Muscle Activation of mTORC1 and Suppression of Autophagy Shortens Lifespan in Two Divergent Model Organisms

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# Abstract

mTORC1 inhibition via either genetic or pharmacological inhibition extends lifespan in multiple species. In this study, we activated mTORC1, specifically in the striated muscle of two distantly related model organisms representing mammals (*Mus musculus*) and insects (*Drosophila melanogaster*). We found a significant reduction in lifespan in both model systems indicating a critical role for the mTORC1 pathway in lifespan conserved across species. We observed reduced autophagy in mouse skeletal muscle with activated mTORC1. Knockdown of the key autophagy gene *Atg8a* in fly muscle also significantly reduced lifespan. These lifespan effects appear to be restricted to knockdown of mTORC1 in skeletal, but not cardiac muscle in flies. Together these data are consistent with a central role for mTORC1 activity in skeletal muscle in the regulation of organismal lifespan.

# Introduction

mTORC1 is a protein kinase which plays important roles in nutrient sensing, regulating the downstream responses to elevated nutrient availability1,2. Several studies have implicated mTORC1 inhibition as a mechanism of organismal lifespan extension in yeast, worms and mammals3–5. While several pathways underlying extended lifespan have been proposed, the tissue or tissues that link mTORC1 activity to extended lifespan have not been identified.

Skeletal muscle is an important potential target tissue for understanding aging, as functional differences in muscle strength predict lifespan in a longitudinal manner6–11. Furthermore, mTORC1 regulates several important, aging related processes in muscle; including oxidative stress, the unfolded protein response, autophagy and lipid metabolism12–14. In this work we tested the hypothesis that activation of mTORC1, specifically within striated muscle tissue can affect organismal lifespan, in concert with impairments in muscle autophagy.

# Methods and Materials

## Animal Housing and Procedures

Floxed *Tsc1* alleles (Tsc1*tm1Djk*/J15 from the Jackson Laboratory) were crossed with *Ckmm-Cre* driver mice (FVB-Tg(*Ckmm-cre*)5Khn/J16 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 littermates that were wild-type at both the *Tsc1* and *Cre* loci (wild-type), homozygous at the *Tsc1* loci (floxed only), hemizygous at the Cre loci (transgene only) or *Tsc1*fl/fl, *Ckmm-Cre*Tg/+. Only male mice were used in this analysis. 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. For molecular analyses, animals at approximately 5 months were fasted overnight then sacrifice via cervical dislocation under isoflurane-induced anesthesia. For aging experiments, animals were allowed to die naturally, or were euthanized at the advice of veterinary staff. Animals were stored in formalin with their visceral cavity opened until analysis by a veterinary pathologist.

## Drosophila Breeding and Maintenance

Fly stocks were purchased from the Bloomington Stock Center (see Table 1) and maintained at 25 °C on standard corn meal food (Bloomington Drosophila Stock Center). Ten females and 3-4 males of the appropriate genotype were chosen from each UAS-TRiP-shRNA17 line as well as a TRiP docking site control (*y1 v1*; P{y[+t7.7]=CaryP}attP2) which contains the genomic insertion site but no shRNA18 . Flies were maintained in a humidified (50–60%) incubator at 25 °C. Newly eclosed adults were separated and sorted according to genotype and gender with 5–10 flies in each vial. Flies bearing balancer chromosomes, which can decrease viability, were discarded from the analysis. Flies were transferred to fresh food twice weekly and deaths noted from each cross. The person handling the flies was blinded to the genotypes of the flies.

## Western Blotting

Muscle samples from 5 month old mice (20-50 ug) were lysed in 20 L/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 (overnight? 1hr?) (Fisher Scientific), blots were probed with anti-LC3 (Cell Signaling #12741S), anti-pS6K (Cell Signaling #9206S), total S6K (Cell Signaling #2708, anti-pS6 (Cell Signaling #2211S) and anti-S6 antibodies (Cell Signaling #2317S). Secondary antibodies were AlexaFlour conjugated anti-mouse (680nm) and anti-rabbit (700nm) secondary antibodies from Life Technologies. Blots were visualized via a LiCOR Odyssey system. Quantification was performed using ImageStudio Lite (LiCOR).

