THE EMBO JOURNAL

Manuscript EMBO-2014-88278

Induction of autophagy supports the bioenergetic demands of quiescent muscle stem cell activation

Ann H. Tang and Thomas A. Rando

Corresponding author: Thomas Rando, Stanford University

Review timeline:

Submission date:	19 February 2014
Editorial Decision:	11 March 2014
Revision received:	09 August 2014
Editorial Decision:	19 August 2014
Revision received:	29 August 2014
Accepted:	01 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: Thomas Schwarz-Romond

1st Editorial Decision 11 March 2014

Thank you very much for the opportunity to consider your results addressing the role of autophagy flux in providing necessary energy for stem cell activation for potential publication in The EMBO Journal.

I received comments from two expert scientists, enabling a formal decision at this stage. Their critiques crystallize two issues that I would be delighted if you were able to sufficiently clarify/extend the current dataset: the first point relates to cause and consequence between autophagy and the onset of the cell cycle (ref#1 major point 1).
Secondly, ref#2 inquires about the possibility to identify/specify SIRT1 targets (direct or transcriptional autophagic components; please see major point 3 of this referee on this).

Overall, I am delighted for the opportunity to invite revisions on your study.

Please notice that The EMBO Journal considers only one round of revisions. I also have to note at this point that the final decision will depend on your responses to the referees that will be involved in assessing your revised paper.

Please do not hesitate to get in touch in case I can be of any assistance. I am very much looking

forward to hear form you and remain with best regards.

REFEREE REPORTS:

Referee #1:

The present study proposes a mechanism that contributes to stem cell (muscle satellite cells) exit from quiescent to an activated/proliferating state. The authors report that autophagosome formation occurs upon the transition to ASC (from QSC) which may serve to provide, in part, the energy necessary for cell proliferation. This work shows that autophagy is only observed in activated/proliferative satellite cells and inhibited in the quiescent state. The provide evidence that SIRT1, a nutrient sensor, is required for the autophagic flux in upon the activation of satellite cells, and can be correlated with an increase of ATP production and mitochondria activity. This study provides new insights about overall regulation of cellular metabolism and it regulation during the transition from quiescent to activated satellite cells. Experiments using siRNA against atg5/7 and sirt1-/- mice to inhibit autophagy as well as the use of pyruvate to circumvent the required autophagy flux step in this transition can restore cell proliferation in inhibited cells. As such, this is a novel and interesting contribution however several issues should be addressed prior to publication and probably should be re-reviewed since some are significant. This stated, the study explores new territory and if the points are addressed, it an important contribution. .

Major comments :

1 A central question still remains: Does autophagy allow satellite cells to enter the cell cycle or is it the entry into the cell cycle that induces autophagy?

The authors have tried to answer this question but data do not support clearly their conclusions stating that "autophagy was induced in ASCs that had not initiated DNA synthesis and hence had not entered the cell cycle". Such a conclusion cannot be made based on the data presented as it is quite difficult to accurately identify the LC3-GFP positive cells. ("3 punctae are considered to have induced autophagic flux"). Furthermore, using chloroquine after 24h, we can see that LC3 is already express and accumulates wherease no positive staining for EdU can be observed.

2 Even if authors have shown that SIRT1 is involved in autophagic flux increases, there are no data related to Mitotracker and ATP production when SIRT1 is knocked down.

As such, we do not know how SIRT1 regulates autophagy. Does SIRT1 induce ATP production and mitochondrial activity? Is SIRT1 activated by some gene known to be activated satellite cells as Myf5/MyoD or is it just a downstream regulator element of the AMPK/NAD+ pathway which is activated in ASCs ?

3 The critical point of this paper is to know how authors have isolated QSC from ASC and by what observation can they say ""QSCs were FACS-sorted from uninjured LC3-GFP mice to a purity of 98%" or "we isolated these cells (QSC, ASC, SC) to a purity of 98% by FACS". Do they mean pax7+/myoD- for QSC and pax7+/myoD+ for ASC? And what marker do they use to isolate them by FACS? This is not mentioned either in the text nor in the material and methods. A figure in supp data of the QSC and ASC sorted by FACS would be appreciated.

4 Authors show that LC3 is increased at the protein level, but do they have looked at the mRNA level ? It would be interesting to add this parameter to support IF pictures.

Minor comments :

1.In the paper has been described the autophagic flux and metabolism in activated satellite cells. The authors assume to sort satellite cells using a specific protocol (cheung et al.) but no pictures of the FACS profile has been shown.

Fig1A and S: It seems that there is a lot of autofluorescence with the GFP and it is not homogeneous

between the different cases (uninjured, 1.5, 2.5 and 5 days after injury). Why wasn't an antibody against LC3 used? Furthermore, the dapi in 2.5 days after injury must be increased in intensity.

Fig 1B: The increase of LC3-GFP is not clear when CQ is compares to -CQ.

Figure 2A should be at higher resolution to really appreciate the LC3-GFP staining in activated satellite cells.

Figure 2B: On what basis do the authors state that "those with greater than 3 punctae are considered to have induced autophagy flux" ? It is unclear regarding the meaning of EdU+ve and -ve in figure legend.

Figure 3B: On what basis to the authors state that "5 punctae are considered to have induced autophagy" and why is it 5 in this case and not 3 as in the figure 2B legend?

Figure 4: Miss data about QSC and ASC with for example their gene expression profile to be sure we are talking about real quiescent and activated satellite cells.

Figure 3D-E-F: The title of figure 3D and text in the manuscript: "Inhibition of activation in fiber-associated..." EdU reveals cell proliferation and not "activation" which occurs before do not agree. This should be fixed.

