THE EMBO JOURNAL

Manuscript EMBO-2014-88658

Parkin-independent mitophagy requires Drp1 and maintains the integrity of mammalian heart and brain

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Review timeline:

Submission date:	16 April 2014
Editorial Decision:	13 May 2014
Revision received:	18 August 2014
Editorial Decision:	19 September 2014
Accepted:	19 September 2014

Transaction Report:

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

Editor: Andrea Leibfried

1st Editorial Decision 13 May 2014

Thank you for submitting your manuscript entitled 'Parkin-independent mitophagy requires Drp1 and maintains the integrity of mammalian heart and brain'. I have now received the reports from all referees.

As you can see below, all referees appreciate your findings very much and referee #1 only suggests only minor amendments of your manuscript. Referee #2 and #3 propose some additional experiments that will further support and develop your data. Given the clear comments provided, I would like to invite you to submit a revised version of the manuscript, addressing the concerns of the referees. Please note that testing whether the synthetic interactions between Drp1 and Parkin reflect redundancy with one of the other mitochondrial ubiquitin ligases as suggested by referee #2 is a further reaching point, which is not essential for publication. Please contact me in case of further questions.

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

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Referee #1:

This is a very interesting manuscript that investigates the physiological and cellular function of the mitochondrial fission regulator, Drp1, in postnatal hearts and cardiomyocytes. The analysis of the effect of Drp1 loss on cardiac function is very thorough and will be of interest to researchers in the field. The additional studies in tissue and cell culture address important mechanistic questions regarding the role of Drp1 relative to Parkin in mitochondrial turnover in both the heart and in purkinje neurons. The finding that mitochondrial p62 accumulation and mitochondrial protein ubiquitination found in the Drp1 knockout is not dependent on Parkin is very important and demonstrates that the current models in the literature have been greatly oversimplified. The work indicates that E3 ligases other than Parkin contribute to mitochondrial ubiquitination prior to turnover. Additional important findings indicate that the role of Drp1 in mitochondrial turnover is not simply to release smaller mitochondrial pieces for engulfment and degradation (as has also been suggested in the literature).

Very minor comments the authors should consider are listed below:

1. There appears to be some residual Drp1 expression in the tissue from Drp1F/F::Myh6-Cre animals. This does not affect the analysis but this reviewer asks the authors to comment on this in the revision response. Does this mean that the Myh6-Cre expression is not sufficient to recombine both Drp1 floxed alleles within the relevant cells and tissues?
2. In the western blot analysis of respiratory components in the double Drp1 Parkin KO, the decrease in protein abundance within the 3x samples provided is not always consistent. The authors should consider activity staining these tissues in addition as they did in the earlier figure. This will likely be more physiologically relevant as I suspect the oxphos activity is much more defective than suggested by the changes in the western blot.

Referee #2:

Kageyama et al. studied the effects of Drp1 deletions on mitochondrial function in mouse heart. The authors have done a thorough job characterizing cardiac function in the mutant. They also show decreased respiration and Ox Phos enzyme levels, consistent with a mitochondrial defect. Apart from survival all the way up to P9-11, these results are not surprising. Mitochondrial morphology defects and increased oxidative damage were also consistent with a mitochondrial fission defect. The novelty here would be that no one really knew how a mitochondrial fission defect would manifest itself in cardiac muscle. So far, the paper is very descriptive, but then it becomes interesting: In figure 4, the authors show a large increase in p62 levels and in ubiquitin-decorated mitochondria. A double mutant shows that these properties are independent of Parkin. The double mutant is also more severely affected in other ways, suggesting that Drp1 and Parkin mutations disrupt different pathways. Similar results were obtained with Purkinje neurons. Finally, MEFs were studied, again showing synergy between Parkin and Drp1 mutations.