## Statistics

Statistical significance was designated at α<0.05 for this study. All statistical tests were performed using the R software package19. 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 Rank Sum 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)20,21. P-values were adjusted for multiple hypothesis testing by the method of Benjamini and Hochberg22. With the exception of western blotting, the experimenter was blinded to the genotypes until data were analysed. 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, 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-B, the LC3-II/I ratio is much lower in *Tsc1* knockout quadriceps, indicating reduced autophagy23. This is consistent with previous reports using a different Cre line24.

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 1C, 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). To test whether the presence of the Cre or floxed *Tsc1* allele alone mediated this effect, we evaluated each of these strains separately. As shown in Figure 1D, all three non-knockout control strains had similar death rates (p=0.77).

A subset of mice were stored in formalin and sent for veterinary pathology, but no consistent cause of death was identified. In animals with histologic evidence of lesions, the predominant process was neoplasia, and the specific etiology was lymphoma/lymphosarcoma affecting multiple organs, though this was only true for wild-type (two out of four) but not knockout animals (none out of three). The lack of a specific diagnosis does not necessarily confirm the lack of lesions in examined animals; rather, autolysis and the small number of animals evaluated may have resulted in loss of identifiable processes or tissues in which an etiology was present in-life.

## Knockdown of *Tsc1* in drosophila muscle tissue reduces lifespan

In a previous study we showed that knockdown of the obligate mTORC1 component Raptor in fly muscles causes early lethality and muscle weakness, but that *Tsc1* knockdown results produced viable adult flies25. To test whether gain of function of dTORC1 decreases lifespan in flies, we used the GAL4/UAS system26 to knock down *Tsc1* in fly muscles with two different shRNA constructs from the Harvard TRiP collection17. These knockdowns were driven by *24B*-GAL4, which is expressed in wing disks and adult fly muscles27. As shown in Figure 2A-B, knockdowns of *Tsc1* increases the rate of natural death for both shRNA constructs and both sexes (shRNA #1, 3.8 fold for females, 3.4 fold for males; shRNA #2: 2.6 fold for females, 1.9 fold for males all with adjusted p-values<1 x10-5).

To test whether cardiac-specific knockdown of *Tsc1* has a similar phenotype we used a *Hand*-GAL4 driver which is expressed in fly cardiac tissue28. In contrast to the *24B* driven knockdowns, we only observed modest increases in mortality (20-30% increase in hazard ratios, Figure 2C). Together, these data support the hypothesis that muscle-specific ablation of *Tsc1* results in early lethality in both mice and flies.

# Knockdown of Atg8a in fly muscles reduces lifespan

To test whether chronically impaired autophagy in muscle tissue can reduce Drosophila lifespan, we used a similar approach to reduce the levels of *Atg5*, *Atg8a* and *Atg8b* in fly muscles using the *24B*-GAL4 driver. We found no obvious effect of *Atg5* or *Atg8b* knockdown driven by *24B*-GAL4, but shortened lifespan in both male (3.4 fold) and female (2.9 fold) *Atg8a* knockdown flies (both with adjusted p-values <0.0001). While ATG5 is upstream of ATG8, this result is consistent with findings showing that *Atg5* knockdown using a ubiquitous GAL4 promoter did not reduce lifespan, but did sensitize the flies to starvation3.

# Discussion

In this study we report that in both mice and flies, deletion or knockdown of *Tsc1* specifically in striated muscle reduces lifespan. Cardiac-specific ablation of Tsc1 using *Tagln-Cre* showed early lethality of these mice, at approximately 3 weeks of age29 or 6 months of age in the case of *Myl-Cre*30. These mice exhibited dramatic cardiac hypertrophy and sudden death at a much earlier age than our animals. However, we observed modest reductions in lifespan in the fly cardiac-specific knockdown. Together these data support the hypothesis that the cause of early lethality may be distinct from cardiac defects in mice and flies, but our approach cannot conclusively show that cardiac *Tsc1* ablation does not play a plays role in reducing lifespan.

Skeletal muscle is an extremely important organ for aging, as humans with high baseline grip strength have 20-217% decreased risk of all-cause mortality, irrespective of gender or body mass index6–11. Candidate gene studies on aging have also implicated genes with important roles in muscle tissue, such as *IGF1R*, *AKT1* and *FOXO3A*31,32. For example, in humans, polymorphisms in *FOXO3A* have been associated with lengthened lifespan32–38. Both mouse and fruit fly models of *FOXO3A* loss of function result in stronger and longer living model organisms39–41.

