Age-dependent effects of reduced mTor signalling on life expectancy through distinct physiology

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Abstract

Research on the mechanisms of ageing has identified ways via which lifespan can be extended in model organisms, increasing the potential for translation of these findings to our own species. However, the large majority of research on animal models involves dietary, genetic or pharmacological treatments throughout life – limiting translational potential and ignoring age-dependent effects. Previously, we have suggested using demographic meta-analysis that reduced mTor signalling has the potential to instantly rejuvenate. We have now tested this prediction experimentally using large-scale demographic data (N > 10,000) combined with conditional knockdown of mTor in *Drosophila melanogaster*. Indeed, reduced mTor decreased mortality rate when applied during old age. Interestingly, we found that transient treatment during early adult life had long-lasting benefits. Age-dependent deep-RNAseq indicated that these effects arose from distinct physiology and implicate alternative splicing as a potential mechanism for the long-lasting benefits of transient mTor reduction. These findings suggest that reducing mTor short term or during old age could be used to combat ageing. In addition, our findings suggest that the results from experimental research on mTor signalling, and potentially other mechanisms of ageing, that employ life-long interventions are likely to be a complex composite of age-dependent effects that counteract or enhance each other.

Introduction

The biology of ageing field has progressed our understanding of mechanisms and interventions that can extend health- and lifespan. The most potent and researched of these are dietary restriction[1–3] and reducing mTor (mechanistic Target of Rapamycin) signalling[4–6]. These interventions we now (only) partially understand and there is a growing wish and reality for translation to our own species[7]. A key factor that hinders the translation of these findings to humans is however that the large majority of the experiments in animal models are carried out for their entire lifetimes, which begs the question whether long-term treatment will be required in humans as well. Any long-term treatment will be hard to apply in our own species and close to impossible to study in clinical trials. More immediate benefits to health- and lifespan are sought for to hold translational promise[8,9]. Notably, dietary restriction can have instant benefits on health indicators[2,10], but trials in humans have only been conducted over a relatively short timespan[10,11]. Animal experiments have shown that in terms of longevity, mortality risk is instantly modulated by diet in flies[12–14], but experiments in other organisms, namely rodents suggest late-life treatment is not necessarily pro-longevity[15,16]. In contrast, short-term energy restriction in early life can result in long-lasting health benefits, as for example in mice by restricting milk access[17].

Reduced mTor signalling has now also been suggested to improve life expectancy after short-term treatment or in old animals. Rapamycin treatment from 600 days of age onwards resulted in longevity benefits (especially in females, as control biased mortality occurred prior to drug treatment in males)[9]. Recently, short term transient rapamycin treatment (of 3 months) of mice aged 2 years led to a lifespan extension, but the authors acknowledge sample size for this study is limited[18]. We have recently also argued using demographic meta-analysis across four model species that reducing mTor signalling at old age could reduce mortality risk[4]. Such inference from demographic models can be informative[4,15,16,19,20], but only by testing different ages of manipulation can such demographic patterns be tested for causality[8,12,13,21]. Here, we present the first of such comprehensive evidence using large-scale demography comprising over 10,000 individual flies (*Drosophila melanogaster*), showing that reduction of mTor in late life results in instant benefits on life expectancy. In addition, we find that short transient mTor knockdown during early adulthood has long-lasting

benefits reducing mortality in late life even when mTor levels are back to unmanipulated levels. Age-dependent RNAseq data from this experiment showed that these two effects originate from differential physiology, potentially derived from differences in alternative splicing.

Results and discussion

Experimental design and GeneSwitch kinetics

Age-dependent knockdown of mTor (using *in vivo* RNAi[22]) was achieved using the well-established conditional GeneSwitch system[23,24], by feeding adult flies mifepristone (RU486). We chose RNAi over rapamycin treatment as genetic suppression of mTor signalling results in larger effects[4] and more potent rapalogs are currently being developed[25]. The ligand RU486 for GeneSwitch allows close experimental control of downstream genetic tools. Our experimental design consisted of four groups. A transient treatment with flies fed food containing RU486 for 12 days during early adult life (starting at age 15±1), a late life group fed RU486 after this timepoint (starting at 27 d old), and two additional groups either fed RU486 from age 15 d until death ('whole life') or fed control food (control, see methods and Figure 1). This age-dependent treatment regime was chosen based on an initial experiment measuring age-specific mortality in response to whole life mTor knockdown, as well as a lower sample size experiment that showed mTor knockdown in late life reduced mortality (both unpublished).

Control experiments confirmed there were no age-dependent changes in the inducibility of the GeneSwitch system (e.g. due to differential feeding or metabolism of RU486) and determined the kinetics of GeneSwitch induction and termination upon RU feeding. With *daughterless* GeneSwitch (*da*-GS, expressed in the whole fly)[23] crossed to overexpression constructs of *hid* and *reaper* (inducers of cell death[26]), we drove and measured the corresponding impact on death relative to RU feeding. Irrespective of age of induction, mortality started to rise after about 2-4 days after initial RU feeding, reached maximum induction after 12 days of RU feeding, and returned to control levels after about 12 day once RU feeding was terminated (Figure 2A, $P < 0.0001$, $N = 3,287$). These RU induction dynamics were used to interpret the kinetic effects of mTor knockdown on mortality and transcriptional profiles (Figure 1).

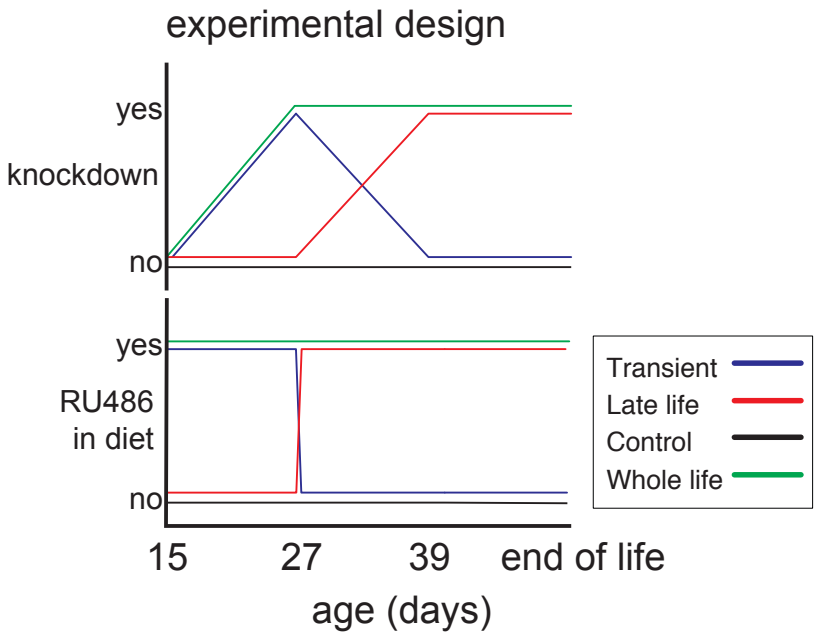

Figure 1. Experimental design. RU486 is fed in an age-dependent manner. Knockdown of mTor is will follow up to maximum induction which is reached after 12 days (Figure 2A). Temporal dynamics of knockdown induction is drawn as linear but could follow other dynamics. Key for interpretation of both mortality and RNAseq data, below, is that maximum response in mortality and associated physiological changes is expected at age 27 and 39.