The Sesaki lab has built a reputation on high quality imaging and thorough phenotypic analyses of mice. I was, however, a bit disappointed by the depth of the conclusions drawn here. The results hint at Parkin independent pathways for mitophagy. That is, however, not new. Previously published effects of Mulan on muscle wasting already show that this is possible. I would have liked to see whether the synthetic interactions between Drp1 and Parkin reflect redundancy with one of the other mitochondrial ubiquitin ligases. I am not sure whether this can easily be done with the available mice, but it would be much more informative. It would also be interesting to see how Drp1 affects one ore more of those other pathways. I don't think that has been done before.

One small point:

Fig. 4b, would like to see Ub double labelling with mitochondrial marker, like in panel c. Preferably also use same enlargement. Otherwise, not clear from these images.

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Referee #3:

This is one of the most interesting and exciting manuscripts I reviewed for this journal.
The authors nicely delineate the roles of DRP1 and Parkin for mitochondrial quality control and function in heart, neurons, and MEF cells using single and double knockout of DRP1 and Parkin. The data demonstrates the importance of both proteins for different steps in promoting mitochondrial turnover and function. Importantly the results also demonstrate Parkin-independent induction of mitophagy in certain cases and how DRP1 synergizes with Parkin to promote mitochondrial turnover. A few results need additional experiments in order to fully characterize the effect of DRP1 and Parkin KO on the specific stages of mitochondrial turnover.

It is very interesting results that Parkin KO alone often does not affect mitochondrial morphology or function in cardiomyocytes, neurons, or MEFs; but in combination with DRP1-KO this worsens the mitochondrial dysfunction. One possible mechanism could be that Parkin-mediated mitochondrial quality control is dispensable when ROS levels are low in a normal condition. However upon high ROS-induced stress (such as with DRP1-KO), Parkin-mediated quality control is more pertinent. The authors could conduct experiments to see if anti-oxidant treatment in DRP1-Parkin-KO cells rescues the additional effect of Parkin KO compared to DRP1-KO alone. In addition the authors could increase ROS (H2O2 or menadione treatment, etc.) in ParkinKO cells to see if this causes mitochondrial dysfunction and decreased turnover.
In Figure 4A, the increase in p62 suggests an inhibition of autophagic flux. To confirm this result, the authors should perform experiments with and without inhibition of autophagic degradation by bafilomycin. This will allow estimation of autophagic flux by quantifying LC3-II signal with and without bafilomycin, and then this can confirm the p62 results, which suggest an inhibition of autophagic flux.

In Figure 4C, the authors should stain for lysosomal markers to demonstrate that mitochondria are not being delivered to lysosomes but show p62 and ubiquitin co-localization.

Additionally, the authors can also test whether arresting autophagic degradation by adding bafilomycin increases the number of p62-mitochondrial colocalized structures or whether there is no additional increase with bafilomycin. No additional increase in the number of structures would support the claim that mitochondrial degradation is arrested although they are being targeted for mitophagy.

In Figure 5A, do the authors detect Parkin localization to mitochondria in DRP1KO hearts or cardiomyocytes (stain for Parkin)?

When the authors detect elongated mitochondria with large spheres at the ends in ParkinKO-DRP1-KO cardiomyocytes, neurons, or MEFs - do these have more or less p62 or ubiquitin localization at the large sphere or elongated portion of the mitochondria? This would be interesting to see especially since the authors detect increased oxidative damage in the large sphere portion of the mitochondria (Figure 6). The phenomenon, shown in figure 6 is strongly supported by the finding shown in Twig et al EMBO 2008, Figure 7b.

In Figure 2E, the cardiomyocyte oxygen consumption under CCCP is not significantly higher than basal oxygen consumption. The authors should provide data showing that the CCCP dose used to measure reserve capacity was titrated to achieve maximal reserve capacity without cell toxicity. Perhaps the CCCP dose was not high enough to stimulate maximal oxygen consumption.
Minor Comments
-Page 10: Line 8 from the top refers to Figure 5G, which doesn't exist. In line 10 from the bottom the first 'f' in the word 'flox' should be upper-case.
-Page 12: line 2: the word MitoTracker is missing the letter 'c'.
-Figure 2: why was respirometry performed on neonatal samples rather than adult?
-Figure 4: The figure legend says mitochondrial protein Tom20 instead of Tim23. Also, how were the bands standardized? By eye, it looks like there may be a difference in LC3-II.

