Raptor plays a role in late muscle development in Drosophila.

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# Abstract

Myogenesis is an important process both during development and in muscle repair. Previous studies suggest that mTORC1 plays a role in the formation of mature muscle from immature muscle precursor cells. Here we show that gene expression for several myogenic transcription factors including *Myf5*, *Myog* and *Mef2c* but not *MyoD* decreases when C2C12 cells are treated with rapamycin, implying a role for mTOR pathway during muscle development. To investigate the possibility that mTOR can regulate muscle *in vivo* we ablated the essential mTORC1 subunit Raptor in *Drosophila melanogaster* and found that muscle-specific ablation of Raptor causes flies to be stuck in their pupal cases prior to eclosion. Furthermore, we found that there is a critical period post-eclosure when *Raptor* ablation causes lethality in flies. Together these results highlight an important new role for mTORC1 in late muscle development, integrity or function.

# Background

The mTOR signaling pathway plays important roles during development in all eukaryotes and mTORC1 is a critical nutrient sensing protein kinase conserved in all eukaryotic organisms1,2. This kinase responds to nutrient and growth hormone signals in the environment and subsequently phosphorylates targets involved in aging, growth, protein lipid and glycogen metabolism3–5. In addition to these effects on differentiated cells, there is an emerging role for mTORC1 in the regulation of cellular differentiation during development including neurogenesis6,7, adipogenesis8 and myogenesis9–11. Consistent with these findings, either loss of the obligate mTORC1 complex members mTOR and Raptor, or treatment with rapamycin induces developmental arrest in mice12–14, worms15 and fruit flies16.

Myogenesis occurs both during development and throughout life via the differentiation of muscle precursor cells called satellite cells17–19. This process involves a cascade of transcription factors including several basic helix-loop-helix transcription factors such as *Myf5, Myog, Myod* and *Mef2c* (reviewed in 20,21). The direct target of mTORC1 on myogenesis has not been clearly established, but recent work has implicated mTORC1 in the regulation of MyoD protein stability, leading to a *miR*-1 dependent effect on myotube fusion22.

To determine the relevance of mTORC1 on muscle differentiation *in vivo* we have examined the effects of loss of TORC1 by both genetic and pharmacological approaches in the fruit fly, *Drosophila melanogaster*. In this study we present data supporting an essential developmental role of TORC1 in late muscle development and/or stability.

# Materials and Methods

## Tissue Culture and Myotube Formation

C2C12 cells were grown in High Glucose Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma-Aldrich) supplemented with penicillin, streptomycin and glutamine (PSG; Life Technologies) and 10% Fetal Bovine Serum (Sigma-Aldrich). Once cells reached >90% confluence, differentiation media (2% Horse Serum (from Sigma-Aldrich) in DMEM with PSG was added as previously described (REF). To determine when specific markers for differentiation were being expressed, cell lysates were generated on days 0, 0.25, 1, 2, 4, 6, 10 and 15 of differentiation. To determine the effects of rapamycin on differentiation, cells were treated every other day for 9 days with either vehicle alone (DMSO; Sigma-Aldrich), or 500nM rapamycin (Cayman chemicals) dissolved in DMSO. Cell lysates were prepared on day 9 of treatment. Cell lysates were generated by washing once with ice-cold PBS followed by the addition of 1 ml of QIAzol (Qiagen) and scraping into a 1.5ml microfuge tube. Lysates were stored at -80°C until RNA was purified.

## Quantitative Real Time PCR

RNA was extracted with the PureLink RNA mini kit (Life Technologies). 1 g of total RNA was used as a template to synthesize cDNA using the High Capacity Reverse Transcription Kit (Life Technologies). cDNA was added to *Power* SYBR Green PCR Master Mix in accordance with the manufacturer’s guidelines (Life Technologies) and qRT-PCR performed on a Roche Lightcycler. A series of control genes including *Gapdh, Rplp0, Actb* and *Rplp13a* were examined, and *Gapdh* was chosen as a control as it was not changed across rapamycin or differentiation conditions. For a complete list of primers and probes used, refer to Table 1. Relative expression was determined via the Ct method as previously described23.