To better understand why ATG8a and ATG8b showed differences in viability, we evaluated the expression of these genes in RNAseq data from the modENCODE consortiumAccording to public RNAseq expression data, *At8a* is widely expressed at higher levels in several tissues relative to *At8b*, including muscle tissues42. *Atg8b* has much more limited expression, and is at lower levels in the fly tissues tested. This may explain the stronger reductions in lifespan in the muscle *Atg8a* knockdown flies.

One potential mechanism linking TORC1 to aging could be impaired protein turnover. TORC1 plays a key role in protein homeostasis, through inhibiting autophagy and proteosomal degradation while promoting protein synthesis in both flies and mice. Drosophila studies have implicated improved muscle proteostasis as a key element coordinating lifespan extension43. Dysregulation of proteostatsis may also underlie the lifespan-restricting effects of high protein diets44. The studies presented here were not designed show that the mTORC1-dependent effects in muscle on aging are upstream of changes in protein turnover and thus, we cannot confirm whether this mechanism is contributing to the phenotypes we observed in our model systems. Indeed, several. Several other mechanisms related to mTORC1 have also been proposed; including oxidative damage, impaired mitochondrial clearance and ER stress. Future studies are necessary to understand the roles of these and other potential downstream effects of TORC1 activity on the aging process. Understanding the tissue-specificity which underlies the some of the effects of TORC1 on aging is an important step to understanding the molecular mechanisms that link TORC1 and rapamycin to organismal lifespan.

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# References

1. Efeyan, A., Comb, W. C. & Sabatini, D. M. Nutrient-sensing mechanisms and pathways. *Nature* **517,** 302–310 (2015).

2. Cornu, M., Albert, V. & Hall, M. N. mTOR in aging, metabolism, and cancer. *Curr. Opin. Genet. Dev.* **23,** 53–62 (2013).

3. Bjedov, I. *et al.* Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. *Cell Metab.* **11,** 35–46 (2010).

4. Harrison, D. E. *et al.* Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* **460,** 392–5 (2009).

5. Kapahi, P. *et al.* Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. *Curr. Biol.* **14,** 885–90 (2004).

6. Rantanen, T. *et al.* Muscle strength and body mass index as long-term predictors of mortality in initially healthy men. *J. Gerontol. A. Biol. Sci. Med. Sci.* **55,** M168–M173 (2000).

7. Ling, C. H. Y. *et al.* Handgrip strength and mortality in the oldest old population: The Leiden 85-plus study. *Cmaj* **182,** 429–435 (2010).

8. Sasaki, H., Kasagi, F., Yamada, M. & Fujita, S. Grip strength predicts cause-specific mortality in middle-aged and elderly persons. *Am. J. Med.* **120,** 337–342 (2007).

9. Gale, C. R., Martyn, C. N., Cooper, C. & Sayer, A. A. Grip strength, body composition, and mortality. *Int. J. Epidemiol.* **36,** 228–235 (2007).

10. Rantanen, T. *et al.* Handgrip strength and cause-specific and total mortality in older disabled women: exploring the mechanism. *J. Am. Geriatr. Soc.* **51,** 636–641 (2003).

11. Metter, E. J., Talbot, L. a, Schrager, M. & Conwit, R. Skeletal muscle strength as a predictor of all-cause mortality in healthy men. *J. Gerontol. A. Biol. Sci. Med. Sci.* **57,** B359–B365 (2002).

12. Ozcan, U. *et al.* Loss of the Tuberous Sclerosis Complex Tumor Suppressors Triggers the Unfolded Protein Response to Regulate Insulin Signaling and Apoptosis. *Mol. Cell* **29,** 541–551 (2008).

13. Bentzinger, C. F. *et al.* Differential response of skeletal muscles to mTORC1 signaling during atrophy and hypertrophy. *Skelet. Muscle* **3,** 6 (2013).

14. Laplante, M. & Sabatini, D. M. mTORC1 activates SREBP-1c and uncouples lipogenesis from gluconeogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 3281–2 (2010).

15. Kwiatkowski, D. J. *et al.* 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.* **11,** 525–34 (2002).

16. Brüning, J. C. C. *et al.* A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol. Cell* **2,** 559–569 (1998).

17. Housden, B. E. *et al.* Loss-of-function genetic tools for animal models: cross-species and cross-platform differences. *Nat. Rev. Genet.* **18,** 24–40 (2016).

