**We thank both reviewers for their insightful and considered comments. We have revised the manuscript, providing explanations and data where requested. Our comments are below in bold, with changes in the manuscript highlighted in red.**

Reviewers' comments:   
  
Reviewer #1 (Technical Comments to the Author):   
  
The experiments are generally well controlled; however, there is a lack of detail provided for many experiments that need addressing prior to publication.   
  
- Details are needed in relation to timepoints used for Rapamycin treatment for the C2C12 cells in Fig1 to ascertain if the effects on target gene expression are acute or chronic.

- Experiments in the C2C12 model profile expression of key myogenic target genes but at a timepoint after the phenotypic effect i.e. assessing these genes could be indicative of the consequence of impaired differentiation, not the effect of Rapamycin itself.

**We have pecified in both the results section and the Figure 1 legend, that the rapamycin was delivered for the entire 9 days of the differentiation time course and therefore represents a chronic treatment.**

- Not stated if data is shown as SD or SEM.

**Pointed out in the methods section (under statistics) and in each of the figure legends that error bars indicate mean +/- SEM.**

- Data is Fig 2 uses the progeny of the cross with Raptor shRNA or TSC1 shRNA as controls when the correct control should be flies expressing control shRNA. Why was this not done?

**We have added this control in the text. It was not included in the figure for a technical reason; the control shRNA was not balanced, so the expected progeny ratio was 1:1 for *24B-*GAL4 > shRNA : TM3,*sb* > shRNA, rather than 1:1:1 which was the case for the balanced shRNA strains. This is described in the results section as:**

**A control strain, expressing no shRNA had a modest decrease in the number of flies with the Tm3,Sb/Control genotype (47% of flies of this genotype, with an expected ratio of 50%, n=537 flies, p=1 by Fisher’s test).**

- Not clear what findings were statistically different since no annotation on graphs.

**Asterisks indicating statistical significance are now included for all figures. Other axis labels**

- No labels on Fig 4-6

**Added these figure labels.**

- No details are included in the methods about the Hand-Gal4 stock.

**Added this to the methods section.**

- Many of the conclusions are too definitive for the data provided (detailed specifically in comments to author) and need to be revised or supported by additional data.

**Adjustments to the interpretation of our data are noted in each section below.**  
  
Reviewer #1 (Remarks to the Author):   
  
The manuscript is interesting and presents some potentially important findings on the effect of mTORC1 and Rapamycin treatment during myogenesis. However, there are a number of scientific and technical points that require addressing to support the conclusions made.   
  
1. During the introduction section, the authors state that myogenesis continues throughout life. I find this a strange statement since myogenesis is defined as the formation of muscle. This statement needs revised. Also, this point is not supported by reference 19, a manuscript specifically showing that muscle mass is maintained and that muscles are capable of hypertrophy in spite of genetic ablation of satellite cells in the adult. 

**This has been revised to now read the following, removing reference 19 (the numbering in this response does not reflect numbering in the manuscript):**

**Myogenesis occurs throughout life via the differentiation of muscle precursor cells called satellite cells**1,2.

2. Can the authors provide more detail about the Rapamycin/C2C12 experiment in Fig 1? Specifically, when on day 9 was the last Rapamycin treatment in relation to the experimental endpoint? It is possible that the changes observed are due to acute treatment of C2C12 MT with Rapamycin. Has this been assessed? 

**We have specified both the methods section and in the figure legend that the most recent rapamycin treatment was one day prior to the cell lysis. This indicates that these changes are reflective of chronic rapamycin administration.**

3. The gene expression studies in Fig 1 do not support the conclusion that mTORC1 is required for transcription downstream of Myod1 as stated. The expression was measured at day 9 of treatment and so could be reflective of the expression level in a cell where differentiation is impaired, not as a direct effect of mTORC1 inhibition. 

**This is an excellent point, our evaluation of a late period of differentiation indeed does not tell us of the timing of these events. To test this further, we have evaluated mTORC1 activity and MyoD protein levels very early in differentiation and observed that inhibition of mTORC1 reduces MyoD protein levels very early in the process. These experiments are described in detail, in response to the second reviewer’s first point. We have also clarified this point in the revised discussion by stating:**

**The decreases in the mRNA levels at the end of the study for *Myog*, *Myf5*, *Mef2c* and *Cdkn1a* are likely reflective of undifferentiated cells, and may not be direct mTORC1 targets. Although these data do not preclude the possibility of other unknown factors, our observations support the hypothesis that mTORC1 is required for MyoD stability, which is then required for activation of the remainder of the myogenic program.**

4. The authors state that differentiation is impaired. It would be advisable for the authors to label cells for myosin heavy chain and count the numbers of myosin heavy chain positive cells. Otherwise, the phenotype could be a result of impaired fusion.