Negative Control

Although no confounding effects of RU486 on mortality have been reported in similar experiments[23,27–30] we conducted negative controls to exclude any such effects. The *da*-GS driver line was crossed to a genetic control from the TRiP collection that included the insertion vector without shRNA. Offspring of this cross were studied directly alongside the kinetic control and age-dependent knockdown experiments. When RU486 was applied in the same regime as all induction experiments, no difference in mortality was seen among any treatment (Figure 2B, $P > 0.56$, $N = 4{,}708$).

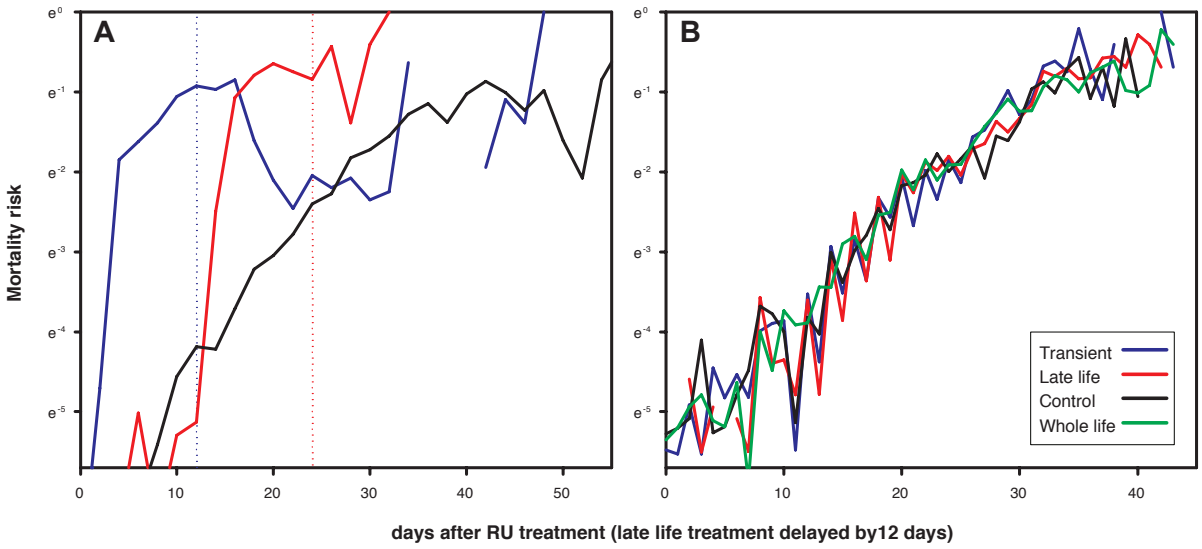

Figure 2. Raw mortality data **A)** *da*-GS (whole fly expression) driving overexpression of *hid* and *rpr* conditionally induced death in the fly, and such induction was reversible in the transient treatment. After the start of RU486 feeding it takes 12 days for full induction of mortality and this drops back to normal levels after removal of RU (see Figure 1 for experimental design). Note control mortality is similar to controls in panel B, suggesting *da*-GS does not cause any leaky expression, i.e. overexpression of *hid* or *reaper* in the absence of RU486. **B)** Negative control: *da*-GS crossed to a TRiP control line (as dTor knockdown, see below, uses TRiP). In none of the timing regimes did RU486 have any effect on mortality ($P > 0.56$), as also reported previously in other studies.

Age-dependent effects of mTor knockdown on mortality

Knockdown of mTor at old age instantly decreased mortality (Figure 3A, Hazard rate (exp) = 0.65 ± 0.06, $P < 0.0001$). Continuous knockdown and transient knockdown of mTor produced similar mortality trajectories where death rate initially increased relative to control but then reduced to a level below that of the control for the remainder of the trial (Figure 3B, Hazard rate (exp) = 0.41—0.43 ± 0.06, $P < 0.0001$). Because early mortality was induced by mTor knockdown, we evaluated if subsequent lifespan extension could be explained by demographic selection acting on phenotypic heterogeneity for frailty. If the frailest flies were killed by the initial induction of mTor RNAi, mortality measured in the remaining cohort could be reduced by the early loss of this frail subset. To determine if demographic selection accounts for our observed late-life mortality pattern, we generated simulated life tables under an assumption that the excess deaths introduced in early life (N = 329) upon induction of mTor RNAi did not act on frailty variation. We simulated life tables beginning at age 39 that now included the 329 individuals that were lost before age 33 days old (as compared to control) in the observed data by uniformly sampling and

reintroduced simulated deaths across this age-interval (ages 39 to 64 days). Note that because mortality increases exponentially, uniform sampling actually biases mortality towards earlier ages and thus simulates the bias induced by phenotypic heterogeneity in frailty. Subsequently we gradually moved and shortened the interval of reintroduction of simulated deaths to earlier ages in subsequent simulations, iteratively increasing demographic selection up to a maximum of all deaths reintroduced at age 39 days. Across all these simulated scenarios, mortality at late ages continued to be significantly reduced in the treatments with early and continuous mTor RNAi ($P < 0.0001$). Thus, transient knockdown of mTor during early life appears to reduce later mortality through long-lasting physiological effects.

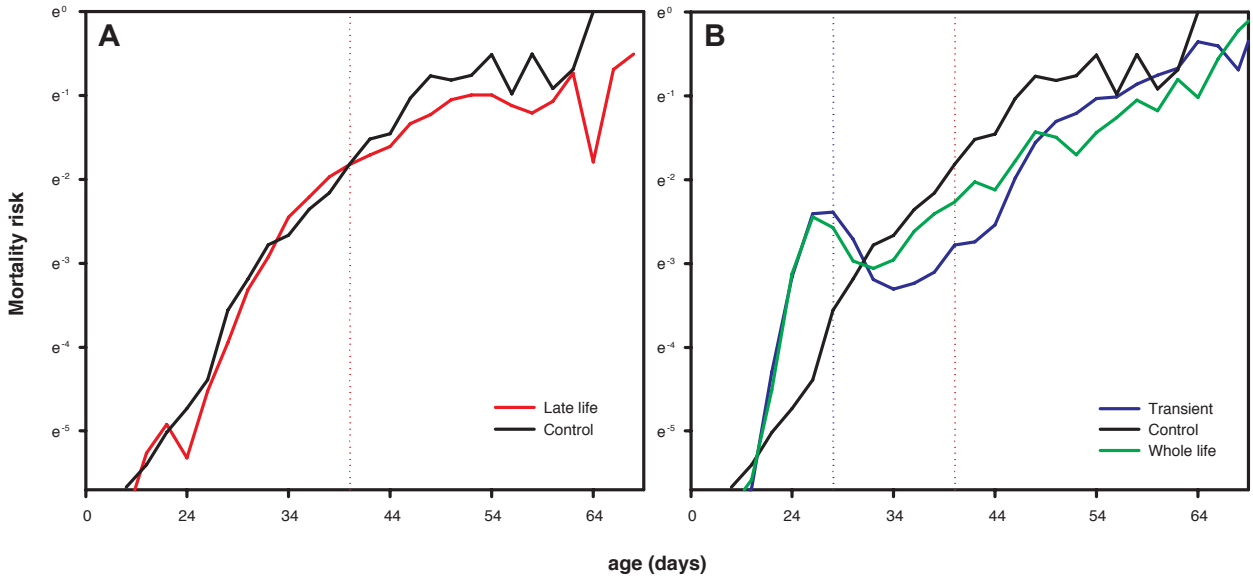

Figure 3. Raw mortality risk plotted on a natural log axis. **A)** Mortality risk is lowered when mTor is knocked down in late life ($P < 0.0001$). The red dotted line indicates when *da*-GeneSwitch is maximally inducing *in vivo* RNAi to knockdown mTor. Data analysed using age-dependent mixed effects cox proportional hazard models correcting for cage effects (see methods[14,31]). **B)** Transient mTor knockdown (maximum induction of GeneSwitch at blue dotted line) resulted in a modest increase in mortality during early life, but subsequently resulted in a sustained mortality reduction throughout life ($P < 0.0001$). Similar effects were seen when mTor was knocked down continuously. The red dotted line now indicated when mTor in the transient treatment is back to control levels. These experiments were all ran together at the same time but are split in two panels for graphical purposes.

