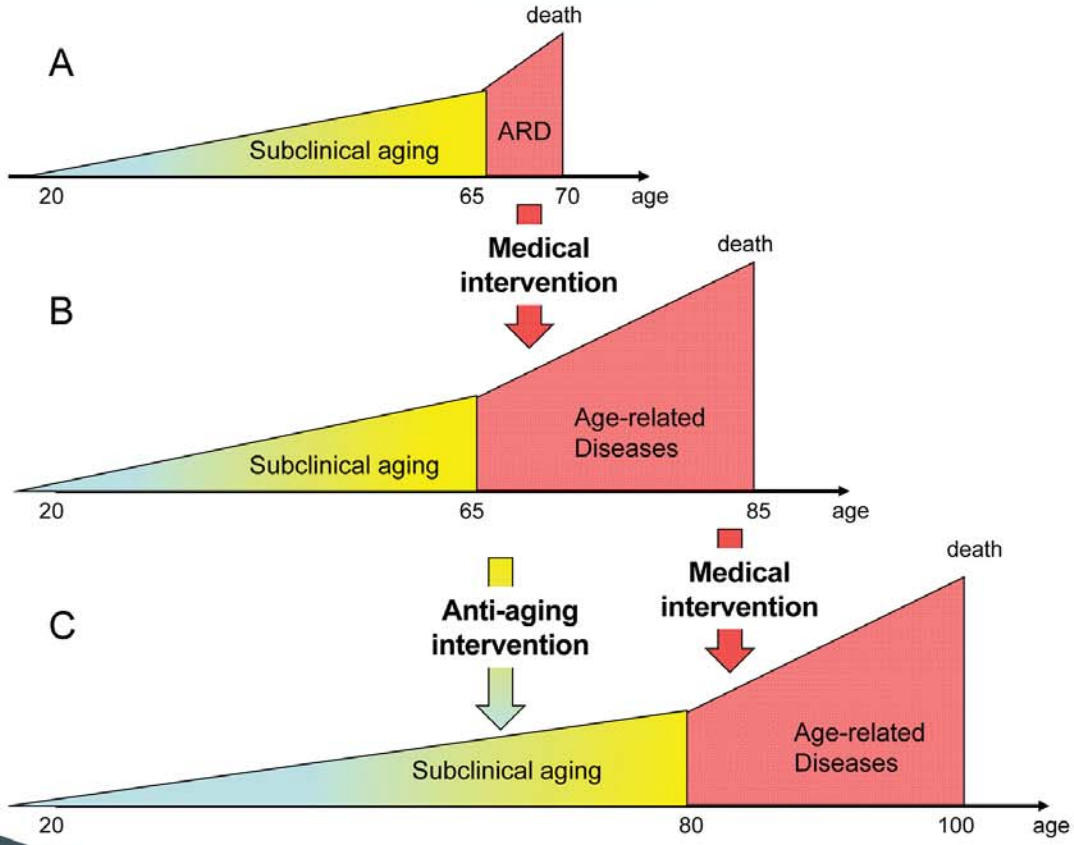


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Research Paper

# Metabolic consequences of long-term rapamycin exposure on common marmoset monkeys (*Callithrix jacchus*)

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**Abstract** **Rapamycin has been shown to extend lifespan in rodent models, but the effects on metabolic health and function have been widely debated in both clinical and translational trials. Prior to rapamycin being used as a treatment to extend both lifespan and healthspan in the human population, it is vital to assess the side effects of the treatment on metabolic pathways in animal model systems, including a closely related non-human primate model. In this study, we found that long-term treatment of marmoset monkeys with orally-administered encapsulated rapamycin resulted in no overall effects on body weight and only a small decrease in fat mass over the first few months of treatment. Rapamycin treated subjects showed no overall changes in daily activity counts, blood lipids, or significant changes in glucose metabolism including oral glucose tolerance. Adipose tissue displayed no differences in gene expression of metabolic markers following treatment, while liver tissue exhibited suppressed G6Pase activity with increased PCK and GPI activity. Overall, the marmosets revealed only minor metabolic consequences of chronic treatment with rapamycin and this adds to the growing body of literature that suggests that chronic and/or intermittent rapamycin treatment results in improved health span and metabolic functioning. The marmosets offer an interesting alternative animal model for future intervention testing and translational modeling.**

## INTRODUCTION

Rapamycin has been found by multiple laboratories to extend mouse lifespan even when mice began receiving rapamycin relatively late in life at 20 months of age, or roughly the equivalent of 55 human years [1]. In addition, rapamycin has been shown to delay the onset of several age-related diseases, including Alzheimer's disease, cardiovascular disease, and cancer in mouse models of these pathologies [2-5]. These findings have led to significant interest in the potential effects of rapamycin as an anti-aging intervention in humans particularly because rapamycin is already approved for use in cancer therapy and as an adjunct immuno-suppressive agent for transplant patients.

However, clinical administration of rapamycin has the potential for several side-effects include hyperlipidemia and hyperglycemia [6], raising concerns as to the potential negative impacts of rapamycin exposure in aged human populations that may have a high underlying prevalence of obesity- and age-induced insulin resistance and hyperlipidemia. However, results from clinical populations suggests that some side effects, such as hypertriglyceridemia, may improve over time and that others may be dose-dependent [6]. The nature of the human data accumulated thus far paints a far from complete picture of why and how rapamycin might affect metabolism and lipid trafficking. Moreover, these clinical studies have focused on patients with pre-existing conditions and have largely utilized combination therapies with other immunosupressants or steroids that can cause metabolic dysfunctions of their own [7]. There have, however, been no long-term studies of the effects of monothereapy with rapamycin or its analogs in populations of otherwise healthy humans. Thus, it is not clear whether the previously reported metabolic risks of rapamycin and its analogs are due to this drug directly or to other confounding factors.

In rodent models, monotherapy with rapamycin has largely, though not equivocally, been associated with impairment of glucose metabolism as measured in glucose tolerance tests. A notable exception is a study by Fang et al. suggesting that chronic treatment with rapamycin has a biphasic effect on glucose metabolism with short-term rapamycin treatment being detrimental to glucose metabolism whereas long-term (20 wk) treatment with rapamycin may promote an insulin-sensitive state in mice with a transition state in between [8]. Further examination has revealed that long term treatment of rapamycin leads to a metabolic switch resulting in enhanced insulin signaling and better triglyceride processing [9]. These rodent studies have largely been performed in animals maintained on a relatively standardized rodent chow, though there is evidence that rapamycin has similar effects on both lifespan and metabolic function in mice fed diets high in caloric content due to increased levels of sugar and/or fat [10,11]. Mice given intermittent treatment of rapamycin and placed on a high fat diet have no gross changes in metabolic markers, and rapa appears to prevent weight gain [12]. Combining the intervention therapies of rapamycin and resveratrol treatment in mice was found to prevent insulin resistance in mice being fed a high fat diet and suggests that combination therapy may be beneficial in a high fat environment [13]. Human glucose metabolism is regulated by a complex interaction of genetics and environment (including diet) that cannot be fully recapitulated in laboratory rodents [14]. Even the timing of or causes of eating/overeating differ between rodent models and humans, further complicating this issue [15, 16]. At the molecular level, there are significant discrepancies between rodents and humans in alterations of gene regulation in metabolic dysfunction suggesting there is little overlap between the two models [17]. Lastly, many of the complications of metabolic dysfunction including nephropathy, neuropathy, and cardiac dysfunction cannot be successfully replicated in single genetic mouse models of metabolic dysfunction or in high fat-fed rodents [18]. Thus, a significant question remains whether the choice of diet (as well as sex of animals or background genetics) could potentially complicate the potential for translation [19-21]. An approach to address whether the effects (and potential side-effects like metabolic dysfunction) of rapamycin in otherwise healthy rodents may also be relevant to humans is to perform such experiments in other species that are predicted to have similar phenotypic metabolic regulation as humans. In other words, studies of rapamycin's effects in a species more closely related to humans can inform as to the generalizability of the rodent findings and issues likely to impede the general use of rapamycin as an anti-aging treatment in humans.

