THE EMBO JOURNAL

Manuscript EMBO-2009-72959

MITOCHONDRIAL FISSION AND REMODELING CONTRIBUTES TO MUSCLE ATROPHY

Vanina Romanello, Eleonora Guadagnin, Ligia Gomes, Ira Roder, Claudia Sandri, Yvonne Petersen, Giulia Milan, Eva Masiero, Paola Del Piccolo, Marc Foretz, Luca Scorrano, Rudige Rudolf, and Marco Sandri

Corresponding author: Marco Sandri, Dulbecco Telethon Institute at Venetian Institute of Molecular Medicine

Review timeline:

Submission date:	26 October 2009
Editorial Decision:	25 November 2009
Revision received:	22 February 2010
Editorial Decision:	09 March 2010
Accepted:	10 March 2010

Transaction Report:

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

1st Editorial Decision 25 November 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Your manuscript has been reviewed by three referees and I enclose their reports below. As you will see from their comments the referees overall express interest in the study and require additional experiments before the study is suitable for the EMBO Journal. The referees ask for a number of experiments and referee #1 specifically raises an number of important issues that must be addressed, referee #2 would like to see more direct evidence for impaired respiratory activity and insight into the dependence of muscular atrophy on mitochondrial dysfunction. Should you be able to address these issues, we would be wiling to consider a revised manuscript.

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. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the 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.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Romanello et al. investigated the role of the mitochondrial network in muscle wasting. The authors first report that after one day of fasting or seven days of denervation there was a disorganized mitochondrial network in the atrophying muscles from mice. Next they show that inhibition of mitochondrial fission protected from the activation of both autophagy and ubiquitin-proteasome-dependent proteolysis in fasting. Conversely, the degree of muscle atrophy correlated with mitochondrial network alterations, but mitochondrial dysfunction by itself was not sufficient for inducing muscle wasting. Finally, it is also shown that mitochondrial fission activated the AMPK-FoXO3 axis, which induces autophagy and ubiquitin-proteasome-dependent proteolysis in muscle wasting. Overall the data are new and potentially interesting, but there are several major problems with the data and their interpretation.

Major problems:

1. It is extremely surprising to see that only one day of starvation resulted in profound changes in the mitochondrial network in vivo (Fig. 1). The perturbations shown most probably reflect not only the effect of starvation but more importantly the effects of chloroquine treatment for 7 days. Indeed it is not clear at all from the Fig. legend whether the control muscles were also treated by chloroquine for 7 days. However, and in any case, the observations reported in Fig. 1 are crucial for the rationale of the paper. Therefore, the authors must provide alternative evidence showing perturbations in the mitochondrial network without any blockade of the lysosomal system.

2. While FoxO3-mediated muscle atrophy is reduced by inhibition of the mitochondrial fission proteins DRP1 (Fig. 3D), muscle atrophy induced by DRP1/hFs1 is conversely blocked by FoxO3 knockdown (Fig. 7D). It is therefore not clear whether FoxO3 is upstream or downstream DRP1/hFs1 in the pathway regulating muscle atrophy. The authors should address and comment this discrepancy.

3. The authors provide indications that Bnip3, like mitochondrial fission proteins DRP1 and hFs1, altered mitochondrial membrane potential (Fig 4). They also show that the regulation of muscle atrophy by Bnip3, DRP1 or hFs1 would involve the stimulation of AMPK and the consequent enhanced binding of FoxO3 to atrogin and MuRF1 promoters. Such stimulation of atrogin and MuRF1 may provide a link with proteasome-dependent proteolysis.
However, since Bnip3-stimulation of atrophy/autophagy does not implicate beclin-1 (see below), the precise mechanisms according to which Bnip3 regulates autophagy remain to be identified.