## Drosophila Stocks and Crosses

The stocks *w1118*, the three muscle GAL4 drivers (*24B*-GAL4, *c179*-GAL4 and *mef2*-GAL4), as well as both the *Raptor* and *mTOR1C* UAS-shRNA TRiP lines used (stock #s) were obtained from the Bloomington Stocks Center (Bloomington, IN). All flies were raised at 25 °C on standard corn meal media with the exception of the 18°C crosses for *24B*-GAL4. Rapamycin was added where indicated after fly food was cooled to below ~50° C**.** To prepare the crosses, virgin females were picked out from each of the GAL4 driver strains. Ten virgin females were used per cross. Males with the appropriate genotype were chosen from each of the lines and crossed to male UAS-TRiP-shRNA lines for *Raptor* (3) or *Tsc1* (3) as well as a UAS-TRiP control which contains the genomic insertion site but no shRNA24. Flies were maintained in a humidified incubator at 25°C. A subset of experiments were also performed at 18°C. Ten days after each cross the F1 progeny began to eclose and were sorted according to phenotype and gender. During each sorting, the number of flies of each phenotype was recorded. The sorted flies were put into new vials, with males and females separated and with 5-10 flies in each vial. Progeny were stored at 25°C until at least 100 flies of each genotype had been collected. At least three independent replicates of each cross were performed.

## Examining Pupal Cases

Twenty days after the *C179-*GAL4>UAS-shRNA-*Raptor* and *Mef2-*GAL4>UAS-shRNA-*Raptor* crosses were made any remaining adult or F1 progeny flies were emptied from the vials. The empty pupal cases were counted and the cases containing dead flies were counted. Pupal cases containing a dead fly were markedly darker in color than the empty cases and contained a visibly formed black, shrunken fly.

## Manual Assistance of Eclosure

## Climbing Assay

To perform the climbing assay flies were tapped to the bottom of a vial and a stopwatch was started simultaneously. The stopwatch was stopped each time a single fly from the group in the vial climbed to a mark at 4cm on the side of the vial. A separate time was recorded for each fly in the vial. This assay was performed within 3 days post eclosure and repeated every ~30 days for a total of 3 trials.

## Statistics

Statistical analyses were performed using the R statistical package, version 3.1.025. Prior to performing ANOVA analyses, normality was assessed by Shapiro-Wilk test and equal variance was tested using a Levene’s test (from the car package, version 2.0-2026). If both these assumptions were met (p>0.05) an ANOVA was performed. If either of these assumptions failed, a Kruskal-Wallis test was performed. If either of those omnibus tests reached significance, then Student’s T-Tests or Wicoxon Rank Sum Tests were performed as indicated, followed by an adjustment for multiple comparisons using the method of Benjamini and Hochberg27. Statistical significance for the manuscript was set at a p or q-value of less than 0.05.

# Results and Discussion

## Rapamycin Inhibits Differentiation of Muscle Cells in Culture

To determine the order in which myogenic markers are induced, we performed a time course experiment in C2C12 cells. We generated cell lysates at various time points between 0 and 15 days of the differentiation process and performed RT-qPCR to measure transcripts of the known differentiation markers including *Myf5*, *Myog*, *Mef2c*, *Cdkn1a*, and *Myod1*. We observed that transcripts for *Myf5*, *Myog*, *Cdkn1a*, and *Myod1* are increased early in the process and continue to increase throughout development with large increases in *Mef2c* not occurring until around day 5 or 6 (Figure 1A). This is consistent previous observations of the transcriptional changes associated with muscle differentiation of cells in culture28.

Next we wanted to determine if rapamycin, a drug known to inhibit TORC1 signaling, had any effects on gene expression during differentiation (Figure 1B and C). Treatment with rapamycin caused significant reductions in mRNA transcript levels detected for all differentiation markers measured (p<0.05), with the exception of *Myod1* (Figure 1B) and prevented the formation of myotubes (Figure 1C).

Since rapamycin does not reduce the differentiation-associated elevation of *Myod1* transcript levels, consistent with previous findings22, it is unlikely that the mTORC1 target is upstream of *Myod1* transcription. Furthermore, since the elevation of several early differentiation targets, including *Myog*, *Myf5*, and *Cdkn1a* are all blocked by rapamycin our data supports the hypothesis that mTORC1 is required downstream of *Myod1* transcription but still quite early in the differentiation process*.* Since *Mef2c* does not increase until late in the differentiation process, and is downstream of *Myog*, reductions in *Mef2c* levels are likely due to defects upstream of *Myog*29.