The common marmoset (*Callithrix jacchus*) is a small monkey with a relatively short lifespan. Both its small size and associated shorter lifespan make this species a valuable nonhuman primate model for the study of aging and chronic disease [22, 23]. Captive marmosets display many similarities to humans in terms of obesity and its sequelae. Spontaneous obesity has been described in multiple captive marmoset colonies that are socially housed and fed a relatively low fat, high fiber diet [22, 24-28]. Obesity in marmosets, defined in a fashion similar to that used in humans, is statistically associated with increased risk to metabolic dysfunction and cardiovascular disease [16]. In addition to displaying evidence of insulin resistance, marmosets at extremely high weights (over 500 grams) show higher age-specific mortality rates as adults when compared to animals of average weight [22]. From 2010-2011, we conducted a year-long study of daily dosing of a group of common marmosets with rapamycin. We previously reported that we were able to maintain circulating blood levels of rapamycin at 5.2 ng/mL by giving the animals a dose of eudragit encapsulated rapamycin in yogurt of 1mg/kg/day. Subjects demonstrated a decrease in mTORC1 after two weeks of treatment. There was no evidence of clinical anemia, mouth ulcers, lung fibrotic changes, significant changes in wound healing, or increased mortality [29]. This report describes a set of metabolic outcomes from this study.

## RESULTS

### Body composition

Rapamycin treated subjects displayed a significant loss of body fat mass at two months post-dosing while control subjects displayed a statistically insignificant change in body fat mass, as illustrated in Fig. 1A (treatment x time interaction, $p < 0.0097$; difference in month 0 and month 2 mean for rapamycin treated subjects, $p < 0.05$, Sidak's multiple comparison test).

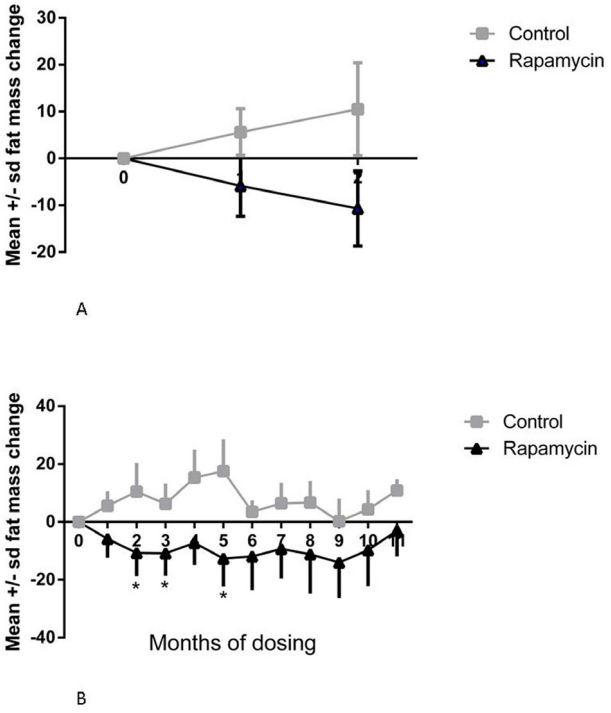



**Figure 1. Change in fat mass.** (**A**) Change in fat mass at 1 and 2 months, post-dosing, from pre-dosing (month 0) measurement. Squares = control subjects; triangles = rapamycin subjects (mean ± SD); treatment x time interaction, $p < 0.0097$; difference in month 0 and month 2 mean for rapamycin treated subjects, $p < 0.05$, Sidak's multiple comparison test. (**B**) Change in fat mass from pre-dosing measurement for months 1-11 for rapamycin subjects. * treatment effect, $F=5.385$, $p=0.018$, Dunnett's multiple comparison test significant, $p < 0.05$, for month 0 versus months 2, 3, and 5.

The rapamycin treated subjects had significantly reduced body fat mass in months 2, 3 and 5, after which their mean fat mass did not differ from the pre-dosing mean (treatment effect, $F=5.385$, $p=0.018$, Dunnett's multiple comparison test significant, $p < 0.05$, for month 0 versus months 2, 3, and 5), resulting in no difference between control and rapamycin treated subjects at the end of the study as illustrated in Fig. 1B. There were no significant differences between controls and rapamycin treated subjects and no effects of rapamycin treated subjects over time on body lean mass.

### Food intake and activity levels

There were no significant differences between controls and rapamycin treated subjects in food intake at two months post-dosing. In rapamycin-treated marmosets, there was a significant increase in food intake at month 5, over the pre-dosing food intake (treatment effect, $F=8.353$, $p=0.001$, Dunnett's multiple comparison test significant, $p < 0.05$, for month 0 versus month 5), as illustrated in Fig. 2.

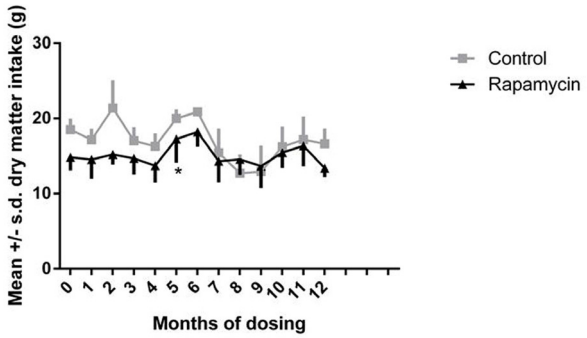
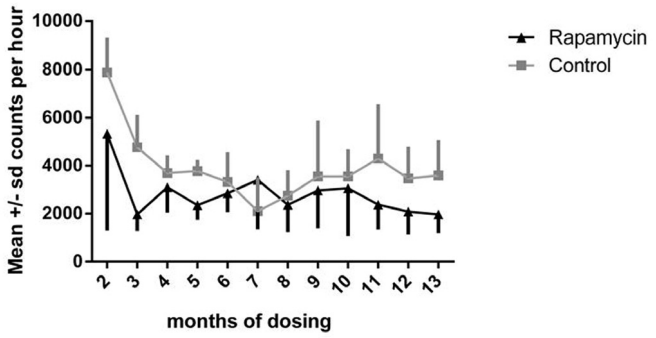



**Figure 2. Food intake.** Daily dry matter intake for months 0-12, month 0 is a pre-dosing measurement. Squares = control subjects; triangles = rapamycin subjects (mean ± SD). * treatment effect, $F=8.353$, $p=0.001$, Dunnett's multiple comparison test significant, $p < 0.05$, for month 0 versus month 5.