Transcriptomes of age-dependent mTor knockdown suggests distinct physiological responses to early and late induction

Conditional knockdown of mTor impacts whole fly mRNA profiles with immediate, reversible and long-lasting changes (Figure 4). Notably, transient mTor reduction induced long-lasting changes in the transcriptome that are distinct from late and whole-life mTor reduction. We used a combined statistical framework (see methods) to distil transcriptional changes that are uniquely associated with the different age-dependent treatment regimes of mTor RNAi (Figure 1). We plotted these effects on the KEGG mTor network to visualize these differences. Knockdown of mTor in late life produced only a limited response across the whole network, whereas transient knockdown in early life induced persistent, substantial differential expressed across the network even though mTor expression was back at control levels (Figure S1).

In the early, transient mTor knockdown cohort, over 6,000 of 10,187 identified transcripts were statistically differentially expressed when compared at old age (model comparison second sampling point at 39 days old, after false-discovery rate Benjamini-Hochberg correction) (Figure 5). Continuous knockdown resulted in over 2,000 differentially expressed genes, whereas late life knockdown resulted in limited differential transcription of around 700 genes. Note, that the comparisons of interest statistically identified here are the additional contribution of the timing of mTor knockdown (early versus late) on top of any effects of whole life knockdown compared to control (for statistical framework see methods). Remarkably, there was only limited overlap in altered transcripts among the treatment categories suggesting the age-dependent effects of mTor knockdown were distinct rather than additive (Figure 5).

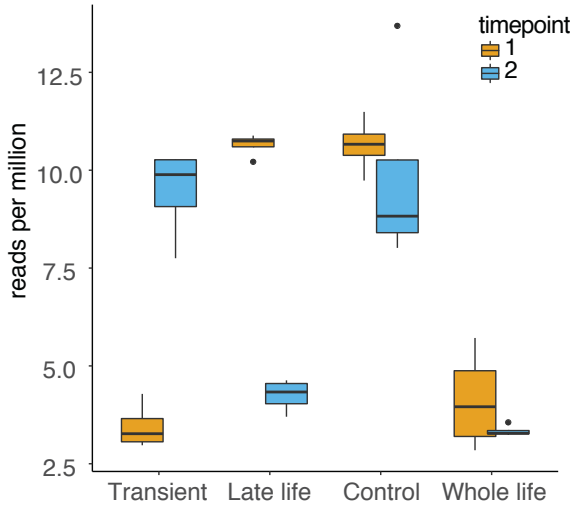

Figure 4. mTor was transiently knocked down, most notably to similar levels in the different treatment categories ($F_{3,24}$= 37.3, P < 0.0001). Blue boxplots indicate the level of mTor expression in RNA from whole flies collected at late life induction (corresponding to red dotted line Figure 3A, see Figure 1). Orange boxplots indicate the same but for early life maximal induction (corresponding to blue line Figure 3B). Transient treatments resulted in immediate experimental changes in transcription in mTor as intended and transient treatment at the respective timepoints where statistically indistinguishable from continuous treatments (P > 0.26).

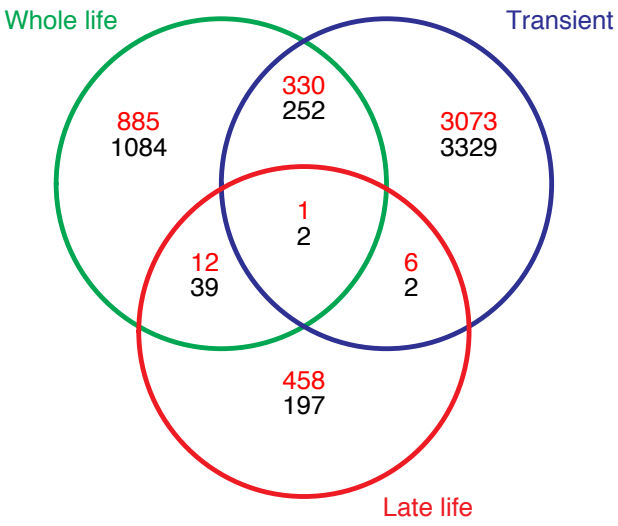

Figure 5. Venn diagram of differentially expressed genes, following false discovery rate correction, compared to the control treatment. Numbers in red are downregulated, black are upregulated, compared to control conditions. Note there is limited overlap between categories suggesting that differences in the transcriptome induced by age-dependent mTor reduction, compared to control, are not additive between treatments, but distinct.

Age-dependent reduction of mTor signalling implicates different physiology

We performed directional KEGG and GO enrichment analyses[32] to provide a generalised view of physiological changes between the transcriptional responses across the three distinct timings of mTor knockdown compared to control (Tables S1-S6). Divergent processes were associated with each of the three timing regimes of mTor reduction.

Whole life mTor reduction was associated with upregulated sugar-based metabolism and amino-acid metabolism (Table S1). In contrast to previous studies where mTor suppression upregulates protein processing and the proteasome[33,34], reduced mTor here downregulated these protein degradation pathways. Variation in temporal dynamics may explain these differences: when the cell is starved it will activate autophagy and protein degradation related mechanisms, but once excess proteins are recycled, the resultant effect is a downregulation of the protein recycling machinery and a shift in total metabolism. The conclusion of the effects of reduced mTor signalling on protein processing and the proteasome might therefore depend on the timing after which this is measured and may depend on the system in which this is studied: cell culture versus whole organism, but this interpretation will require future testing. In line with the interpretation that metabolism is downregulated when mTor is reduced for a prolonged time is that DNA replication and RNA transport are downregulated (Table S1).

In contrast to continuous mTor knockdown, transient depletion of mTor in early adult life upregulated cellular activity at terms representing DNA replication, RNA transport and basal transcription factors, and for ubiquitin mediated proteolysis at old age. Lysosome-associated terms are downregulated as are as elements for metabolism, mainly related to fats (Table S2). We find terms for the spliceosome are widely upregulated, which may explain why transient mTor knockdown has systemic long-term effects on mortality rate and gene expression (Table S2). Notably, work with C. elegans recently suggests that splicing regulation is a potential key mediator of ageing during dietary restriction and by control of mTor[35–38]. We detected limited KEGG enrichment with genes uniquely differentially expressed in the late life mTor knockdown regime suggesting these effects are similar to knockdown of mTor throughout life. Of note, is the partial downregulation of the spliceosome (15 out of

115 genes in this category, Table S3) compared to upregulation in the transient mTor knockdown treatment (84 out of 115). These interpretations based on KEGG were likewise seen in GO analysis, although at lower resolution (Tables S4-S6).

