

LETTERS

Rapamycin fed late in life extends lifespan in genetically heterogeneous mice

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Inhibition of the TOR signalling pathway by genetic or pharmacological intervention extends lifespan in invertebrates, including yeast, nematodes and fruitflies^{1–5}; however, whether inhibition of mTOR signalling can extend lifespan in a mammalian species was unknown. Here we report that rapamycin, an inhibitor of the mTOR pathway, extends median and maximal lifespan of both male and female mice when fed beginning at 600 days of age. On the basis of age at 90% mortality, rapamycin led to an increase of 14% for females and 9% for males. The effect was seen at three independent test sites in genetically heterogeneous mice, chosen to avoid genotype-specific effects on disease susceptibility. Disease patterns of rapamycin-treated mice did not differ from those of control mice. In a separate study, rapamycin fed to mice beginning at 270 days of age also increased survival in both males and females, based on an interim analysis conducted near the median survival point. Rapamycin may extend lifespan by postponing death from cancer, by retarding mechanisms of ageing, or both. To our knowledge, these are the first results to demonstrate a role for mTOR signalling in the regulation of mammalian lifespan, as well as pharmacological extension of lifespan in both genders. These findings have implications for further development of interventions targeting mTOR for the treatment and prevention of age-related diseases.

Because incidences of most diseases rise rapidly with age⁶, interventions that delay ageing would greatly benefit health^{7,8}. So far, dietary additives that delay ageing and increase lifespan in rodent models have shown only weak effects^{9–11}. Before clinical studies are considered, anti-ageing interventions must be repeatable and effective in many mouse genotypes, and not merely postpone strain-specific diseases^{12–14}.

The National Institute on Aging Interventions Testing Program (ITP) evaluates agents that may delay ageing and increase lifespan in genetically heterogeneous mice^{15–17}. Agents are chosen as summarized at <http://www.nia.nih.gov/ResearchInformation/ScientificResources/InterventionsTestingProgram.htm>. Studies are simultaneously replicated at three test sites: The Jackson Laboratory (TJL), the University of Michigan (UM), and the University of Texas Health Science Center (UT). BALB/cByJ × C57BL/6J F1 (CB6F1) females and C3H/HeJ × DBA/2J F1 (C3D2F1) males are supplied to each site by The Jackson Laboratory, and mated to produce genetically heterogeneous populations in which each animal is genetically unique, but a full

sibling of all other mice in the population¹⁸. Sufficient mice are used to provide 80% power to detect a 10% increase (or decrease) in mean lifespan with respect to unmanipulated controls of the same sex, even if data from one of the three test sites were to be unavailable. Here we report that dietary encapsulated rapamycin increases mouse survival, including survival to the last decile, a measure of maximal lifespan.

Rapamycin reduces function of the rapamycin target kinase TOR and has anti-neoplastic activities; genetic inhibition of TOR extends lifespan in short-lived model organisms. In male and female mice at each of three collaborating research sites, median and maximum lifespan were extended by feeding encapsulated rapamycin starting at 600 days of age (Fig. 1). We analysed the data set as of 1 February 2009, with 2% (38 of 1,901) of mice still alive. For data pooled across sites, a log-rank test rejected the null hypothesis that treatment and control groups did not differ ($P < 0.0001$); mice fed rapamycin were longer lived than controls ($P < 0.0001$) in both males and females. Expressed as mean lifespan, the effect sizes were 9% for males and 13% for females in the pooled data set. Expressed as life expectancy at 600 days (the age of first exposure to rapamycin), the effect sizes were 28% for males and 38% for females. Mice treated with other agents (enalapril and CAPE (caffeic acid phenethyl ester)) evaluated in parallel did not differ from controls at the doses used (Supplementary Fig. 1).