Figure 5A: Why do the authors examine ATP synthesis in QSC and not in ASC since authors have shown that ATP levels are higher in ASC and SC progeny ? (previous figure; low basal level of ATP in QSC).

In the paragraph "inhibition of autophagy leads to a delay in SC activation" there are some mistakes: this is not Fig.4S but Fig.3S

In the material and methods : "SC progeny isolation" authors say "The muscle were digested and sorted as described for SC isolation". Please add references or more details.

Referee #2:

Tang and Rando report that macroautophagy (autophagy) and its induction by the nutrient sensor SIRT1 are required to generate energy for quiescent muscle satellite cells (QSCs) to become activated SCs (ASCs) that proliferate, self-renewal, or differentiate. When an increased autophagic flux is inhibited or sirt1 is knocked out, QSC activation is delayed and this delay correlates with lower intracellular ATP. This delay in activation can be partially rescued by adding pyruvate to the tissue culture medium.

Overall the study is interesting but requires attention to several issues:

1. Page 5, Introduction and elsewhere in the text- The comment(s) that stem cell mitochondria are immature is misleading in that it suggests impaired or lack of functional mitochondria and the word "immature" is imprecise in this context. Even early stage pluripotent stem cells have mitochondria with functional respiratory complexes that consume oxygen, albeit at a lower level than differentiated cells, and they have an active TCA cycle. This comment requires modification to more accurately reflect the state of mitochondrial energetic and biosynthetic function beyond "immature mitochondria" in stem cells, especially for adult-type stem and precursor cells such as SCs, which are the focus of this study.

2. Page 5: The statement that stem cells "generate ATP primarily through glycolysis rather than the more productive oxidative phosphorylation" requires either a modification or broader referencing. The modification could be a change to "hematopoietic stem cells", which is the subject of the two cited references, or if the intent is more inclusive, then referencing of additional types of quiescent adult stem cells should be included to support the broadness of the statement.

3. SIRT1 is a NAD(+) dependent histone (and other protein) deacetylase with functions beyond being a nutrient (NAD+ level) sensor for cells and has been shown to control autophagic flux in several other studies including in starvation, a severe form of metabolic stress. Therefore, a SIRT1 role in energetic and biosynthetic stress of QSC activation is a reasonable idea to examine, as done here. However, as a histone/protein deacetylase, its direct role in regulating autophagy by siRNA knockdown or knockout is not revealed by the current studies and there are leading candidate pathways for this that have been reported in other contexts. How difficult would it be to determine which if any of the autophagy components/regulators are SIRT1 deacetylase targets in QSCs? Also, SIRT1 activity on gene expression connected to autophagy activation could be deciphered and, if so, does manipulation of such SIRT1 candidates affect the activation of autophagy? This information would provide a more insightful understanding for how SIRT1 regulates autophagic flux in QSCs beyond induction of autophagosome assembly or markers and cell cycle recruitment.

4. Figure S1B- are there staining panels missing compared to what is described in the Figure S1B legend?

5. Figure 2- how were fiber explants activated? Was this induced by muscle injury before excision and ex vivo culture, or does the isolation procedure induce QSC activation? This information should be briefly provided for experts and non-experts alike.

6. Figure 3, S3- Do autophagy inhibited, activation delayed fiber SCs or activated QSCs undergo a higher rate of apoptosis compared to activated WT fiber SCs and QSCs?

7. Page 9 text- change (Figs 3E; S4B-D) to (Figs 3E; S3B-D) and (Fig. S4E,F) to (Fig. S3E,F).

8. If QSC activation is delayed with CQ, 3-MA, or atg5/7 knockdown, as measured by cell cycle recruitment with EdU incorporation (Fig. 3D,E), then why would autophagy inhibited cells catch up to WT cells at 36 hours (Fig. S3E,F) instead of simply maintaining this difference at the later time point (in other words, why would EdU incorporation in autophagy inhibited QSCs catch up to WT QSCs from 24 to 36 hours instead of maintaining a constant ratio of EdU incorporation over the time course?)?

9. Page 10- change (Fig. S4G) to (Fig. S3G).

10. Page 10- Cell size changes measured by volume changes include osmotic contributions and can be an inaccurate measure of cell biosynthetic/degradative processes- assessments of cell biomass are more precise for this purpose.

11. The suggestion from data in Fig. 4 on mitotracker staining intensity increase and ATP increase is that increased mitochondrial activity is the source for increased cellular ATP. However, the coupling efficiency for mitochondria was not measured, mitotracker staining could increase through increased ATP hydrolysis in complex V boosting the H+ gradient and mitochondrial membrane potential, and increased cell ATP levels could be from increased glycolytic flux as well as from increased autophagy. The data could therefore support the authors' interpretation or alternative interpretations such as this, and experiments were not provided to distinguish between these two or other possibilities.

12. Page 11- Autophagy was blocked by atg5 and atg7 siRNAs causing a delay in ASC activation, which was partially rescued by added sodium pyruvate in the medium. How did the level of cellular ATP change during this partial rescue?

13. Although the focus of the study is on autophagy, it seems to be only part of the energetic and biosynthetic picture since autophagy inhibition by chemicals or knockdowns/knockouts does not completely eliminate QSC activation and recruitment into the cell cycle. Glycolytic flux, fatty acid oxidation, and anapleurotic fuels could have similarly important roles in activation but were not examined. Perhaps the delay in activation of QSCs with blocked autophagy is because that process occurs first, before the other processes mentioned are ramped up, or the combined processes are required and if one is inhibited it takes time for the others to reach the needed threshold for activation?