Alternative splicing

Noting that the spliceosome was upregulated in the transient mTor group, we analysed the RNAseq data set for alternative splicing using exon-level based reads. In the transient mTor knockdown group, 327 genes were significantly differentially spliced, compared to 17 of continuous mTor knockdown and 4 specific to mTor knockdown in late life. KEGG analysis in the transient mTor knockdown group were enriched for differentially spliced variants associated with endocytosis[39] , the lysosome[40], Hippo signalling (potentially mediating longevity through autophagy[41] and FOXO[42]) and mTor signalling itself (Table S7). Thus, potentially the long-lasting mortality benefits from transient mTor reduction are mediated by long-lasting changes in alternative splicing, as predicted from the upregulation of the spliceosome[43].

Conclusion

Short-term treatments that extend lifespan will be key to translate findings from the field of ageing biology to actual medical applications. These experiments provide the evidence, together with earlier findings from late-life and transient treatment with rapamycin (inhibitor of mTor) in mice[9,18], that short-term or timed suppression of mTor signalling can have beneficial effects on life/health-span[4] – a treatment regime that might be practical for humans. While the magnitude of reduced mortality produced by mTor inhibition are less than those we report for diet restriction in Drosophila[1,13], they are large relative to those gained in humans when key environmental factors are modulated, such as by cessation of smoking[44]. Furthermore, long-term mortality benefits of early, transient mTor depletion appears to operate through different transcriptional changes compared to how mTor affects older animals, and the early impacts appear to involve alternative splicing. The long-lasting benefits from transient treatment could arise from metabolic or signalling reprogramming or hormesis[45].

These novel insights will help inform future application and potential side effects of drugs targeting mTor. Future work will benefit from uncovering if tissue-specific effects of mTor signalling underlie these age-dependent dynamics and will also need to experimentally test which physiological mechanisms hypothesised from the transcriptome profiles cause the observed mortality differences. These mechanisms will be complex because our data suggest that reduced mTor throughout life probably affects a composite of two (or more) age-dependent processes.

Methods

Fly husbandry and mortality measurement

Flies were grown and kept on our standard rich diet (8% autolysed yeast, 13% table sugar, 6% cornmeal, 1% agar and nipagin 0.225% [w/v], with only growing bottles containing 0.4% [v/v] propanoic acid)[14]. Media for vials was cooked and then spilt to allow preparation of drug or control food from the same batch of fly food, controlling for variation in cooking batch. RU486 (Generon UK, dissolved in absolute ethanol at a stock solution of 10mg/ml) was added to the media, during cooling for dispensing into vials, to give a final concentration of 200μM in the fly food. An equal volume of absolute ethanol was added to the control food.

Flies for experiments were grown in controlled density bottles by using a set amount of 10 virgins mated with males. Offspring of each cross was age standardised by transferring all newly eclosed adult flies each day to a new bottle (Flystuff square bottles) for two days of subsequent mating. After mating, flies were sorted into vials, using light CO_2 anaesthesia, of 25 females each and transferred the same day to a demography cage to contain 125 flies each (for a detailed description see[14]). The cage design[12] allows the removal of dead flies and changing of food, every other day, without physically transferring the flies, to allow for as little disturbance to them as possible. A low frequency of accidental deaths (stuck to the food or killed accidentally) and escapees were righthand censored in the analyses.

Statistical analysis of mortality

All mortality data was analysed using mixed effects cox-proportional hazard models using age-dependent covariates[46] to test for the age-dependent changes in mortality. These models are conservative as they correct for pseudoreplication cause by cage effects (similar to vial to vial error and critical in reducing type I error). Age-dependent covariates allow a comparison during specific ages, specifically relevant as the experimental design induced age-dependent modulation of mTor (Figure 1)[14]. Coefficients are reported in the text and are in comparison to the control treated flies. These coefficients are shown on the linear scale, with standard errors on the log-scale to maintain symmetric errors. Models also included transfer day in the model to correct for any variation between day or growing conditions (although omission of

this correction did not change any of the results). All experiments had a maximum of four transferring days into the cages and ages at RU486 treatment thus maximally differed 1-2 days around the mean age of induction. The x-axis, age, in the raw mortality graphs is corrected for such differences in transfer data as all flies (across all the data presented) were given RU486 on the same calendar date. In the graphs this allows an appreciation of the changes in mortality in response to RU486 timing. All analyses included actual non-corrected ages.

Transcriptome

RNA was extracted from a lysate (generated by bead milling) of ~4 whole flies per sample (4 flies per treatment per timepoint, total 32 samples) using a Qiagen RNeasy mini kit. Samples were shipped on dry ice to the Oxford Genomics Centre where samples were reverse transcribed and an equal concentration was polyA enriched library prepped and deep-sequenced in full multiplex using Illumina HiSeq4000 with 75bp paired ends. Average mapped reads across samples was 176 million. Reads were mapped to the Drosophila melanogaster genome (Release 6) using annotated (and thus not *de novo* assembled) transcripts using *hisat2*[47], formatted into read counts using *stringtie*[47] to be used in *ballgown*[47] in R, and analysed for differential expression using *edgeR* using the general linear modelling framework in *glmQLFit*. We used a full model design correcting for age-dependent changes of mTor knockdown to distil the effects of the timing regimes specifically at late age on the total transcriptome (y ~ knockdown * timepoint + timing regime at old age). This statistical framework therefore identifies differential transcription specific to the timing regime by which mTor is conditionally knocked down compared to control conditions when mortality benefits where observed (Figure 3). Differential splicing was analysed using exome mapping and the function *diffSpliceDGE* from *edgeR*. KEGG and GO enrichments[32] were conducted using the *limma*[48] package in R. For plotting of the KEGG mTor pathway, the pathway was updated manually using the most recent fly literature for plotting purposes only (but not for enrichment analyses).

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355 **References**

356 1. Tatar, M. The plate half-full: Status of research on the mechanisms of dietary
357 restriction in Drosophila melanogaster. *Exp. Gerontol.* **46**, 363–368 (2011).
358 2. Fontana, L. & Partridge, L. Promoting health and longevity through diet: From
359 model organisms to humans. *Cell* **161**, 106–118 (2015).
360 3. Simons, M. J. P., Koch, W. & Verhulst, S. Dietary restriction of rodents
361 decreases aging rate without affecting initial mortality rate - a meta-analysis.
362 *Aging Cell* **12**, 410–414 (2013).
363 4. Garratt, M., Nakagawa, S. & Simons, M. J. P. Comparative idiosyncrasies in
364 life extension by reduced mTOR signalling and its distinctiveness from dietary
365 restriction. *Aging Cell* **15**, (2016).
366 5. Swindell, W. R. Meta-Analysis of 29 Experiments Evaluating the Effects of
367 Rapamycin on Life Span in the Laboratory Mouse. *Journals Gerontol. - Ser. A
368 Biol. Sci. Med. Sci.* **72**, 1024–1032 (2017).
369 6. Johnson, S. C., Rabinovitch, P. S. & Kaeberlein, M. MTOR is a key modulator
370 of ageing and age-related disease. *Nature* **493**, 338–345 (2013).
371 7. Partridge, L., Deelen, J. & Slagboom, P. E. Facing up to the global challenges
372 of ageing. *Nature* **561**, 45–56 (2018).
373 8. Vaupel, J. W., Carey, J. R. & Christensen, K. It's never too late. *Science* **301**,
374 1679–1681 (2003).
375 9. Harrison, D. E. *et al.* Rapamycin fed late in life extends lifespan in genetically
376 heterogeneous mice. *Nature* **460**, 392–395 (2009).
377 10. Fontana, L. *et al.* Decreased Consumption of Branched-Chain Amino Acids
378 Improves Metabolic Health. *Cell Rep.* **16**, 520–530 (2016).
379 11. Rizza, W., Veronese, N. & Fontana, L. What are the roles of calorie restriction
380 and diet quality in promoting healthy longevity? *Ageing Res. Rev.* **13**, 38–45
381 (2014).
382 12. Good, T. P. & Tatar, M. Age-specific mortality and reproduction respond to
383 adult dietary restriction in Drosophila melanogaster. *J. Insect Physiol.* **47**,
384 1467–1473 (2001).
385 13. Mair, W., Goymer, P., Pletcher, S. D. & Partridge, L. Demography of dietary
386 restriction and death in Drosophila. *Science* **301**, 1731–3 (2003).
387 14. McCracken, A. W., Adams, G., Hartshorne, L. & Simons, M. J. P. The hidden
388 costs of dietary restriction: implications for its evolutionary and mechanistic