In both control and rapamycin-treated subjects activity scored as accelerometer counts per hour declined significantly after the second month of dosing, as illustrated in Fig. 3, then remained stable over the remainder of the study. There were no significant differences between controls and rapamycin treated subjects. Because both groups were affected, these data suggest that this decline was a result of habituation to wearing the harness holding the accelerometer.

### Lipid and glucose metabolic measures

There were no significant differences between controls and rapamycin-treated subjects in pre- versus post-dosing mean circulating triglyceride concentrations.




**Fiure 3. Daily activity.** Accelerometer counts per hour for months 2-13 of dosing. Squares = control subjects; triangles= rapamycin subjects (mean ± SD).

There was, however considerable inter-individual variation in both baseline triglyceride concentration and in change over time, as illustrated in Fig. 4. Two of the rapamycin treated subjects that were borderline hypertriglyceridemic (476 and 402 mg/dl, with > 400 mg/dl defined as hypertriglyceridemic, [24] before dosing, displayed dramatic increases in circulating triglyceride concentration at month 2 (702 and 1,359 mg/dl); however, their triglyceride concentrations then varied considerably over the next 7 months. There was no consistent hypertriglyceridemia caused by rapamycin among subjects who began with normal circulating triglyceride concentrations There were two subjects (one control and one rapamycin treated) that displayed severe hypertriglycerimedia before dosing (603 and 1,611 mg/dl respectively). They both remained hypertriglyceridemic through the study.

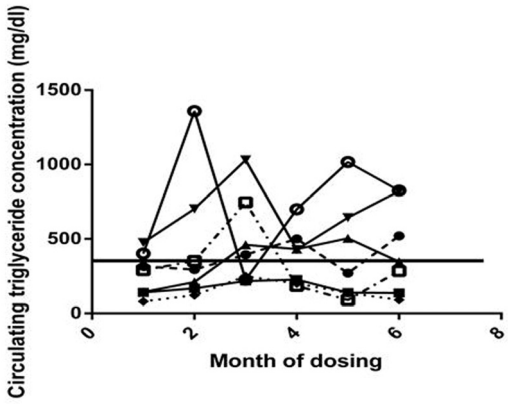



**Figure 4. Circulating triglyceride.** Circulating triglyceride concentrations for each rapamycin subject for months 0-6, month 0 is a pre-dosing measurement. The solid horizontal line represents the previously established cut-off point for normal triglyceride concentrations in this species.

Three measures of glucose metabolic function were assessed: fasting blood glucose, QuickI index, and AUC. There were no significant changes in fasting glucose concentrations as illustrated in Fig. 5A. The QuickI index, calculated as 1/[log(fasting insulin) + log(fasting glucose)], is the typical measure reported in nonhuman primate studies to provide an estimate of insulin sensitivity, with higher values indicating more insulin sensitivity. The area under the curve (AUC) for the glucose tolerance tests represents the relative glucose excursion caused by a consistent dose of glucose and is a measure of the efficiency with which the entire system can remove glucose from the circulation. As illustrated in Fig. 5D, the mean QuickI index for the control group was higher than that for the rapamycin treated group prior to treatment ($F=5.396$, $p = 0.0453$, Sidak's multiple comparison test, $p < 0.05$ for month 0 control vs rapamycin treated), suggesting that the animals that became the control group were, on average, more insulin sensitive than those in the group randomly selected to be treated with rapamycin. However, the rapamycin-treated group displayed a reduced QuickI measurement even prior to treatment that was not altered during these first two months of dosing as indicated by the lack of a significant interaction effect. We also found that QuickI did not differ among rapamycin-treated animals through 8 months of rapamycin treatment as illustrated in Fig. 5C. There were no differences between control and rapamycin treated subjects and no interaction effect on the glucose AUC. There were also no significant differences in post-dosing average glucose AUC in the rapamycin treated subjects through 8 months of dosing, as illustrated in Fig. 5B. Together, these data suggest limited to no impairment of glucose metabolism in healthy marmosets treated with daily administration of rapamycin at doses sufficient to reduce mTOR signaling.

**Assessment of molecular effects**

The long-term administration of rapamycin in rodents has been associated with hyperglycemia caused in part by increased gluconeogenesis [27-29]. In the liver of rapamycin treated animals, we found significant upregulation of the expression of phosphoenolpyruvate carboxykinase (PCK1), indicative of altered gluconeogenic capacity. Surprisingly, we found that glucose 6 phosphatase expression in rapamycin treated animals was significantly reduced (Fig. 6). Rapamycin did not alter the expression of other markers of gluconeogenesis. The lack of a consistent alteration in the expression of gluconeogenic effectors may explain why rapamycin-treated marmosets showed no significant change in fasting blood glucose levels.