**Based on the data presented in Figure 1e (formerly Figure 1C), we see no tubulation of rapamycin treated cells, a stage that precedes fusion at either day 5 or 10. To demonstrate this more clearly, we present below images from an even earlier timepoint (2 days) as Figure 1 of this response. At this stage, DMSO treated cells have begun to efficiently form fused myocytes but the rapamycin treated cells do not. These findings are now described in the results section:**

**Macintosh HD:Users:davebridges:Documents:Source:DrosophilaMuscleFunction:Manuscripts:Hatfield et al Submission:Reviews:Response Mhc Figure.pdf**

***Mhc* isoforms are reduced during differentiation of C2C12 in the presence of rapamycin.** These genes were tested in the same lysates presented in Figure 1b and were not included to do lack of space. Asterisk indicates p<0.05

**We did not observe any fused myocytes in the rapamycin treated cells, suggesting that the earliest rapamycin-sensitive event is prior to myocyte fusion, which results in impaired myotubule formation. This is consistent with previous studies examining the effects of rapamycin on myoblast differentiation**3–7**.**

**We have also presented new data showing that the major myosin heavy chain isoforms (*Mhc1*) is expressed later than the myogenic transcription factors including *Mef2c* (Figure 1a) and these myosin heavy chain transcripts are reduced with rapamycin treatment during differentiation (Figure 1b). Although we didn’t include this for space reasons, we also evaluated the lower expressed Mhc isoforms and those were also not induced in the presence of rapamycin (see figure to the right).**

**To address the tubulation question, we now present another series of images after 2 days of differentiation in Figures 1e and f. As these images show, there is a clear shift way from fused myotubes even early in differentiation process. We have also made these images much larger for clarity. We attempted to differentiate between multinucleate and single nuclei containing tubes at each of these stages, but were unable to confidently quantify these effects.**

5. The authors state that the differences in phenotype are due to a less efficient effect (presumably they mean expression of the shRNA?) by c179 Gal4 compared to mef2-Gal4 yet no data is actually provided to show the efficiency of knockdown with the shRNA due to the different Gal4 lines so the conclusion can not be made. 

**We have included this caveat and the possibility that there are different anatomical locations which underlie the differences between the drivers in the revised discussion:**

**Alternately, it is possible that the differences observed between muscle drivers are due to differences in knockdown efficiency, or different anatomical locations in which these drivers are active.**

6. Have the authors assessed the effect of their intervention on the imaginal myoblasts associated with the wing discs. These cells, which form the indirect flight muscles, will express the mef2-shRNA and so presumably are affected by the treatment. This is an important point because this could explain why the flies are unable to enclose (weakness in the IFM) and why the mef2-GAL4 effect is stronger than c179-GAL4. This would have an important impact on the conclusions made. Have the authors looked at these cells and/or used a functional test that assesses the function of these muscles directly? 

**All three of *24B*-GAL4**8**, *Mef2*-GAL4**9 **and *C179*-GAL4**10,11 **are reported to be expressed in wing disks, so it is unlikely that that is the difference between these lines, although the exact timing of wing disk expression of these lines is not known. This is described in the discussion section:**

**All three of of *24B*-GAL4**8**, *Mef2*-GAL4**9 **and *c179*-GAL4**10,11 **are reported to be expressed in wing disks and *Mhc* has been shown to be expressed in the developing embryo**12**. A complete evaluation of the precise timing of activation of these drivers is beyond the scope of this study, but one possibility is that *Raptor* is required for efficient muscle development at a stage corresponding to the *Mef2/24B* promoter activation, but is no longer required by the time *Mhc* is expressed.**

**Futhermore, we examined the modENCODE Cell Line Expression Data**

**mRNA profiling data available on Flybase and found that *Mef2* is expressed in wing disk cell lines, but *Mhc* is undetectable. We have mentioned this in the discussion:**