origins. *bioRxiv* **Preprint**, doi:10.1101/533711 (2019).

15. Simons, M. J. P., Koch, W. & Verhulst, S. Dietary restriction of rodents decreases aging rate without affecting initial mortality rate - a meta-analysis. *Aging Cell* **12**, 410-414 (2013).
16. Merry, B. J. Dietary restriction in rodents - Delayed or retarded ageing? *Mech. Ageing Dev.* **126**, 951–959 (2005).
17. Sun, L., Sadighi Akha, A. A., Miller, R. A. & Harper, J. M. Life-span extension in mice by preweaning food restriction and by methionine restriction in middle age. *Journals Gerontol. - Ser. A Biol. Sci. Med. Sci.* **64**, 711–722 (2009).
18. Bitto, A. *et al.* Transient rapamycin treatment can increase lifespan and healthspan in middle-aged mice. *Elife* **5**, 1–17 (2016).
19. Garratt, M., Nakagawa, S. & Simons, M. J. P. Life-span Extension With Reduced Somatotrophic Signaling: Moderation of Aging Effect by Signal Type, Sex, and Experimental Cohort. *J. Gerontol. A. Biol. Sci. Med. Sci.* **72**, 1620–1626 (2017).
20. Pletcher, S. D. Model fitting and hypothesis testing for ages-specific mortality data. *J. Evol. Biol.* **12**, 430–439 (1999).
21. Partridge, L., Pletcher, S. D. & Mair, W. Dietary restriction, mortality trajectories, risk and damage. *Mech. Ageing Dev.* **126**, 35–41 (2005).
22. Ni, J. Q. *et al.* A genome-scale shRNA resource for transgenic RNAi in Drosophila. *Nat. Methods* **8**, 405–407 (2011).
23. Tricoire, H. *et al.* The steroid hormone receptor EcR finely modulates Drosophila lifespan during adulthood in a sex-specific manner. *Mech. Ageing Dev.* **130**, 547–552 (2009).
24. Osterwalder, T., Yoon, K. S., White, B. H. & Keshishian, H. A conditional tissue-specific transgene expression system using inducible GAL4. *Proc. Natl. Acad. Sci.* **98**, 12596–12601 (2001).
25. Schreiber, K. H. *et al.* A novel rapamycin analog is highly selective for mTORC1 in vivo. *Nat. Commun.* **10**, 3194 (2019).
26. Goyal, L., McCall, K., Agapite, J., Hartwieg, E. & Steller, H. Induction of apoptosis by Drosophila reaper, hid and grim through inhibition of IAP function. *EMBO J.* **19**, 589–97 (2000).
27. Hwangbo, D. S., Garsham, 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).

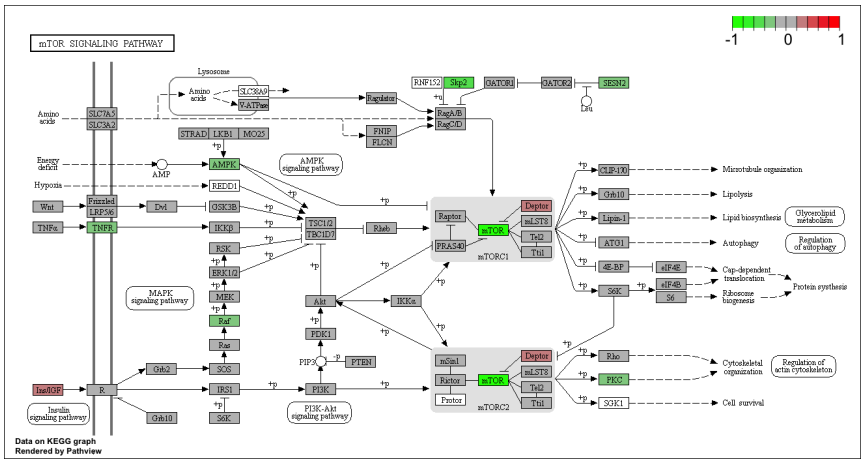
28. Giannakou, M. E. *et al.* Long-lived Drosophila with over-expressed dFOXO in adult fat body. *Science* **305**, 361 (2004).
29. Gorsky, M. K., Burnouf, S., Dols, J., Mandelkow, E. & Partridge, L. Acetylation mimic of lysine 280 exacerbates human Tau neurotoxicity in vivo. *Sci. Rep.* **6**, 1–12 (2016).
30. Rera, M. *et al.* Modulation of longevity and tissue homeostasis by the drosophila PGC-1 homolog. *Cell Metab.* **14**, 623–634 (2011).
31. Therneau, T. M., Grambsch, P. M., Shane Pankratz, V. Penalized Survival Models and Frailty. *J. Comput. Graph. Stat.* **12**, 156–175 (2003).
32. Young, M. D., Wakefield, M. J., Smyth, G. K. & Oshlack, A. Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biol.* **11**, R14 (2010).
33. Zhao, J., Zhai, B., Gygi, S. P. & Goldberg, A. L. mTOR inhibition activates overall protein degradation by the ubiquitin proteasome system as well as by autophagy. *Proc. Natl. Acad. Sci.* **112**, 15790–15797 (2015).
34. Zhang, Y. *et al.* Coordinated regulation of protein synthesis and degradation by mTORC1. *Nature* **513**, 440–443 (2014).
35. Meshorer, E., Soreq, H., Meshorer, E. & Soreq, H. Pre-mRNA splicing modulations in senescence. *Aging Cell* **1**, 10-16 (2002).
36. Heintz, C. *et al.* Splicing factor 1 modulates dietary restriction and TORC1 pathway longevity in C. elegans. *Nature* **541**, 102–106 (2017).
37. Tabrez, S. S., Sharma, R. D., Jain, V., Siddiqui, A. A. & Mukhopadhyay, A. Differential alternative splicing coupled to nonsense-mediated decay of mRNA ensures dietary restriction-induced longevity. *Nat. Commun.* **8**, 306 (2017).
38. Lee, G. *et al.* Post-transcriptional Regulation of De Novo Lipogenesis by mTORC1-S6K1-SRPK2 Signaling. *Cell* **171**, 1545-1558.e18 (2017).
39. Park, S.-K., Link, C. D. & Johnson, T. E. Life-span extension by dietary restriction is mediated by NLP-7 signaling and coelomocyte endocytosis in *C. elegans*. *FASEB J.* **24**, 383–392 (2010).
40. Bouska, M., Huang, K., Kang, P. & Bai, H. Organelle aging: Lessons from model organisms. *J. Genet. Genomics* **46**, 171–185 (2019).
41. Wilkinson, D. S. *et al.* Phosphorylation of LC3 by the Hippo Kinases STK3/STK4 Is Essential for Autophagy. *Mol. Cell* **57**, 55–68 (2015).

