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p110α of PI3K is Necessary and Sufficient for Quiescence Exit in Adult Muscle Satellite Cells

Gang Wang, Han Zhu, Chenghao Situ, Lifang Han, Youqian Yu, Tom H. Cheung, Kai Liu, and Zhenguo Wu

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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 25th October 2017

Thank you for the submission of your manuscript (EMBOJ-2017-98239) to The EMBO Journal. Your study has been sent to three referees, and we have received reports from all of them, which I copy below.

As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of concerns that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In particular, referee #3 points out that your claims on a role of PI3K/p110a in satellite cell quiescence exit are not sufficiently well supported by the current data and thus states the need for you to corroborate your analysis of YFP-positive cells derived through lineage tracing. This referee also asks you to consolidate the link between mTORC1 and Jun. Referees #1 and #2 agree in that the contribution of p110a and Jun to the muscle stem cell quiescent state should be analysed in greater detail. In addition, the referees list a number of technical issues on assays used and controls made, that need to be addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and we are the in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. I agree with the referees that the manuscript would strongly benefit from more refined characterization of the downstream signalling.

REFEREE REPORTS

Referee #1:

Wu et al in the manuscript titled "p110α of PI3K Is Indispensable for Quiescence Exit and the Cell Cycle Reentry in Adult Muscle Satellite Cells" described a previously undocumented function of p110α subunit of the PI3K in satellite cell activation and subsequent quiescence state exiting. Although both MTORC1 / FoxOs signaling pathways in satellite cells biology have been extensively studied, this manuscript is the first-time investigation of their upstream kinase (specifically the PI3K catalytic subunit p110α) function in such biological context using in vivo mouse models. In particular, the study reveals molecular mechanism of how PI3K signaling regulating quiescence exit through mTORC1-AKT and/or FOXO signaling. The manuscript is well written and offers high level of novelty. Several questions need to be addressed and clarified before publishing.
Major:
1. The author claimed that p110α regulates satellite cell quiescence exit (see their title). However, I feel that the data presented here cannot fully distinct the effects of PI3K on quiescence exit vs activated cell proliferation and downstream differentiation. Particularly, the severe muscle regeneration defects in p110α iKO mice likely due to the combinatory effects on satellite cell activation and proliferation. Can the author clarify what is the difference between quiescence exiting and satellite cell activation? How does the data in the manuscript support p110α role specifically in quiescence exit but not in mediating cell activation? Several reports have indicated downstream effectors of PI3K kinase signaling pathway (such as ERk signaling) also actively participate in satellite cell activation (for example in Perez-Ruiz A, Zammit PS 2007 and von Maltzahn J, Rudnicki MA 2011). As a matter of fact, most experiment results are based on culture of isolated satellite cells or acute muscle injury both of which rapidly activate satellite cells, thus the data presented cannot formally exclude the intrinsic function of PI3K-mTOR pathway in directly regulating myoblast activation, proliferation and subsequently differentiation. It would greatly benefit readers if the authors can elaborate on this point in their discussion.
2. It is interesting that the authors observed an almost completely depletion of Pax7 protein but not RNA level. In line of recent reports that pax7 protein level is actively regulated by phosphorylation and methylation by variety of kinases and methyltransferases, the author should address if p110α directly functions upstream of pax7 phosphorylation? And how such negative effects on pax7 protein level affects satellite cell identity maintaining?
3. It is interesting to see protein level of Jun, Fos and Fosb in iKO and idKO comparing to control (specifically in Figure 2F and 4D). This would provide mechanistic insight of how PI3K regulate mitogen activated signaling in satellite cells in vivo. In addition, MAPK, ERK and p38 signaling should also be examined in FACS sorted satellite cells from iKO and idKO muscles. To test the possibility that PI3K may regulate cell proliferation through activating mitogen signaling pathway.

Minor issues:
1) Figure 1B and D, quantification of regenerating myofibers should base on the staining of eMHC and not based on H&E sections. It is important that the authors present data to demonstrate that there is similar extend of CTX induced damages in wt and iKO muscle. If possible, the authors should present a panel of images covering the whole timeline of muscle regeneration process (from day 1 to 3 weeks).
2) Fig 2D, Vcam+ gating does not seem to be assigned by cell population. The authors may need to provide the parental gating information and flow cytometry data to support the gating rationale. Fig2F, WB probing of p110α is needed to demonstrate the knockout efficiency of such cells. The Myf5 blot images is not clear as the last band is cutoff. Did author also probe for myogenin or MHC to exclude the possibility of premature differentiation of p110α KO satellite cells?
3) Figure 3D, why was the gate for pyronin Y cut through the cell population in the middle? What would be the percentage if gate is set at 100?
4) Fig 5B enrichment plot resolution is too low to be read.
5) Fig6E, myoD staining is not clear, and MusC cannot be clearly identified. Also, the wild type MuSCs morphology does not looks like normal fresh isolated MuSCs. Adding Pax7 staining would also help to identify and confirm previous results of P100a on pax7 protein levels in MuSCs.

Referee #2:

In this manuscript the authors show that genetic deficiency of the catalytic sub-unit of PI3K - p110alpha - impairs satellite cell activation upon muscle injury. This defect could be rescued by genetic re-activation of mTORC1, c-Jun overexpression or FoxOs knockdown. Moreover, constitutive activation of PI3K promotes spontaneous exit from quiescent in satellite cells from unperturbed muscles.
Overall, this study provides evidence for a role of PI3K in regulating the decision of satellite cell to break quiescence and undergo activation. The evidence is based on a great combination of genetic and transcriptomic approaches that revealed a number of novel key regulators, including the catalytic sub-unit of PI3K (p110alpha) and previously unrecognized downstream effectors, such as Jun.
Interestingly, this network could to be implicated as general regulator of quiescence in many cell types, as the authors pointed in the discussion.
The experiments are logically connected and straightforward, and the data are convincing.

Below are some minor concerns that the authors may want to address to further improve the quality of the manuscript.