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1st Revision - authors' response 18 August 2014

Point-by-point Responses to Reviewers' Comments

Referee #1:

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Very minor comments the authors should consider are listed below:

1. There appears to be some residual Drp1 expression in the tissue from Drp1F/F::Myh6-Cre animals. This does not affect the analysis but this reviewer asks the authors to comment on this in the revision response. Does this mean that the Myh6-Cre expression is not sufficient to recombine both Drp1 floxed alleles within the relevant cells and tissues?

Response: We are grateful for the supportive and insightful comments. We agree with this reviewer that the remaining Drp1 likely results from incomplete recombination of the Drp1 floxed allele. In addition, because isolated hearts contain cardiac valves, coronary vessels, and the aorta, all of which do not express Myh6-Cre, these portions may also affect the amount of Drp1 in immunoblotting. We have included these discussions in the revised manuscript.

2. In the western blot analysis of respiratory components in the double Drp1 Parkin KO, the decrease in protein abundance within the 3x samples provided is not always consistent. The authors should consider activity staining these tissues in addition as they did in the earlier figure. This will likely be more physiologically relevant as I suspect the oxphos activity is much more defective than suggested by the changes in the western blot.

Response: We have measured the activity of the electron transport chain complex I *in vivo* in the new Fig. S4. Although the data confirm decreases in mitochondrial respiration in ParkinDrp1KO hearts, it appears that this assay does not distinguish further decreases in ParkinDrp1KO mice compared to those in Drp1KO mice, possibly due to the limited range of detection for the activity.

Referee #2:

Kageyama et al. studied the effects of Drp1 deletions on mitochondrial function in mouse heart. The authors have done a thorough job characterizing cardiac function in the mutant. They also show decreased respiration and Ox Phos enzyme levels, consistent with a mitochondrial defect. Apart from survival all the way up to P9-11, these results are not surprising. Mitochondrial morphology defects and increased oxidative damage were also consistent with a mitochondrial fission defect. The novelty here would be that no one really knew how a mitochondrial fission defect would manifest itself in cardiac muscle. So far, the paper is very descriptive, but then it becomes interesting: In figure 4, the authors show a large increase in p62 levels and in ubiquitin-decorated mitochondria. A double mutant shows that these properties are independent of Parkin. The double mutant is also more severely affected in other ways, suggesting that Drp1 and Parkin

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mutations disrupt different pathways. Similar results were obtained with Purkinje neurons. Finally, MEFs were studied, again showing synergy between Parkin and Drp1 mutations. The Sesaki lab has built a reputation on high quality imaging and thorough phenotypic analyses of mice. I was, however, a bit disappointed by the depth of the conclusions drawn here. The results hint at Parkin independent pathways for mitophagy. That is, however, not new. Previously published effects of Mulan on muscle wasting already show that this is possible. I would have liked to see whether the synthetic interactions between Drp1 and Parkin reflect redundancy with one of the other mitochondrial ubiquitin ligases. I am not sure whether this can easily be done with the available mice, but it would be much more informative. It would also be interesting to see how Drp1 affects one ore more of those other pathways. I don't think that has been done before.

Response: We appreciate the positive and valuable comments from this referee. We agree that identifying E3 ligases that ubiquitinate mitochondrial proteins in Drp1-mediated mitophagy is extremely interesting and that Mulan is a strong candidate. However, testing this model will require a substantial number of new experiments and the development of new tools. We therefore feel that these experiments are appropriate for the subject of another study, as suggested by the Editor.

One small point:
Fig. 4b, would like to see Ub double labelling with mitochodrial marker, like in panel c. Preferably also use same enlargement. Otherwise, not clear from these images.