14. Figure 6C LC3B-II WB band is not convincingly less in the sirt1-/-CQ+ lane from that seen in the sirt1+/+CQ+ lane- the band seems to have run oddly.

15. Does the ATP level fail to increase in activated QSCs from sirt1-/- compared to sirt1+/+ mice?

1st Revision - authors' response 09 August 2014

Based on the constructive comments by the Referees, we have made extensive revisions of the manuscript in terms of additional experimental studies, modifications of text and figures, and reworking of Discussion points. We have addressed each Reviewer's comments, major and minor, and we acknowledge that the manuscript is substantially improved as a result. Response to each Reviewer's comments and suggestions are detailed below. Among the most significant revisions that address the comments of one or more Reviewers are as follows:

- Demonstration that inhibition of autophagy delays QSC activation and entry into the cell cycle before the G1 checkpoint (new Figure S6);
- Evidence that SIRT1 interacts with and deacetylates ATG7 in SC progeny as a mechanism for the regulation of autophagy (new Figure 8A, B);
- Evidence that SIRT1 activates autophagy through the AMPK pathway and not through the mTOR pathway in SC progeny (new Figure 8C);

Together, these additional data more firmly support the model we propose of an induction of autophagy being necessary for the energetic demands for a quiescent stem cell to activate and enter the cell cycle. We have, in addition, made revisions to the figures as per the Reviewers' suggestions, performing additional studies to provide more convincing and compelling data in support of the interpretations presented. Below we present a more detailed accounting of the revisions outlined as a point-by-point response to each comment by each Reviewer.

REFEREE #1

Major comments

1. "*A central question still remains: Does autophagy allow satellite cells to enter the cell cycle or is it the entry into the cell cycle that induces autophagy? The authors have tried to answer this question but data do not support clearly their conclusions stating that "autophagy was induced in ASCs that had not initiated DNA synthesis and hence had not entered the cell cycle". Such a conclusion cannot be made based on the data presented as it is quite difficult to accurately identify the LC3-GFP positive cells. ("3 punctae are considered to have induced autophagic flux"). Furthermore, using chloroquine after 24h, we can see that LC3 is already expressed and accumulates whereas no positive staining for EdU can be observed*."

These are important issues for consideration. Just to clarify, our observation that "*autophagy was induced in ASCs that had not initiated DNA synthesis and hence had not entered the cell cycle"* **was not the basis of our conclusion as to whether autophagy is necessary for satellite cells to enter the cell cycle. This is, as the Referee notes, only a correlation. Rather, it is specifically the studies in which *inhibition* of autophagy (e.g. either by blocking autophagy, Figure 3D, or by downregulation of *atg5/7*, Figure 3E) delays satellite cells from entering S phase that we draw the conclusion of the causal relationship. The referee's own observation that "…** *using chloroquine after 24h, we can see that LC3 is already expressed and accumulates whereas no positive staining for EdU can be*

observed" **was indeed the basis for our statement that "*autophagy was induced in ASCs that had not initiated DNA synthesis and hence had not entered the cell cycle*".**

Nevertheless, to test whether blocking autophagy inhibits cell cycle entry even more rigorously in response to the Referee's concern, we have conducted experiments to examine G1 checkpoint proteins as a measure of entry into the cell cycle after siRNA knockdown of *atg5/7*. Progression through G1/S is characterized by increasing levels of Cyclin A, Cyclin E, and phospho-Rb and decreasing levels of p27. QSCs treated with *atg5/7* siRNAs and cultured for 24 hours were found to have decreased levels of Cyclin A, Cyclin E, and phospho-Rb and increased levels of p27 relative to those in control cultures, indicating a delay in cell cycle progression at the G1/S boundary.

These experiments therefore provide corroborating data that inhibition of autophagy inhibits cell cycle entry. We present these data in Figure S6.

The concern that punctae are difficult to identify is well-taken. Indeed, fiber-associated SCs, in contrast to those grown on a culture dish, have a more compact morphology and less spread out cytoplasm for the visualization of punctae. The presence of punctae, nonetheless, is easily distinguishable from a cell that contains none. We have provided higher resolution images, now shown in Figure 2B, to better illustrate the punctae in fiber-associated cells.

2. *"Even if authors have shown that SIRT1 is involved in autophagic flux increases, there are no data related to Mitotracker and ATP production when SIRT1 is knocked down. As such, we do not know how SIRT1 regulates autophagy. Does SIRT1 induce ATP production and mitochondrial activity? Is SIRT1 activated by some gene known to be activated satellite cells as Myf5/MyoD or is it just a downstream regulator element of the AMPK/NAD+ pathway which is activated in ASCs?"*

SIRT1 has been reported to regulate autophagy in numerous systems [Lee et al, 2008; Hariharan et al, 2010; Takeda-Watenabe, et al, 2012], including those focusing on mitochondrial homeostasis and function [Jeong et al, 2013]. We had thus not sought to confirm these specific pathways per se but rather to extend the results reported by other investigators and referenced in our manuscript.

However, as Referee #2 also raised a similar point with regards the regulation of autophagy by SIRT1, we investigated the interaction between SIRT1 and components of the autophagy machinery in SC progeny. We found that SIRT1 physically interacts with ATG7, but not ATG5, and that ATG7 is hyperacetylated in the absence of SIRT1. In addition, we analyzed two pathways that likely link SIRT1 to autophagy, the mTOR and AMPK pathways. We found that SIRT1 does signal through the AMPK pathway but not the mTOR pathway during SC activation. We present these new data in Fig. 8 with additional text in the Results and Discussion sections.