1) Figure 1. Muscle regeneration is assessed by central nucleation, which is a vague morphological sign of regeneration. A more rigorous assessment should be provided by immunostaining for embryonal MyHC at different time points following injury - 3,7 and 14 days.
2) How does PI3K signaling affect Pax7 protein levels? The authors should be at least attempt to consolidate this evidence by experiments in cultured cells, as they allude to a reasonable hypothesis (autophagy-mediated control of Pax7 degradation)
3) Jun overexpression experiments. : It is not specified what Jun variant has been used in the experiment. It would actually be informative to compare cJun, JunB and JunD, as they have been shown to exert different effects on skeletal myogenesis
4) The contribution of Jun in PI3K-mediated regulation of satellite cell quiescence is unclear, as it is the connection with other components of this network. The authors provide initial evidence that Jun and FoxO3 pathways are connected, by showing changes in FoxO3 sub-nuclear localization changes in response to Jun activation in p110alpha null satellite cells. It would be interesting to test the effect of Jun mutants - e.g. those that do not respond to JNK signaling, by mutation of phosphorylated serines.

Referee #3:

In this report, Wang and colleagues explore the function of p110α, a catalytic subunit of phosphatidylinositol 3-kinase (PI3K), in adult muscle satellite cells (MuSCs). The authors nicely show that upon conditional ablation of p110α, MuSCs are unable to activate and fail to regenerate skeletal muscle following injury. Moreover the authors analyze the functional relationship of p110α with downstream pathways mTORC1, Jun, or FoxOs and show partial rescue. Finally the authors show that activation of PI3K in adult MuSCs leads to their gradual loss. The findings are very relevant for fundamental knowledge of MuSCs activation and homeostasis.

A major point is that by using a YFP lineage tracing the authors show that the YFP populations is not significantly different between p110a control and KO, while Pax7 is no longer expressed. The authors use this YFP+ fraction for all subsequent experiments as freshly isolated or quiescent MuSCs. This is an issue as this population if far from being MuSCs (90% Pax7- in resting state). The manuscript aim to describe a critical role of p110a in exit-of-quiescence but loss of p110a impacts MuSCs biology. How to describe the exit-of-quiescence of something so altered that we cannot know if it is quiescent anymore? By using YFP+ cells for every subsequent experiment the authors fails to ask the appropriate biological question and miss the most exciting aspect of their phenotype. It is therefore absolutely required to further characterize YFP+/PAX7- cells in order understand the phenotypes analyzed. RNAseq would be for instance required in this context.

Other points :

1) In Figure 2, the authors disrupt the expression of p110a in Pax7+ cells and observe a drastic loss of PAX7 in tissue sections and isolated fibers. Yet this phenotype is contradictory with the claim that these cells are in a more « deep » quiescence state as Pax7 is a marker of MuSCs quiescence.

2) In figure 5 the authors observe similar levels of Pax7 mRNA in p110aKO cells versus control which is highly surprising given the protein results. How do the authors explain this observation?

3) In Figure 3 the authors should not consider the cells WT MuSCs if they use the heterozygote control. They perform the comparison of EdU incorporation on isolated fibers from control and p110aKO (Figure 3C) using a picture of single fibers without Pax7 on the KO, which is an issue for the analysis. The authors should use YFP instead of PAX7 as done in other experiments.

4) Figure 3G, the authors describe cells shape only with FACS plots while a staining with a membrane marker in vitro or in vivo would be more informative. Alternatively, cell diameter in brightfield could be analyzed as done in Rodgers et al. 2014.

5) The link mTORC1 -> Jun is unclear. The fact tha JUN is upregulated in RAPTOR inactivation does not mean that mTORC1 is a direct « transcriptional regulator » of JUN, RAPTOR is an inhibitor of mTORC1 but could also act independently and the JUN transcriptional modification could be totally correlative.

6) The authors describe an AP-1 family upregulation at the transcript level in cells isolated from p110a versus control. These data needs to be revisited in light of recent findings (Van den Brink et al. 2017) showing AP-1 as a family highly induced by the dissociation procedure.

7) Following this comment, the authors should be cautious regarding the identity of isolated "quiescent" MuSCs (figure S1 for example). They state in the manuscript that the isolation procedure could impact these cells biology and the aforementioned reference proves that. When the observed phenomenon are related to RTK signaling and phosphorylation cascades, given the kinetics of such signaling events, researchers should be cautious about isolating cells to study their properties and maybe focus on in vivo experiments.

8) Figure 2A : 3 random field per replicate with the reported values is no more than 30 cells counted per replicate, on the low side for strong confidence.

9) Figure 2D : photo quality is low and there are no statistics on the % of VCAM+ cells

10) « In addition, MyoD is also an established marker to differentiate ASCs from QSCs as MyoD is only expressed in ASCs but not QSCs (Sambasivan and Tajbakhsh, 2015; Yin et al., 2013). » In the light of this sentence, how can they explain the presence of MYOD protein in QSCs measured by western blot in Figure 2 ?

11) Figure 4 : The authors re-induce mTor (by disrupting its inhibitor RAPTOR), a pro-activation factor that have been previously shown to be involved in quiescence exit (Rodgers et al. 2014) and they observe a rescue of PAX7 levels. How does this fits with the observed phenotype ? PAX7 being a quiescence maker, if p110a activates mTORC1 to exit quiescence as the authors states, mTORC1 levels rescue should diminish PAX7 levels.

12) Figure 7 : the authors observe a nice phenotype. However, they emits an hypothesis regarding the presence of EdU+/YFP- cells inside the myofibers. If there is an increase fusion of YFP+ cells, the authors should observe YFP+ myofibers, which will actually be a proof of concept of their phenotype. This is routinely observed when knocking off a quiescence factor (Bjornson et al.2012 ; Mourikis et al. 2012) or when YFP+ MuSCs are transplanted inside a WT muscle. In the latter case, the observation of fluorescent myofiber is actually the read-out of transplant efficiency. Also, if the MuSCs fused with the myofiber the authors would observe central nuclei, not lateral myonuclei requiring dystrophin staining to be shown inside myofibers.