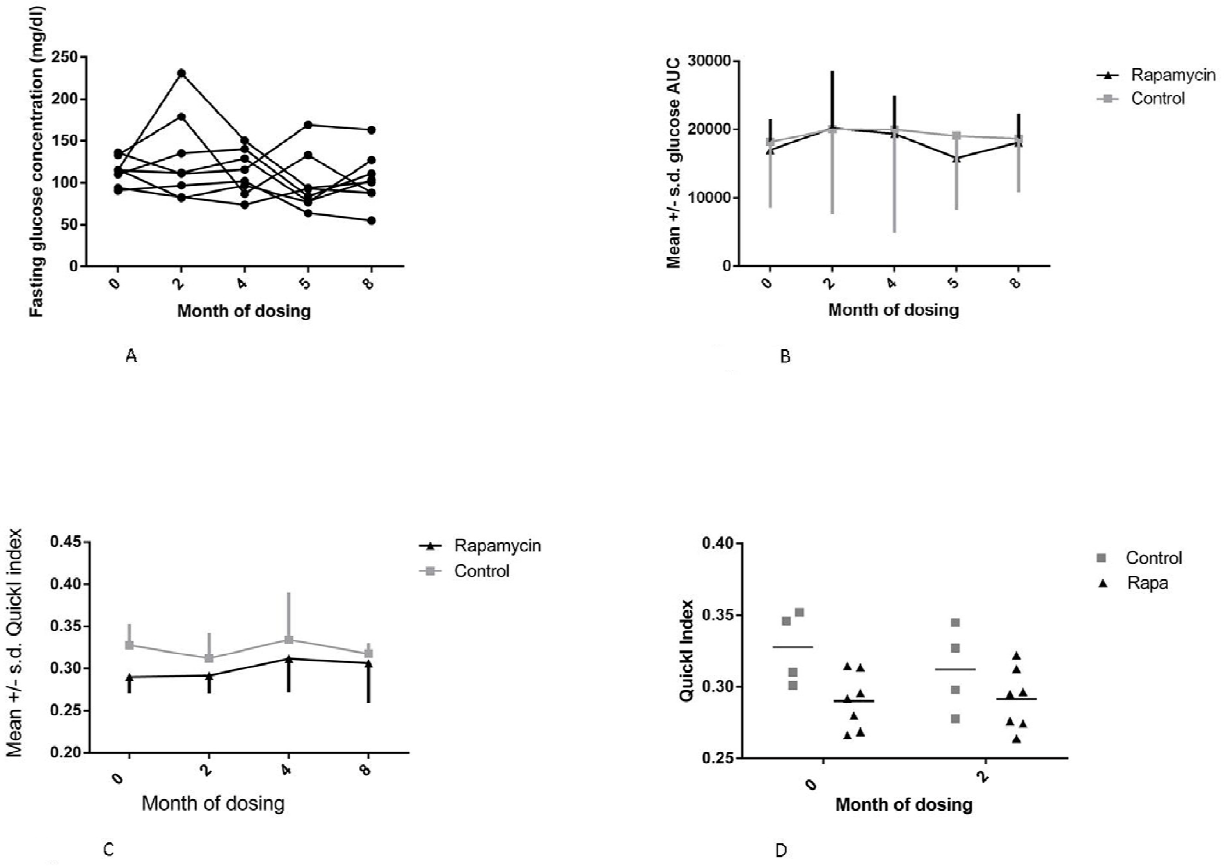



**Figure 5. Metabolic measures.** (**A**) Fasting glucose concentration for months 0-8 for rapamycin subjects, month 0 is a pre-dosing measurement. (**B**) Glucose area under the curve (AUC) for months 0-8 for rapamycin subjects. (C) QuickI index for rapamycin subjects for months 0-8. (**D**) QuickI index - 1/[log(fasting insulin) + log(fasting glucose)] for months 0 and 2 of dosing, *(F=5.396, p = 0.0453, Sidak's multiple comparison test, p < 0.05 for month 0 control vs rapamycin treated). For all panels squares = control subjects; triangles = rapamycin subjects (mean ± SD).

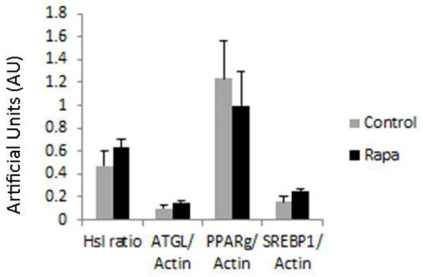
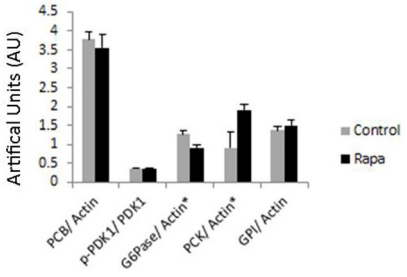



Figure 6A




Because rapamycin modulated fat content of marmosets in the early periods of treatment, we assessed the potential modulation of effectors of lipolysis/lipogenesis in adipose. In visceral adipose samples, we found no significant effects on the phosphorylation or expression of any of these markers, suggesting little effect of rapamycin. However, at the time of sacrifice, rapamycin-treated marmosets were not significantly leaner than control animals. Due to limitations of the design of this study, we could not determine whether these effectors were altered by rapamycin at earlier time points when fat mass was reduced by treatment (Fig. 1B).

**Figure 6. Immunoblot results.** Immunoblot results for the following: adipose triglyceride lipase (ATGL), pyruvate carboxylase (PCB), glucose-6-phosphatase α (G6Pase), glucose-6-phosphate isomerase (GPI), peroxisome proliferator-activated receptor γ (PPARγ), phospho-pyruvate dehydrogenase kinase (p-PDK1), pyruvate dehydrogenase kinase (PDK1), phosphoenol-pyruvate carboxykinase 1 (PCK1), sterol regulatory element-binding protein 1 (SREBP1) corrected by actin. A. Adipose tissue collected at sacrifice following 14 months of rapamycin (black) or control dosing (grey) (mean ± SE) B. Liver tissue collected at sacrifice following 14 months of rapamycin (black) or control dosing (grey) (mean ± SE) *indicates significance p<0.05.

## DISCUSSION

Prior to assessing the effectiveness of rapamycin as an anti-aging treatment in humans, it is first necessary to elucidate the potential effects on long term health outcomes. In particular, there has been a great deal of controversy and inconsistent results in the clinical studies of rapamycin, which have highlighted the potential increased risk for metabolic defects such as hyperlipidemia and hyperglycemia that are consistent with increasing risk of cardiovascular disease and type 2 diabetes [30]. However, the effects of this drug on relatively healthy humans are largely unknown. A recent short term study (6 weeks) of elderly patients given doses of a rapamycin analog found few side effects significant from placebo control subjects and reported a significant increase in serological response to flu vaccination, however, this study did not examine any markers of metabolic health in these subjects during treatment [31]. In order to assess potential consequences of long term rapamycin treatment on primate metabolic health we tested the effects of rapamycin on a group of healthy, aged, non-human primates, the common marmoset. We previously reported the ability to reliably and routinely dose socially housed marmoset monkeys with yogurt mixtures containing eudragit encapsulated rapamycin [29]. Dosing with 0.4 mg/day resulted in average blood rapamycin levels of 5.2 ng/mL, which is well within the range found in studies of other model species, and humans [1-5]. Further, we previously reported evidence of suppressed phospho-rpS6 in PBMC samples of rapamycin subjects suggesting down-regulation in mTORC1. In this study we reported several markers that suggest that chronic oral dosing with eudragit-encapsulated rapamycin has little impact on the metabolic status of marmosets.

Many have proposed that rapamycin is a mimic of calorie restriction which is the gold-standard for anti-aging intervention resulting in both extended life span and health span in many rodent models. Rapamycin has been shown to suppress mTOR activity, potentially decrease weight and fat mass, and extend healthspan and life span in a similar manner as calorie restriction [1-4]. In marmosets we demonstrated no overall change in body weight while being treated with rapamycin, but the marmosets did have significant loss of body fat. However, the loss of body fat stabilized at approximately five months of treatment and this time point was associated with an increase in food intake of rapamycin subjects. While we are unable to elucidate the underlying mechanisms for the sudden shift in dietary intake at 5 months of dosing, it is interesting to consider the possibility that the rapamycin-dosed animals altered caloric intake in response to the fat mass loss [32].

One of the major reported risks of rapamycin administration clinically is the development of new-onset type 2 diabetes as shown in clinical studies of kidney transplant patients treated with rapamycin analogs [33, 34]. However, the interpretation of these data is complicated by several factors including the impaired health status of the subjects and the use of combination therapies using additional drugs that are known to cause metabolic impairment on their own. Recent clinical studies of kidney transplant patients suggest that Tacrolimus rather than Sirolimus may be the leading cause of new onset diabetes within 10 weeks of treatment, however all research suggests that continued examination of monotherapies are needed to elucidate the side effects of each immunosuppressant [35, 36]. In rodents, the chronic administration of rapamycin as a mono-therapy has often been shown to impair glucose metabolism. For example, both inbred and genetically heterogeneous mouse strains develop glucose intolerance with oral administration of encapsulated rapamycin [11, 37, 38]. In inbred C57BL/6 mice, but not genetically heterogeneous mice, rapamycin is also associated with the development of insulin resistance. Interestingly, these effects of rapamycin on metabolism are dependent on both dose of rapamycin and sex of subjects [38] and do not appear to be permanent alterations as the metabolic defects can be reversed by ending rapamycin treatment [11]. Similarly, rapamycin treatment to normoglycemic, pre-diabetic *P. obesus* treated with rapamycin display heightened hyperglycemia and increased insulin resistance in part by reducing pancreatic β-cell function. In this rodent model it was suggested that rapamycin exacerbated the pre-existing diabetic symptoms and metabolic disorder in high risk animals [39]. Alternatively the effect of rapamycin is thought to mimic the metabolic changes associated with starvation diabetes or Type 0 diabetes [40, 41, 42]; which is often thought to be a positive, adaptive form of metabolic changes associated with enhanced metabolic efficiency and decreased risk of true diabetes. Evidence of enhanced insulin signaling following long term rapa [9], and intermittent rapa [11], as well as little evidence for detrimental mitochondrial function following rapamycin [42], supports the hypothesis that rapamycin may in fact be inducing changes similar to starvation diabetes [13, 40-42]. Interestingly, in our current study we found no evidence that rapamycin negatively impairs glucose metabolism in marmosets. One possible interpretation of these findings could be that rapamycin treatment might negatively and significantly affect only subjects that are predisposed to metabolic disease. This also