4. The authors indicate that Bnip3-BH3 mutant, like native Bnip3, does induce autophagy and muscle atrophy (Fig. 5D, E). Therefore, induction of autophagy and muscle atrophy by Bnip3 does not require disruption of beclin-1/Bcl2 by Bnip3-BH3 domain and does not implicate activation of autophagy via beclin-1. Conversely, the authors provide indications (Fig 5S) that Bnip3 regulates muscle atrophy via its localization signal to mitochondria. This central observation should be emphasized in the manuscript.

5. The authors should provide a scheme of the mechanisms identified in the manuscript.

Minor problems:

1. Delete r after Rudolf in the authors' list.

2. Please amend the title page 6. Indeed the data do not show that 'Induction of mitochondrial fission and dysfunction is SUFFICIENT to active an atrophy program'.

3. Bar scales are too faint in Figs. 1 and 4 to reproduce correctly. Scale bars are also missing in several panels of Fig. 2.

4. Statistics are missing in Fig. 5C, D and 7A.

5. There are several typescript errors in the Chip Assay paragraph page 11.

Referee #2 (Remarks to the Author):

The authors have examined the role of alterations in mitochondrial morphology during muscle atrophy. Induction of muscle atrophy by fasting or denervation in mice is accompanied by the disorganization of the mitochondrial network and FoxO-triggered lysosomal degradation of mitochondria. Downregulation of Fis1 or Bnip1 or expression of a dominant negative variant of Drp1 prevented the expression of ubiquitin ligases required for autophagy and protected against atrophy, suggesting the fission is required for muscle atrophy. On the other hand, transfection of muscles with fission components altered - as expected - the mitochondrial network, activated AMPK and induced muscle atrophy. Using a variant of Fis1 in these experiments, the authors provide evidence that both fission and mitochondrial dysfunction are required for muscle atrophy. This is an interesting and in most parts technical sound study providing compelling evidence for a role of mitochondrial fission for muscle atrophy. the findings will be of considerable interest to the field, although several studies have already addressed the role of mitochondrial fragmentation and autophagy in other contexts. However, the relative dependence of atrophy on mitochondrial dysfunction and fission remains rather speculative and unclear. The authors do not provide direct evidence for an impaired respiratory activity under the conditions used, but use only AMPK activation as an indirect read-out. What do the authors mean with a dissociation of mitochondrial fission and dysfunction by Fis1 K148R (p. 7)? As a dysfunction of mitochondria induces mitochondrial fragmentation, the manuscript would benefit from a clarification of this point. Another point concerns the degree of autophagy during muscle atrophy, as only a rather limited amount of autophagosomes with engulfed mitochondria appear to be detectable (e.g. Fig. 2B). Finally, the quantification of the western blots after downregulation of fission components (Fig. 3A and Suppl. Fig. S1) is not convincing (e.g. for oligo 3). Documentation of the efficiency of the RNAi by RT-PCR would be advisable.

Referee #3 (Remarks to the Author):

In this manuscript, Romanello and colleagues investigate the role of mitochondrial fusion and fission events in skeletal muscle autophagy and subsequent atrophy.

COMMENTS

1. - The authors demonstrate in their supplemental data (Supp Fig S6) that mitochondrial remodeling per se is not sufficient to induce muscle atrophy, rather mitochondrial dysfunction appears to be pre-requisite. This important distinction does not currently receive sufficient attention, this figure should be presented and discussed in detail, and further experiments should be conducted using the mutant hFisK148R.
2. Due to differences in mitochondrial content, it would have been interesting to examine the effects of DRP1 and Fis1 inhibition in fast vs. slow fibers.
3. It would be useful to have a measure of apoptosis in the atrophying muscles to confirm that the observed muscle atrophy is a result of lysosomal/proteasomal degradation.
4. In Figure 1, the authors use Chloroquine to block lysosomal activity and show dramatic differences in the size of the autophagosomes, what is this difference a result of?
5. Page 3, line 30: To this reviewers knowledge, endurance training has not been found to prevent age-related muscle wasting and weakness.
6. Figure 1: Why does the control fiber not stain for LC3?
7. Figure 1: Scale bars are different for each of the conditions, it would be useful if control, denervated and starved muscles were presented at the same magnification
8. Figure 2D: How many sections were examined for EM? Are the images presented representative? It appears that subsarcolemmal mitochondria are still present in the caFOXO3 muscles, although the number does appear reduced
9. Are total Fis1 and/or DRP1 levels altered during starvation and denervation?
10. Figure 5E: The legend states that Bnip3 induced atrophy is not affected by the mutation of the BH3 domain, however, the figure clearly indicates a decrease in atrophy?