The point raised by the Referee about alterations mitochondrial activity by changes in SIRT1 activity is directly relevant to the studies of our manuscript. As such, we have measured mitochondrial activity in QSCs in which SIRT1 levels were knocked out by genetic means. These results revealed that mitochondrial activity is lower in *sirt1*$^{-/-}$ QSCs than *sirt1*$^{+/-}$ QSCs, confirming that SIRT1 induces mitochondrial function. These data are presented in Fig. S10.

3. *"The critical point of this paper is to know how authors have isolated QSC from ASC and by what observation can they say "QSCs were FACS-sorted from uninjured LC3-GFP mice to a purity of 98%" or "we isolated these cells (QSC, ASC, SC) to a purity of 98% by FACS". Do they mean*

pax7+/myoD- for QSC and pax7+/myoD+ for ASC? And what marker do they use to isolate them by FACS? This is not mentioned either in the text or in the material and methods. A figure in supp data of the QSC and ASC sorted by FACS would be appreciated."

We apologize for not including more data in support of these methodologies which are in such routine use in our laboratory that we neglected to include the level of detail that is requested by the Referee and will be important for any reader who is not intimately familiar with the muscle stem cell literature. We have included references to our previously published work in which the sorting schemes are described and the yield and purity are presented [Cheung et al, 2012; Liu et al, 2013]. In addition, we have included FACS plots in the Fig. S2 to illustrate in this manuscript the methodologies employed and added additional details in the Methods section.

4. *"Authors show that LC3 is increased at the protein level, but have they looked at the mRNA level? It would be interesting to add this parameter to support IF pictures."*

The accumulation of lipidated LC3B by WB and autophagosomes by imaging LC3-GFP punctae are the most well accepted methods to measure autophagic flux. In fact, the levels of the transcripts of most *atg* genes do not change substantially upon induction of autophagy [Klionsky et al, 2008]. We therefore focused on quantifying LC3B-II and autophagosomes rather than transcript levels.

Minor comments

5. *"In the paper has been described the autophagic flux and metabolism in activated satellite cells. The authors assume to sort satellite cells using a specific protocol (cheung et al.) but no pictures of the FACS profile has been shown."*

As mentioned in response to Point #3 above, we have now included FACS plots in Figure S2 and additional details in the Methods section in order to provide more complete methodological information for anyone reading this paper and wanting to replicate any of the studies.

6. *"Fig. 1A and S: It seems that there is a lot of autofluorescence with the GFP and it is not homogeneous between the different cases (uninjured, 1.5, 2.5 and 5 days after injury). Why wasn't an antibody against LC3 used? Furthermore, the dapi in 2.5 days after injury must be increased in intensity."*

We realize that we should have clarified that the high level of GFP intensity in fibers of LC3-GFP mice is not autofluorescence. Rather, the level of LC3 increases substantially during myogenic differentiation and, thus, the high level of GFP is related to the high level of LC3 expression. We have now made this clear in the Figure Legend for Fig. 1A.

Although we did not emphasize this in the original submission, we did in fact use an antibody to LC3 to corroborate the findings with the LC3-GFP mouse. These data were included in Figure S1B. We have now emphasized this point in the manuscript.

We have, as per the Referee's suggestion, increased the intensity of the DAPI staining of the panel illustrating 2.5 days post-injury.

7. "Fig 1B: The increase of LC3-GFP is not clear when CQ is compares to -CQ."

Whereas the intensity of GFP in cells with or without CQ does not appreciably change, as pointed out by the Referee, it is not the overall level of LC3 that is important for assessing the induction of autophagic flux but rather, as noted above, the formation of autophagosomes as illustrated by the change in the number of punctae (which we highlight by arrows) that demonstrates the effects of CQ.

8. "Figure 2A should be at higher resolution to really appreciate the LC3-GFP staining in activated satellite cells."

We have included (now as a new Figure 2B), as per the Referee's suggestion, higher resolution images of the images in panel 2A in which LC3-GFP fluorescence is used to illustrate the change in LC3 distribution during the process of SC activation after injury.

9. "Figure 2B: On what basis do the authors state that "those with greater than 3 punctae are considered to have induced autophagy flux"? It is unclear regarding the meaning of EdU+ve and -ve in figure legend."

The use of a specific number of punctae as a read-out of autophagic flux is something that is standard in the field as reflected in several publications on autophagy that we have referenced in the manuscript [Mizushima et al, 2004; Klionsky et al, 2008; Mizushima et al, 2010]. We have clarified this point in the Methods and in the Legend for Figure 1B. Because of the extremely small size of QSCs and even early ASCs on fibers, we used the number of 3 punctae since this allows for direct comparisons since even a few punctae are significant early during the activation process, before which there are none.

We apologize for this oversight. We have clarified the meaning of EdU^{+ve} and EdU^{-ve} in the in the Legend for Figure 2C. Clearly, our goal is to illustrate that the induction of autophagic flux coincides with, and in fact precedes, the entry into S phase since there are clearly cells that are exhibit induction of autophagic flux prior to the incorporation of EdU. Over time, virtually all cells become positive for both.

10. "Figure 3B: On what basis to the authors state that "5 punctae are considered to have induced autophagy" and why is it 5 in this case and not 3 as in the figure 2B legend?"

As noted in Point #9 above, the use of the number of punctae is standard for assessment of autophagic flux [Mizushima et al, 2004; Klionsky et al, 2008; Mizushima et al, 2010]. For cells in culture for 12 or 24 hours, as in panel 3A, their attachment onto a flat tissue culture substrate creates a more flattened morphology and hence spreading out of the cytoplasm. This increased area of cytoplasm allows for clearer viewing of a greater number of punctae than on fiber-associated SCs, so the distinction between cells with IAF and those without is clear even with a cut-off of 5 punctae.