These data show that the mTORC1 pathway is required to be active at a very early time point during the differentiation in C2C12 cells into myotubes. Additionally, these data indicate that *Myog*, *Myf5*, *Cdkn1a* and *Mef2c* all require mTORC1 function for their transcriptional activation during myogenesis. These data are consistent with the hypothesis that one role of mTORC1 in differentiation is through the stabilization of MyoD as previously suggested22, though whether there are other mTORC1 targets in early differentiation is not clear. Since the primary effect of miRNA-1 is on myotube fusion, it is likely that there are other mTORC1 dependent effects, as the morphological changes prior to myotube fusion are also disrupted by rapamycin 11.

## Muscle Specific Knockdown of *Raptor* Leads to Lethality in Drosophila

In order to study the role of TORC1 signaling on muscle development *in vivo*, we manipulated dTORC1 function in fruit flies. First, we tested whether inhibition of the dTORC1 pathway affected the development of these flies. As previously reported, high doses of rapamycin prevents egg laying by the maternal fly 16. We performed dose curves and found that at much lower doses (EC50 of ~860 nM) there was an absence of pupae and flies (Supplementary Figure 1). There was no obvious distinction between inhibition of pupal lethality and prevention of fly eclosure. These data suggest that rapamycin inhibits fly development, similar to what has been observed in mice 12. It also supports studies showing that whole animal knockout of *Raptor* leads to developmental lethality in several model organisms 13–15,30.

To look specifically at the role of dTORC1 in muscle, we knocked out either *Tsc1* or *Raptor* to generate constitutive gain and loss of function alleles in fly muscles using the *GAL4*-UAS system 31. We used several *GAL4* drivers that cause expression of the UAS driven shRNA cassettes in both skeletal muscle and cardiac muscle. Skeletal muscle was targeted through the use of *24B-GAL4, C179-GAL4,* *Mef2-GAL4* and *Mhc-GAL4* drivers, while cardiac muscle was targeted using the *Hand-GAL4* driver. To minimize potential off target effects, three different shRNAs for each of the two genes (*Raptor* and *Tsc1*) were used.

First, we crossed heterozygous, balanced *24B*-*GAL4* flies with heterozygous, balanced UAS-shRNA transgenic flies. The flies expressing both balancer chromosomes had decreased viability, and this genotype was excluded from the analysis. The flies eclosed from crosses using the *Hand-GAL4* driver occurred in roughly equal ratios (Figure 2A), indicating there is no obvious effect of manipulating dTORC1 with the cardiac *GAL4* driver. Similarly, *24B-GAL4* driven expression of *Tsc1* shRNA had no significant effect on birth rates. However, when the driver was used to express *Raptor* shRNA, the progeny exhibited a dramatic decrease in the number of eclosed flies (Figure 2B). This indicates that *24B-GAL4* driven expression of *Raptor* shRNA is lethal at some point prior to eclosure. Similarly, by using another muscle specific driver, *C179-GAL4* crossed to heterozygous UAS-shRNA/TM6b flies we also observed reduced muscle-specific *Raptor* knockdown flies, although in this case some knockdown flies were able to eclose (Figure 2C).

We attempted to rescue the lethality phenotype of *Raptor* suppression in skeletal muscle by repeating the *24B-GAL4* x *Raptor* shRNA crosses in a colder environment. Colder temperatures are which decreases GAL4 driver expression 31. This was unable to rescue the lethality of the *24B*-*GAL4/Raptor* shRNA flies, and the birth rates of the two control genotypes were congruent with those produced at 25° C (Supplementary Figure 2).

To test for the stage under which these flies fail to eclose next used *C179-GAL4* and *Mef2-GAL4* drivers and repeated did simiar studies. In these experiments, we crossed flies which were homozygous for the *GAL4* with flies that were homozygous for the UAS-*Raptor* shRNA transgene. As a control, we used a fly line that was identical to the TRiP fly lines, but did not have a shRNA inserted (see Table 2). The *Mef2-GAL4* driver resulted in complete lethality of *Raptor* knockdown flies (see Figure 3A). There was partial lethality in the three the *C179-GAL4* mediated *Raptor* knockdown flies (p-value < 0.005 for those shRNA strains, with a 73-92% decrease in the number of flies depending on the strain, see Figure 3B). These results indicate that the *C179-GAL4* driver is less efficient at mediating *Raptor*-specific lethality than the *Mef2-GAL4* and the *24B-GAL4* drivers. Interestingly, this was not observed in mice where muscle-specific *Raptor* knockout mice are viable 32,33. One key difference here is that in those models, which use a late-expressed muscle *Cre* driver, *Raptor* is not ablated until very late in muscle differentiation.