might explain some of the slight differences in rapamycin's effect on glucose metabolic dysfunction in C57BL/6 but not genetically mixed mice. Another possible explanation could be the length of treatment utilized here. Recent studies have suggested a bi-phasic effect of rapamycin on glucose metabolism, with long-term administration of rapamycin to mice associated with increased, rather than reduced insulin sensitivity [8, 9, 44]. However, others have shown rapamycin in eudragit-encapsulated form (as we used in this study) does not show this bi-phasic effect, but rather continuously impairs glucose metabolism in mice [11,37]. In part, the metabolic impairments of rapamycin are thought to be due to inhibition of mTORC2 rather than mTORC1 [45]; while we previously showed that this rapamycin dose was sufficient to inhibit mTORC1 [29], it may not be sufficient to inhibit mTORC2 signaling in the marmoset and thus minimize the presumed metabolic defects of rapamycin treatment.

The increased risk of hyperglycemia with rapamycin treatment has been attributed to increased hepatic gluconeogenesis in rodent studies. In both mice and rats, rapamycin treatment significantly increases hepatic glucose production following injection with pyruvate and increases the expression of the gluconeogenic effectors like PCK1 and G6Pase [45, 46]. In this study, we also confirmed that a significant increase in PCK1 with rapamycin treatment, but paradoxically, found a significant decrease in G6Pase. While the rise in PCK1 would be consistent with increased gluconeogenesis, the decrease in G6Pase might be interpreted as inhibiting this process. It is not clear why rapamycin treatment has this contrasting effect in marmosets, but this might explain why marmosets did not display hyperglycemia with this treatment.

In addition to its well-known roles in cell survival and growth, recent studies have linked the mTOR signaling pathway with the regulation of lipid metabolism [47]. However, the direct effects of rapamycin on lipid metabolism have often been contradictory in published reports. For example, rapamycin has been reported to both improve and impair fatty acid oxidation in skeletal muscle cell lines [44, 48]. Reports regarding rapamycin's effects on lipid utilization in vivo in rodents are similarly inconsistent; rapamycin has been reported to decrease, increase or not effect fat accumulation among several different studies [1, 11, 37, 38, 49, 50]. Here, we found that rapamycin significantly reduced fat mass in marmosets at early time points in our treatment regime. However, after approximately 5 months of treatment, fat mass no longer differed between rapamycin-treated and control marmosets and we found no evidence for differences in lipolysis or lipogenesis in adipose tissue collected at the end of this study. Interestingly, this change coincided with a significant increase in food consumption among the rapamycin-treated marmosets. This alteration could represent a compensatory effect for the long-term inhibition of mTOR signaling. Further temporal studies regarding the effect of rapamycin in this model will be necessary to address this possibility.

Treatment with rapamycin as an intervention in the aging process for humans offers many possibilities but some studies have reported deleterious side effects that raise concern regarding the efficacy of this treatment. This study represents the first to examine the metabolic consequences of rapamycin dosing in healthy non-human primates. We have reported here evidence that long term rapamycin treatment at a dose that has been used in previous studies and reduces mTOR signaling in marmosets [29] does not result in notable negative side-effects on metabolic function in healthy marmosets. We believe that marmosets offer a unique non-human primate model that will allow detailed evaluation of the effect of potential anti-aging treatments on primate metabolic function, dietary intake, and activity patterning.

## MATERIALS AND METHODS

Subjects. The subjects for this study were common marmosets (*Callithrix jacchus*) housed at the Southwest National Primate Research Center. Basic husbandry and housing for this colony have been described previously [51]. Thirteen subjects between the ages of 7.1 and 9.1 years were housed as female-vasectomized male pairs. Four pairs received daily oral dosing of 1.0 mg/kg/day (0.40 mg/day) rapamycin in a yogurt vehicle via syringe for 14 months as described [29]. Two pairs (5 subjects, one male died mid-way through the study and was replaced with another male) received daily doses of empty eudragit capsules in yogurt as control. Throughout the long term dosing regimen several markers of metabolic health were assessed.

Body composition. Marmoset lean and fat mass was assessed monthly via quantitative magnetic resonance (QMR) imaging using an Echo MRI unit [26]. Unsedated animals were placed in a plastic tube which was then inserted into the magnetic chamber with scans taking less than 2 minutes on average for each animal. Animals were weighed biweekly throughout the project by placing a scale within the cage and rewarding the animal's for maintaining position on the scale.

Caloric intake. Subjects participated in a 2 day food intake trial once per month for the length of the trial [28]. For these trials the subjects were separated from each other within the cage and fed their daily base diet consisting of two feed types from Harlan Teklad and Purina. Samples of each diet were taken from each prepared batch, frozen and stored until analysis. Diet fed to the subjects was weighed prior to feeding. After 24 hours all remaining food was removed and weighed, and fresh food was weighed and fed. After 48 hours all remaining food was removed and weighed and the subjects were returned to normal housing and feeding schedule. Samples were dried and dry weight consumption and caloric consumption was calculated and averaged over the 48 hour period.

Activity. Daily activity patterns were assessed with the Mini actiwatch (CamNtech) which were placed in a marmoset pouch (Lomar) on a ferret harness (Petco). Subjects were gradually habituated to the ferret harnesses over the course of three weeks, increasing time in the harness incrementally throughout training until 24 hours in the harness had been achieved. The miniwatches are data loggers that batch data in 15 second epochs. For these trials animals were separated from each other within the cage and placed in harnesses with the actiwatch in the pouch secured across the back of the animal. Animals remained in the harness for 48 hours of data collection during which normal husbandry and feeding continued. At the end of the trial the animals were captured in transfer boxes and the harnesses were removed. Data was downloaded from the device. The activity counts from the first 15 minutes and last 15 minutes of the collection were removed from analysis as these represented handling and cage manipulation.

Blood chemistry. Each month animals were fasted overnight and 2 ml of blood were drawn to assess circulating triglyceride concentrations, fasting glucose and insulin concentrations. Fasting glucose was determined immediately following the blood collection via glucometer. Blood was collected into serum separator tubes, spun and frozen in -80°C until further analysis. Triglyceride concentrations were assessed at the SNPRC clinical pathology lab. Samples were shipped to Wisconsin for analysis of insulin concentrations as described [52].