Nevertheless, for consistency we have gone back to re-analyze these data to use the same cut-off of 3 punctae for the cells in culture. The data are very similar and lead to the same conclusion, but the criteria are now identical for fiber-associated SCs and SC progeny grown on tissue culture dishes.

11. "Figure 4: Miss data about QSC and ASC with for example their gene expression profile to be sure we are talking about real quiescent and activated satellite cells."

As noted above in response to Points #3 and #5, we have published previously on the characteristics of the SCs obtained by FACS, and thus it was an oversight that we prepared this manuscript without considering the importance of including this information, which as the Referee points out, is central to our experimental design and interpretations. In a recent manuscript [Liu et al, 2013], we actually addressed in detail the evidence that this sorting scheme results in the purification of QSCs and ASCs based on their gene expression profiles. We have specifically included this now in the Results section and a reference to the publication that specifically addresses it.

12. *"Figure 3D-E-F: The title of figure 3D and text in the manuscript: "Inhibition of activation in fiber-associated..." EdU reveals cell proliferation and not "activation" which occurs before do not agree. This should be fixed."*

We completely agree with the referee that EdU incorporation is a marker of DNA synthesis, which is a late component of the more general process of SC activation. As we are primarily interested in the process by which a SC breaks quiescence and enters the cell cycle (which we consider to be the process of activation), we have focused on the very early changes and not, for example, on other aspects of the proliferative activity of activated SCs. To clarify, we have defined what we mean by "activation" more explicitly, and we have likewise clarified our use of EdU incorporation as a measure of the SCs having "activated" and entered the cell cycle. We are grateful to the referee for highlighting this important distinction.

13. *"Figure 5A: Why do the authors examine ATP synthesis in QSC and not in ASC since authors have shown that ATP levels are higher in ASC and SC progeny? (previous figure; low basal level of ATP in QSC)."*

We apologize that the Legend was unclear on this point. In fact, these *are* ASCs (as the Referee suggests they should be). The Legend mentioned that "FACS-sorted QSCs" were plated, but they then activate in culture and are indeed ASCs at the time of analysis. We have modified the Legend for Figure 5A so as to avoid this confusion.

14. *"In the paragraph "inhibition of autophagy leads to a delay in SC activation" there are some mistakes: this is not Fig. 4S but Fig. 3S."*

We apologize for this mistake as well, and again we thank the Referee to for bringing it to our attention. The mislabeling of figures has been corrected.

15. *"In the material and methods: "SC progeny isolation" authors say "The muscle were digested and sorted as described for SC isolation". Please add references or more details."*

We have, as per the Referee's suggestion, included more details as well as references to this methodology.

REFEREE #2

1. *"Page 5, Introduction and elsewhere in the text- The comment(s) that stem cell mitochondria are immature is misleading in that it suggests impaired or lack of functional mitochondria and the word "immature" is imprecise in this context. Even early stage pluripotent stem cells have mitochondria with functional respiratory complexes that consume oxygen, albeit at a lower level than differentiated cells, and they have an active TCA cycle. This comment requires modification*

to more accurately reflect the state of mitochondrial energetic and biosynthetic function beyond "immature mitochondria" in stem cells, especially for adult-type stem and precursor cells such as SCs, which are the focus of this study."

We appreciated the Referee's attention to detail with regard to this wording, and we are in complete agreement that our use of the term "immature" is imprecise. We have changed it to "lower mitochondrial content and activity" throughout the manuscript in order to present a more accurate description of the state of mitochondria as measured by the mitochondrial activity assays employed.

2. *"Page 5: The statement that stem cells "generate ATP primarily through glycolysis rather than the more productive oxidative phosphorylation" requires either a modification or broader referencing. The modification could be a change to "hematopoietic stem cells", which is the subject of the two cited references, or if the intent is more inclusive, then referencing of additional types of quiescent adult stem cells should be included to support the broadness of the statement."*

This point is well taken and we acknowledge that this generalization is unfounded until studied in detail in a wide variety of adult, somatic stem cells. We have modified the statement specifically to refer to the findings in hematopoietic stem cells.

3. *"SIRT1 is a NAD(+) dependent histone (and other protein) deacetylase with functions beyond being a nutrient (NAD+ level) sensor for cells and has been shown to control autophagic flux in several other studies including in starvation, a severe form of metabolic stress. Therefore, a SIRT1 role in energetic and biosynthetic stress of QSC activation is a reasonable idea to examine, as done here. However, as a histone/protein deacetylase, its direct role in regulating autophagy by siRNA knockdown or knockout is not revealed by the current studies and there are leading candidate pathways for this that have been reported in other contexts. How difficult would it be to determine which if any of the autophagy components/regulators are SIRT1 deacetylase targets in QSCs? Also, SIRT1 activity on gene expression connected to autophagy activation could be deciphered and, if so, does manipulation of such SIRT1 candidates affect the activation of autophagy? This information would provide a more insightful understanding for how SIRT1 regulates autophagic flux in QSCs beyond induction of autophagosome assembly or markers and cell cycle recruitment."*

Referee #1 also raised the issue of the role of SIRT1 in regulating autophagy (Point #2), and as we responded there we would also respond here, namely that Lee et al (2008) examined interaction of SIRT1 with three ATG proteins and their acetylation status and that others have reported the regulation of autophagy by SIRT1 [Hariharan et al, 2010; Takeda-Watenabe, et al, 2012; Jeong et al, 2013]. It was not our intention to replicate those studies. As the Referee points out, there are some candidates that have been revealed in other studies that could be examined here to confirm the alteration in autophagy genes and regulators when SIRT1 is knocked out. Toward that end, we pursued two lines of investigation to expand the mechanistic link between SIRT1 activity and autophagy.