1st Revision - authors' response 23rd January 2018

Referee #1:

Wu et al in the manuscript titled "p110α of PI3K Is Indispensable for Quiescence Exit and the Cell Cycle Reentry in Adult Muscle Satellite Cells" described a previously undocumented function of p110α subunit of the PI3K in satellite cell activation and subsequent quiescence state exiting. Although both MTORC1 / FoxOs signaling pathways in satellite cells biology have been extensively studied, this manuscript is the first-time investigation of their upstream kinase (specifically the PI3K catalytic subunit p110α) function in such biological context using in vivo mouse models. In particular, the study reveals molecular mechanism of how PI3K signaling regulating quiescence exit through mTORC1-AKT and/or FOXO signaling. The manuscript is well written and offers high level of novelty. Several questions need to be addressed and clarified before publishing.
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Our reply: I am sorry that we did not make our points clearer in the manuscript. Broadly speaking, "quiescence exit" and "satellite cell activation" describe the same process, which is the transition between the "quiescent satellite cells" (or QSCs) and the "activated satellite cells (or ASCs), and can often be used interchangeably. In my personal view based on our work here, I think the two terms describe two ends of the same process described above. "Satellite cell activation" specifically refers to the "**late**" point (i.e., close to ASC) when MuSCs start to express MyoD protein, which normally peaks at 24 hrs after the injury (Yin et al., Physiol Rev. 2013. **93**:23-67). By this time, MuSCs already increase in size and express many other RNAs required for the cell cycle re-entry (e.g., those encoding cyclins, Cdks, and E2Fs). By contrast, "quiescence exit" refers to the "**early**" point (close to QSC). We showed here that there is a PI3K-dependent "checkpoint" associated with "quiescence exit": when MuSCs pass this point, they can proceed to become fully activated and re-enter the cell cycle. If they fail to pass this point, they will remain in the quiescent state. As to the two references mentioned by the reviewer, they are quite different from our studies in that one describes the signaling pathways that regulate *Myf5* gene expression in myonuclei of differentiated myofibers (Perez-Ruiz et al., 2007) and the other describes the role of Wnt7a on myofiber hypertrophy. As to the last point raised by the reviewer, we fully agree with the reviewer that the data from our current study do not exclude the possibility that PI3K can also regulate other aspects of MuSC behavior (e.g., proliferation or differentiation) as the p110a-null MuSCs are already arrested in the quiescent state, which prevents us from further examining its impact on the subsequent steps (e.g., MuSC proliferation, differentiation, or self-renewal). As we already mentioned in the "Discussion" (the last sentence of the 1st paragraph on p14), it requires the use of additional Cre driver lines (e.g., MyoD-CreER or myogenin-CreER) to address such issues.

2. It is interesting that the authors observed an almost completely depletion of Pax7 protein but not RNA level. In line of recent reports that pax7 protein level is actively regulated by phosphorylation and methylation by variety of kinases and methyltransferases, the author should address if p110α directly functions upstream of pax7 phosphorylation? And how such negative effects on pax7 protein level affects satellite cell identity maintaining?

Our reply: In wild-type MuSCs, it is well established that the mRNA and protein levels of Pax7 are high in the quiescent MuSCs but low in the activated and proliferating MuSCs (Mourikis and Tajbakhsh, 2014. BMC Dev Biol. **14**: 2). However, in p110a-null MuSCs, even though the Pax7 protein levels were very low, they failed to exit quiescence. Thus, although our observation of the downregulation of Pax7 protein but not its mRNA in p110a-null MuSCs is interesting, the current knowledge could not explain why the mutant cells failed to exit quiescence. As to the potential connection of p110a to Pax7 phosphorylation, we felt it is premature to pursue this line now based on the following reasons: firstly, it remains to be confirmed whether the endogenous Pax7 in MuSCs is indeed phosphorylated by casein kinase 2 (CK2) or not, as such phosphorylation was only detected in the overexpressed Pax7 in immortalized C2C12 myoblast cell lines (Gonzalez et al., PLoS ONE, 2016. **11**: e0154919). Secondly, CK2 is not a known component directly functioning in the canonical PI3K/Akt/mTORC1 pathway. Even if there is a crosstalk between PI3K and CK2, most likely, it is indirect. Thus, although it would be interesting to study whether and how the PI3K signaling impacts on the post-translational modification status of Pax7, we feel that it is beyond the scope of our current study.

3. It is interesting to see protein level of Jun, Fos and Fosb in iKO and idKO comparing to control (specifically in Figure 2F and 4D). This would provide mechanistic insight of how PI3K regulate mitogen activated signaling in satellite cells in vivo. In addition, MAPK, ERK and p38 signaling should also be examined in FACS sorted satellite cells from iKO and idKO muscles. To test the possibility that PI3K may regulate cell proliferation through activating mitogen signaling pathway.

Our reply: Indeed, in our original manuscript, we only examined the mRNA levels of Jun and Fos, but not their protein levels. Therefore, we followed the reviewer's advice and probed for the protein levels of Jun and Fos in the control and p110a-null MuSCs. Consistently, the protein levels of Jun were downregulated in freshly-isolated p110a-null MuSCs (revised Fig.5C), but that of Fos in freshly-isolated MuSCs was too low to be detected. This result also explains why re-expression of Jun but not Fos in p110a-null MuSCs could rescue their defects in quiescence exit and the cell cycle reentry. When we induced *p110a* deletion in cultured MuSCs with 4-hydoxyltamoxifen, we found that Fos protein was also downregulated in p110a-null myoblasts (Fig.EV7). Although it would be interesting to study potential crosstalk between the PI3K pathway and other signaling pathways including various MAPK pathways, the main objective of our current study is to firmly establish a role for the PI3K/mTORC1/FoxOs pathway in regulating quiescence exit. Thus, we think it would be better to address such crosstalk in a future project. Nevertheless, as an initial effort, we followed the reviewer's advice and examined the protein levels of the active (i.e., the dually-phosphorylated form) and total MAPKs in the control and iKO cells (see Fig A. below). We found that the protein levels of the total and active ERK and p38 MAPK were not obviously affected, but that of active JNK was reduced in p110a-null MuSCs. It is unclear why this occurs and remains to be investigated in the future.