Glucose challenge. Animals were fasted overnight prior to an oral glucose tolerance test [24], and placed in a restraint device used for blood collection to which they had previously been habituated. An EDTA coated needle and syringe were used to collect 0.5 ml of blood from the femoral vein for the baseline bleed. The animals were then dosed orally with a 40% dextrose solution receiving a calculated glucose dose equal to 0.5% of their current body weight. Subjects remained in the restraint for a 15 and 30 minute post dose blood sample drawn from the tail vein via an EDTA coated butterfly needle. Subjects were removed from the restraint device following the 30 minute sample and placed in a transport box until the 60 minute sample, and this was repeated for the 120 minute sample. The 15, 60 and 120 minute samples were glucometer reads only. For the 30 minute sample 0.5 ml of blood was collected for further analysis. Following the 120 minute bleed the animals were returned to their home cage and fed. The 0 and 30 minute samples were spun and frozen until shipment to Wisconsin for insulin assay analysis.

Immunoblots. Total protein extracts were isolated from liver and visceral fat tissue that had been snapped frozen in liquid nitrogen after sacrifice and stored at -80°C until use. Protein extracts were homogenized in RIPA buffer with additional protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL, USA), centrifuged at 14,000g at 4°C for 15 minutes, and then stored at -80°C until needed. Equal amounts of protein samples were separated electrophoretically by SDS-PAGE and then transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Primary antibodies and their sources used in this study: adipose triglyceride lipase (ATGL), pyruvate carboxylase (PCB), glucose-6-phosphatase α (G6Pase), glucose-6-phosphate isomerase (GPI), and actin from Santa Cruz (Santa Cruz CA), peroxisome proliferator-activated receptor γ (PPARγ), phospho-pyruvate dehydrogenase kinase (p-PDK1), pyruvate dehydrogenase kinase (PDK1), phosphoenolpyruvate carboxykinase 1 (PCK1) from Cell Signaling (Beverly MA), sterol regulatory element-binding protein 1 (SREBP1) from Abcam (Cambridge MA) and deptor from Millipore with all alkaline phosphatase-conjugated secondary antibodies (anti-rabbit and anti-mouse) from Santa Cruz. Protein bands on immunoblots were detected using ECL reagent and analyzed using ImageJ.

Analyses. Variables of interest included body mass, fat mass, fat-free mass, 24 hour total actimeter counts, 24 hour caloric intake, triglyceride concentration, fasting glucose concentration, fasting insulin concentration, and glucose AUC following an oral glucose challenge. For each variable, the change in pre- Rapa dose value to post- Rapa dose value, measured following (one month) of dosing was compared for control subjects versus rapamycin-treated subjects in a two-way, repeated measures ANOVA. An additional repeated measures ANOVA was used to compare values within each rapamycin-treated subject over time for the entire

dosing period. Comparisons of tissue protein activity were done using MANOVA with Bonferroni corrections. Analyses were conducted using GraphPad/ Prism and SPSS 13.0.

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## Conflict of interest statement

The authors have no conflict of interests to declare.

## REFERENCES

**1.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009; 460:392-395.

**2.** Wilkinson JE, Burmeister L, Brooks SV, Chan CC, Friedline S, Harrison DE, Hejtmancik JF, Nadon N, Strong R, Wood LK, Woodward MA, Miller RA. Rapamycin slows aging in mice. Aging Cell. 2012; 11:675–682.

**3.** Zhang Y, Bokov A, Gelfond J, Soto V, Ikeno Y, Hubbard G, Diaz V, Sloane L, Maslin K, Treaster S, Réndon S, van Remmen H, Ward W, et al. Rapamycin extends life and health in C57Bl/6 mice. The Journals of Gerontology. Series A, Biological sciences and medical sciences. 2013; 69A:119–130.

**4.** Fok WC, Chen Y, Bokov A, Zhang **Y**, Salmon AB, Diaz V, Javors M, Wood WH 3rd, Zhang **Y**, Becker KG, Pérez VI, Richardson A. Mice fed rapamycin have an increase in lifespan associated with major changes in the liver transcriptome. PLoS One. 2014; 9:e83988.

**5.** Halloran J, Hussong SA, Burbank R, Podlutskaya N, Fischer KE, Sloane LB, Austad SN, Strong R, Richardson A, Hart MJ, Galvan V. Chronic inhibition of mammalian target of rapamycin by rapamycin modulates cognitive and non-cognitive components of behavior throughout lifespan in mice. Neuroscience. 2012; 223:102-113.

**6.** Stallone G, Infante B, Grandaliano G, Gesualdo L. Management of side effects of sirolimus therapy. Transplantation. 2009; 87:S23–S26.

**7.** Montalbano M, Neff GW, Yamashiki N, Meyer D, Bettiol M, Slapak-Green G, Ruiz P, Manten E, Safdar K, O'Brien C, Tzakis AG. A retrospective review of liver transplant patients treated with sirolimus from a single center: an analysis of sirolimus-related complications. Transplantation. 200;78:264–268.

**8.** Fang Y, Westbrook R, Hill C, Boparai RK, Arum O, Spong A, Wang F, Javors MA, Chen J, Sun LY, Bartke A. Duration of rapamycin treatment has differential effects on metabolism in mice. Cell Metabolism. 2013; 17:456-462.

**9.** Fang Y, Bartke A. Prolonged rapamycin treatment led to beneficial metabolic switch. Aging. 2013;5:328-329.

**10.** Leontieva OV, Paszkiewicz GM, Blagosklonny MV. Weekly administration of rapamycin improves survival and biomarkers in obese male mice on high-fat diet. Aging Cell. 2014; 13:616-622

**11.** Liu Y, Diaz V, Fernandez E, Strong R, Ye L, Baur JA, Lamming DW, Richardson A, Salmon AB. Rapamycin-induced metabolic defects are reversible in both lean and obese mice. Aging (Albany NY). 2014; 6:742-754.

**12.** Leontieva OV, Pasckiewicz GM, Blagosklonny MV. Comparison of rapamycin schedules in mice on high-fat diet. Cell Cycle. 2014; 13:3350-3356.

**13.** Leontieva OV, Paszkiewicz G, Demidenko ZN, Blagosklonny MV. Resveratrol potentiates rapamycin to prevent hperinsulinemia and obesity in male mice on high fat diet. Cell Death and Disease. 2013; 4:e472.

**14.** Grarup N, Andersen G. Gene–environment interactions in the pathogenesis of type 2 diabetes and metabolism. Current Opinion in Clinical Nutrition & Metabolic Care. 2007; 10:420-426.

**15.** Halford JC, Boyland EJ, Blundell JE, Kirkham TC, Harrold JA. Pharmacological management of appetite expression in obesity. Nature Reviews Endocrinology. 2010; 6:255-269.

**16.** Jensen T, Kiersgaard M, Sørensen D, Mikkelsen L. Fasting of mice: a review. Laboratory Animals. 2013; 47:225-240.

**17.** Li S, Zhang HY, Hu CC, Lawrence F, Gallagher KE, Surapaneni A, Estrem ST, Calley JN, Varga G, Dow ER, Chen Y. Assessment of diet-induced obese rats as an obesity model by comparative functional genomics. Obesity. 2008; 16:811-818.

**18.** Lai M, Chandrasekera PC, Barnard ND. You are what you eat, or are you? The challenges of translating high-fat-fed rodents to human obesity and diabetes. Nutrition & Diabetes. 2014; 4:e135.