11. Figure 6B: Do the authors have data for dnAMPK alone? In this figure it almost looks as though dnAMPK+Bnip3 results in an increase in fiber CSA

1st Revision - Authors' Response 22 February 2010

Reviewer #1:

1. It is extremely surprising to see that only one day of starvation resulted in profound changes in the mitochondrial network in vivo (Fig. 1). The perturbations shown most probably reflect not only the effect of starvation but more importantly the effects of chloroquine treatment for 7 days. Indeed it is not clear at all from the Fig. legend whether the control muscles were also treated by chloroquine for 7 days. However, and in any case, the observations reported in Fig. 1 are crucial for the rationale of the paper. Therefore, the authors must provide alternative evidence showing perturbations in the mitochondrial network without any blockade of the lysosomal system.

- The referee correctly noted that our language about chloroquine treatment was ambiguous. Therefore we changed the text and the figures to emphasize that changes in mitochondrial network already happened in muscles of untreated animals. Chloroquine exacerbates the abnormalities of mitochondrial morphology since mitochondria are not anymore destroyed into lysosomes and accumulate in clusters into the autophagolysosomes. To further support the concept that autophagy flux is the reason of mitochondrial clustering in chloroquine treated mice and of mitochondrial network remodeling in atrophying muscles we have analysed the mitochondrial network of autophagy knockout mice. We have recently generated muscle specific autophagy knockout mice. As depicted in Supplementary Fig. S1 mitochondrial network is already altered in fed Atg7 deficient muscle due to the fact that abnormal mitochondrial are not removed anymore and accumulate into the myofibers (Masiero et al. Cell Metab. 2009). However the mitochondrial network does not show the dramatic changes observed during fasting in control and chloroquine treated mice. Mitochondria are more close to each other as a consequence of the degradation of contractile proteins via proteasome, but are not removed and do not form the big clusters observed in chloroquine treated mice

2. While FoxO3-mediated muscle atrophy is reduced by inhibition of the mitochondrial fission proteins DRP1 (Fig. 3D), muscle atrophy induced by DRP1/hFs1 is conversely blocked by FoxO3 knockdown (Fig. 7D). It is therefore not clear whether FoxO3 is upstream or downstream DRP1/hFs1 in the pathway regulating muscle atrophy. The authors should address and comment this discrepancy."

- The reviewer underlines an important aspect, in fact FoxO is part of a positive feedback loop. The new findings of mitochondrial function and our previous results demonstrate that i) mitochondrial dysfunction activates FoxO via AMPK and that ii) FoxO itself induces mitochondrial fragmentation and dysfunction that maintains FoxO3 active in the nucleus (New Fig 6A-D.). This circuit is self-sustaining and keeps FoxO in a condition of hyperactivation leading to muscle loss. This loop can be interrupted by blocking the effects either on mitochondria or on FoxO. We have tried to summarize this concept in the scheme depicted in Supplementary Figure S14

3. The authors provide indications that Bnip3, like mitochondrial fission proteins DRP1 and hFs1, altered mitochondrial membrane potential (Fig 4). They also show that the regulation of muscle atrophy by Bnip3, DRP1 or hFis1 would involve the stimulation of AMPK and the consequent enhanced binding of FoxO3 to atrogin and MuRF1 promoters. Such stimulation of atrogin and MuRF1 may provide a link with proteasome-dependent proteolysis. However, since Bnip3-stimulation of atrophy/autophagy does not implicate beclin-1 (see below), the precise mechanisms according to which Bnip3 regulates autophagy remain to be identified.