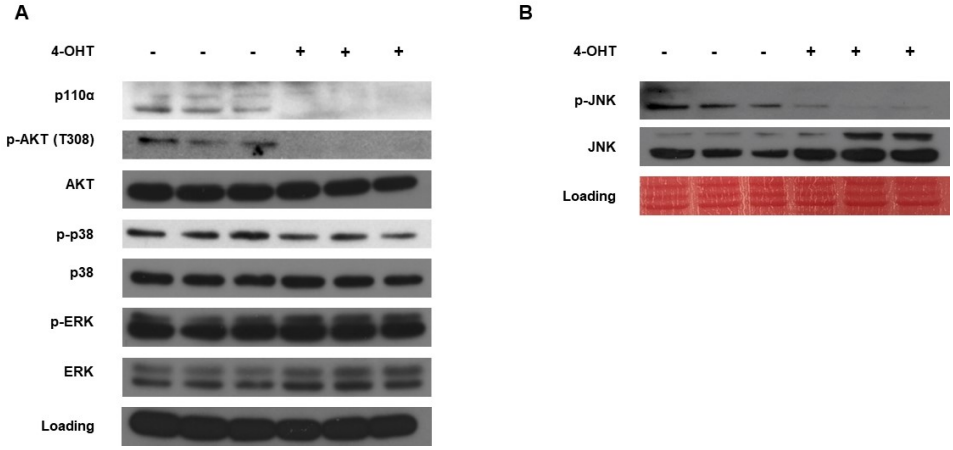

Figure A. FACS-isolated MuSCs from iKO mice (without prior Tmx treatment) were cultured for 2 days followed by 2 days of 4-OHT treatment before harvest. Whole cell lysates were subjected to western blotting. (A) Probing for ERK and p38. (B) Probing for JNK.

Minor issues:
1) Figure 1B and D, quantification of regenerating myofibers should base on the staining of eMHC

and not based on H&E sections. It is important that the authors present data to demonstrate that there is similar extend of CTX induced damages in wt and iKO muscle. If possible, the authors should present a panel of images covering the whole timeline of muscle regeneration process (from day 1 to 3 weeks).

Our reply: We followed the advice of the reviewer and examined muscle regeneration of the control and p110a-null iKO mice at more time points (i.e., days 2, 5, 7, 14, and 28). The whole panel of images covering different time points of regeneration is now presented in the revised Fig.1A. As eMHC is only detectable in newly-formed myotubes during the early phase of regeneration (typically between days 4-6 after injury), thus, we subjected tibialis anterior (TA) muscle sections from the control and iKO mice after 5 days of injury to immunostaining for eMHC and quantified the extent of regeneration based on eMHC staining. While the eMHC+ nascent myotubes were abundantly present in TA muscle sections of the control mice, they were barely detectable in that of the p110a-null iKO mice (revised Fig.1B, C).

2) Fig 2D, Vcam+ gating does not seem to be assigned by cell population. The authors may need to provide the parental gating information and flow cytometry data to support the gating rationale. Fig2F, WB probing of p110α is needed to demonstrate the knockout efficiency of such cells. The Myf5 blot images is not clear as the last band is cutoff. Did author also probe for myogenin or MHC to exclude the possibility of premature differentiation of p110α KO satellite cells?

Our reply: In the revised Fig.2D, we provided new FACS plots showing the percentage of cells (i.e., MuSCs) specifically gated by anti-Vcam1 staining among all the mononucleated $CD31^-/CD45^-/Sca1^-$ cells from the control and p110a-null mice. As a negative control, in the absence of anti-Vcam1 staining, very few cells were gated in the same region (see Fig B below). This sorting protocol for isolation of mouse MuSC was developed by Tom Rando's group (Liu, et al. *Cell Rep*. 2013), which is very robust and reproducible. We routinely get more than 300,000 MuSCs from one mouse following such a protocol. Consistently, more than 90% of such $Vcam1^+/Sca1^-$ cells are $Pax7^+$ MuSCs. In the revised Fig.2F, we repeated the experiment and probed for p110a, MyoD, and Myf5. We showed that p110a was efficiently deleted in culture. Importantly, we showed that the protein levels of Pax7 were obviously reduced in p110a-null cells. MyoD protein levels were also reduced in mutant cells, but Myf5 protein levels remained unchanged. We did not probe for myogenin or MHC in the mutant MuSCs, as p110a KO cells did not have premature differentiation based on the fact that such mutant cells were arrested in the quiescent state and that the transcriptome of the mutant cells did not even contain meaningful levels of mRNA transcripts for either *myogenin* or *Mhc*.

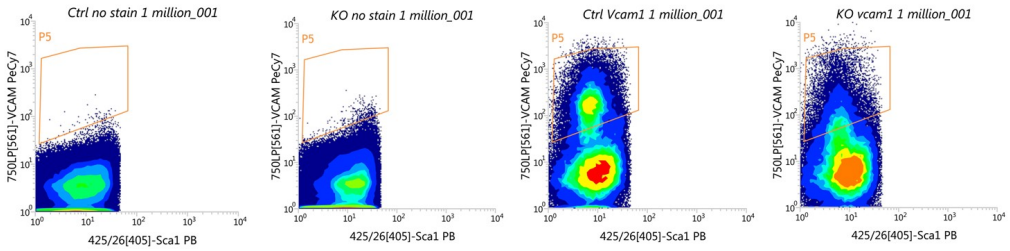

Figure B. Representative FACS plots showing the percentage of $Vcam1^+$ MuSCs (boxed areas) among the $CD31^-CD45^-Sca1^-$ cell populations from uninjured muscles of the Ctrl and iKO mice

3) Figure 3D, why was the gate for pyronin Y cut through the cell population in the middle? What would be the percentage if gate is set at 100?