Response: We thank this reviewer for pointing this out. In the original manuscript, Fig. 4B only includes low-magnification images showing that p62 and ubiquitin are colocalized in Myh6-Drp1KO hearts. Fig. 4C provides additional high-magnification images that more clearly show that these two proteins are located on the mitochondria. We have now added magnified images to the new Fig. 4B that show colocalization of p62 and ubiquitin at a high magnification.

Referee #3:

This is one of the most interesting and exciting manuscripts I reviewed for this journal. The authors nicely delineate the roles of DRP1 and Parkin for mitochondrial quality control and function in heart, neurons, and MEF cells using single and double knockout of DRP1 and Parkin. The data demonstrates the importance of both proteins for different steps in promoting mitochondrial turnover and function. Importantly the results also demonstrate Parkin-independent induction of mitophagy in certain cases and how DRP1 synergizes with Parkin to promote mitochondrial turnover. A few results need additional experiments in order to fully characterize the effect of DRP1 and Parkin KO on the specific stages of mitochondrial turnover. It is very interesting results that Parkin KO alone often does not affect mitochondrial morphology or function in cardiomyocytes, neurons, or MEFs; but in combination with DRP1-KO this worsens the mitochondrial dysfunction. One possible mechanism could be that Parkin-mediated mitochondrial quality control is dispensable when ROS levels are low in a normal condition. However upon high ROS-induced stress (such as with DRP1-KO), Parkin-mediated quality control is more pertinent. The authors could conduct experiments to see if anti-oxidant treatment in DRP1-Parkin-KO cells rescues the additional effect of Parkin KO compared to DRP1-KO alone. In addition the authors could increase ROS (H2O2 or menadione treatment, etc.) in ParkinKO cells to see if this causes mitochondrial dysfunction and decreased turnover.

Response: We thank this referee for the very encouraging comments and for the constructive suggestions for experiments that could strengthen the manuscript. As recommended, we treated ParkinDrp1KO MEFs with an antioxidant, Nacetylcysteine, and examined its effect on mitochondrial morphology. We found that the defects in mitochondrial morphology were significantly rescued, to a level similar to that seen in Drp1KO MEFs (new Fig. S6). Therefore, as this reviewer suggested, the loss of Drp1 in ParkinKO MEFs increases the level of oxidative stress, which can be counteracted by antioxidants.

In Figure 4A, the increase in p62 suggests an inhibition of autophagic flux. To confirm this

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result, the authors should perform experiments with and without inhibition of autophagic degradation by bafilomycin. This will allow estimation of autophagic flux by quantifying LC3-II signal with and without bafilomycin, and then this can confirm the p62 results which suggest an inhibition of autophagic flux.

Response: Although blocking autophagy degradation by bafilomycin A *in vivo* could be a very interesting experiment, it is technically difficult to treat neonatal mice with bafilomycin A due to their small sizes. Therefore, we analyzed the accumulation of LC3-II in WT and Drp1KO MEFs in the presence and absence of bafilomycin A. Results showed that the rate of LC3-II accumulation was not decreased in Drp1KO MEFs (new Fig. S5), suggesting that the loss of Drp1 does not inhibit autophagic flux.

In Figure 4C, the authors should stain for lysosomal markers to demonstrate that mitochondria are not being delivered to lysosomes but show p62 and ubiquitin co-localization. Additionally, the authors can also test whether arresting autophagic degradation by adding bafilomycin increases the number of p62-mitochondrial colocalized structures or whether there is no additional increase with bafilomycin. No additional increase in the number of structures would support the claim that mitochondrial degradation is arrested although they are being targeted for mitophagy.

Response: We performed triple immunostaining using antibodies to a lysosomal marker (Lamp1), ubiquitin, and a mitochondrial protein (PDH) in the new Fig. 4D. We found that ubiquitin-decorated mitochondria were not colocalized with Lamp1 in Drp1KO hearts. Interestingly, many lysosomes were found near ubiquitin-decorated mitochondria. These data show that mitochondria were not transported to lysosomes. Regarding the second point, bafilomycin treatment is difficult to perform on neonatal Myh6-Drp1KO mice, as described above. We are currently generating tamoxifen-inducible, cardiac-specific Drp1KO mice, which will allow us to use adult animals for this experiment. We hope to address this question in our future studies.