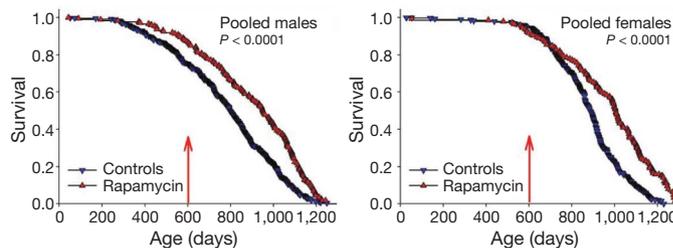


Figure 1 | Survival plots for male and female mice, comparing control mice to those fed rapamycin in the diet starting at 600 days of age, pooling across the three test sites. P values were calculated by the log-rank test. Four per cent of the control mice and three per cent of rapamycin-assigned mice were removed from the experiment for technical reasons. Only five animals (three controls, two rapamycin) were removed after the start of rapamycin treatment at 600 days. Thus, there were no significant differences between groups in censoring.

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Rapamycin-fed and control mice were then compared separately for each combination of site and gender. Rapamycin had a consistent benefit, compared with controls, with P values ranging from 0.03 to 0.0001 (Fig. 2).

Female mice at all three sites had improved survival after rapamycin feeding (Fig. 2). Mean lifespan increases for females were 15%, 16% and 7% (TJL, UM and UT, respectively), and life expectancy at 600 days increased by 45%, 48% and 22% for females at the three sites. Median lifespan estimates of control females were consistent across sites (881–895 days), and were similar to values noted in Cohort 2004, which ranged from 858 to 909 days¹⁵. Thus, the improvement in survival seen in the rapamycin-fed females is not an artefact of low survival for the control females.

Male mice at all three sites also had improved survival after rapamycin feeding (Fig. 2). Mean lifespan increases for males were 5%, 8% and 15% (TJL, UM and UT, respectively), and male life expectancy at 600 days increased by 16%, 23% and 52%. Interpretation is complicated by differences among sites in survival of control males, and because mice assigned to the rapamycin-fed group at UT and perhaps at UM had lower mortality before 600 days than controls. Control mice at UT and UM differed from those fed rapamycin not only in exposure to rapamycin from 600 days of age but also in specific formulation of the mouse chows (all based on the NIH-31 standard) used between weaning and 600 days. We thus cannot rule out the possibility that improved survival among males in the rapamycin group, at UT and at UM, might reflect differences in nutritional or health status between control and rapamycin groups before 600 days, rather than solely the effects of rapamycin. Notably, the significant benefits of rapamycin on male (and female) survival at TJL could not have been affected by diet before drug administration, because at TJL both control and rapamycin-fed mice received the same chow (Purina 5LG6) throughout this period.

Maximum lifespan was increased by rapamycin feeding. Table 1 shows the ages at the 90th percentile for control and rapamycin-treated mice, along with the 95% upper confidence bound for the controls. For each site and sex, the 90th percentile age for rapamycin-treated mice is higher than the upper limit for the corresponding control group, showing that rapamycin increases the age for 90th percentile survival.

To determine whether increases in maximal lifespan due to rapamycin feeding are statistically significant, we compared the proportion of

living mice in each group after 90% had died in the joint life table¹⁹ (details in Supplementary Table 1). Summing across the three sites, 4.8% of the female control mice were alive at these ages, compared with 21.5% of the rapamycin-treated females ($P < 0.0001$). For males, the corresponding values were 5.9% of controls and 20.2% of rapamycin-treated mice ($P < 0.0001$). The site-specific calculations documented a significant effect on females at both TJL ($P = 0.0006$) and UM ($P = 0.0001$); for males, we noted a significant effect at both TJL ($P = 0.008$) and UT ($P = 0.0001$), with a marginal effect at UM ($P = 0.07$). Rapamycin feeding initiated at 600 days of age thus leads to a significant increase in maximal lifespan.

To test if the spectrum of lesions was altered by dietary rapamycin, complete necropsies were conducted on 31 control and 40 rapamycin-fed mice that were either found dead or killed when moribund (details in Supplementary Table 2). Although rapamycin postpones death, it did not change the distribution of presumptive causes of death.

