 indeed opposes the fusion inhibitory activity of αS in such in vitro fusion experiments. However, in line with the editor's opinion we believe that this issue is outside of the topic of the current manuscript, and we would prefer to perform a much deeper investigation of this interesting observation for a future publication.

....to demonstrate the effect of synuclein, the authors should use another cell line which does not have synuclein.

We have performed such experiments by using siRNA to suppress αS expression. This revealed the expected opposite mitochondrial phenotype (Fig. 8). We are not aware of cells lacking endogenous αS expression.

The concept of this manuscript is interesting, however, it would be better if the authors confirmed that this phenomenon applied to other mutations as well, in order to confirm the true ortholog of synuclein.

As suggested by the reviewer we also overexpressed the αS homologue b-synuclein (ßS) and observed very similar findings, i.e. mitochondrial fragmentation (new Fig. 2D & E). This further confirms our findings on the effects of αS on mitochondrial fragmentation.

In Fig. 1, the authors should monitor fusion by the increase in static light scatting upon the addition of aliquot of C12E8 including synuclein, PINK1, Parkin, DJ1 and each mutants.

Unfortunately, nobody so far succeeded in recombinant expression of soluble and biological functional PINK1, Parkin. Nevertheless, we performed the requested experiment with purified recombinant DJ-1. Interestingly, this revealed that DJ-1 can reverse the fusion inhibitory function of αS. We would prefer to pursue this project in a more detailed future project.

In Fig.1D, the authors should add other synuclein deletion mutants including N-terminal and both deletion

We performed additional experiments including peptides comprising the C-terminus and the central region of the protein. As expected, these peptides failed to inhibit membrane fusion. This is shown in the new Figure 1D.

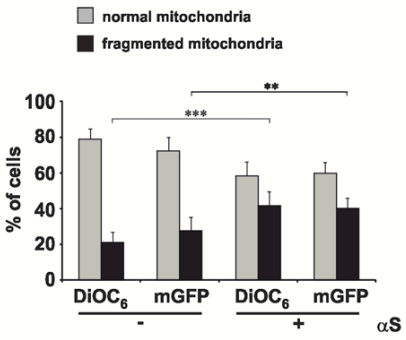
In Fig. 2B, we do not understand how synuclein mutants differ from wt synuclein. This phenomenon does not explain why these mutants are pathogenic to PD. The authors should explain the reason.

Our findings indeed demonstrate that pathogenic mutations do not alter the biological function of αS in inhibition of membrane fusion. This is described in the Results section: "Expression of similar amounts of mutant αS A30P or A53T lead to fragmentation of mitochondria to the same extent as the wild-type protein (Figure 2A, B & C). This is consistent with the finding that mutants of αS also bind to model membranes (Giannakis et al., 2008; Karpinar et al., 2009; Nuscher et al., 2004; Perlmutter et al., 2009; Ramakrishnan et al., 2006)". Our findings rather suggest that αS gene duplications or triplications lead to increased binding of αS binding to mitochondria, which then inhibits mitochondrial fusion

and would therefore trigger disease pathology. The missense mutations may affect different cellular pathways such as aggregation, see discussion page 19.

Taken together, we strongly believe that we have addressed all points raised by the reviewers with additional experimental evidence.

Additional Information 1



Additional Information 1: Comparison of two different methods to fluorescently labeled mitochondria. SH-SY5Y cells were control transfected (-) or transfected with αS. Living cells were stained with the fluorescent dye DiOC6 and imaged with a fluorescence microscope or cells were cotransfected with mito-GFP, fixed and imaged with a confocal microscope. For all conditions, three independent experiments were performed in triplicates. The amount of cells with normal or fragmented mitochondrial morphology was quantified and statistically analyzed. With both methods, the same increase in the number of cells with fragmented mitochondria was observed.

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2nd Editorial Decision 02 August 2010

Thank you for sending us your revised manuscript. Our original referees have now seen it again, and you will be pleased to learn that in their view you have addressed their criticisms in a satisfactory manner and that they now support publication in The EMBO Journal.

Still, I was wondering whether you would like to consider addressing the minor issues suggested by referee 2 (see below). In addition, there is one remaining editorial issue that needs further attention.

Prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) to ask for the original scans (for our records).

In the case of the present submission there are a number of panels that appear to not fully meet these requirements: figure 5D, supplementary figure S5C

I therefore like to kindly ask you to send us a new version of the manuscript that contains suitably amended versions of these figures. I think that it would also be important to explain in the figure legends that all lanes come from the same gel. Please be reminded that according to our editorial

policies we also need to see the original scans for the figures in question.

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final version of the paper.

Thank you very much for your cooperation.

Sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

Authors did a great job in exhaustively addressing all my comments. Their revision is a remarkable one. I am persuaded that a-synuclein inhibits mitochondrial fusion and that the other PD genes can have a counteracting effect on this. Further work will be clearly needed to address the molecular details of this pathway but this is going to be a foundng paper for this avenue of research.