a. First, we have examined directly the interaction between SIRT1 with ATG5 and ATG7 and the acetylation status of ATG5 and ATG7 in *sirt1*$^{+/+}$ and *sirt1*$^{-/-}$ SC progeny. These studies revealed an interaction between SIRT1 and ATG7 but not ATG5. Furthermore, higher levels of acetylated ATG7 were detected in *sirt1*$^{-/-}$ than *sirt1*$^{+/+}$ SC progeny, supporting the model that deacetylation of this protein by SIRT1 is a mechanism by which autophagy is regulated in SC progeny. We present these new data in Figure 8A, B.

b. Second, we have looked specifically at a series of candidate genes that regulate autophagy in the progeny of SCs. We looked for the effect of loss of *sirt1* on the AMPK

and mTOR pathways. In the former case, we found profound hypophosphorylation of AMPK in *sirt1*$^{-/-}$ SC progeny. In contrast, mTOR pathway targets, S6 and 4EBP, were similarly phosphorylated in both *sirt1*$^{-/-}$ and *sirt1*$^{+/+}$ cells. These data suggest that SIRT1 signals through AMPK to activate autophagy rather than through the mTOR pathway in SCs undergoing activation. The data are presented in Fig. 8C.

4. *"Figure S1B- are there staining panels missing compared to what is described in the Figure S1B legend?"*

We apologize for this mistake and we thank the Referee to for bringing it to our attention. This has now been corrected.

5. *"Figure 2- how were fiber explants activated? Was this induced by muscle injury before excision and ex vivo culture, or does the isolation procedure induce QSC activation? This information should be briefly provided for experts and non-experts alike."*

We have, as per the Referee's suggestion, included more details as well as references to this methodology.

6. *"Figure 3, S3- Do autophagy inhibited, activation delayed fiber SCs or activated QSCs undergo a higher rate of apoptosis compared to activated WT fiber SCs and QSCs?"*

We had not specifically examined this issue. In response to the Referee's questions, we have gone back to examine activated Caspase-3 staining in ASCs in which autophagy is inhibited. These studies revealed that apoptosis does not increase significantly in cells treated with *atg5/7* siRNAs, demonstrating that treatments inhibiting autophagy are not toxic to the cells. These data are now included in Fig. S5H.

7. *"Page 9 text- change (Figs 3E; S4B-D) to (Figs 3E; S3B-D) and (Fig. S4E,F) to (Fig. S3E,F)."*

We apologize for this mistake also and we again thank the Referee to for bringing it to our attention.

8. *"If QSC activation is delayed with CQ, 3-MA, or atg5/7 knockdown, as measured by cell cycle recruitment with EdU incorporation (Fig. 3D,E), then why would autophagy inhibited cells catch up to WT cells at 36 hours (Fig. S3E,F) instead of simply maintaining this difference at the later time point (in other words, why would EdU incorporation in autophagy inhibited QSCs catch up to WT QSCs from 24 to 36 hours instead of maintaining a constant ratio of EdU incorporation over the time course?)?"*

This is an astute observation and in fact relates to several points, the first two are technical and the last is more conceptual. First, the EdU incorporation in these studies is in response to continuous EdU exposure and thus represents a cumulative measure, not a snapshot. Thus, all the cells that will divide, even if delayed in their activation, will become EdU^{+ve} over time. Second, the reason that these populations do not become 100% EdU^{+ve} appears to be due to the fact that, even in control conditions, only ~75% of the cells incorporate EdU over several days in culture because a subset of cells either assumes a quiescent state (a "reserve cell" model) or differentiates without undergoing cell division.

In terms of the conceptual point, from our data, and probably related to Point #13 below, we suspect that inhibition of autophagy is not sufficient to prevent, only delay, SC entry

into the cell cycle. As such, we would expect that such cumulative measures would indeed reveal that autophagy-inhibited cells would catch up with control cells in such assays. Ultimately, a more complete picture of the energetic sources for QSCs to break quiescence and enter the cell cycle might reveal interventions that would completely block the cell cycle entry by inhibiting all major sources of the energetic requirements. However, it is not clear that such treatment would be compatible with the survival of cells even in the quiescent state.

We recognize the importance of these considerations in terms of the interpretation of the data presented. We have thus added paragraph to the Discussion to address these issues.

9. "Page 10- change (Fig. S4G) to (Fig. S3G)."

We apologize for this mistake also and we again thank the Referee to for bringing it to our attention. This has now been corrected.

10. "Page 10- Cell size changes measured by volume changes include osmotic contributions and can be an inaccurate measure of cell biosynthetic/degradative processes- assessments of cell biomass are more precise for this purpose."

We acknowledge that osmotic contributions would influence cell volume as measured in our assays. However, all populations are treated identically so that one population is not exposed to different osmotic pressures than any other. Thus, we do not expect that these changes are merely technical ones. In fact, in another project in the lab that was just recently published (Rodgers et al (2014) Nature, *510*: 393-396., we found that small changes in cell size among QSCs reflects surprising functional differences that lead to more rapid cell cycle entry. These size changes are purely correlative (as they are in our study here), but there is a tight association with a variety of functional read-outs including mitochondrial activity and levels of transcription and translation.

For the purposes of the study presented here, we present these volume changes purely as correlations and we interpret them as such. We have gone back through the manuscript to make sure that, at no point, is any further conclusion drawn and present them as interesting correlates of the entry of QSCs into the cell cycle. We have now included a reference to our other project in the lab as a potential source of comparison for assessing cell volume and its functional correlates, and we trust that we have been very careful in terms of not overstating any of the conclusions.