Our reply: We think the reviewer meant "Fig.3G" here instead of "Fig.3D". The gate for pyronin Y was set mainly based on the plots of the freshly-sorted MuSCs from uninjured muscles (Fig.3G, top panels). When the gate for pyronin Y was set at 30, most (i.e., ~97%) of the p110a-null MuSCs fell in the lower left quadrant (i.e., the G_0 state), while a lower percentage (i.e., 69%) of wild-type MuSCs were in the G_0 state due to partial activation of MuSCs during the isolation process (Brink et al., Nat Methods. 2017; van Velthoven et al., Cell Rep. 2017; Machado et al., Cell Rep. 2017). The bottom panels represent the FACS plots of activated MuSCs at 36 hrs after injury. By this time, most wild-type MuSCs already became activated and some started to re-enter the cell cycle. Here, the gate for pyronin Y was set at 50 in order to gate for those MuSCs with very low RNA contents that were comparable to the quiescent MuSCs from uninjured muscles. If the gate for pyronin Y were set at 100, then the percentage of G_0 cells would have been inappropriately larger for both control and mutant MuSCs.

4) Fig 5B enrichment plot resolution is too low to be read.
Our reply: This is mainly due to the fact that the figure was converted to the low-resolution pdf format for the review purpose. The figure in the original tiff format had much higher resolution. We re-made the figure with higher resolution to make it clearer (see revised Fig.5B).

5) Fig6E, myoD staining is not clear, and MusC cannot be clearly identified. Also, the wild type MuSCs morphology does not looks like normal fresh isolated MuSCs. Adding Pax7 staining would also help to identify and confirm previous results of P100a on pax7 protein levels in MuSCs.

Our reply: Indeed, the cells in Fig.6E were not freshly isolated MuSCs. Instead, they were cultured for 36 hrs to make sure that FoxO3 was cytoplasmic or pan-cellular in most wild type MuSCs. As the p110a-null MuSCs were arrested in quiescence, FoxO3a in these mutant MuSCs remained mostly nuclear. However, such cultured cells were not suitable for Pax7 staining anymore, as the protein levels of Pax7 were already very low even in wild type MuSCs by this time. That is why we stained for MyoD instead in the wild-type cells. To make the MyoD staining signals clearer, we presented a separate set of images with the MyoD staining only (revised Fig.6E, middle panels). It is noteworthy that the MyoD signal was barely detectable in p110-null cells, consistent with the fact that the mutant cells were arrested in quiescence.

Referee #2:

In this manuscript the authors show that genetic deficiency of the catalytic sub-unit of PI3K - p110alpha - impairs satellite cell activation upon muscle injury. This defect could be rescued by genetic re-activation of mTORC1, c-Jun overexpression or FoxOs knockdown. Moreover, constitutive activation of PI3K promotes spontaneous exit from quiescent in satellite cells from unperturbed muscles.
Overall, this study provides evidence for a role of PI3K in regulating the decision of satellite cell to break quiescence and undergo activation. The evidence is based on a great combination of genetic and transcriptomic approaches that revealed a number of novel key regulators, including the catalytic sub-unit of PI3K (p110alpha) and previously unrecognized downstream effectors, such as Jun.
Interestingly, this network could to be implicated as general regulator of quiescence in many cell types, as the authors pointed in the discussion.
The experiments are logically connected and straightforward, and the data are convincing.

Below are some minor concerns that the authors may want to address to further improve the quality of the manuscript.

1) Figure 1. Muscle regeneration is assessed by central nucleation, which is a vague morphological sign of regeneration. A more rigorous assessment should be provided by immunostaining for embryonal MyHC at different time points following injury - 3,7 and 14 days.
Our reply: This comment is similar to that (i.e., minor issue No.1) raised by reviewer 1. We already repeated this experiment following the advice from both reviewers. Please refer to our reply above and the revised Fig.1A.
2) How does PI3K signaling affect Pax7 protein levels? The authors should be at least attempt to consolidate this evidence by experiments in cultured cells, as they allude to a reasonable hypothesis (autophagy-mediated control of Pax7 degradation)
Our reply: We already performed some preliminary experiments using FACS-sorted MuSCs in culture. Our data showed that the reduction in Pax7 protein levels in p110a-null MuSCs was mainly caused by enhanced autophagy (which was known to be induced by inhibition or loss of class I PI3K), as inhibition of autophagy by either an autophagy inhibitor (i.e., LY294002), or an Atg5-siRNA, all led to partial restoration of Pax7 protein levels in p110a-null MuSCs (Fig.EV3)..

3) Jun overexpression experiments. : It is not specified what Jun variant has been used in the experiment. It would actually be informative to compare cJun, JunB and JunD, as they have been shown to exert different effects on skeletal myogenesis

Our reply: In fact, Jun is now used by NCBI as a standard nomenclature for cJun. Based on our RNA-seq data, only the mRNA levels of Jun, but not that of JunB or JunD, were found to be obviously downregulated in the mutant MuSCs. Thus, to specifically correct the defect in the mutant MuSCs, we only performed the rescue experiments by re-expressing Jun.

4) The contribution of Jun in PI3K-mediated regulation of satellite cell quiescence is unclear, as it is the connection with other components of this network. The authors provide initial evidence that Jun and FoxO3 pathways are connected, by showing changes in FoxO3 sub-nuclear localization changes in response to Jun activation in p110alpha null satellite cells. It would be interesting to test the effect of Jun mutants - e.g. those that do not respond to JNK signaling, by mutation of phosphorylated serines.

Our reply: In our current manuscript, we demonstrated that *Jun* represents a key transcriptional target of the PI3K/mTORC1 axis in MuSCs. Consistently, several recent papers also found that Jun mRNA is rapidly upregulated in "freshly-ioslated" MuSCs induced by the isolation process (van den Brink, et al., Nat Methods. 2017. 14: 935-6; van Velthoven et al., Cell Rep. 2017; Machado et al., Cell Rep. 2017). Such findings are consistent with the established role of Jun in cell proliferation and the cell cycle re-entry in serum-starved cells that were re-stimulated with serum (Shaulian and Karin, 2001. Oncogene. **20**: 2390-400). It remains unclear whether the JNK pathway also regulates quiescence exit by phosphorylating the Jun protein in MuSCs. Our initial analysis showed that the active JNK was also reduced in p110a-null MuSCs (see Fig A above). This suggests that there is crosstalk between the PI3K/Akt/mTORC1 pathway and the JNK pathway. However, the details of such crosstalk remain unclear and need to be further addressed in a future project.