A separate group of mice was used to evaluate the effects of encapsulated rapamycin initiated at 270 days of age (Fig. 3a). At the time of analysis, 51% of the females and 68% of the males had died, and a stratified log-rank test showed significantly lower mortality risk in the rapamycin-treated mice compared to controls, pooling across the three test sites ($P = 0.0002$ for males and $P < 0.0001$ for females). When each site was evaluated separately, the beneficial effect of rapamycin for females was significant at each site ($P < 0.005$); for males, the effect was significant ($P < 0.025$) at UM and UT, but not at TJL. Rapamycin seems to reduce mid-life mortality risk when started at 270 days of age, but additional data are needed to provide an accurate estimate of effect size, and to evaluate effects on maximal longevity.

To document biochemical effects of rapamycin at the dose used for the lifespan studies, we evaluated the phosphorylation status of ribosomal protein subunit S6 (rpS6)—a target substrate of S6 kinase 1 in the mTOR signalling pathway²⁰—in visceral white adipose tissue (a sensitive indicator of mTOR inhibition by rapamycin treatment *in vivo*). Figure 3b shows that rapamycin feeding reduced the levels of phosphorylated rpS6 4–5-fold when fed from 270 to about 800 days of age. Blood levels of rapamycin in the treated mice were equivalent in males and females, between 60 and 70 ng ml⁻¹.

Initial evidence that reduced TOR function can extend longevity came primarily from studies in yeast^{1,2} and invertebrates^{3–5}. Beneficial effects of diet restriction²¹ and dwarf mutations, both of which extend lifespan in rodents, may, to some degree, result from

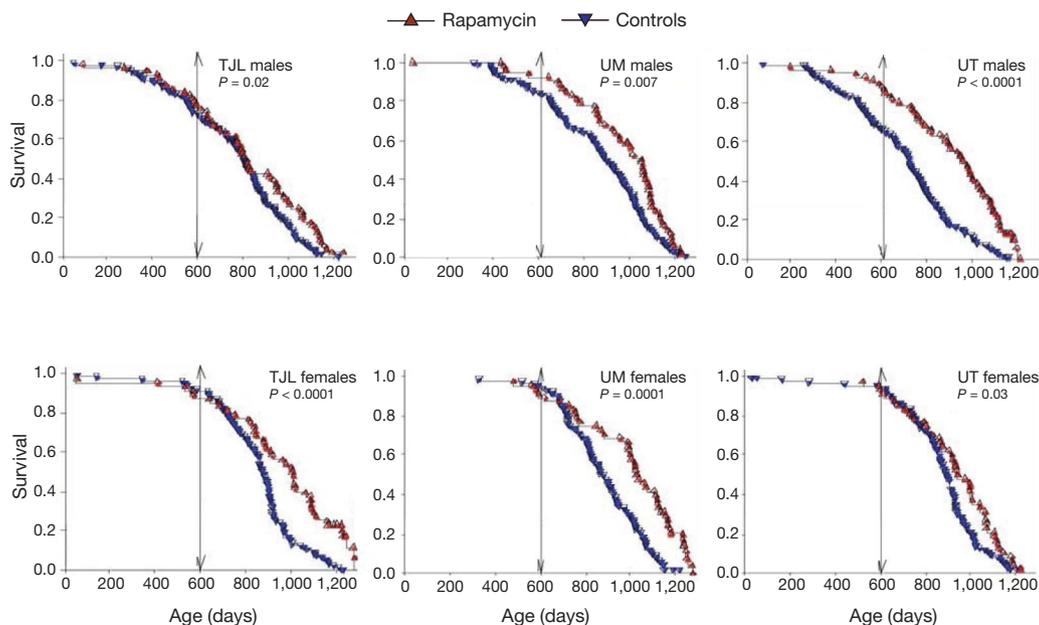


Figure 2 | Survival of control and rapamycin-treated mice for males and females for each of the three test sites separately. P values represent results of log-rank calculations. Vertical lines at age 600 days indicate the age at which the mice were first exposed to rapamycin.