11. "The suggestion from data in Fig. 4 on mitotracker staining intensity increase and ATP increase is that increased mitochondrial activity is the source for increased cellular ATP. However, the coupling efficiency for mitochondria was not measured, mitotracker staining could increase through increased ATP hydrolysis in complex V boosting the H+ gradient and mitochondrial membrane potential, and increased cell ATP levels could be from increased glycolytic flux as well as from increased autophagy. The data could therefore support the authors' interpretation or alternative interpretations such as this, and experiments were not provided to distinguish between these two or other possibilities.

We thank the Referee for pointing out this caveat of our studies looking at, and correlating, ATP levels and mitochondrial activity. We had not pursued detailed metabolic studies to differentiate among these possibilities. Thus, we have carefully gone through our interpretations to add the caveats of the alternative explanations that have not been ruled out by any of the studies presented.

12. *"Page 11- Autophagy was blocked by atg5 and atg7 siRNAs causing a delay in ASC activation, which was partially rescued by added sodium pyruvate in the medium. How did the level of cellular ATP change during this partial rescue?"*

At the suggestion of the Referee, we have gone back to test the effect of exogenous sodium pyruvate on *atg5/7* siRNA treated SCs on the changes in cellular ATP. These studies revealed a significant increase in the ATP content in pyruvate-treated cultures transfected with *atg5/7* siRNA, further confirming pyruvate to be a metabolic intermediate that aids in SC activation. The data are now presented in Fig. S8.

13. *"Although the focus of the study is on autophagy, it seems to be only part of the energetic and biosynthetic picture since autophagy inhibition by chemicals or knockdowns/knockouts does not completely eliminate QSC activation and recruitment into the cell cycle. Glycolytic flux, fatty acid oxidation, and anapleurotic fuels could have similarly important roles in activation but were not examined. Perhaps the delay in activation of QSCs with blocked autophagy is because that process occurs first, before the other processes mentioned are ramped up, or the combined processes are required and if one is inhibited it takes time for the others to reach the needed threshold for activation?"*

As noted in our response to Point #8 above, this is an important conceptual point that we had not addressed in our original submission. We have now added a paragraph to the Discussion to discuss specifically the potential sources of energy that QSCs may use, besides autophagy, to generate the ATP necessary for the energetic requirements for this massive change in cell function. Indeed, the point about the delay versus the complete inhibition of autophagy suggests that there are (not surprisingly) other sources of energy, including those mentioned by the Referee. We thank the Referee for bringing this important point to the forefront and we believe that the manuscript is strengthened by inclusion of a discussion of this more general topic.

14. *"Figure 6C LC3B-II WB band is not convincingly less in the sirt1-/-CQ+ lane from that seen in the sirt1+/+CQ+ lane- the band seems to have run oddly."*

In order to provide more convincing evidence, we have measured the fold change in LC3B-II in the *sirt1*$^{-/-}$ cells and in WT cells in response to CQ treatments in a series of experiments. These analyses confirmed that autophagic flux is indeed significantly reduced in *sirt1*$^{-/-}$ cells. The data are presented in Figure 6D.

15. *"Does the ATP level fail to increase in activated QSCs from sirt1-/- compared to sirt1+/+ mice?"*

We did indeed observe a reduction in the increase in ATP levels in *sirt1*$^{-/-}$ cells compared *sirt1*$^{+/+}$ cells, but the difference did not reach statistical significance. However, we would predict that this genetic intervention that is at a point far upstream of the regulation of substrates for metabolism likely reflects redundancies in the system in terms of nutrient sensing mechanisms and effectors for generation of substrates for energy production during SC activation. It may be necessary to inhibit multiple nutrient sensing pathways to completely phenocopy the effects of inhibition of the downstream autophagic processes in terms of the all of the functional changes seen when autophagy is inhibited directly. Consistent with the observed reduction in ATP levels in *sirt1*$^{-/-}$ cells, we found that mitochondrial activity was reduced in *sirt1*$^{-/-}$ QSCs relative to control QSCs, now shown in Fig. S10.

2nd Editorial Decision 19 August 2014

I am writing to inform you that based on a second round of referee commenting I am in principle prepared to accept your fantastically revised paper.

(i) I do however enclose the final remarks from an expert referee and would like to give you the opportunity to position yourself/possibly integrate some of the remarks in a constructive manner as you see fit and/or relevant.

(ii) Please also notice that The EMBO Journal encourages submission of source data, as to increase data reliability and reproducibility. We would kindly ask for a single PDF for every figure with uncropped/unprocessed gels, at least for those supporting the main conclusions of the paper. In case of graphs/data-quantifications, we also offer to host the underlying excel sheets as supporting information. This policy is for the moment voluntary. While we would appreciate your contribution, I would not insist on providing this information.

(iii) Please provide a 2-up to 4 bullet point synopsis that emphasizes the major advance from your study. Please use short and concise terms.

(iv) Lastly, we have the opportunity to graphically feature the work. In case you would have/could easily generate a 'graphical abstract' of the size 550x150(max 400) pixels that would be much appreciated.

I hope that this message will be received as good news and would be grateful for your timely attention and response as to facilitate rapid production/publication.

I take the liberty to congratulate you already on this occasion to a fine study!

REFEREE REPORTS:

Summary

The added studies in this revised manuscript increasing the link between SIRT1 to the regulation of atg7 and AMPK pathways adds value for its role in autophagy during activation of QSCs.