**19.** Ikemoto S, Takahashi M, Tsunoda N, Maruyama K, Itakura H, Ezaki O. High-fat diet-induced hyperglycemia and obesity in mice: differential effects of dietary oils. Metabolism. 1996; 45:1539-1546.

**20.** Gallou-Kabani C, Vigé A, Gross MS, Rabès JP, Boileau C, Larue-Achagiotis C, Tomé D, Jais JP, Junien C. C57BL/6J and A/J mice fed a high-fat diet delineate components of metabolic syndrome. Obesity. 2007; 15:1996-2005.

**21.** Warden CH, Fisler JS. Comparisons of diets used in animal models of high-fat feeding. Cell Metabolism. 2008; 7:277.

**22.** Tardif SD, Mansfield K, Ratnam R, Ross CN, Ziegler TE. The marmoset as a model of aging and age related disease. Institute for Laboratory Animal Research Journal. 2011; 52:54–65.

**23.** Ross CN, Davis K, Dobek G, Tardif SD. Aging phenotypes of common marmosets (*Callithrix jacchus*). Journal of Aging Research. 2012; 2012:567143.

**24.** Tardif SD, Power ML, Ross CN, Rutherford JN, Layne-Colon DG, Paulik MA. Characterization of obese phenotypes in a small

nonhuman primate, the common marmoset (*Callithrix jacchus*). Obesity. 2009; 17:1499-1505.

**25.** Wachtman LM, Kramer JA, Miller AD, Hachey AM, Curran EH, Mansfield KG. Differential contribution of dietary fat and monosaccharide to metabolic syndrome in the common marmoset (*Callithrix jacchus*). Obesity. 2011; 19:1145-1156.

**26.** Power ML, Ross CN, Schulkin J, Tardif SD. The development of obesity begins at an early age in captive common marmosets (*Callithrix jacchus*). American Journal of Primatology. 2012; 74:261–269.

**27.** Power ML, Ross CN, Schulkin J, Ziegler TE, Tardif SD. Metabolic consequences of the early onset of obesity in common marmoset monkeys. Obesity. 2013; 21:E592–E598.

**28.** Ross CN, Power ML, Artavia J, Tardif SD. Relation of food intake behaviors and obesity development in young common marmoset monkeys. Obesity. 2013; 21:1891-1899.

**29.** Tardif SD, Ross CN, Bergman P, Fernandez E, Javors M, Salmon A, Spross J, Strong R, Richardson A. Testing efficacy of administration of the antiaging drug rapamycin in a nonhuman primate, the common marmoset. Journals of Gerontology: Biological Sciences. 2015; 70:577-587.

**30.** Verges B, Walter T, Cariou B. Effects of anti-cancer targeted therapies on lipid and glucose metabolism. European Journal of Endocrinology. 2014; 170:R43-55.

**31.** Mannick JB, Giudice G, Lattanzi M, Valiante NM, Praestgaard J, Huang B, Lonetto MA, Maexker HT, Kovarik J, Carson S, Glass DJ, Kickstein LB. mTOR inhibition improves immune function in the elderly. Science Translational Medicine. 2014, 268:268ra179.

**32.** Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central nervous system control of food intake and body weight. Nature. 2006; 443:289-295.

**33.** Teutonico A, Schena PF, Di Paolo S. Glucose metabolism in renal transplant recipients: effect of calcineurin inhibitor withdrawal and conversion to sirolimus. Journal of the American Society of Nephrology. 2005; 16:3128-3135.

**34.** Johnston O, Rose CL, Webster AC, Gill JS. Sirolimus is associated with new-onset diabetes in kidney transplant recipients. Journal of the American Society of Nephrology. 2008;197:1411-1418.

**35.** Yates CJ, Fourlanos S, Hjelmesaeth J, Colman PG, Cohney SJ. New-onset diabetes after kidney transplantation-changes and challenges. American Journal of Transplantation. 2012; 12:820-828.

**36.** Pavlakis M, Goldfarb-Rumyantzev AS. Diabetes after transplantation and sirolimus: what's the connection? Journal of the American Society of Nephrology. 2008; 19:1255-1256.

**37.** Lamming DW, Ye L, Astle CM, Baur JA, Sabatini DM, Harrison DE. Young and old genetically heterogeneous HET3 mice on a rapamycin diet are glucose intolerant but insulin sensitive. Aging Cell. 2013; 12:712-718.

**38.** Miller RA, Harrison DE, Astle CM, Fernandez E, Flurkey K, Han M, Javors MA, Li X, Nadon NL, Nelson JF, Pletcher S, Salmon AB, Sharp ZD, Van Roekel S, Winkleman L, Strong R. Rapamycin-mediated lifespan increase in mice is dose and sex dependent and metabolically distinct from dietary restriction. Aging Cell. 2014; 13:468-477.

**39.** Fraenkel M, Ketzinel-Gilad M, Ariav Y, Pappo O, Karaca M, Castel J, Berthault MF, Magnan C, Cerasi E, Kaiser N, Leibowitz G. mTOR inhibition by rapamycin prevents cell adaptation to hyperglycemia and exacerbates the metabolic state in Type 2 diabetes. Diabetes. 2008; 57:945-957.

**40.** Blagosklonny MV. Rapamycin-induced glucose intolerance: hunger or starvation diabetes. Cell Cycle. 2011; 10:4217-4224.

**41.** Blagosklonny MV. Once again on rapamycin-induced insulin resistance and longevity: despite of or owing to. Aging. 2012; 4: 350-358.

**42.** Blagosklonny MV. TOR-centric view on insulin resistance and diabetic complications: perspecitve for endocrinologists and gerontologists. Cell Death and Disase. 2013; 4: e964.

**43.** Ye L, Widlund AL, Sims CA, Lamming DW, Guan Y, Davis JG, Sabatini DM, Harrison DE, Vang O, Baur JA. Rapamycin doses sufficient to extend lifespan do not compromise muscle mitochondrial content or endurance. Aging. 2013; 5:539-550.

**44.** Ye L, Varamini B, Lamming DW, Sabatini DM, Baur JA. Rapamycin has a biphasic effect on insulin sensitivity in C2C12 myotubes due to sequential disruption of mTORC1 and mTORC2. Frontiers in Genetics. 2012; 3:177.

**45.** Lamming DW, Ye L, Katajisto P, Goncalves MD, Saitoh M, Stevens DM, Davis JG, Salmon AB, Richardson A, Ahima RS, Guertin DA, Sabatini DM, Baur JA. Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. Science. 2012; 335:1638-1643.

**46.** Festuccia WT, Blanchard PG, Belchior T, Chimin P, Paschoal VA, Magdalon J, Hirabara SM, Simões D, St-Pierre P, Carpinelli A, Marette A, Deshaies Y. PPARγ activation attenuates glucose intolerance induced by mTOR inhibition with rapamycin in rats. American Journal of Physiology-Endocrinology and Metabolism. 2014; 306:E1046-E1054.

**47.** Lamming DW, Sabatini DM. A central role for mTOR in lipid homeostasis. Cell Metabolism. 2013; 18:465-469.

**48.** Sipula IJ, Brown NF, Perdomo G. Rapamycin-mediated inhibition of mammalian target of rapamycin in skeletal muscle cells reduces glucose utilization and increases fatty acid oxidation. Metabolism. 2006; 55:1637-1644.