Table 1 | The effect of rapamycin on maximum lifespan

Comparison	Sites	Age in days at 90th percentile for controls (upper confidence limit)*	Age in days at 90th percentile for rapamycin-treated mice	Percentage increase
Females				
Rapamycin versus controls	All sites	1,094 (1,136)	1,245	14
Rapamycin versus controls	TJL	1,100 (1,165)	1,282	17
Rapamycin versus controls	UM	1,094 (1,149)	1,250	14
Rapamycin versus controls	UT	1,089 (1,159)	1,179	8
Males				
Rapamycin versus controls	All sites	1,078 (1,111)	1,179	9
Rapamycin versus controls	TJL	1,035 (1,091)	1,142	10
Rapamycin versus controls	UM	1,141 (1,177)	1,188	4
Rapamycin versus controls	UT	1,020 (1,101)	1,179	16

* The upper limit of the 95% confidence interval for control mice is indicated in parentheses. For example, in the top row, for females pooled across sites, the 95% confidence interval for controls goes up to 1,136 days, and the estimate for 90th percentile survival for the rapamycin-treated mice is 1,245 days. This gives good evidence that the 90th percentile survival for rapamycin-treated mice (1,245) is substantially above that for controls (1,094).

repression of the mTOR complex 1 (mTORC1) pathway^{22,23}. It is not yet known to what extent inhibition of mTOR will recapitulate other aspects of the phenotypes associated with diet restriction or dwarf mutations. Our demonstration that rapamycin feeding increases lifespan even when started late in life, as well as the absence of changes in body weight (data not shown), distinguishes our results from studies using diet restriction: in all cases diet restriction reduces body weight, and in most reports²¹, although not all²⁴, diet restriction produces little, if any, benefit if started after about 550 days of age.

Rapamycin may extend lifespan in old, genetically heterogeneous mice through a combination of anti-neoplastic effects^{25,26} and effects on cellular stress resistance and response to nutrient dynamics^{27–29}. The increase in both median and maximum lifespan seen in rapamycin-fed mice is consistent with the hypothesis that inhibiting the mTORC1 pathway retards mammalian ageing, but is not compelling proof that ageing rates are altered; this would require testing whether the

intervention decelerates age-dependent changes in multiple organs, cell types and intracellular and extracellular processes¹⁴. Comparing the effects of rapamycin treatment and other models of decelerated ageing will help narrow the list of possible mechanisms for longevity extension.

At the cellular level, mTORC1 helps to coordinate growth and survival responses induced by alterations in nutrient availability, energy status, growth factor stimuli and exposure to potentially lethal cell stresses^{27–29}; this strategic position at the nexus of nutrient/stress sensing pathways may contribute to the importance of TOR function in regulating lifespan in invertebrates and in mammals as well. It is especially noteworthy that rapamycin feeding can extend mouse lifespan even when started late in life; in terms of the percentage of the maximal lifespan, a 600-day-old mouse is roughly the equivalent of a 60-year-old person¹⁴. An effective anti-ageing intervention that could be initiated later than the midpoint of the lifespan could prove to be