42. Lehtinen, M. K. *et al.* A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. *Cell* **125**, 987–1001 (2006).
43. Pleiss, J. A., Whitworth, G. B., Bergkessel, M. & Guthrie, C. Rapid, Transcript-Specific Changes in Splicing in Response to Environmental Stress. *Mol. Cell* **27**, 928–937 (2007).
44. Jacobs, D. R. Cigarette Smoking and Mortality Risk. *Arch. Intern. Med.* **159**, 733 (1999).
45. Rattan, S. I. S. Hormesis in aging. *Ageing Res. Rev.* **7**, 63–78 (2008).
46. Therneau, T. & Atkinson, E. Using Time Dependent Covariates and Time Dependent Coefficients in the Cox Model Time dependent covariates. 1–24 (2016).
47. Pertea, M., Kim, D., Pertea, G. M., Leek, J. T. & Salzberg, S. L. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nat. Protoc.* **11**, (2016).
48. Ritchie, M. E. *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* **43**, e47–e47 (2015).

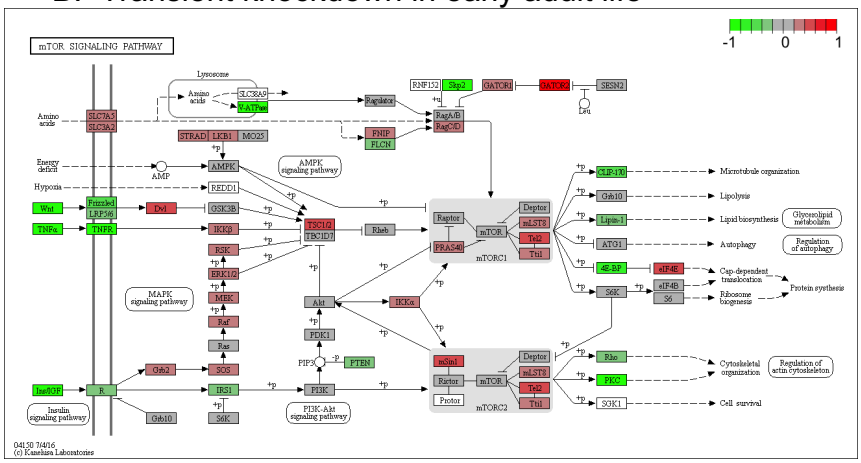
Supplementary Figure S1

Differential expression of mTor knockdown age-dependent regimes plotted on the wider KEGG mTor network (at late age). Intensity of greener colours indicate reduced transcription relative to control. Intensity of red colours indicate increased transcription relative to control. White boxes indicate genes for which there is no clear paralog in *Drosophila melanogaster*.

A. Whole life knockdown



B. Transient knockdown in early adult life



C. Knockdown in late life only

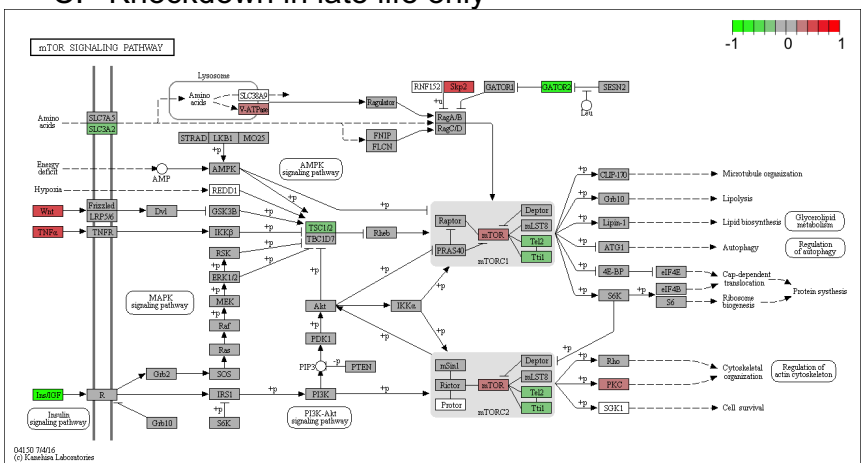


Table S1. KEGG analysis of transcriptome resulting from mTor knockdown

Pathway	N	Up	Down	P Up	P Down
Metabolic pathways	912	172	86	**<0.001**	0.996
Galactose metabolism	29	13	3	**<0.001**	0.697
Starch and sucrose metabolism	28	12	0	**<0.001**	1
Toll and Imd signaling pathway	63	20	6	**<0.001**	0.788
Carbon metabolism	91	25	11	**<0.001**	0.546
Glycolysis / Gluconeogenesis	40	14	7	**<0.001**	0.201
Pentose and glucuronate interconversions	42	14	4	**0.001**	0.763
Glycine, serine and threonine metabolism	25	10	1	**0.001**	0.96
Pentose phosphate pathway	19	8	2	**0.002**	0.687
Drug metabolism - other enzymes	84	21	9	**0.003**	0.698
Proteasome	38	0	33	1	**<0.001**
Protein processing in endoplasmic reticulum	115	6	32	0.999	**<0.001**
DNA replication	34	0	14	1	**<0.001**
mRNA surveillance pathway	60	4	15	0.971	**0.004**
Ubiquitin mediated proteolysis	84	4	19	0.998	**0.005**
Synthesis and degradation of ketone bodies	7	0	4	1	**0.005**
Mucin type O-glycan biosynthesis	15	1	6	0.888	**0.006**
Nucleotide excision repair	37	1	10	0.995	**0.01**
RNA transport	121	7	23	0.998	**0.017**
Mismatch repair	20	0	6	1	**0.027**

Table S2. KEGG analysis of transcriptome resulting from Early adult transient mTor knockdown

Pathway	N	Up	Down	P Up	P Down
Spliceosome	115	84	8	**<0.001**	1
DNA replication	34	33	1	**<0.001**	1
Nucleotide excision repair	37	35	1	**<0.001**	1
RNA transport	121	81	4	**<0.001**	1
Basal transcription factors	37	33	1	**<0.001**	1
Fanconi anemia pathway	26	24	0	**<0.001**	1
Mismatch repair	20	19	1	**<0.001**	1
Homologous recombination	23	21	0	**<0.001**	1
Ubiquitin mediated proteolysis	84	54	4	**<0.001**	1
mRNA surveillance pathway	60	41	4	**<0.001**	1
Neuroactive ligand-receptor interaction	42	0	37	1	**<0.001**
Biosynthesis of unsaturated fatty acids	21	2	17	0.999	**<0.001**
Lysosome	97	12	53	1	**<0.001**
ECM-receptor interaction	12	0	10	1	**0.001**
Insect hormone biosynthesis	24	1	16	1	**0.001**
Metabolic pathways	912	227	348	1	**0.001**
Fatty acid metabolism	49	6	27	1	**0.001**
Ascorbate and aldarate metabolism	27	3	17	0.999	**0.002**
Glycerolipid metabolism	39	7	22	0.995	**0.003**
Caffeine metabolism	5	0	5	1	**0.004**