Bnip3 and its homologous Bnip3l contain an atypical BH3 domain (Zhang J and Ney PA, Cell Death Differ. 2009) but recent evidence suggests that they different from the classical BH3 only

proteins (Bid, Bim, Bik….). For instance, Bcl2 can not prevent the mitochondrial swelling induced by Bnip3 (Quinsay MN et al., 2010 Journal of Molecular and Cellular Cardiology). Moreover Bnip3 action requires the decrease of the pro-fusion protein OPA1 (Quinsay MN et al., 2010 Journal of Molecular and Cellular Cardiology) and can interact with the fission protein DRP1 (Kubli et al., 2007, Biochem J). Indeed others have reported that mitochondrial localization signal is critical for Bnip3 dependent autophagy (Hamacher-Brady, A. et al. Cell Death Differ. 2007).

The mechanism that can induce mitophagy independent of beclin1 has been recently revealed for Bnip3l. Bnip3l is able to bind the lipidated form of Atg8 homologues, LC3 and Gabarap, recruiting the autophagosomes on mitochondria. This action is similar to p62-mediated delivery of ubiquitinated proteins to autophagosomes. In fact both Bnip3 and Bnip3l contain two conserved LIR (LC3 Interacting Region) domains that are located at the N-terminus (amino acid 35-38 of murine Bnip3l) and adjacent to the BH3 domain (amino acid 138-143) (Novak I et al, 2010 EMBO Reports). The most critical domain for LC3 interaction is the one located at the NH2-terminal. We have explored this possibility and we have found that Bnip3 wild type and the BH3 mutant but not the mutant that lacks the mitochondrial signal colocalize with LC3. We have now added the new data in Supplementary Fig S10

4. The authors indicate that Bnip3-BH3 mutant, like native Bnip3, does induce autophagy and muscle atrophy (Fig. 5D, E). Therefore, induction of autophagy and muscle atrophy by Bnip3 does not require disruption of beclin-1/Bcl2 by Bnip3-BH3 domain and does not implicate activation of autophagy via beclin-1. Conversely, the authors provide indications (Fig 5S) that Bnip3 regulates muscle atrophy via its localization signal to mitochondria. This central observation should be emphasized in the manuscript.

As suggested by the reviewer we have emphasized our observations of Bnip3 in results and discussion sections. We have added new findings (see above point) in Supplementary Fig S10 to support the Bnip3-dependent recruitment of autophagy machinery on mitochondria as mechanism of mitophagy.

5. The authors should provide a scheme of the mechanisms identified in the manuscript.

As requested by the reviewer we have added a scheme (Supplementary Fig S14) that summarizes the signaling triggered by mitochondrial dysfunction/damage. The positive loop between mitochondrial damage-AMPK activation-FoxO induction-mitochondrial damage creates a vicious circle that leads to muscle atrophy.

Minor Points

1. Delete r after Rudolf in the authors' list.

We have deleted the r after Rudolf in the authors' list

2. Please amend the title page 6. Indeed the data do not show that 'Induction of mitochondrial fission and dysfunction is SUFFICIENT to active an atrophy program'.

We have removed the word SUFFICIENT from the title.

3. Bar scales are too faint in Figs. 1 and 4 to reproduce correctly. Scale bars are also missing in several panels of Fig. 2.

We have added and improved the scale bars in the figures 1, 2 and 4.