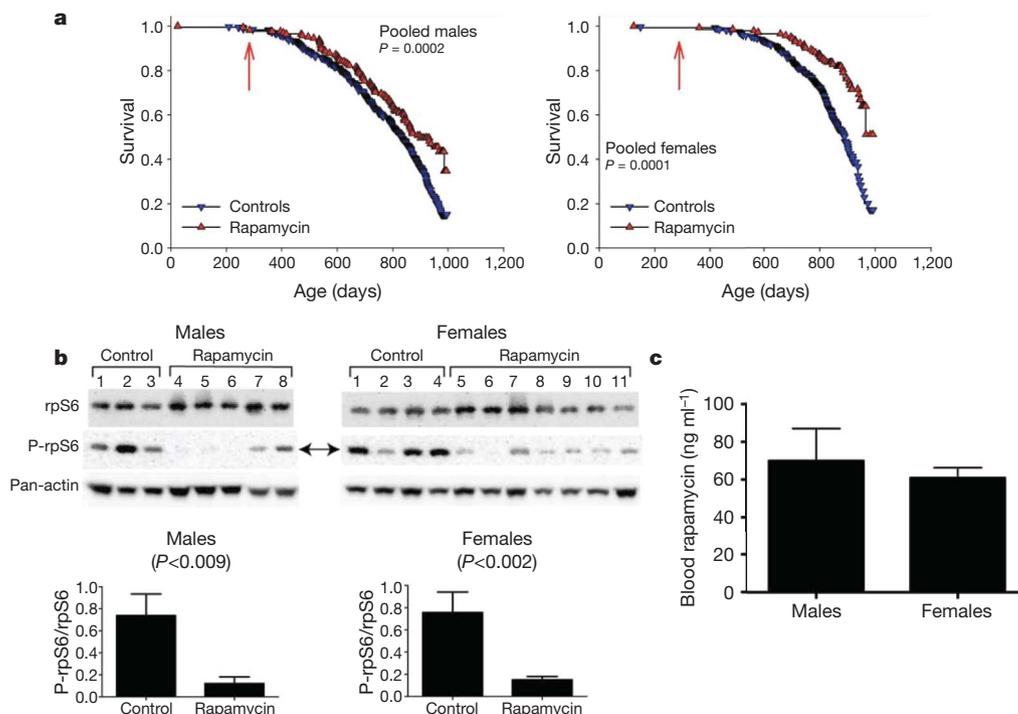


Figure 3 | Characterization of mice receiving rapamycin from 270 days of age. **a**, Survival plots for male and female mice, comparing control mice to rapamycin-treated mice of a separate (Cohort 2006) population, in which mice were treated with rapamycin from 270 days of age. Because at the time of the interim analysis all live mice were between 800 and 995 days of age, we have only limited information about the shape of the survival curve at ages above 900 days, and the apparent change in slope at the oldest ages (>990 days) reflects this experimental uncertainty. P values were calculated by the log-rank test. **b**, Effects of dietary rapamycin on an mTORC1 effector in

the visceral fat pads from 750-day-old to 880-day-old male and female mice. Ribosomal subunit protein S6 (rpS6) and its phosphorylation status (P-rpS6, double arrow) were immunoassayed in tissue lysates prepared from mice consuming microencapsulated rapamycin-containing or control diets. Antibodies used are shown to the left. The ratio of intensity values for P-rpS6/rpS6 is shown in the graphs for female and male mice. Pan-actin was also immunoassayed in the blots to provide an indication of protein loading for each lane. **c**, Whole blood rapamycin content in 750-day-old to 880-day-old male and female mice. In **b** and **c**, error bars show standard errors of the mean.

especially relevant to clinical situations, in which the efficacy of anti-ageing interventions would be particularly difficult to test in younger volunteers. Our data justify special attention to the role of the TOR pathway in control of ageing in mammals and in the pathogenesis of late-life illnesses.

METHODS SUMMARY

Mice. Specific pathogen-free (SPF) mice were produced at each of the three test sites by mating CB6F1 females with C3D2F1 males to produce a genetically heterogeneous population¹⁵. Weanlings at the three sites were fed similar diets (but not identical; see Supplementary Information for details) until they were started on food containing rapamycin, in Purina 5LG6, at 600 days of age. A second independent study was begun 1 year later, with rapamycin initiated at 270 days rather than at 600 days of age. The principal endpoint was age at death (for mice found dead at daily inspections) or age at euthanasia (for mice deemed unlikely to survive for more than an additional 48 h).