Referee #2 (Remarks to the Author):

The authors have adequately addressed my previous concerns and suggestions. Two minor points can be easily addressed before publication of this interesting manuscript.

1. Mitochondria look rather pale in the fluorescent micrographs in Fig. 8A. I guess this happened during conversion to black and white images. Fluorescent micrographs in 8D are rather small and dark. Can these images be improved?

2. Methods, p. 26. The LSM510 confocal microscope is manufactured by Zeiss, not by Leica.

2nd Revision - authors' response 11 August 2010

Editor:

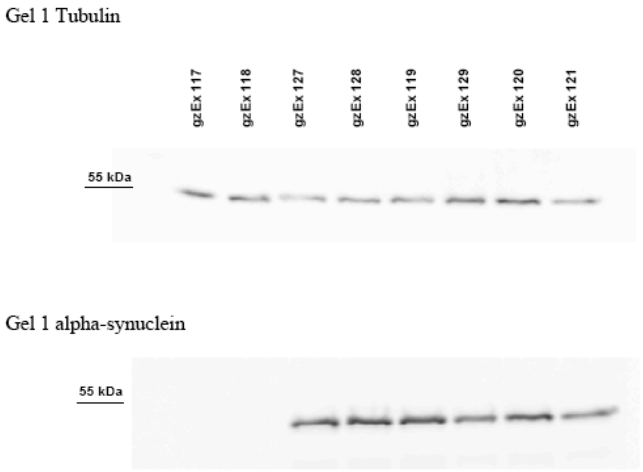
In the case of the present submission there are a number of panels that appear to not fully meet these requirements: figure 5D, supplementary figure S5C. I therefore like to kindly ask you to send us a new version of the manuscript that contains suitably amended versions of these figures. I think that it would also be important to explain in the figure legends that all lanes come from the same gel. Please be reminded that according to our editorial policies we also need to see the original scans for the figures in question.

We now present amended versions of Figure 5D and Figure S5C.

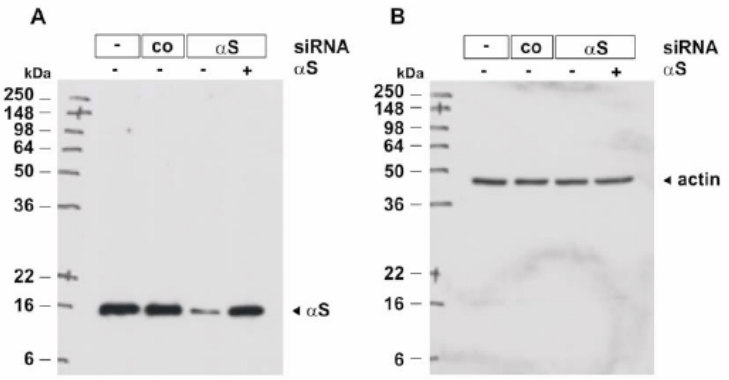
In Figure 5D, gray bars were introduced where lanes had been cropped. In the figure legend we added the remark: 'All lanes originate from the same gel. Only the lanes of those transgenic lines, that were chosen for imaging due to good penetrance and fluorescent signal, are shown here.'

Please find below the original scans of the experiments shown in Figure 5D and in the new Figure S5C.

Original Scan Fig. 5D



Original Scan Fig. S5C



In addition, we noted that the size differences of mitochondria in Figure 5C & E could lead to some confusion. We therefore added the following short paragraph:

"Strikingly, a similar mitochondrial fragmentation was observed in aged 7-day-old worms in the absence of ectopic αS expression (Figure 5E), suggesting that mitochondrial fragmentation also happens during the normal ageing process of the BWM tissue. *C. elegans* BWMs are particularly susceptible to ageing and have been shown to gradually and progressively deteriorate with age (Herndon *et al.*, 2002). *C. elegans* mean life span is about 12-18 days. After reaching adulthood *C. elegans* hermaphrodites lay all their eggs within approximately 3 days and then persist through a post-reproductive period where senescent decline is evident (Herndon *et al.*, 2002). Since *C. elegans* animals still grow after reaching adulthood, aged BWMs were bigger in size (Fig. 5E)."

Referee #2 (Remarks to the Author):

1. Mitochondria look rather pale in the fluorescent micrographs in Fig. 8A.
I guess this happened during conversion to black and white images.
Fluorescent micrographs in 8D are rather small and dark. Can these images be improved?

We now adjusted brightness and contrast of the fluorescent micrographs to present a better visibility of the mitochondria.
Similarly, we also adjusted brightness and contrast of the fluorescent micrographs shown in Figure S5A.

2. Methods, p. 26. The LSM510 confocal microscope is manufactured by Zeiss, not by Leica.

This was corrected accordingly.