4. Statistics are missing in Fig. 5C, D and 7A.

We have added statistics to Fig 5C, D and 7A

5. There are several typescript errors in the Chip Assay paragraph page 11. .

We have corrected the typescript errors in the ChIP Assay paragraph

Reviewer #2:

"The authors have examined the role of alterations in mitochondrial morphology during muscle atrophy. Induction of muscle atrophy by fasting or denervation in mice is accompanied by the disorganization of the mitochondrial network and FoxO-triggered lysosomal degradation of mitochondria. Downregulation of Fis1 or Bnip3 or expression of a dominant negative variant of Drp1 prevented the expression of ubiquitin ligases required for autophagy and protected against atrophy, suggesting the fission is required for muscle atrophy. On the other hand, transfection of muscles with fission components altered - as expected - the mitochondrial network, activated AMPK and induced muscle atrophy. Using a variant of Fis1 in these experiments, the authors

provide evidence that both fission and mitochondrial dysfunction are required for muscle atrophy. This is an interesting and in most parts technical sound study providing compelling evidence for a role of mitochondrial fission for muscle atrophy. the findings will be of considerable interest to the field, although several studies have already addressed the role of mitochondrial fragmentation and autophagy in other contexts. However, the relative dependence of atrophy on mitochondrial dysfunction and fission remains rather speculative and unclear. The authors do not provide direct evidence for an impaired respiratory activity under the conditions used, but use only AMPK

activation as an indirect read-out. What do the authors mean with a dissociation of mitochondrial fission and dysfunction by Fis1 K148R (p. 7)?"

Because of the reviewer's request we have performed new experiments to better clarify the contribution of mitochondrial fission versus mitochondrial dysfunction. We quantified mitochondrial membrane potential in isolated adult myofibers which were transfected with the different constructs for the fission machinery. Mitochondrial potential was monitored by TMRM staining in presence of oligomyicin treatment. The new Fig. 6B shows that inhibition of the F1F0-ATP synthase in control myofibers does not affect the mitochondrial transmembrane potential, as expected. However expression of hFis1 caused an important and significant mitochondrial depolarization after oligomycin treatment. This finding suggests that the ATP synthase is operating in a reverse mode consuming ATP to pump protons in order to mantain mitochondrial membrane potential. On the other hand, expression of $hFis1^{K148R}$ does not affect mitochondrial membrane potential, confirming our results of two photon microscopy and of AMPK activation and our previously published data that $hFis1^{K148R}$ fragments mitochondria preserving membrane potential and mitochondrial function. Moreover, expression of $hFis^{K148R}$ in adult fibers does not activate the expression of the atrophy-related ubiquitin ligase MuRF1 (Figure 6 D) confirming that only dysfunctional fragmented mitochondria can trigger an atrophy program.

As a dysfunction of mitochondria induces mitochondrial fragmentation, the manuscript would benefit from a clarification of this point."

As suggested by the reviewer we have added new data of mitochondrial function to clarify the point that mitochondrial dysfunction is critical for FoxO activation and muscle atrophy.

Another point concerns the degree of autophagy during muscle atrophy, as only a rather limited amount of autophagosomes with engulfed mitochondria appear to be detectable (e.g. Fig. 2B).?

We agree with the reviewer that only a fraction of autophagosomes are used for mitophagy while the remaining vesicles are utilized to remove proteins and other organelles including sarco/endoplasmic reticulum. However, because of the reviewer's request we have used our muscle-specific autophagy knockout mice to confirm that autophagy is involved in mitochondrial changes. We have recently generated muscle specific Atg7 null mice (Masiero et al. Cell Metab. 2009) which have been already characterized. Autophagy deficient muscles do not show the dramatic changes in mitochondrial network observed during fasting in wild type animals. There is a tendency of mitochondria clumping, as a consequence of the degradation of contractile proteins via proteasome, but the pattern and the localization of mitochondria is well preserved in Atg7 knockout muscles. The new data are depicted in Supplementary Fig S1.

Finally, the quantification of the western blots after downregulation of fission components (Fig. 3A and Suppl. Fig. S1) is not convincing (e.g. for oligo 3). Documentation of the efficiency of the RNAi by RT-PCR would be advisable.