Diet preparation. Rapamycin (from LC Labs) was microencapsulated by Southwest Research Institute (San Antonio, Texas), using a spinning disk atomization coating process with the enteric coating material Eudragit S100 (Röhm Pharma). This coating increased the fraction of rapamycin that survived the food preparation process by three- to fourfold, and protected the agent from digestion in the stomach¹⁶. Encapsulated rapamycin was then incorporated into 5LG6 mouse chow and distributed to all three test sites. Rapamycin in blood and diet was measured by high-performance liquid chromatography (HPLC) with ultraviolet detection as detailed in Supplementary Information.

Rapamycin effectiveness. To test effects of rapamycin on mTORC1 targets, we measured phosphorylation of ribosomal protein S6 (at Ser 240 and Ser 244), a substrate of S6 kinase 1, in visceral adipose tissue. Adipose tissue was dissected from mice that had been fed a rapamycin diet for 420 days; lysates were loaded on a 4–12% gradient PAGE and electrophoresed overnight at 5V. After transfer to membranes, blocking and incubation with appropriate antibodies, total amounts of rpS6 and amounts of phosphorylated rpS6 were quantified by chemiluminescence. Techniques are detailed in Supplementary Information.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions D.E.H., R.S. and R.A.M. serve as the principal investigators at the three collaborating institutions; they were responsible for project design, supervision of technical personnel, interpretation of results, and preparation of manuscript drafts. Z.D.S. proposed rapamycin for the study, and was responsible for the measures of mTOR function. J.F.N. and K. Flurkey provided advice on experimental design and interpretation, and comments on the manuscript. Lab manager C.M.A. provided advice, and supervised laboratory procedures and data collection at The Jackson Laboratory site. N.L.N. served as the project officer for the National Institute on Aging, and contributed to program development, experimental design and analysis. J.E.W. conducted and helped interpret the necropsy analyses. K. Frenkel recommended CAPE for the study, and advised on dose and route of administration. C.S.C. and M.P. recommended enalapril for the study, and advised on dose and route of administration. M.A.J. was responsible for the pharmacological analyses. E.F. supervised and conducted laboratory procedures and data collection at the University of Texas site.

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METHODS

Mouse production, maintenance and estimation of lifespan. Mice were produced at each of the three test sites by mating CB6F1 females with C3D2F1 males to produce a genetically heterogeneous population. Details of the methods used for health monitoring were provided previously¹⁵; in brief, each of the three colonies was evaluated four times each year for infectious agents, including pinworm. All such tests were negative throughout the entire study period. Each test site enrolled approximately equal numbers of 19- to 21-day-old weanlings each month over a 6-month period, housing three males or four females per cage. Each site used diets that the manufacturer stated were based on the NIH-31 standard for breeding cages, and the period between weaning and the initiation of experimental diets is given below. For breeding cages, UM used Purina 5008, UT used Teklad 7912 and TJL used Purina 5K52. For weanlings before 120 days of age, UM used Purina 5008, UT used Teklad 7912 and TJL used Purina 5LG6. Starting when 120 days old, mice in the control, enalapril and CAPE groups received Purina 5LG6 at all three sites, without additives (control group) or with the test agent. Mice in the rapamycin group remained on the weanling diet until they began to receive rapamycin, in Purina 5LG6, at 600 days of age. Separate cohorts of control and rapamycin-treated mice were established in the same way 1 year later, again at each test site, but with rapamycin initiated at 270 days rather than at 600 days of age. Additional husbandry details, including accounts of tests for T-cell subset distribution and activity administered to a subset of each group, are provided elsewhere^{16,17}.

Removal of mice from the longevity population. The Cohort 2005 study population, distributed almost equally among the three test sites, consisted initially of 1,960 mice, of which 674 were assigned to the control group and 317–328 to each of the four treatment groups. Of these, 51 mice were removed from the study because of fighting (31 mice), accidental death (such as chip implantation or cage flooding; 13 mice), or because of technical error (error in gender assignment or diet selection; 7 mice). For survival analyses, mice were treated as alive at the date of their removal from the protocol, and lost to follow-up thereafter. These censored mice were not included in calculations of median longevity.