Referee #3:

In this report, Wang and colleagues explore the function of p110α, a catalytic subunit of phosphatidylinositol 3-kinase (PI3K), in adult muscle satellite cells (MuSCs). The authors nicely show that upon conditional ablation of p110α, MuSCs are unable to activate and fail to regenerate skeletal muscle following injury. Moreover the authors analyze the functional relationship of p110α with downstream pathways mTORC1, Jun, or FoxOs and show partial rescue. Finally the authors show that activation of PI3K in adult MuSCs leads to their gradual loss. The findings are very relevant for fundamental knowledge of MuSCs activation and homeostasis.

A major point is that by using a YFP lineage tracing the authors show that the YFP populations is not significantly different between p110a control and KO, while Pax7 is no longer expressed. The authors use this YFP+ fraction for all subsequent experiments as freshly isolated or quiescent MuSCs. This is an issue as this population if far from being MuSCs (90% Pax7- in resting state). The manuscript aim to describe a critical role of p110a in exit-of-quiescence but loss of p110a impacts MuSCs biology. How to describe the exit-of-quiescence of something so altered that we cannot know if it is quiescent anymore? By using YFP+ cells for every subsequent experiment the authors fails to ask the appropriate biological question and miss the most exciting aspect of their phenotype. It is therefore absolutely required to further characterize YFP+/PAX7- cells in order understand the phenotypes analyzed. RNAseq would be for instance required in this context.

Our reply: Although Pax7 is arguably the most prominent molecular marker for MuSCs, it is not absolutely required to specify the fate or identity of MuSCs. The number of the Pax7-null MuSCs was similar to that of the wild-type MuSCs in new-born pups (Oustanina, et al., EMBO J. 2004. 23: 3430-9; Relaix et al., J Cell Biol. 2006. **172**: 91-102). When Pax7 was deleted in adult mice, the Pax7-null MuSCs were still present in the mutant mice in the first few weeks but their total number slowly declined (Gunther et al., Cell Stem Cell. 2013. **13**: 590-601). This result indicates that Pax7 regulates the long-term maintenance, instead of the identity, of the adult MuSCs. Moreover, our findings that deletion of *Tsc1* in YFP^{+} p110a-null MuSCs partially restored the functions of MuSCs (Fig.4) further indicates that the satellite cell identity of such p110a-null $YFP^{+}/Pax7^{low}$ cells did not change. Due to the reduction (instead of complete absence) in Pax7 protein levels, the levels of some additional markers of MuSCs (e.g., Vcam1) also dropped (Fig 2D). This indicates that we cannot use our routine sorting scheme to isolate the mutant MuSCs (for normal MuSCs without YFP, we routinely sort them based on Vcam1 as shown in Fig.2D). Thus, in our opinion, the use of an YFP "tracer" that is expressed in MuSCs independently of the Pax7 expression status is the best

way to ensure that we were comparing the control MuSCs with the mutant 'MuSCs" (i.e., comparing apple to apple). As suggested by the reviewer, we already obtained the transcriptome profile of such $YFP^{+}/Pax7^{low}$ cells by RNA-seq and compared it to that of the control MuSCs ($YFP^{+}/Pax7^{+}$) (see summary in Fig.5). We have deposited our RNA-seq data to GEO (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109472, Accession number: GSE109472, passcode: wvsjegsqhrerxcp), which can now be accessed and viewed by reviewers. We will make such data publicly available upon the acceptance of our manuscript.
Other points :

1) In Figure 2, the authors disrupt the expression of p110a in Pax7+ cells and observe a drastic loss of PAX7 in tissue sections and isolated fibers. Yet this phenotype is contradictory with the claim that these cells are in a more « deep » quiescence state as Pax7 is a marker of MuSCs quiescence.
Our reply: Indeed, as we already indicated in our response above (see our response to major point 2 from reviewer 1), Pax7 mRNA and protein levels are high in the quiescent MuSCs but low in activated and proliferating MuSCs. However, even though Pax7 protein levels were obviously downregulated in p110a-null MuSCs, such mutant cells were unable to exit from quiescence. This further echoes our previous point above that the levels of Pax7 protein alone does not determine whether cells will be in the quiescent state or not.

2) In figure 5 the authors observe similar levels of Pax7 mRNA in p110aKO cells versus control which is highly surprising given the protein results. How do the authors explain this observation?
Our reply: As we described in our "Discussion" (last sentence on p14) and our response above (see our response to point 2 made by reviewer 2), p110a does not affect the transcription of the *Pax7* gene, but does regulate its protein turnover through autophagy (Fig EV3).

3) In Figure 3 the authors should not consider the cells WT MuSCs if they use the heterozygote control. They perform the comparison of EdU incorporation on isolated fibers from control and p110aKO (Figure 3C) using a picture of single fibers without Pax7 on the KO, which is an issue for the analysis. The authors should use YFP instead of PAX7 as done in other experiments.
Our reply: Following the advice of the reviewer, we now used more accurate terms in the revised text and figures to describe whether the control mice were "heterozygous" or "wild-type". For Fig.3C, although we only showed the EdU incorporation in MuSCs of one myofiber, we actually counted more than 20 such myofibers per mouse for three pairs of mice and presented the quantitative data in Fig.3D. We agree with the reviewer that it would be better to stain for YFP in such experiments considering the low expression of Pax7. We repeated this experiment and the new data were presented in the revised Fig.3C, D.

4) Figure 3G, the authors describe cells shape only with FACS plots while a staining with a membrane marker in vitro or in vivo would be more informative. Alternatively, cell diameter in brightfield could be analyzed as done in Rodgers et al. 2014.
Our reply: We followed the reviewer's advice by staining freshly-sorted MuSCs for CD34, a membrane marker and then measured the areas of such stained cells to determine their sizes. The new data were presented in the revised Fig EV4D, E.