Table S3. KEGG analysis of transcriptome resulting from Late adult mTor knockdown

Pathway	N	Up	Down	P Up	P Down
Folate biosynthesis	34	5	0	**0.001**	1
Thiamine metabolism	16	3	0	**0.006**	1
ABC transporters	19	3	0	**0.01**	1
Arginine biosynthesis	13	2	0	**0.037**	1
Fatty acid elongation	13	2	0	**0.037**	1
2-Oxocarboxylic acid metabolism	14	2	1	**0.042**	0.49
Amino sugar and nucleotide sugar metabolism	40	3	2	0.068	0.567
Biosynthesis of unsaturated fatty acids	21	2	0	0.087	1
Longevity regulating pathway - multiple species	47	3	0	0.099	1
Caffeine metabolism	5	1	0	0.113	1
Ribosome biogenesis in eukaryotes	72	0	18	1	**<0.001**
Aminoacyl-tRNA biosynthesis	41	0	10	1	**<0.001**
RNA polymerase	28	0	7	1	**<0.001**
Spliceosome	115	2	15	0.76	**<0.001**
Pentose phosphate pathway	19	0	4	1	**0.011**
RNA transport	121	0	12	1	**0.011**
Homologous recombination	23	0	4	1	**0.021**
Glycine, serine and threonine metabolism	25	1	4	0.451	**0.028**
Non-homologous end-joining	6	0	2	1	**0.029**
Fanconi anemia pathway	26	0	4	1	**0.032**

Table S4. GO analysis of transcriptome resulting from mTor knockdown

Term	N	Up	Down	P Up	P Down
Biological Process					
response to biotic stimulus	329	102	43	**<0.001**	0.307
response to external biotic stimulus	329	102	43	**<0.001**	0.307
response to other organism	329	102	43	**<0.001**	0.307
response to bacterium	211	76	23	**<0.001**	0.73
ubiquitin-dependent protein catabolic process	229	15	73	1	**<0.001**
protein catabolic process	291	19	85	1	**<0.001**
modification-dependent macromolecule catabolic process	241	17	75	1	**<0.001**
eggshell formation	118	13	48	0.826	**<0.001**
modification-dependent protein catabolic process	235	16	73	1	**<0.001**
chorion-containing eggshell formation	116	13	47	0.808	**<0.001**
defense response	358	103	47	**<0.001**	0.286
proteolysis involved in cellular protein catabolic process	263	19	77	1	**<0.001**
proteasome-mediated ubiquitin-dependent protein catabolic process	179	13	60	0.997	**<0.001**
proteolysis	665	112	147	**0.007**	**<0.001**
cellular protein catabolic process	265	19	77	1	**<0.001**
defense response to other organism	247	78	32	**<0.001**	0.359
macromolecule catabolic process	447	41	109	0.998	**<0.001**
female gamete generation	676	49	147	1	**<0.001**
proteasomal protein catabolic process	191	14	61	0.998	**<0.001**
cellular macromolecule catabolic process	377	30	95	1	**<0.001**
Molecular Function					
threonine-type endopeptidase activity	16	0	14	1	**<0.001**
threonine-type peptidase activity	16	0	14	1	**<0.001**
peptidase activity, acting on L-amino acid peptides	385	78	90	**<0.001**	**<0.001**
peptidase activity	395	79	90	**<0.001**	**<0.001**
endopeptidase inhibitor activity	48	24	3	**<0.001**	0.94
endopeptidase regulator activity	50	24	4	**<0.001**	0.869
serine-type endopeptidase inhibitor activity	34	19	3	**<0.001**	0.795
peptidase inhibitor activity	51	24	4	**<0.001**	0.878
peptidase regulator activity	59	25	6	**<0.001**	0.731
endopeptidase activity	259	62	61	**<0.001**	**<0.001**
catalytic activity, acting on a protein	1093	143	185	0.696	**<0.001**
DNA replication origin binding	8	0	7	1	**<0.001**
hydrolase activity	1465	230	231	**0.006**	**<0.001**
3'-5' DNA helicase activity	17	0	10	1	**<0.001**
enzyme inhibitor activity	79	26	4	**<0.001**	0.99
oxidoreductase activity	506	103	46	**<0.001**	0.987
proteasome-activating ATPase activity	7	0	6	1	**<0.001**
thiol-dependent ubiquitin-specific protease activity	35	3	14	0.871	**<0.001**
thiol-dependent ubiquitinyl hydrolase activity	35	3	14	0.871	**<0.001**
ubiquitinyl hydrolase activity	35	3	14	0.871	**<0.001**
Cellular Component					
endopeptidase complex	47	0	37	1	**<0.001**
proteasome complex	47	0	37	1	**<0.001**
peptidase complex	73	1	45	1	**<0.001**
extracellular region	635	171	78	**<0.001**	0.447
external encapsulating structure	43	1	29	0.998	**<0.001**
chorion	38	0	26	1	**<0.001**
proteasome regulatory particle	23	0	19	1	**<0.001**
proteasome accessory complex	24	0	19	1	**<0.001**
proteasome core complex	15	0	14	1	**<0.001**
extracellular space	351	96	48	**<0.001**	0.192
nucleus	2236	176	357	1	**<0.001**
extracellular region part	441	106	60	**<0.001**	0.171
protein-containing complex	2320	160	361	1	**<0.001**
proteasome core complex, alpha-subunit complex	8	0	8	1	**<0.001**
proteasome regulatory particle, lid subcomplex	10	0	9	1	**<0.001**
cell	6094	635	817	1	**<0.001**
cell part	6094	635	817	1	**<0.001**
cytosolic proteasome complex	9	0	8	1	**<0.001**
proteasome regulatory particle, base subcomplex	12	0	9	1	**<0.001**
microtubule cytoskeleton	244	19	56	0.998	**<0.001**

Table S5. GO analysis of transcriptome resulting from Early adult transient mTor knockdown