Estimation of age at death (lifespan). Mice were examined at least daily for signs of ill health, and were killed for humane reasons if they were so severely moribund that they were considered, by an experienced technician, unlikely to survive for more than an additional 48 h. A mouse was considered severely moribund if it showed more than one of the following clinical signs: (1) inability to eat or to drink; (2) severe lethargy, as indicated by a lack of response such as a reluctance to move when gently prodded with a forceps; (3) severe balance or gait disturbance; (4) rapid weight loss over a period of 1 week or more; or (5) a severely ulcerated or bleeding tumour. The age at which a moribund mouse was killed was taken as the best available estimate of its natural lifespan. Mice found dead were also noted at each daily inspection. Bodies were fixed for later necropsy analysis.

Control and experimental diets. TestDiet (Richmond) prepared batches of Purina 5LG6 food containing each of the test substances, as well as control diet batches, at intervals of approximately 120 days, and shipped each batch of food at the same time to each of the three test sites. Enalapril was purchased from Sigma (catalogue E6888-5G) and used at 120 mg per kg food; on the assumption that the average mouse weighs 30 g and consumes 5 g of food per day, this dose supplies 20 mg enalapril per kg body weight per day. CAPE, caffeic acid phenethyl ester, was purchased from Cayman (catalogue 70750), and used at either of two doses: the high dose was 300 mg per kg food (50 mg per kg body weight per day), and the low dose was 30 mg per kg food (5 mg per kg body weight per day). Enalapril was tested because in aged humans and in rodent models of hypertension, obesity, diabetes and congestive heart failure, it has been reported to improve many of these conditions. CAPE was tested because this agent has been reported to possess antioxidant, anti-inflammatory and immunomodulatory capabilities, as well as specific toxicity to transformed and tumour cells. Lifespans of mice given enalapril or CAPE are compared with controls and those given rapamycin in Supplementary Fig. 1. Rapamycin was purchased from LC Labs. The rapamycin was microencapsulated by Southwest Research Institute (San Antonio, Texas), using a spinning disk atomization coating process with the enter coating material Eudragit S100 (Röhm Pharma). This thermoplastic coating material increased the fraction of rapamycin that survived the food preparation process by three- to fourfold¹⁶. Because the coating material is water soluble only in non-acidic conditions, the encapsulated rapamycin is released in the small intestine rather than in the stomach. A pilot study showed that encapsulated rapamycin led to blood concentrations approximately tenfold higher than achieved by equivalent doses of non-encapsulated rapamycin¹⁶. The encapsulated rapamycin was administered at 14 mg per kg food (2.24 mg of rapamycin per kg body weight per day).

Measurement of rapamycin. Rapamycin was obtained from LC Laboratories. 32-desmethoxyrapamycin (32-RPM) was obtained from Sigma Chemical Company. HPLC grade methanol and acetonitrile were purchased from Fisher. All other reagents were purchased from Sigma Chemical Company. Milli-Q water was used for preparation of all solutions. The HPLC system consisted of a Waters 510 HPLC pump, Waters 717 autosampler, Waters 2487 UV detector, and Waters Empower chromatographic software (Waters). The HPLC analytical column was a Grace Alltima C18 (4.6 × 150 mm, 5 μm) purchased from Alltech. The mobile phase was 64% (v/v) acetonitrile and 36% water. The flow rate of the mobile phase was 1.5 ml min⁻¹ and the wavelength of absorbance was 278 nm. The temperature of the HPLC analytical column was maintained at 70 °C during the chromatographic runs using an Eppendorf CH-30 column heater.

Rapamycin and 32-RPM powder were dissolved in methanol at a concentration of 1 mg ml⁻¹ and stored in aliquots at -80 °C. A working stock solution was prepared each day from the methanol stock solutions at a concentration of 1 μg ml⁻¹ and used to spike the calibrators. Calibrator samples were prepared daily by spiking either whole blood or mouse food with stock solutions to achieve final concentrations of 0, 4, 8, 12, 24, 100 and 200 ng ml⁻¹.