5) The link mTORC1 -> Jun is unclear. The fact that JUN is upregulated in RAPTOR inactivation does not mean that mTORC1 is a direct « transcriptional regulator » of JUN, RAPTOR is an inhibitor of mTORC1 but could also act independently and the JUN transcriptional modification could be totally correlative.
Our reply: I think the reviewer actually meant "Tsc1" here instead of "Raptor" as Raptor is an indispensable component of the mTORC1 complex. We agree with the reviewer that we have not got solid evidence to claim that mTORC1 is a "direct" transcription regulator of Jun. Rather, we simply proposed in the manuscript that Jun is a transcription target of mTORC1 mainly based on the following findings (Fig.5B, C, and F): inactivation of mTORC1 by p110a-deletion reduced the mRNA and protein levels of Jun, while restoration of the mTORC1 activity in p110a-null MuSCs by further deleting *Tsc1* partially restored the mRNA levels of Jun. We further provided new data to show that the elevated Jun mRNA levels in MuSCs from idKO mice could be suppressed again by rapamycin, a known inhibitor of mTORC1 (see revised Fig.5G), which further supported our claim that Jun is indeed a transcriptional target of mTORC1.

6) The authors describe an AP-1 family upregulation at the transcript level in cells isolated from

p110a versus control. These data needs to be revisited in light of recent findings (Van den Brink et al. 2017) showing AP-1 as a family highly induced by the dissociation procedure.
Our reply: We thank the reviewer for bringing this new paper to our attention (it came out after we submitted our manuscript. That is why we did not cite this paper in the original manuscript. Now, we have discussed this paper along with two other related papers in our revised manuscript, see page 15 in "Discussion"). In fact, as correctly pointed out by authors in those three recent papers, the wild-type MuSCs freshly-isolated by FACS already contained a subpopulation of early activated MuSCs (as manifested by higher expression of Jun, Fos, and other immediate early genes) that were induced by the cell dissociation and FACS isolation procedures. By contrast, our p110a-null MuSCs were arrested in quiescence irrespective of the dissociation procedures. That is why both Jun and Fos mRNAs were downregulated in our mutant MuSCs compared to that in the control MuSCs.

7) Following this comment, the authors should be cautious regarding the identity of isolated "quiescent" MuSCs (figure S1 for example). They state in the manuscript that the isolation procedure could impact these cells biology and the aforementioned reference proves that. When the observed phenomenon are related to RTK signaling and phosphorylation cascades, given the kinetics of such signaling events, researchers should be cautious about isolating cells to study their properties and maybe focus on in vivo experiments.
Our reply: We fully agree with the reviewer on this point. With the publication by van den Brink and two other related papers in Cell Rep (van Velthoven et al., Cell Rep. 2017; Machado et al., Cell Rep. 2017), the muscle stem cell community now starts to recognize that the "freshly-isolated MuSCs" from uninjured muscles are not as "quiescent" as it originally thought. Instead, some of the MuSCs are already partially activated during the cell dissociation and isolation process. Our p110a-null MuSCs are interesting in that they fail to exit quiescence, and thus not affected by different isolation procedures. Moreover, some of the key immediate-early genes (like Jun) that are involved in quiescence exit in normal wild-type MuSCs can be easily recognized when we compared the transcriptome profile of the freshly-isolated wild-type MuSCs with that of the mutant MuSCs. As suggested by the reviewer, for key experiments (e.g., Figs.1, 3A, E, and 7A, C, EV1B, and EV8A, B), we also drew conclusion from in vivo experiments.

8) Figure 2A : 3 random field per replicate with the reported values is no more than 30 cells counted per replicate, on the low side for strong confidence.
Our reply: We apologize for not making the figure legend much clearer, which may lead to some misunderstanding here. In fact, we examined the number of $Pax7^+$ cells on three randomly-chosen fields per TA section (about 10 $Pax7^+$ cells per field on TA sections from wild-type mice). We analyzed three TA sections per mouse and we used three pairs of mice. Thus, in total, we counted about 10 x 3 (No of fields/TA section) x 3 (No of TA sections/mouse) x 3 (three mice per group)=270 cells in the control group.

9) Figure 2D : photo quality is low and there are no statistics on the % of VCAM+ cells
Our reply: We repeated the experiment and provided the statistical information requested by the reviewer (see the revised Fig.2D,E).

10) « In addition, MyoD is also an established marker to differentiate ASCs from QSCs as MyoD is only expressed in ASCs but not QSCs (Sambasivan and Tajbakhsh, 2015; Yin et al., 2013). » In the light of this sentence, how can they explain the presence of MYOD protein in QSCs measured by western blot in Figure 2 ?
Our reply: The cells used in Fig.2F were isolated from the Pax7-CreER:$p110a^{f/f}$:R26R-YFP mice without prior tamoxifen treatment and then **cultured** in vitro. After 48 hrs of culturing, the p110a gene was induced for deletion by adding 4-hydroxyltamoxifen (4-OHT) into the culture media. After additional 48 hrs of culturing, the cells were harvested for western blot analysis. The main purpose of this experiment was to obtain enough cells through cell culturing so that we could confirm by western blot that Pax7 protein levels were indeed decreased upon deletion of p110a (which induced autophagy in such mutant cells, and in turn promoted Pax7 protein degradation). The cultured cells used in Fig.2F already exit the quiescence and re-entered the cell cycle by the time p110a was deleted. Therefore, the experimental scheme used in Fig.2F is quite different from those used in most of the other experiments in our manuscript in which p110a was deleted by tamoxifen in vivo before MuSCs were isolated.

