Activation of mTORC1 and Suppression of Autophagy in Muscle 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. 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 25C on standard corn meal food. For crosses, virgin females were collected from the GAL4 driver strains. Ten females and 3-4 males with the appropriate genotype were chosen from each 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 separated and sorted according to visible markers and gender with 5–10 flies in each vial. Flies bearing the balancer markers were discarded from the analysis. 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. P-values were adjusted for multiple hypothesis testing by the method of Benjamini and Hochberg7. 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-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 line8.

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).

As previous reports implicated cardiac hypertrophy in cardiac muscle-specific knockout of *Tsc1*9,10 we evaluated cardiac mass and histology from these mice. We did not observer any evidence of rhabdomyomas, as was present in previous reports, but we did observe increased cardiac mass (Figure 1E). 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

Our previous studies had shown that knockdown of the obligate mTORC1 component Raptor in fly muscles causes early lethality and muscle weakness, but that *Tsc1* knockdown resulted in viable adult flies11. To test whether gain of function of dTORC1 decreases lifespan in flies, we used the UAS-shRNA/GAL4 system to knockdown *Tsc1* in fly muscles. These knockdowns were driven by *24B*-GAL4 which is expressed in wing disks and adult fly muscles12. As shown in Figure 2A-D, knockdowns driven by two shRNA’s targeting *Tsc1* increase the rates of natural deaths for both strains 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 tissue13. In contrast to the *24B* driven knockdowns, we only observed modest increases in mortality (20-30% increase in hazard ratios, Figure 2E-F). 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 that there

# Discussion

In this study we report that in both mice and flies, deletion or knockdown of *Tsc1* specifically in muscle tissue reduces lifespan. Cardiac-specific ablation of Tsc1 using *Tagln-Cre* showed early lethality of these mice, at approximately 3 weeks of age10 or 6 months of age in the case of *Myl-Cre*9. These mice exhibited dramatic cardiac hypertrophy and sudden death at a much earlier age than our animals. 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 plays no role in reductions in lifespan.

Skeletal muscle tissue is an extremely important organ for aging, as participants with the highest baseline grip strength had 20-217% decreased risk of all-cause mortality, irrespective of gender or body mass index14–19. Candidate gene studies on aging have also implicated genes with important roles in muscle tissue such as *IGF1R*, *AKT1* and *FOXO3A*20,21. For example, in humans, polymorphisms in *FOXO3A* have been associated with lengthened lifespan21–27. Both mouse and fruit fly models of *FOXO3A* loss of function result in stronger and longer living model organisms28–30.

One potential mechanism linking TORC1 to aging could be protein overproduction. 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 extension31. Dysregulation of proteostatsis may also underlie the lifespan-restricting effects of high protein diets32. The studies presented here were not designed show that the mTORC1-dependent effects in muscle on aging are upstream of changes in protein turnover. Several other mechanisms related to mTORC1 have also been proposed including oxidative damage, impaired mitochondrial clearance and ER stress. Future studies will be needed to understand the roles of these and other potential downstream effects. 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. 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 MDD, 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. 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.

8. Castets P, Lin S, Rion N, Di Fulvio S, Romanino K, Guridi M, Frank S, Tintignac LAA, 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.

9. Meikle L, McMullen JR, Sherwood MC, Lader AS, Walker V, Chan JA, Kwiatkowski DJ. A mouse model of cardiac rhabdomyoma generated by loss of Tsc1 in ventricular myocytes. Hum Mol Genet 2005; 14:429–35.

10. Malhowski AJ, Hira H, Bashiruddin S, Warburton R, Goto J, Robert B, Kwiatkowski DJ, Finlay GA. Smooth muscle protein-22-mediated deletion of Tsc1 results in cardiac hypertrophy that is mTORC1-mediated and reversed by rapamycin. Hum Mol Genet 2011; 20:1290–305.

11. Hatfield I, Harvey I, Yates ER, Redd JR, Reiter LT, Bridges D. The role of TORC1 in muscle development in Drosophila. Sci Rep 2015; 5:9676.

12. Luo L, Joyce Liao Y, Jan LY, Jan YN. Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. Genes Dev 1994; 8:1787–802.

13. Han Z, Yi P, Li X, Olson EN. Hand, an evolutionarily conserved bHLH transcription factor required for Drosophila cardiogenesis and hematopoiesis. Development 2006; 133:1175–82.

14. Rantanen T, Harris T, Leveille SG, Visser M, Foley D, Masaki K, Guralnik JM. Muscle strength and body mass index as long-term predictors of mortality in initially healthy men. J Gerontol A Biol Sci Med Sci 2000; 55:M168–73.