Because of reviewer request we have analysed the efficiency of RNAi by quantitative RT-PCR the efficiency of RNAi. We have now added the new panel in Supplemetary Fig S3

Reviewer #3:

1. The authors demonstrate in their supplemental data (Supp Fig S6) that mitochondrial remodeling per se is not sufficient to induce muscle atrophy, rather mitochondrial dysfunction appears to be pre-requisite. This important distinction does not currently receive sufficient attention, this figure should be presented and discussed in detail, and further experiments should be conducted using the mutant hFisK148R.

-Because of the referee's comment, we have moved the panels of Supplementary Fig S6 into the new Figure 6 and we have added new experiments to address the role of hFisK148R on mitochondrial function and signaling.

We monitored mitochondrial membrane potential in isolated adult myofibers which were transfected with the different constructs for the fission machinery. Mitochondrial potential was monitored by TMRM staining in presence of oligomyicin treatment. The new Fig. 6B shows that inhibition of the F1F0-ATP synthase in control myofibers does not affect the mitochondrial transmembrane potential, as expected. However expression of hFis1 caused an important and significant mitochondrial depolarization after oligomycin treatment. This finding suggests that the ATP synthase is operating in a reverse mode consuming ATP to pump protons in order to mantain mitochondrial membrane potential. On the other hand, expression of $hFis1^{K148R}$ does not affect mitochondrial membrane potential, confirming our results of two photon microscopy and of AMPK activation and our previously published data that $hFis1^{K148R}$ fragments mitochondria preserving membrane potential and mitochondrial function. Moreover, expression of $hFis^{K148R}$ in adult fibers does not activate the expression of the atrophy-related ubiquitin ligase MuRF1 (Figure 6 D) confirming that only dysfunctional fragmented mitochondria can trigger an atrophy program.

2. Due to differences in mitochondrial content, it would have been interesting to examine the effects of DRP1 and Fis1 inhibition in fast vs. slow fibers.

- Fast and slow muscles show different susceptibility to atrophy. We have recently shown that b-oxidative fibers are preserved by atrophy during fasting due to the high level of expression of PGC1a (Sandri M et al., PNAS. 2006). Therefore we can not use this model of atrophy in slow muscles such as soleus. However, slow fibers are more prone to lose proteins during disuse/denervation when PGC1a expression dramatically falls. Thus, in response to the reviewer's requests we have studied the effect of inhibition of the fission machinery in Soleus muscle during denervation. Knocking down Fis1 and Bnip3 significantly protected, although to a lesser degree than fasting, this muscle from atrophy. The new data are depicted in Supplementary Fig S4

3. It would be useful to have a measure of apoptosis in the atrophying muscles to confirm that the observed muscle atrophy is a result of lysosomal/proteasomal degradation.

-Because of reviewer's request we have analyzed the level of apoptosis under different conditions of atrophy. As already well described, apoptosis occurs upon denervation (Sandri M, *Curr Opin Clin Nutr Metab Care*. 2002), but is not a general process that occurs in atrophying muscles (Sandri M, *Physiology,* 2008). We confirmed an increase of apoptotic nuclei in denervated muscles and a trend of increase when FoxO3 is overexpressed. However fasting and overexpression of fission machinery components did not induce apoptosis. Thus apoptotic signalling can contribute to muscle loss in some conditions but it is not a general mechanism of muscle wasting in catabolic conditions. The new data are depicted in Supplementary Fig S6

4. In Figure 1, the authors use Chloroquine to block lysosomal activity and show dramatic differences in the size of the autophagosomes, what is this difference a result of?.

-The difference of LC3 positive vesicles size is caused by the kinetics of the different experiments. Chloroquine increases the low pH of lysosome therefore inhibiting the hydrolytic capability of this organelle. However it does not affect the autophagy flux which still delivers organelles and proteins into the lysosome. Amongst the different components that are degraded into the lysosomes there are also some autophagy proteins such as LC3, Gabarap and p62 that are attached to the inner membrane of the vesicle. Thus, the LC3 positive vesicles of Figure 1 are mainly autophagolysosomes and the difference in size of these vesicles between fasted and denervated muscles reflects the timing of autophagy inhibition during catabolic conditions.. Fasting lasted for 24 hours while denervation lasted for 7 days. Thus, during the 7 days of denervation LC3 as well as mitochondria were continuously delivered to the lysosomes, as a consequence of the autophagy activation, and were stocked into the autophagolysosomes forming big vacuoles. n fasting the time period of autophagy activation was much shorter (24 hours) and therefore the autophagolysosomes that accumulate were of smaller size.