**Furthermore, mRNA profiling studies of wing disc derived cells lines show expression of *Mef2* but not *Mhc* in these developing organs**13**.**

**We also addressed this point indirectly by presenting the effects of driving *Raptor* knockdown late in development using a *Mhc-*GAL4 driver (see the new Figure 7). This driver is expressed in differentiated muscles, from embryonic stages forward. When we knock down *Raptor* using this driver, we do not observe any reductions in fly eclosure, suggesting that inhibiting *Raptor* later in the myogenic program has no effect on the ability of the flies to eclose. Importantly, *Mhc* is also expressed in developing embryos**12**, suggesting that knocking down *Raptor* in wing disks is not driving the eclosure defect, but more likely, the defect is in wing disk myogenesis. These new data are described in the results section as:**

**In order to evaluate the effects of *Raptor* knockdown later during differentiation, we next utilized a *Mhc*-GAL4 driver. *Mhc* expression occurs quite late in the differentiation process relative to *Mef2* in differentiating C2C12 cells (Figure 1a). In contrast to the other, earlier GAL4 lines, we did not observe any defects in eclosure with *Raptor* knockdown using the *Mhc*-GAL4 driver (Figure 7a). We then evaluated the eclosed flies for climbing activity, and did not observe any significant differences between these flies and control flies, although there was a slight trend towards decreased climbing activity (Figure 7b).**

Reviewer #2 (Remarks to the Author):   
  
Summary:   
This manuscript describes studies that attempt to investigate the necessity of mTORC1 (more specifically dTORC1) in the development of skeletal muscle in the model system Drosophila melanogaster in vivo. Specifically, the authors have used the shRNA to knockdown the mTORC1 component, Raptor, specifically in skeletal muscle during development. The main novel findings are that the developmental knockdown of Raptor in skeletal muscle leads to reduced muscle function which, if severe enough results in an inability to eclose from pupal cases and subsequent death. If the knockdown is less efficacious and eclosure is possible, impaired muscle function is still evident across the lifespan and, especially in males, and is associated with reduced longevity.   
  
Major comments:   
The C2C12 time course experiments that examine the effect of the allosteric mTORC1 inhibitor, rapamycin, are novel especially with regard to Myf5, Mef2c and Cdkn1a (p21) mRNA expression. Given the emphasis placed in this section on the role of mTORC1 on MyoD stability and its subsequent effects on Myf5, Mef2c and Cdkn1a expression, examination of the changes in the time course of protein levels of MyoD, with and without rapamycin, would be relatively simple and provide more solid support for the proposed role that mTORC1 regulates MyoD protein stability.

**We assessed the levels of MyoD and mTORC1 activity during the early phases of differentiation, and have presented these data in the new panels 1C and 1D. These data support the hypothesis that MyoD levels are decreased at the protein but not the mRNA level. In addition to modified methods, and figure legend sections we have described these data in the results section as:**

**Since *Myod* mRNA levels were unchanged, we next tested whether MyoD protein levels are altered by rapamycin treatment. We added the differentiation media for 4h in the presence of DMSO or rapamycin and observed that rapamycin reduced MyoD protein levels by 47% (Figures 1c-d).**

**In addition to this finding, we have also observed activation of mTORC1 signaling, as measured by S6 phosphorylation after 4h of differentiation media. These findings are described as:**

**We also observed elevations in mTORC1 activity 4h after the transition to differentiation media, as shown by increased S6 (Figure 1c-d). These data suggest that activation of mTORC1 signaling occurs during differentiation, consistent with previous reports** 4**. Furthermore, this activation is independent of Akt signaling, which was actually decreased during the transition from 10% FBS to 2% horse serum.**

The in vivo experiments appear to be well performed, however, I have some issue with the conclusions drawn from them. i.e. The main conclusion, that the developmental loss of Raptor, and thus dTORC1, results in a "developmental problem in myogenesis", are based on muscle function parameters (e.g. an inability to eclose and the 4 min walking test). In the absence of mRNA/protein data and/or histological data on muscle fiber numbers and morphology, how can the authors separate developmental issues with myogenesis per se from post-development myogenesis-independent but dTORC1-dependent events e.g. changes in energy metabolism resulting in increased weakness and fatiguability, changes in protein turnover that results in muscle atrophy and/or the development of a postnatal dystrophic-like phenotype similar to what happens in Raptor knockout mice?