15. Ling CHY, Taekema D, De Craen AJM, Gussekloo J, Westendorp RGJ, Maier AB. Handgrip strength and mortality in the oldest old population: The Leiden 85-plus study. Cmaj 2010; 182:429–35.

16. Sasaki H, Kasagi F, Yamada M, Fujita S. Grip strength predicts cause-specific mortality in middle-aged and elderly persons. Am J Med 2007; 120:337–42.

17. Gale CR, Martyn CN, Cooper C, Sayer AA. Grip strength, body composition, and mortality. Int J Epidemiol 2007; 36:228–35.

18. Rantanen T, Volpato S, Ferrucci L, Heikkinen E, Fried LP, Guralnik JM. Handgrip strength and cause-specific and total mortality in older disabled women: exploring the mechanism. J Am Geriatr Soc 2003; 51:636–41.

19. Metter EJ, 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 2002; 57:B359–65.

20. Suh Y, Atzmon G, Cho M-O, Hwang D, Liu B, Leahy DJ, Barzilai N, Cohen P. Functionally significant insulin-like growth factor I receptor mutations in centenarians. Proc Natl Acad Sci U S A 2008; 105:3438–42.

21. Pawlikowska L, Hu D, Huntsman S, Sung A, Chu C, Chen J, Joyner AH, Schork NJ, Hsueh W-CC, Reiner AP, et al. Association of common genetic variation in the insulin/IGF1 signaling pathway with human longevity. Aging Cell 2009; 8:460–72.

22. Willcox BJ, Donlon T a, He Q, Chen R, Grove JS, Yano K, Masaki KH, Willcox DC, Rodriguez B, Curb JD. FOXO3A genotype is strongly associated with human longevity. Proc Natl Acad Sci U S A 2008; 105:13987–92.

23. Bao J-M, Song X-L, Hong Y-Q, Zhu H-L, Li C, Zhang T, Chen W, Zhao S-C, Chen Q. Association between FOXO3A gene polymorphisms and human longevity: a meta-analysis. Asian J Androl 2014; 16:446–52.

24. Anselmi CV, Malovini A, Roncarati R, Novelli V, Villa F, Condorelli G, Bellazzi R, Puca AA. Association of the FOXO3A locus with extreme longevity in a southern Italian centenarian study. Rejuvenation Res 2009; 12:95–104.

25. Flachsbart F, Caliebe A, Kleindorp R, Blanché H, von Eller-Eberstein H, Nikolaus S, Schreiber S, Nebel A. Association of FOXO3A variation with human longevity confirmed in German centenarians. Proc Natl Acad Sci U S A 2009; 106:2700–5.

26. Li Y, Wang WJ, Cao H, Lu J, Wu C, Hu FY, Guo J, Zhao L, Yang F, Zhang YX, et al. Genetic association of FOXO1A and FOXO3A with longevity trait in Han Chinese populations. Hum Mol Genet 2009; 18:4897–904.

27. Soerensen M, Dato S, Christensen K, McGue M, Stevnsner T, Bohr V a., Christiansen L. Replication of an association of variation in the FOXO3A gene with human longevity using both case-control and longitudinal data. Aging Cell 2010; 9:1010–7.

28. Giannakou ME, Goss M, Jünger MA, Hafen E, Leevers SJ, Partridge L. Long-lived Drosophila with overexpressed dFOXO in adult fat body. Science (80- ) 2004; 305:361.

29. Hwangbo DS, Gershman B, Tu M-P, Palmer M, Tatar M. Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature 2004; 429:562–6.

30. Milan G, Romanello V, Pescatore F, Armani A, Paik J-H, Frasson L, Seydel A, Zhao J, Abraham R, Goldberg AL, et al. Regulation of autophagy and the ubiquitin-proteasome system by the FoxO transcriptional network during muscle atrophy. Nat Commun 2015; 6:6670.

31. Demontis F, Perrimon N. FOXO/4E-BP signaling in Drosophila muscles regulates organism-wide proteostasis during aging. Cell 2010; 143:813–25.

32. Solon-Biet SM, McMahon AC, Ballard JWO, Ruohonen K, Wu LE, Cogger VC, Warren A, Huang X, Pichaud N, Melvin RG, et al. The ratio of macronutrients, not caloric intake, dictates cardiometabolic health, aging, and longevity in ad libitum-fed mice. Cell Metab 2014; 19:418–30.

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