In Figure 5A, do the authors detect Parkin localization to mitochondria in DRP1KO hearts or cardiomyocytes (stain for Parkin)?

Response: We performed immunofluorescence microscopy of Drp1KO hearts with anti-parkin antibodies and found no recruitment of parkin to enlarged mitochondria or p62- and ubiquitin-positive mitochondria (new Fig. 5G and H). Considering previous reports showing that parkin is translocated to mitochondria when the membrane potential is dissipated by CCCP, it is possible that mitochondria in Drp1KO hearts still maintain some membrane potential, albeit decreased, which may preclude the recruitment of parkin.

When the authors detect elongated mitochondria with large spheres at the ends in ParkinKODRP1-KO cardiomyocytes, neurons, or MEFs - do these have more or less p62 or ubiquitin localization at the large sphere or elongated portion of the mitochondria? This would be interesting to see especially since the authors detect increased oxidative damage in the large sphere portion of the mitochondria (Figure 6). The phenomenon, shown in figure 6 is strongly supported by the finding shown in Twig et al EMBO 2008, Figure 7b.

Response: This is an excellent point. The accumulation of ubiquitin and p62 was almost exclusively observed on the spherical part of mitochondria in Drp1KO cardiomyocytes and in Purkinje neurons. As pointed out by this reviewer, our findings and those made by Orian Shirihai's laboratory (Tiwg et al., 2008), provide a tight correlation between oxidative damage, morphological changes, and the accumulation of mitophagy markers.

In Figure 2E, the cardiomyocyte oxygen consumption under CCCP is not significantly higher than basal oxygen consumption. The authors should provide data showing that the CCCP dose used to measure reserve capacity was titrated to achieve maximal reserve capacity without cell toxicity. Perhaps the CCCP dose was not high enough to stimulate maximal oxygen

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consumption.

Response: We used CCCP at 1 μM to measure oxygen consumption rates. Although this concentration is widely used, as the referee correctly pointed out, it is possible that it did not achieve maximal respiratory capacity in our experiments. Because we could not obtain sufficient numbers of cardiomyocytes to optimize the condition within the time available for revision, we have removed the respiratory capacity data from Fig. 2 and show only the basal respiration data in the revised manuscript (Fig. 2E). This presentation does not affect our conclusion that Drp1KO cardiomyocytes have decreased mitochondrial respiration.

Minor Comments

-Page 10: Line 8 from the top refers to Figure 5G, which doesn't exist. In line 10 from the bottom the first 'f' in the word 'flox' should be upper-case.

Response: We thank the referee for noticing these errors. We have corrected them.
-Page 12: line 2: the word MitoTracker is missing the letter 'c'.

Response: We corrected this.

-Figure 2: why was respirometry performed on neonatal samples rather than adult?

Response: We used neonatal cardiomyocytes because we analyzed P1-7 mice in most of the experiments, and Myh6-Drp1KO mice die at P9-11. Inducible knockout mice for cardiac Drp1, as mentioned above, will make it possible to measure oxygen consumption rates in Drp1KO adult cardiomyocytes in our future study.

-Figure 4: The figure legend says mitochondrial protein Tom20 instead of Tim23. Also, how were the bands standardized? By eye, it looks like there may be a difference in LC3-II.

Response: We corrected Tom20 to Tim23. The bands were normalized for the amount of protein loaded in each lane. We carefully quantified band intensity and found no significant difference.

2nd Editorial Decision 19 September 2014

Thank you for submitting the revised version of your manuscript entitled 'Parkin-independent mitophagy requires Drp1 and maintains the integrity of mammalian heart and brain'. I have now received feedback from referee #3, who appreciates the introduced changes as you can see below.

I am thus happy to accept your manuscript for publication here.

Referee #3:

I went over the manuscript and my previous comments.
The authors addressed all of my comments appropriately. The paper can now be published.

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