11) Figure 4 : The authors re-induce mTor (by disrupting its inhibitor RAPTOR), a pro-activation factor that have been previously shown to be involved in quiescence exit (Rodgers et al. 2014) and they observe a rescue of PAX7 levels. How does this fits with the observed phenotype? PAX7 being a quiescence maker, if p110a activates mTORC1 to exit quiescence as the authors states, mTORC1 levels rescue should diminish PAX7 levels.
Our reply: We think that the reviewer actually meant "Tsc1" instead of "Raptor" in the first sentence above. Indeed, Pax7 mRNA and protein levels are known to correlate with the quiescent state of MuSCs: they are high in the quiescent MuSCs but low in the activated and proliferating MuSCs. However, Pax7 itself is not an absolute determining factor for the quiescent state. In support of this argument, our mutant p110a-null MuSCs had very low levels of Pax7 protein, yet they failed to exit quiescence (Figs.2 and 3). Further deletion of *Tsc1* in p110a-null MuSCs partially restored Pax7 protein levels both in vivo (as manifested in Fig.4F by enhanced Pax7 staining in the MuSCs from idKO mice) and in culture (as manifested in Fig.4D by western blot). This result indicates that the Pax7 protein levels in quiescent MuSCs are sensitive to the status of the autophagy machinery that in turn is regulated by the mTORC1 activity: loss of p110a activates autophagy due to decreased mTORC1 activity, while restored mTORC1 activity by *Tsc1* deletion inhibits autophagy. However, the Pax7 protein levels in proliferating MuSCs are not determined by such a post-translational protein stabilization mechanism. Instead, they are determined by the levels of Pax7 mRNA. Thus, in proliferating MuSCs, even though the PI3K/mTORC1 activity is high, the Pax7 protein levels remain low due to the low levels of Pax7 mRNA.

12) Figure 7 : the authors observe a nice phenotype. However, they emits an hypothesis regarding the presence of EdU+/YFP- cells inside the myofibers. If there is an increase fusion of YFP+ cells, the authors should observe YFP+ myofibers which will actually be a proof of concept of their phenotype. This is routinely observed when knocking off a quiescence factor (Bjornson et al.2012 ; Mourikis et al. 2012) or when YFP+ MuSCs are transplanted inside a WT muscle. In the latter case, the observation of fluorescent myofiber is actually the read-out of transplant efficiency. Also, if the MuSCs fused with the myofiber the authors would observe central nuclei, not lateral myonuclei requiring dystrophin staining to be shown inside myofibers.

Our reply: In uninjured muscles, the number of MuSCs is very low on myofibers (~5-6/myofiber from EDL muscles). Thus, the fusion of a few YFP^+ MuSCs into a myofiber would make it very hard to turn the whole fiber yellow! This is also the case in a reference cited by the reviewer (e.g., Fig.3A in the paper by Bjornson et al., 2012). By nature, our experiment in Fig.7 is quite different from a transplantation experiment where a large number of MuSCs are typically used. As to the presence of central nuclei, indeed, they are frequently found in regenerating muscles after injury. However, the fusion of $YFP^+/Pax7^{H1047R}$ cells to their associated myofibers occurred under normal condition without any muscle injury. This result was also consistent with that in the paper by Bjornson et al (see Fig.6 on p239).

In summary, in this revised manuscript, we have tried our best to address all the questions/concerns raised by three reviewers. We sincerely hope that our revised manuscript is now satisfactory to you and the reviewers and is now in a form ready to be published in EMBO J.

2nd Editorial Decision 9th Febuary 2018

Thank you for submitting your revised manuscript for consideration by The EMBO Journal, and your patience with our response. Your revised manuscript has now been seen by the three original referees, whose comments are enclosed below. As you will see, all referees find that their concerns have been sufficiently addressed and are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues on material & methods and formal formatting as outlined below, which need to be adjusted at re-submission.

REFEREE REPORTS.

Referee #1:

The authors have addressed all my comments. No further concern.

Referee #2:

In my opinion the authors have been satisfactorily responsive to my comments, as well as to the large majority of the points raised by the other reviewers. Therefore, the manuscript has been further improved. I especially appreciate the effort to provide further convincing evidence (and mechanistic explanation) in support to the novel finding that there is a PI3K-dependent "checkpoint" that controls MuSCs "quiescence break" and that is connected with downregulation of Pax7 protein and upregulation (and likely activation) of Jun. Data provided only to reviewers (in the rebuttal letter) suggest a crosstalk between the PI3K/Akt/mTORC1 pathway and the Jun kinases JNK, which should inspire future follow-up studies. Overall, this is a manuscript of high scientific quality and impact, and deserves to be published in EMBO J. with no further reservations.

Referee #3:

The revised manuscript from Dr Zhenguo Wu and colleagues has addressed all the concerns previously raised, and I believe it is appropriate for publication in its current form. I also would like to congratulate the authors for a very nice study.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Zhenguo Wu
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2017-98239

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical methods were used to pre-determine the sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For all experiments involving mice, we used at least 3 mice for each group.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We only made sure that we used age-matched mice with appropriate genotypes for comparison. No other criteria were used to include or exclude mice.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Due to obvious differences between the mutant MuSCs and the control, no particular steps were taken to minimize the effects of subjective bias when allocating mice to treatment.
For animal studies, include a statement about randomization even if no randomization was used.	For experiments involving mice, no randomization was used.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Due to obvious differences between the mutant MuSCs and the control, no particuular steps were taken to minimize the effects of subjective bias during animal group allocation.
4.b. For animal studies, include a statement about blinding even if no blinding was done	In experiments involving mice, no blinding was done.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	yes

C- Reagents

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Please refer to details of the antibodies used under 'Materials and Methods"
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	C2C12 line was purchased from ATCC

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	p110αflox/flox (Stock No: 017704), Tsc1flox/flox (Stock No: 005680), and R26R-p110αH1047R (Stock No: 016977), Pax7CreERT(GaKa)/+ (Stock No: 017763), and R26R-EYFP (Stock No: 006148) mice were all from the Jackson Laboratory. In this study, we generated the following three strains: p110αf/f:Pax7CreER/CreER:R26RYFP/YFP, Tsc1f/f:p110αf/f:Pax7CreER/CreER:R26RYFP/YFP, and R26RH1047R/YFP:Pax7CreER/+ . All mice were kept in IVC cages in our animal facility.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All the mice were maintained and handled according to the protocols approved by the Animal Ethics Committee at HKUST.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N.A.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N.A.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N.A.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N.A.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N.A.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N.A.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N.A.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N.A.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	We did. Our RNA-seq data were deposited to GEO: GSE109472.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N.A.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N.A.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N.A.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N.A.