Additional non-Essential Comments for the Authors

1. Why knockout atg5 in SCs in Figure S5E when the revised studies show that SIRT1 binds to atg7 and not to atg5 as the mechanism for inducing autophagy with activation of QSCs? Are floxed atg7 mouse cells not available to connect this mechanism better/more directly to SIRT1 activity?

2. Figure 6C- SIRT1, even bound to or in a complex with atg7, is not the only mechanism for inducing autophagy in QSCs, as measured by LC3B-II induction in sirt1-/- SCs induced to undergo autophagy by CQ. So while it appears necessary (page 14 top text), it is not the only pathway/mechanism inducing, cooperating with, or augmenting autophagosome assembly.

3. Is SIRT1 bound in a complex containing both atg7 and AMPKa? Are these processes linked or separate signaling arms connecting SIRT1 to autophagy induction?

4. An interesting unexplored question is how an activation stimulus (injury) given to QSCs is connected to SIRT1 activity in regulating atg7 and AMPKa pathway(s) induction of autophagy.

5. Comment / alternative interpretation- It is curious that autophagy activation drives QSCs into ASCs and proliferation and the data presented suggest this is by providing breakdown products for energy production and biosynthetic metabolites. However, as noted by the authors, FAO, increased glycolytic flux, and other nutrient sources can also increase energy production within cells. It is not exhaustively shown here whether autophagy is actually used to selectively remove protein inhibitors of cellular activation, coupled to other forms of energy and intermediate metabolite generation.

Superficially, these combined processes (alternative sources of energy production coupled to selective autophagic destruction of activation inhibitors) would seem more logical for cells about to undergo rapid expansion than would wholesale destruction of cellular components to generate additional energy and metabolites for this transition.

2nd Revision - authors' response 29 August 2014

Based on the constructive comments by the Referee, we have revised our text accordingly. We hope that our conclusions are presented with sufficient care and accuracy and that we avoid any overinterpretation by providing the caveats and limitations of our studies and alternate interpretations.

REFEREE

1. Why knockout atg5 in SCs in Figure S5E when the revised studies show that SIRT1 binds to atg7 and not to atg5 as the mechanism for inducing autophagy with activation of QSCs? Are floxed atg7 mouse cells not available to connect this mechanism better/more directly to SIRT1 activity?

We had been generating the conditional ATG5 KO based on the evidence in the literature that ATG5 would be an important component of the autophagy machinery. Whereas this appears to be upheld by our data, we discovered only later from biochemical studies of SIRT1 interaction components of the autophagy pathway that SIRT1 binds to ATG7 but not ATG5. As the knockout of SIRT1 in SCs only partially inhibits autophagy, we would thus conclude that ATG5 is an important component of the autophagy machinery that is not directly regulated by SIRT1. We have modified the text to reflect this conclusion (highlighted on page 14).

2. Figure 6C- SIRT1, even bound to or in a complex with atg7, is not the only mechanism for inducing autophagy in QSCs, as measured by LC3B-II induction in sirt1-/- SCs induced to undergo autophagy by CQ. So while it appears necessary (page 14 top text), it is not the only pathway/mechanism inducing, cooperating with, or augmenting autophagosome assembly.

As all of our data using pharmacologic and genetic interventions to inhibit autophagy result in only a partial inhibition, we certainly did not mean to imply that there are not additional mechanisms for inducing and regulating autophagy. We have modified the text on this page (highlighted on page 14) and other sections of the manuscript (highlighted on pages 20 and 21) to reflect this point.

3. Is SIRT1 bound in a complex containing both atg7 and AMPKa? Are these processes linked or separate signaling arms connecting SIRT1 to autophagy

We did not test for any complex containing SIRT1, ATG7, and AMPKα. We undertook 2 simultaneous approaches in seeking a mechanism by which SIRT1 may regulate autophagy during SC activation. In one approach, we looked for a physical interaction between SIRT1 and ATG7 and ATG5 based on the findings of Lee et al (2008). In a second approach, we looked at pathways known to regulate autophagy downstream of SIRT1. We apologize for the misleading section header on page 14, which we have now modified, and we have reworked the last paragraph of the Results (page 15) and the first full paragraph on page 20 of the Discussion to clarify these issues.

4. An interesting unexplored question is how an activation stimulus (injury) given to QSCs is connected to SIRT1 activity in regulating atg7 and AMPKa pathway(s) induction of autophagy.

We had alluded to this issue in our Discussion but we have now added additional text (highlighted on page 19) directly stating the hypothesis that the activation out of quiescence is a process akin to nutrient deprivation, and in that sense would be the signal that leads to the activation of SIRT1.

5. Comment / alternative interpretation- It is curious that autophagy activation drives QSCs into ASCs and proliferation and the data presented suggest this is by providing breakdown products for energy production and biosynthetic metabolites. However, as noted by the authors, FAO, increased glycolytic flux, and other nutrient sources can also increase energy production within cells. It is not exhaustively shown here whether autophagy is actually used to selectively remove protein inhibitors of cellular activation, coupled to other forms of energy and intermediate metabolite generation. Superficially, these combined processes (alternative sources of energy production coupled to selective autophagic destruction of activation inhibitors) would seem more logical for cells about to undergo rapid expansion than would wholesale destruction of cellular components to generate additional energy and metabolites for this transition.

This point is well taken. We had based much of our conclusions on the rescue experiments in which an exogenous metabolite could partially rescue the activation delay imposed by the inhibition of autophagy. We completely agree that we have not ruled out this alternate explanation that the selective degradation of inhibitors of the activation process could account for the importance of the induction of autophagy in the process of SC activation. We have included this caveat (highlighted on page 16) now in the Discussion.