Term	N	Up	Down	P Up	P Down
Biological Process					
nucleic acid metabolic process	1858	1107	259	**<0.001**	1
cellular macromolecule metabolic process	2860	1519	499	**<0.001**	1
macromolecule metabolic process	3655	1815	770	**<0.001**	1
nucleobase-containing compound metabolic process	2131	1161	359	**<0.001**	1
RNA metabolic process	1656	952	252	**<0.001**	1
heterocycle metabolic process	2202	1184	385	**<0.001**	1
cellular aromatic compound metabolic process	2259	1196	414	**<0.001**	1
cellular metabolic process	4287	1973	964	**<0.001**	1
organic cyclic compound metabolic process	2309	1210	432	**<0.001**	1
gene expression	2035	1096	308	**<0.001**	1
cellular process	6226	2636	1688	**<0.001**	1
cellular nitrogen compound metabolic process	2586	1317	450	**<0.001**	1
nitrogen compound metabolic process	4076	1885	957	**<0.001**	1
primary metabolic process	4262	1926	1012	**<0.001**	1
chromosome organization	567	401	32	**<0.001**	1
regulation of macromolecule metabolic process	1685	907	346	**<0.001**	1
regulation of metabolic process	1814	954	381	**<0.001**	1
regulation of nitrogen compound metabolic process	1591	859	335	**<0.001**	1
cell cycle	720	465	68	**<0.001**	1
regulation of primary metabolic process	1607	864	338	**<0.001**	1
Molecular Function					
nucleic acid binding	1464	812	222	**<0.001**	1
binding	4407	1894	1219	**<0.001**	1
heterocyclic compound binding	2319	1100	521	**<0.001**	1
organic cyclic compound binding	2335	1101	533	**<0.001**	1
DNA binding	717	421	152	**<0.001**	1
transmembrane transporter activity	530	63	313	1	**<0.001**
inorganic molecular entity transmembrane transporter activity	378	35	237	1	**<0.001**
protein binding	2023	930	535	**<0.001**	1
molecular transducer activity	208	18	147	1	**<0.001**
signaling receptor activity	208	18	147	1	**<0.001**
ion transmembrane transporter activity	393	42	235	1	**<0.001**
transporter activity	623	109	333	1	**<0.001**
transcription factor activity, protein binding	180	129	19	**<0.001**	1
transmembrane signaling receptor activity	169	14	120	1	**<0.001**
chromatin binding	186	131	17	**<0.001**	1
transcription factor activity, transcription factor binding	166	116	19	**<0.001**	1
channel activity	147	12	103	1	**<0.001**
passive transmembrane transporter activity	147	12	103	1	**<0.001**
G-protein coupled receptor activity	82	2	67	1	**<0.001**
cation transmembrane transporter activity	267	28	160	1	**<0.001**
Cellular Component					
nucleus	2236	1370	291	**<0.001**	1
intracellular part	5305	2521	1090	**<0.001**	1
intracellular	5350	2534	1109	**<0.001**	1
intracellular membrane-bounded organelle	3636	1870	632	**<0.001**	1
intracellular organelle	4273	2102	811	**<0.001**	1
organelle	4328	2115	837	**<0.001**	1
membrane-bounded organelle	3844	1933	709	**<0.001**	1
nuclear part	1154	781	58	**<0.001**	1
intracellular organelle part	2481	1317	319	**<0.001**	1
cell	6094	2628	1562	**<0.001**	1
cell part	6094	2628	1562	**<0.001**	1
organelle part	2542	1327	352	**<0.001**	1
protein-containing complex	2320	1232	333	**<0.001**	1
intracellular organelle lumen	1012	631	57	**<0.001**	1
membrane-enclosed lumen	1012	631	57	**<0.001**	1
organelle lumen	1012	631	57	**<0.001**	1
nuclear lumen	827	539	43	**<0.001**	1
chromosome	521	370	31	**<0.001**	1
intrinsic component of plasma membrane	446	42	322	1	**<0.001**
integral component of plasma membrane	433	39	315	1	**<0.001**

Table S6. GO analysis of transcriptome resulting from Late adult mTor knockdown

Term	N	Up	Down	P Up	P Down
Biological Process					
ncRNA metabolic process	321	0	67	1	**<0.001**
gene expression	2035	9	192	1	**<0.001**
RNA metabolic process	1656	8	166	1	**<0.001**
nucleic acid metabolic process	1858	9	178	1	**<0.001**
ncRNA processing	245	0	55	1	**<0.001**
RNA processing	569	0	86	1	**<0.001**
cellular nitrogen compound metabolic process	2586	18	216	1	**<0.001**
nucleobase-containing compound metabolic process	2131	17	187	1	**<0.001**
heterocycle metabolic process	2202	18	189	1	**<0.001**
organic cyclic compound metabolic process	2309	23	193	1	**<0.001**
cellular aromatic compound metabolic process	2259	21	189	1	**<0.001**
transmembrane transport	487	47	10	**<0.001**	0.999
ribosome biogenesis	212	0	43	1	**<0.001**
rRNA metabolic process	151	0	36	1	**<0.001**
ribonucleoprotein complex biogenesis	293	1	50	0.999	**<0.001**
macromolecule metabolic process	3655	45	255	1	**<0.001**
rRNA processing	141	0	34	1	**<0.001**
nitrogen compound metabolic process	4076	60	270	1	**<0.001**
primary metabolic process	4262	60	278	1	**<0.001**
tRNA metabolic process	131	0	29	1	**<0.001**
Molecular Function					
catalytic activity, acting on RNA	246	0	50	1	**<0.001**
RNA binding	582	0	79	1	**<0.001**
nucleic acid binding	1464	5	140	1	**<0.001**
heterocyclic compound binding	2319	27	187	1	**<0.001**
organic cyclic compound binding	2335	28	187	1	**<0.001**
transmembrane transporter activity	530	46	11	**<0.001**	1
secondary active transmembrane transporter activity	124	23	1	**<0.001**	0.998
transporter activity	623	46	19	**<0.001**	0.986
active transmembrane transporter activity	229	27	5	**<0.001**	0.985
organic anion transmembrane transporter activity	126	20	0	**<0.001**	1
anion transmembrane transporter activity	157	21	1	**<0.001**	0.999
sodium-independent organic anion transmembrane transporter activity	27	10	0	**<0.001**	1
ion transmembrane transporter activity	393	32	3	**<0.001**	1
catalytic activity, acting on a tRNA	90	0	20	1	**<0.001**
helicase activity	93	0	20	1	**<0.001**
chitin binding	62	12	1	**<0.001**	0.95
inorganic molecular entity transmembrane transporter activity	378	29	4	**<0.001**	1
ATP-dependent helicase activity	71	0	16	1	**<0.001**
purine NTP-dependent helicase activity	71	0	16	1	**<0.001**
catalytic activity	3401	85	211	0.284	**<0.001**
Cellular Component					
nuclear part	1154	2	116	1	**<0.001**
nucleus	2236	10	180	1	**<0.001**
intracellular	5350	71	330	1	**<0.001**
intracellular part	5305	71	328	1	**<0.001**
intracellular organelle lumen	1012	2	98	1	**<0.001**
membrane-enclosed lumen	1012	2	98	1	**<0.001**
organelle lumen	1012	2	98	1	**<0.001**
intracellular membrane-bounded organelle	3636	35	244	1	**<0.001**
nucleolus	209	0	36	1	**<0.001**
nuclear lumen	827	2	82	1	**<0.001**
membrane-bounded organelle	3844	37	246	1	**<0.001**
preribosome	72	0	19	1	**<0.001**
ribonucleoprotein complex	570	0	61	1	**<0.001**
integral component of membrane	1057	55	17	**<0.001**	1
protein-containing complex	2320	17	161	1	**<0.001**
intrinsic component of membrane	1077	55	17	**<0.001**	1
cell	6094	113	342	1	**<0.001**
cell part	6094	113	342	1	**<0.001**
integral component of plasma membrane	433	31	3	**<0.001**	1
aminoacyl-tRNA synthetase multienzyme complex	10	0	7	1	**<0.001**

Table S7. KEGG analysis of alternative splicing at old age induced by transient mTor knockdown

Pathway	N	Differentially expressed	P Enrichment
Endocytosis	122	11	**0.002**
Hippo signaling pathway - fly	61	6	**0.013**
Glycerophospholipid metabolism	63	6	**0.015**
mTOR signaling pathway	96	7	**0.035**
Lysosome	118	8	**0.036**