Rapamycin was quantified in mouse blood using HPLC with ultraviolet detection. Briefly, 0.5 ml of calibrators and unknown samples were mixed with 75 μl of 1.0 μg ml⁻¹ 32-desmethoxy rapamycin (internal standard), 1.0 ml ZnSO₄ (50 g l⁻¹) and 1.0 ml of acetone. The samples were vortexed vigorously for 20 s, then centrifuged at 2,600g at 23 °C temperature for 5 min (subsequent centrifugations were performed under the same conditions). Supernatants were transferred to clean test tubes, then 200 μl of 100 mM NaOH was added, followed by vortexing. Then, 2 ml of 1-chlorobutane was added and the samples were capped, vortexed (1 min), and centrifuged. The supernatants were transferred to 10 ml glass tubes and dried to residue under a stream of nitrogen at ambient temperature. The dried extracts were dissolved in 750 μl of mobile phase and then 2 ml of hexane was added to each tube. The tubes were capped, vortexed for 30 s, and centrifuged for 2 min. The hexane layers were removed and discarded. The remaining extracts were dried under nitrogen and reconstituted in 250 μl of mobile phase, and then 200 μl of the final extracts were injected into the HPLC. The ratio of the peak area of rapamycin to that of the internal standard (response ratio) for each unknown sample was compared against a linear regression of calibrator response ratios to quantify rapamycin. The concentration of rapamycin was expressed as ng per ml whole blood.

Rapamycin content of mouse chow was verified using HPLC with ultraviolet detection. Briefly, 100 mg of chow for spiked calibrators and unknown samples was crushed with a mortar and pestle, then vortexed vigorously with 20 μl of 100 μg ml⁻¹ 32-RPM (internal standard) and 0.5 ml methanol. The samples were then mechanically shaken for 10 min. Next, 0.5 ml of Millipore water was added and the samples were vortexed vigorously for 20 s. The samples were centrifuged for 10 min and then 40 μl were injected into the HPLC. The ratio of the peak area of rapamycin to that of the internal standard (response ratio) was compared against a linear regression of calibrator response ratios at rapamycin concentrations of 0, 2, 4, 8, 10 and 20 ng mg⁻¹ of food to quantify rapamycin. The concentration of rapamycin in food was expressed as ng per mg food (parts per million).

Rapamycin effectiveness. To assay for the status of an mTORC1 downstream effector, we measured phosphorylation of ribosomal protein S6 (Ser 240 and Ser 244), a substrate of S6 kinase 1, in visceral adipose tissue lysates in mice fed an encapsulated rapamycin diet for 420 days or a control diet with empty microcapsules. Tissues were dissected and snap frozen in liquid nitrogen for storage at -80 °C, ground into powder under liquid nitrogen and dissolved in 10 volumes of buffer (50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 1% NP-40, 1 mM EDTA, 50 mM NaF, 40 mM 2-glycerophosphate, 0.1 mM Na orthovanadate (pH 10), 1 mM benzamide, and 1× Complete protease inhibitor cocktail (Roche)). After sonication and microcentrifugation, lysates were quantified³⁰. Forty micrograms of soluble protein from each extract was loaded on a 4–12% gradient PAGE and electrophoresed overnight at 5V. Gels were then transferred to nitrocellulose membranes (dry procedure), blocked and incubated with the primary antibodies (S6 ribosomal protein (5G10) rabbit monoclonal antibody, catalogue number 2217; phospho-S6 ribosomal protein (Ser235/236) antibody, catalogue number 2215; pan-actin antibody, catalogue number 4968; Cell Signaling Technologies), followed by secondary antibody (anti-rabbit IgG (H+L) peroxidase conjugated antibody, catalogue number 31460; Pierce) for detection by chemiluminescence. Signal intensities for each immunoblot were captured using a Kodak Image Station, and these were analysed using Kodak 1D image analysis software.

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