5. Page 3, line 30: To this reviewers knowledge, endurance training has not been found to prevent age-related muscle wasting and weakness.?

We agree with the reviewer that resistance training is more important to preserve muscle mass and force in elderly persons than endurance. However a recent paper has shown that forced expression of PGC1ą the master gene of mitochondrial biogenesis that is induced by endurance training, in skeletal muscles of old mice ameliorates loss of muscle mass (Wenz, T. et al. PNAS. 2009). We have added this reference and modified the sentence to underline a possible beneficial effect of endurance training in controlling PGC1a levels.

6. Figure 1: Why does the control fiber not stain for LC3?

We have changes Figure 1 with a picture that retains LC3-YFP fluorescence. The previous one was too weak and the green fluorescence was lost during the conversion in PDF format.

7. Figure 1: Scale bars are different for each of the conditions, it would be useful if control, denervated and starved muscles were presented at the same magnification

Because of reviewer's request we have changed as much as possible the magnification in order to maintain the same scale bar but also to preserve details. However it is worth to underline that it is difficult to visualize mitochondria and vesicles when myofibers shrink and become atrophic such as during denervation or FoxO3 overexpression.

8. Figure 2D: How many sections were examined for EM? Are the images presented representative? It appears that subsarcolemmal mitochondria are still present in the caFOXO3 muscles, although the number does appear reduced

This methodological issue should have been included earlier. We had analyzed five transfected muscles and more than 100 fibers were looked at electron microscopy. The image is representative of the atrophic fibers. We have specified the numbers in methods section and clarified that this image is representative in figure caption. We agree with reviewer that subsarcolemmal mitochondria are reduced in number and size and we changed accordingly the text in the figure caption

9. Are total Fis1 and/or DRP1 levels altered during starvation and denervation?

As suggested by the reviewer we have monitored the Fis1 and DRP1 expression level. The new data are depicted in Supplementary Fig S2

10. Figure 5E: The legend states that Bnip3 induced atrophy is not affected by the mutation of the BH3 domain, however, the figure clearly indicates a decrease in atrophy?

The difference between Bnip3 and BH3 mutant is not statistically significant. In order to better clarify this issue we changed the graphics of our statistical analysis in Figure 5E.

11. Figure 6B: Do the authors have data for dnAMPK alone? In this figure it almost looks as though dnAMPK+Bnip3 results in an increase in fiber CSA

Because of the reviewer's request we added in Supplementary Figure S12 the data of dnAMPK alone. Indeed, accordingly to our published results dnAMPK alone promotes an increase of CSA (Aguilar V. et al. Cell Metabolism, 2007). However there was no significant difference between fibers expressing d.n.AMPK alone and fibers cotranfected with either d.n.AMPK and Bnip3 or d.n. AMPK and DRP1/hFis1 that are shonw in Figure 7B and 7C respectively. These findings were confirmed by the RNAi experiments shown in Figure 7D and 7E.

2nd Editorial Decision 09 March 2010

I am please to inform you that all three referees that have reevaluated your manuscript are satisfied with your revisions and recommend publication in the EMBO Journal. I enclose their comments below. You will receive the official acceptance email in the next day or so.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

None

Referee #2 (Remarks to the Author):

The authors have addressed carefully my concerns raised and clarified further the relative importance of mitochondrial dysfunction and fission. I therefore can recommend publication.

Referee #3 (Remarks to the Author):

The authors have satisfactorily addressed our previous concerns

