Activation of mTORC1 and Suppression of Autophagy in Muscle Tissue Shortens Lifespan in Mice and Flies

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# Abstract

# Introduction

# Methods and Materials

## Animal Housing and Procedures

Floxed *Tsc1* alleles (Tsc1*tm1Djk*/J1 from the Jackson Laboratory) were crossed with *Ckmm-Cre* driver mice (FVB-Tg(*Ckmm-cre*)5Khn/J2 from the Jackson Laboratory) to generate animals that were heterozygous for the floxed allele, and either hemizygous for the *Cre* allele or wild-type at this locus. These mice were used to generate male littermates that were wild-type at both the *Tsc1* and *Cre* loci, homozygous at the *Tsc1* loci (floxed only), hemizygous at the Cre loci (transgene only) or *Tsc1*fl/fl, *Ckmm-Cre*Tg/+. Animals were housed in a 12h light/dark cycle animal facility with ad libitum access to food (Harlan Teklad) and water according to procedures approved by the University of Michigan University Committee on Use and Care of Animals. Animals were allowed to die naturally, or were euthanized at the advice of veterinary staff.

## Drosophila Breeding and Maintenance

Fly stocks (see Table 1) were purchased from the Bloomington Stock cCnter and maintained and were raised at 25C on standard corn meal food. For crosses, virgin females were collected from 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 as well as a UAS-TRiP control which contains the genomic insertion site but no shRNA3
. Flies were maintained in a humidified (50–60%)
incubator at 25C. Ten days after each cross the F1 progeny began to eclose and adults were sorted according to phenotype and gender. Flies bearing the balancer markers were discarded from the analysis. Sorted flies were put into new vials, with males and females separated and with 5–10 flies in each vial. Flies were transferred to fresh food twice weekly with deaths noted from each cross. The person handling the flies was blinded to the genotype of the flies.

## Western Blotting

Muscle samples (20-50 ug) were lysed in 20 uL/mg of RIPA buffer using a Qialyser (5 minutes at 30 Hz). After centrifugation (15 minutes at 14 000 RPM), lysates were boiled in a final concentration of 1X SDS-PAGE sample buffer (BioRad). Proteins were separated on pre-cast gradient gels from BioRad and transferred to nitrocellulose membranes. After blocking in 2% bovine serum albumin (Fisher), proteins were probed with anti-LC3, anti-pS6K, total S6K, anti-pS6 and anti-S6 antibodies. Secondary antibodies were alexa 680/700 conjugated anti-mouse and anti-rabbit secondary antibodies and blots were visualized via a LiCOR Odyssey system. Quantification was performed using ImageStudio Lite (LiCOR).

## Statistics

All statistical tests were performed using the R software package4. For pairwise tests, normality was tested via Shapiro-Wilk tests, and equal variance was tested using Levene’s test. Based on these results, either Wilcoxon tests, Student’s *t* or Welch’s *t*-tests were performed. For mouse studies, the three non-knockout genotypes (floxed allele alone, transgene alone and wild-type at both loci) were analyzed separately and then combined and labeled as “Controls” if not significantly different. For survival analyses and cox proportional hazard tests, the survival package was used (version 2.38-3)5,6. All raw data and reproducible code is available at http://bridgeslab.github.io/DrosophilaMuscleFunction

# Results

## Deletion of *Tsc1* in muscle tissue causes shortened lifespan and increased autophagy

To evaluate the effects of chronic mTORC1 elevation on aging in mice, we deleted the negative regulator of mTORC1, TSC1 via a floxed/Cre recombinase system. As shown in Figure 1A-B, deletion of TSC1 causes elevations in mTORC1 activity in quadriceps from these animals as determined by increased phosphorylation of the mTORC1 targets S6K and S6. To evaluate the effects of mTORC1 activation on autophagy in skeletal muscle, we evaluated the levels of LC3-I and II by western blotting. As shown in Figures 1A/C, the LC3-II/I ratio is much lower in *Tsc1* knockout quadriceps, consistent with previous reports using a different Cre line7.

We next observed these animals without manipulation as they aged. We observed increased signs of aging including hunched and scruffy appearances at an earlier age in the knockout animals but not any of the control littermates. As shown in Figure 1D, muscle-specific *Tsc1* knockout mice died of natural causes at a higher rate. Based on a Cox-proportional hazard model the hazard ratio was 4.17 compared to non-knockout littermates (p=2.0 x 10-5).

# Discussion

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# References

1. Kwiatkowski DJ, Zhang H, Bandura JL, Heiberger KM, Glogauer M, el-Hashemite N, Onda H. A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. Hum Mol Genet 2002; 11:525–34.

2. Brüning JC, Michael MD, Winnay JN, Hayashi T, Hörsch D, Accili D, Goodyear LJ, Kahn CR. A Muscle-Specific Insulin Receptor Knockout Exhibits Features of the Metabolic Syndrome of NIDDM without Altering Glucose Tolerance. Mol Cell 1998; 2:559–69.

3. Ni J, Zhou R, Czech B, Liu L, Holderbaum L, Yang-Zhou D, Shim H, Tao R, Handler D, Karpowicz P, et al. A genome-scale shRNA resource for transgenic RNAi in Drosophila. Nat Methods 2011; 8:405–7.

4. R Core Team. R: A Language and Environment for Statistical Computing. 2013;

5. Therneau TM, Grambsch PM. Modeling Survival Data: Extending the Cox Model. New York, NY: Springer New York; 2000.

6. Therneau T. A Package for Survival Analysis in S. R package version. Survival (Lond).2012;

7. Castets P, Lin S, Rion N, Di Fulvio S, Romanino K, Guridi M, Frank S, Tintignac LA a, Sinnreich M, Rüegg MA, et al. Sustained activation of mTORC1 in skeletal muscle inhibits constitutive and starvation-induced autophagy and causes a severe, late-onset myopathy. Cell Metab 2013; 17:731–44.

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