# Abstract

# Background

* TOR
* why muscle and aging
* Flies
* GAL4/UAS

# Methods

## Materials

Fly stocks were ordered from the Bloomington stock center or were described previously (see Supplementary Table 1). Food was made prepared according to XXX **.** Rapamycin (Cayman Chemicals) was added where indicated when food was ~50° C**.**

## Tissue Culture and Myotube Formation

## Real Time Quantitative PCR

We did real time qPCR

## Fly Breeding

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 shRNA strains, and four males were used per cross. Flies were maintained in an incubator at 25° C. When cooler temperatures were required, flies were maintained at 18° C. The parents were flipped into a new vial every five days. The progeny began to eclose after ten days, at which point the progeny was 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. The progeny was stored in an incubator at 25° C. This process was continued until at least 100 flies of each genotype had been collected. At least three replicates of each cross was performed.

## Examining Pupal Cases

Twenty days after the *C179-GAL4/Raptor* and *Mef-GAL4/Raptor* crosses were made, the progeny flies were emptied from the vials. The empty pupal cases were counted and the cases containing dead flies were counted. A pupal case with a dead fly in it was markedly darker in color than the empty cases, and contained a black, shrunken fly.

## Crawling Assay

To perform the crawling assay, the flies were tapped to the bottom of the vial at the same time a stopwatch was started. The stopwatch was stopped each time a fly in the vial crawled 4 cm. up the side of the vial. A separate time was recorded for each fly in the vial. This was performed soon after eclosure and repeated every ~30 days for a total of 3 trials.

## Statistics

Statistical analyses were performed using the R statistical package, version 3.1.0 [1]. 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 2.0-20 [2]). 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 comparasons using the method of Benjamini and Hochberg [3]. 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

## 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 prevent egg laying by the maternal fly [4]. We performed dose curves and found that at much lower doses (EC50 of ~860 nM) there was an absence of pupae and flies (Supplmentary 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 [5]. It also supports studies showing that whole animal knockout of *Raptor* leads to developmental lethality in several model organisms [6–9].

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 [10]. 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,* *Mef-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 examined this per gender, and with the exception of shRNA #2, in this case, males and female *Raptor* knockdown flies were approximately equally reduced (Figure 2D).

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 [10]. 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 *Mef-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 *Mef-GAL4* driver resulted in complete lethality of *Raptor* knockdown flies (see Figure 3A). The *C179-GAL4* mediated Raptor knockdown flies, although partial lethality was still demonstrated in the significantly lower birth rate of the knockdown genotype (p-value < 0.002) (see Figure 3B). These results indicate that the *C179-GAL4* driver is less efficient at mediating *Raptor*-specific lethality than the *Mef-GAL4* and the *24B-GAL4* drivers.

This was not observed in mice where muscle-specific *Raptor* knockout mice are viable [11, 12].

## 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 *Mef-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 *Mef-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 *Mef-GAL4* driven *Raptor* knockdown nearly 100% of the pupal cases contained dead flies (Figure 3C). There was also a significant number of dead flies in pupal cases from the *Cl79-GAL4/Raptor* crosses (Figure 3D). The relative number of dead flies in the different *Cl79-GAL4/Raptor* crosses corresponds to the number of knockdown flies produced from those crosses (Figure 2C-D); the fewer the number of live knockdown flies from a cross, the more dead flies produced from that cross. These results indicate that *Raptor* knockdown in skeletal muscle produced lethality after pupal development, but prior to eclosure.

## Co-Knockdown of Raptor and Atg5 in Drosophila Muscles Does not Rescue Lethality

Inhibition of dTORC1 in muscle alters the activity of its various downstream targets. One major target is that when dTORC1 is suppressed, autophagy levels are increased [13]. To determine whether an increase in autophagy was the source of the lethality in the *Raptor* knockdown genotype, crosses were made in which both *Raptor* and autophagy were simulataneously knocked down in skeletal muscle. The co-knockdown of *Atg5* along with *Raptor* did not lead to any rescue of *Raptor* mediated lethality (Supplementary Figure 3).

## 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 the *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, but interestingly, among the flies that survived, they generally had quite normal lifespan (see Figure 4). 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 generally lived to reach the same age as the controls. This was especially dramatic for male flies for each of these crosses, consistent with male flies being smaller and weaker than female flies.

## Effects of Muscle Specific Raptor Knockdown on Muscle Function

To study the effects of TORC1 suppression on muscle function, a crawling 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 5. 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 efficienty 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 a *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 by XXXX (LTR)

# References

1. R Core Team: **R: A Language and Environment for Statistical Computing**. 2013.

2. Fox J, Weisberg S: *An {R} Companion to Applied Regression*. Second. Thousand Oaks {CA}: Sage; 2011.

3. Benjamini Y, Hochberg Y: **Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing**. *J R Stat Soc Ser B* 1995, **57**:289–300.

4. Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, Partridge L: **Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster.** *Cell Metab* 2010, **11**:35–46.

5. Hentges KE, Sirry B, Gingeras a C, Sarbassov D, Sonenberg N, Sabatini D, Peterson a S: **FRAP/mTOR is required for proliferation and patterning during embryonic development in the mouse.** *Proc Natl Acad Sci U S A* 2001, **98**:13796–801.

6. Jia K, Chen D, Riddle DL: **The TOR pathway interacts with the insulin signaling pathway to regulate C. elegans larval development, metabolism and life span.** *Development* 2004, **131**:3897–906.

7. Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown MSS, Fitzgerald KJ, Sabatini DMM: **Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1**. *Dev Cell* 2006, **11**:859–871.

8. Gangloff Y, Mueller M, Dann SG, Svoboda P, Sticker M, Spetz J, Um SH, Brown EJ, Cereghini S, Thomas G, Kozma SC: **Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development.** *Mol Cell Biol* 2004, **24**:9508–16.

9. Murakami M, Ichisaka T, Maeda M, Oshiro N, Hara K, Edenhofer F, Kiyama H, Yonezawa K, Yamanaka S: **mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells.** *Mol Cell Biol* 2004, **24**:6710–8.

10. Duffy JB: **GAL4 System in Drosophila : A Fly Geneticist ’ s Swiss Army Knife**. 2002, **15**:1–15.

11. Bentzinger CF, Romanino K, Cloëtta D, Lin S, Mascarenhas JB, Oliveri F, Xia J, Casanova E, Costa CF, Brink M, Zorzato F, Hall MN, Rüegg MA: **Skeletal muscle-specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy.** *Cell Metab* 2008, **8**:411–24.

12. Bentzinger CF, Lin S, Romanino K, Castets P, Guridi M, Summermatter S, Handschin C, Tintignac L a, Hall MN, Rüegg M a: **Differential response of skeletal muscles to mTORC1 signaling during atrophy and hypertrophy.** *Skelet Muscle* 2013, **3**:6.

13. Neufeld TP: **TOR-dependent control of autophagy: biting the hand that feeds.** *Curr Opin Cell Biol* 2010, **22**:157–68.

# Figure Legends

Figure 1.

**Figure 2. Skeletal muscle specific Raptor knockdown causes lethality.** A) Proportion of progeny born from a Hand-GAL4/Tm3,sb x shRNA/TM6B. The progeny that are TB6B/Tm3, are excluded 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 cross. C) Proportion of progeny born from a C179/C179 x shRNA/TM6B cross. In this case half the progeny should be knockdown, so the expected ratio is 0.5. D) Proportion of flies born from crosses in C, separated by Gender. 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-GAL4* mediated 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 Wilcoxon-rank-sum test, followed by an adjustment for multiple comparisons. 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. The figure on the right shows dead flies stuck in their pupal cases, and the figure on the left shows empty pupal cases post-eclosure. The pictures were taken 20 days after the crosses were made. The control is using 36304 shRNA, which is a control line for TRiP RNAi lines. We found that the lethality effect of Raptor knockdown in skeletal muscle was occurring in the pupal stage.

Figure 6. The graphs on the left show the number of total pupal cases produced in the Mef-GAL4/shRNA and C179-GAL4/shRNA crosses. The graphs on the right show the percentage of the cases that contained dead flies. The cases were counted around 20 days after the crosses were made. Homozygous strains of both the GAL4 driver and the shRNA lines were used for the crosses. Nearly 100% of the pupal cases in the Mef-GAL4/Raptor shRNA crosses contained dead flies. There was also a high percentage of dead flies in the cases of the C179-GAL4/Raptor shRNA crosses, and the percentage of dead flies correlates with the relative strengths of the three different Raptor shRNA lines.

**Figure 5: 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.

Figure 8. The line graphs show the survival rate of flies with Tsc and Raptor knockdown in skeletal muscle. The graph on the left shows the effects of Tsc knockdown using the 24B-GAL4 driver, and the graph on the right shows the effects of Raptor knockdown using the C179-GAL4 driver. The crosses were flipped twice a week to count for dead flies. Both Tsc and Raptor knockdown in skeletal muscle result in decreased longevity. The controls contained either the GAL4 driver strain over a marker, or the shRNA strain over a marker. Knocking down Tsc and Raptor in cardiac muscle using the Hand-GAL4 driver had no significant effects on longevity.

# 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: The graph depicts the relative birth rates of the progeny produced when the 24B-GAL4/Raptor shrRNA 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.