## Muscle *Raptor* Knockdown Flies Fail to Eclose from Pupae

To determine at which point prior to eclosure the *Raptor* knockdown flies dying, we examined the pupal cases on the sides of the vials from the *Cl79-GAL4 > Raptor* shRNAcrosses and the *Mef2-GAL4 > Raptor* shRNAcrosses. Twenty days after the crosses were prepared the empty pupal cases were counted and the cases containing dead flies were counted. We observed no significant differences in the total number of pupal cases from either of these crosses (Figure 3C-D, p=0.416 and p=0.066 from ANOVA respectively). In fact, we observed a slightly increased number of pupae from the *Mef2-GAL4* > *Raptor* shRNA crosses. These data support the hypothesis that lethality occurs after pupal development.

We next visually examined the pupal cases for the presence of alive or dead flies (Figure 3E). After blind scoring, we noted that for the *Mef2-GAL4* driven *Raptor* knockdown nearly 100% of the pupal cases contained dead flies (15 fold more dead pupae than controls; Figure 3F). There was also a significant number of dead flies in pupal cases from the *Cl79-GAL4/Raptor* crosses (Figure 3G). For *C179-*GAL4 driven *Raptor* knockdown flies there was 3 to 11 times more dead pupae depending on the shRNA strain. These results indicate that *Raptor* knockdown in skeletal muscle produced lethality after pupal development, but prior to eclosure.

## Lethality of Muscle *Raptor* knockdown flies is in part due to an inability to eclose

To test whether the muscle *Raptor* knockdown-mediated lethality is due to a muscle weakness that prevents eclosure, we carefully examined fly morphology within pupal cases. As shown in Figure 4A, at day 10 a fully formed fly is apparent within the pupal case, but by day 14 the fly has not yet eclosed and by day 20 the fly has degraded to the stage observed in Figure 3E. To determine if this is due to an inability to exit the pupal case, we gently opened 5 pupal cases from *Mef2-*GAL4>*Raptor* knockdown flies to assist in eclosure. In 4 of 5 cases the fly eclosed and three flies survived >3 weeks. To test if these eclosed flies had muscle weaknesses we performed climbing assays on these eclosed flies and as shown in Figure 4B, the *Mef2-*GAL4>*Raptor* flies exhibited dramatically reduced climbing ability.

## Effects of Muscle-Specific *Raptor* Knockdown on Longevity

We next turned our attention to the few flies that survived in the case of the *C179-GAL4* cross. The lifespan of these *Raptor* knockdown flies was measured to determine the effects of dTORC1suppression on longevity. When *Raptor* was knocked down in skeletal using the *Cl79-GAL4* driver, even after the decrease in viability, a large proportion of the flies died shortly after eclosure. Interestingly, among the flies that survived, they generally had normal lifespan (see Figure 5). This suggests that there is a critical period of about 20 days after eclosure during which the *Raptor* knockdown flies are still prone to early death. The flies that lived past this critical period lived to reach approximately the same age as the controls. This was especially dramatic for male flies for each of these crosses (Figure 5A, C and E compared to Figure 5B, D and F).

## Effects of Muscle Specific *Raptor* Knockdown on Muscle Function

To study the effects of TORC1 suppression on muscle function, a climbing assay was performed on the *Raptor* knockdown flies driven by the *C179-GAL4* driver at several ages. The progeny flies from the crosses were each timed for how long it took them to climb 4 cm. up the side of the vial. The average times for each cross are shown in Figure 6. The results indicate that dTORC1 suppression leads to reduced muscle function in the flies that eclose even very early, consistent a developmental problem in myogenesis. Interestingly, these problems persist throughout the lifespan of the fly, even in those that reach adulthood. Also interesting, is that there was a correspondence between the efficiency of the shRNA strain to cause lethality and its effects on muscle function, indicating a potential gene-dosage effect on both of these phenotypes.