18. Ni, J. *et al.* A genome-scale shRNA resource for transgenic RNAi in Drosophila. *Nat. Methods* **8,** 405–7 (2011).

19. R Core Team. R: A Language and Environment for Statistical Computing. (2013).

20. Therneau, T. M. & Grambsch, P. M. *Modeling Survival Data: Extending the Cox Model*. (Springer New York, 2000). doi:10.1007/978-1-4757-3294-8

21. Therneau, T. A Package for Survival Analysis in S. R package version. *Survival* (2012).

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

23. Klionsky, D. J. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* **12,** 1–222 (2016).

24. Castets, P. *et al.* Sustained activation of mTORC1 in skeletal muscle inhibits constitutive and starvation-induced autophagy and causes a severe, late-onset myopathy. *Cell Metab.* **17,** 731–44 (2013).

25. Hatfield, I. *et al.* The role of TORC1 in muscle development in Drosophila. *Sci. Rep.* **5,** 9676 (2015).

26. Duffy, J. B. GAL4 System in Drosophila : A Fly Geneticist ’ s Swiss Army Knife. **15,** 1–15 (2002).

27. Luo, L., Joyce Liao, Y., Jan, L. Y. & Jan, Y. N. Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Dev.* **8,** 1787–1802 (1994).

28. Han, Z., Yi, P., Li, X. & Olson, E. N. Hand, an evolutionarily conserved bHLH transcription factor required for Drosophila cardiogenesis and hematopoiesis. *Development* **133,** 1175–82 (2006).

29. Malhowski, A. J. *et al.* Smooth muscle protein-22-mediated deletion of Tsc1 results in cardiac hypertrophy that is mTORC1-mediated and reversed by rapamycin. *Hum. Mol. Genet.* **20,** 1290–305 (2011).

30. Meikle, L. *et al.* A mouse model of cardiac rhabdomyoma generated by loss of Tsc1 in ventricular myocytes. *Hum. Mol. Genet.* **14,** 429–435 (2005).

31. Suh, Y. *et al.* Functionally significant insulin-like growth factor I receptor mutations in centenarians. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 3438–42 (2008).

32. Pawlikowska, L. *et al.* Association of common genetic variation in the insulin/IGF1 signaling pathway with human longevity. *Aging Cell* **8,** 460–72 (2009).

33. Willcox, B. J. *et al.* FOXO3A genotype is strongly associated with human longevity. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 13987–13992 (2008).

34. Bao, J.-M. *et al.* Association between FOXO3A gene polymorphisms and human longevity: a meta-analysis. *Asian J. Androl.* **16,** 446–52 (2014).

35. Anselmi, C. V. *et al.* Association of the FOXO3A locus with extreme longevity in a southern Italian centenarian study. *Rejuvenation Res.* **12,** 95–104 (2009).

36. Flachsbart, F. *et al.* Association of FOXO3A variation with human longevity confirmed in German centenarians. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 2700–2705 (2009).

37. Li, Y. *et al.* Genetic association of FOXO1A and FOXO3A with longevity trait in Han Chinese populations. *Hum. Mol. Genet.* **18,** 4897–4904 (2009).

38. Soerensen, M. *et al.* Replication of an association of variation in the FOXO3A gene with human longevity using both case-control and longitudinal data. *Aging Cell* **9,** 1010–1017 (2010).

39. Giannakou, M. E. *et al.* Long-lived Drosophila with overexpressed dFOXO in adult fat body. *Science (80-. ).* **305,** 361 (2004).

40. Hwangbo, D. S., Gershman, B., Tu, M.-P., Palmer, M. & Tatar, M. Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. *Nature* **429,** 562–566 (2004).

41. Milan, G. *et al.* Regulation of autophagy and the ubiquitin-proteasome system by the FoxO transcriptional network during muscle atrophy. *Nat. Commun.* **6,** 6670 (2015).

42. Brown, J. B. *et al.* Diversity and dynamics of the Drosophila transcriptome. *Nature* **512,** 1–7 (2014).

43. Demontis, F. & Perrimon, N. FOXO/4E-BP signaling in Drosophila muscles regulates organism-wide proteostasis during aging. *Cell* **143,** 813–25 (2010).

44. Solon-Biet, S. M. *et al.* The ratio of macronutrients, not caloric intake, dictates cardiometabolic health, aging, and longevity in ad libitum-fed mice. *Cell Metab.* **19,** 418–430 (2014).

45. Levine, M. E. E. *et al.* Low protein intake is associated with a major reduction in IGF-1, cancer, and overall mortality in the 65 and younger but not older population. *Cell Metab.* **19,** 407–417 (2014).

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