**To address this point, we have added a new figure to the manuscript describing similar experiments performed in *Mhc*-GAL4 driven *Raptor* knockout flies. As shown in this new Figure 7 we did not observe any reductions in the number of eclosed flies when *Raptor* knockdown was driven using a driver that expresses GAL4 late in myocyte differentiation. Furthermore, we did not observe any significant defects in climbing ability in these flies.**

**We did however note that these flies have reduceed lifespan, even without apparent early life muscle defects. This is consistent with the Bentzinger et al paper mentioned below. We have discussed these findings as such:**

**Furthermore, even outside of the context of reduced viability/climbing ability *Mhc-*GAL4 driven *Raptor* knockdown flies still died earlier than control flies (Figure 7c). The *ACTA1-Cre* driven *Rptor* knockout studies did not evaluate mTORC1-dependent myogenesis in mice. Another study implicated mTORC1 in the differentiation of ES cells into satellite cells, a process which is likely upstream of our model system**14**. Together these results implicate mTORC1 as essential at multiple steps of myogenesis and maintenance of muscle function in both flies and mice.**

Indeed, the reduced lifespan is similar to what is seen in the muscle-specific Raptor knockout mouse (Bentzinger et al., Cell Metab, 2008).

**We have now cited this effect in our expanded discussion section (see below).**

**These findings are consistent with mouse studies in which human skeletal actin-driven (*ACTA1)* Cre expression drove the knockout of muscle *Rptor*. These mice were observed to be weaker than littermate controls, and prone to early death**15***,* similar to our observations of the *c179*-GAL4 and *Mhc*-GAL4 driven *Raptor* knockout flies.**

Based on the current data in the manuscript, it seems that the most appropriate conclusion is that Raptor and/or dTORC1 is necessary for skeletal muscle function. Additional data is needed to make the conclusion that dTORC1 is necessary for normal myogenesis per se. Additional data would also better integrate the C2C12 data with the in vivo data.

**We have provided additional data now, describing a late-acting Raptor knockdown, driven by an alternate GAL4 driver. We have also looked more specifically at myotube fusion (see point 4 from reviewer 1) and MyoD protein levels (see first point from this reviewer).**

One further issue is the idea, based on the effect of rapamycin on MRFs in C2C12 and in vivo, that "mTORC1 is required to be active". This statement implies that mTOR kinase activity is necessary; however, there is certainly evidence for mTOR kinase-independent but rapamycin-sensitive mechanisms in myogenesis (see Ge et al., 2009, Am J Physiol; Erbay and Chen, 2001, J Biol Chem; Park and Chen, 2005, J Biol Chem). This possibility needs to be discussed in the manuscript.

**This has been added to the discussion:**

**Although mTORC1 is the primary target of rapamycin, studies using rapamycin-insensitive analogs of mTOR have suggested there may be alternative rapamycin-sensitive targets of rapamycin at other stages of myogenesis**4,7,16**.**

In fact, the manuscript would benefit greatly from the addition of a Discussion section, separate from the Results, where the above issues can be addressed and a better discussion of how these results compare with data from the mouse muscle-specific Raptor knockout. 

**A separate discussion section has been added including (as stated above) an integration of the data from Raptor knockout mice.**

Minor comments:   
Abstract   
The last sentence is a little too strong. Unless Drosophila is specifically being referred to here, this is not a new mechanism. This has been shown before, although not in vivo (see Park and Chen, 2005, J Biol Chem)

**This sentence has been revised to read the following:**

**Together these results highlight an important role for TORC1 in late muscle development, integrity or function in both Drosophila and mammalian cells.**

Background   
Page 2, para 1, line 4 - minor point -reference 16 did not investigate developmental arrest in in flies

**Bjedov et al show in Figure 2D of their manuscript that rapamycin blocks fecundity (eggs laid per female) which although is not developmental arrest, implies embryonic lethality. We have modified that sentence to now read:**

**…loss of the obligate mTORC1 complex members mTOR and Raptor, or treatment with rapamycin induces developmental lethality in mice**17–19**, worms**20 **and fruit flies**21**.**

Materials and Methods

Climbing assay section, last line - should this be 30 days or 3 days?

**This has been clarified to read:**

**This assay was first performed within 3 days post eclosure and repeated every ~30 days for a total of 3 trials.**

# References

Note: These numbers do not match the numbering in the manuscript.

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