## Summary

In this work, we have described a role for TORC1 in the regulation of myogenesis *in vitro* and shown in fruit flies, that inhibition of the dTORC1 complex results in lethality and weakened muscle function. Together these data support an *in vivo*, evolutionarily conserved role for the TORC1 complex in the early stages of muscle development.

# Acknowledgements

The authors would like to thank the members of the Bridges and Reiter lab for insightful discussions. This work was supported in part by a Dean’s Neurology Support Fund (LTR).

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# Table Legends

**Table 2:** Forward and reverse primers used in qPCR experiments. All primers are based on mouse sequences.

**Table 2:** Fly stocks used in this study.

# Figure Legends

**Figure 1. Rapamycin blocks C2C12 differentiation.** A) The order of appearance of myotube differentiation markers over the course of 15 days in differentiation media only B) Differences in differentation marker transcripts when treated with DMSO (vehicle) or 500nM rapamycin for 9 days. Transcripts from both A) and B) were measured by RT-qPCR and normalized to *Gapdh*. C) Images of morphological changes in C2C12 myoblasts in response to 9 days of DMSO or rapamycin treatment (500nM).

**Figure 2. Skeletal muscle specific Raptor knockdown causes lethality.** A) Proportion of progeny born from a *Hand*-GAL4/TM3, Sb x shRNAi/TM6b, *Tb, Hu*. The progeny that are TM6b/TM3 are excluded due to known reduced viability so the expected ratios (as indicated by the dotted line) are 0.33. Knockdown flies are shown in red throughout. B) Proportion of progeny born from a *24B*-GAL4/TM3, *Sb* x shRNA/TM6b, *Tb, Hu* cross. C) Proportion of progeny born from a *C179*-GAL4/*C179*-GAL4 x shRNAi/TM6b, *Tb, Hu* cross. In this case half the progeny should be knockdown, so the expected ratio is 0.5. Error bars indicate sampling standard error, with >195 flies examined for each cross.

**Figure 3. Muscle-specific Raptor knockdown flies die post-pupal formation but pre-eclosure.** The graph depicts the birthrates of the progeny from crosses of homozygous *GAL4* drivers with homozygous *Raptor* shRNA transgenic flies or a control TRiP line. Panels A, C and F indicate *Mef2-*GAL4 driven knockdowns while B, D and G indicate *C179-*GAL4mediated knockdowns. A and B) show the total number of flies eclosed; C and D) indicate the total number of pupae after 20 days, and F and G) show the percentage of dead pupae. Panel E shows a representative example of dead flies, still within their pupal cases. Asterisks indicate p<0.05 by ANOVA followed by Dunnett’s test (B, C and D) or Kruskal-Wallis tests then Wilcoxon-rank-sum tests followed by an adjustment for multiple comparisons (A, F and G). Each of these analyses describe the average 5-9 independent crosses, with error bars indicating standard error of the mean between replicate crosses.

**Figure 5. Lifespan of *C179-GAL4* Driven Raptor Knockout Flies.** Dashed lines indicate two control strains. Each panel shows a control of *C179*-GAL4 crossed to the control shRNA strain, as well as the balancer containing progeny of the *C179*-GAL4 homozygotes crossed to the heterozygous UAS-*Raptor* shRNA/TM6b flies.

**Figure 6: Muscle-specific Raptor knockdown flies have reduced climbing rate.** Average climbing rate as measured during three age range intervals (in days) for *C179-*GAL4 driven *Raptor* knockdown flies. Asterisk indicates p<0.05 based on a Wilcoxon Rank-Sum test relative to the control flies, and adjusted for multiple observations. Note that the different abscissa indicates age-related slowing of climbing speed.

# Supplementary Tables and Figures

**Supplementary Figure 1: Dose response of rapamycin on fly eclosure and larvae development.** Flies were mated in the presence of varying doses of rapamycin. After 7 days, the parental flies were removed. After 21 days, the number of progeny and larval cases were counted.

**Supplementary Figure 2:** **Effects of lowered temperatures on eclosure of *24B-GAL4* driven *Raptor* knockdowns.** The graph depicts the relative birth rates of the progeny produced when the *24B-GAL4/Raptor* shRNA crosses were repeated at 18° C. The double balancer genotype is excluded from the graph. The dashed line represents the expected birth rates of the three genotypes. The colder environment was unable to rescue the lethality effect of Raptor knockdown in skeletal muscle.