**49.** Yu Z, Wang R, Fok WC, Coles A, Salmon AB, Perez VI. Rapamycin and dietary restriction induce metabolically distinctive changes in mouse liver. The Journals of Gerontology. Series A, Biological sciences and medical sciences. 2015; 70:410-420.

**50.** Houde VP, Brule S, Festuccia WT, Blanchard PG, Bellmann K, Deshaies Y, Marette A. Chronic Rapamycin treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and impairing lipid deposition in adipose tissue. Diabetes. 2010; 59:1338-1348.

**51.** Layne DG, Power RA. Husbandry, handling, and nutrition for marmosets. Comparative Medicine. 2003; 53:351–359.

**52.** Ziegler TE, Colman RJ, Tardif SD, Sosa ME, Wegner FH, Wittwer DJ, Shrestha H. Development of metabolic function biomarkers in the common marmoset, *Callithrix jacchus*. American Journal of Primatology. 2013; 75:500-508.

Research Paper

# Rapamycin-induced metabolic defects are reversible in both lean and obese mice

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**Abstract: The inhibition of mTOR (mechanistic target of rapamycin) by the macrolide rapamycin has many beneficial effects in mice, including extension of lifespan and reduction or prevention of several age-related diseases. At the same time, chronic rapamycin treatment causes impairments in glucose metabolism including hyperglycemia, glucose intolerance and insulin resistance. It is unknown whether these metabolic effects of rapamycin are permanent or whether they can be alleviated. Here, we confirmed that rapamycin causes glucose intolerance and insulin resistance in both inbred and genetically heterogeneous mice fed either low fat or high fat diets, suggesting that these effects of rapamycin are independent of genetic background. Importantly, we also found that these effects were almost completely lost within a few weeks of cessation of treatment, showing that chronic rapamycin treatment does not induce permanent impairment of glucose metabolism. Somewhat surprisingly, chronic rapamycin also promoted increased accumulation of adipose tissue in high fat fed mice. However, this effect too was lost when rapamycin treatment was ended suggesting that this effect of rapamycin is also not permanent. The reversible nature of rapamycin's alterations of metabolic function suggests that these potentially detrimental side-effects might be managed through alternative dosing strategies or concurrent treatment**

## INTRODUCTION

The mTOR (mechanistic target of rapamycin) signaling pathway serves as a central regulator of cell metabolism in response to nutrient and growth factor stimuli. The serine/threonine protein kinase mTOR acts as a catalytic core unit of both mTORC1 and mTORC2. Each mTORC complex likely plays discrete roles in metabolic function: mTORC1 integrates nutrient, growth factor and cellular energy status to regulate cell

proliferation, growth and metabolism, whereas mTORC2 acts upon several downstream kinases including the Akt/PKB to regulate cell metabolism and survival [reviewed in [1]]. Due to its role in regulating cellular metabolism, aberrant mTOR signaling may be fundamental to the development of metabolic disease and dysfunction. For example, chronic activation of mTORC1 signaling in obesity is thought to play a significant role in the development of insulin resistance in muscle, adipose and liver tissue [2-4].

As the name implies, mTOR signaling is targeted by the bacterial macrolide rapamycin which interacts with the binding protein FKBP12 to inhibit some, but not all, mTOR functions [5]. While rapamycin is thought to primarily inhibit mTORC1 signaling through a direct mechanism, recent studies suggest chronic rapamycin treatment also down-regulates mTORC2 activity [6, 7]. Rapamycin and its analogues are approved for treatment of some forms of cancer and as immunosuppressants following organ transplantation. Rapamycin is also the first pharmacological agent capable of extending lifespan in both male and female mice according to the rigorous criteria established by the NIA's Intervention Testing Program [8-11]. Chronic rapamycin treatment has been shown to slow the progression of some, but not all, of the physiological declines associated with mouse aging [12-14]. Surprisingly, chronic rapamycin also promotes metabolic changes generally thought to be unfavorable, including glucose intolerance, insulin resistance and dyslipidemia, in several different rodent models [6, 15-20]. There is some evidence that the degree of metabolic dysfunction caused by rapamycin may be dependent on genetic background, length and means of administration of treatment and diet [6, 17, 18]. This is also a consideration in the clinical administration of rapamycin as the incidence of insulin resistance and new onset diabetes was shown to be significantly elevated in kidney transplant patients receiving rapamycin therapy [21, 22].

These potentially detrimental effects glucose regulation (glucose intolerance, insulin resistance, etc.) are concerns that currently may preclude the use of rapamycin and its analogues to treat and prevent age-related diseases. An important question heretofore unaddressed is whether chronic rapamycin treatment induces permanent alterations to metabolic function *in vivo*. In this study, we tested whether cessation of chronic rapamycin treatment could reverse its impairment of glucose metabolism in mice. In addition, we tested whether feeding mice a high fat diet, which also impairs glucose regulation, would exacerbate the impairment caused by rapamycin. Our results suggest that the metabolic effects of chronic rapamycin treatment are not permanent but rather dependent on its continued presence and activity suggesting that these adverse effects may be reduced or prevented through alternative treatment plans.

## RESULTS

### Chronic oral delivery of encapsulated rapamycin impairs glucose metabolism

Chronic rapamycin treatment has been shown to impair several measurements of glucose metabolism including increased circulating levels of glucose and insulin and impaired glucose and insulin tolerance [6, 17, 18, 23, 24]. At least some of these effects seem to be dependent on genetic strain of the model system, means of administration and length of treatment [6, 17, 18, 23, 24]. Here, we found that chronic treatment with enteric rapamycin (eRAPA) significantly impairs glucose intolerance and promotes insulin resistance in C57BL/6 mice when given in combination with either a low fat or a high fat diet (Figure 1). The same group of mice were tested longitudinally, first after 2 months and then after 4 months of eRAPA treatment. Both high fat diet and eRAPA impaired glucose and insulin tolerance at each time points, but we found no significant interaction effect suggesting that eRAPA promoted metabolic dysfunction equally in both low fat and high fat diets (Figure 1B, D). Moreover, time on diet had no significant effect on either markers suggesting that the effects of eRAPA occur quickly but do not become progressively worse at least in the time frame at which we studied. Fasting blood glucose levels were unaffected by eRAPA at both time points on both diets, though high fat diet did significantly increase these levels (Figure 2A). We also measured glucose-stimulated insulin secretion to determine if this might explain the impaired glucose tolerance. While high fat diet significantly increased blood insulin levels in both fasted and glucose-stimulated mice, these levels were unaffected by treatment with eRAPA on both diets (Figure 2B).

There is some evidence that the effects of rapamycin on insulin sensitivity differs among genetic strains of mice. For example, while inbred mice like C57BL/6 tend to become insulin resistant with rapamycin treatment, mice with heterogeneous genetic background have shown variable effects in terms of insulin sensitivity [6, 17, 18]. Here, we found that genetically heterogeneous UT-HET3 mice respond in a similar manner as C57BL/6 mice to chronic eRAPA in both diet formulations we used. In UT-HET3 mice, eRAPA impaired glucose tolerance and caused insulin resistance in combination with both low fat and high fat diets but

did not affect fasting glucose or insulin levels (Figure 4). The degree of glucose intolerance caused by eRAPA in UT-HET3 mice was similar to that of C57BL/6 while insulin resistance was milder in the genetically heterogeneous mice. Together, these data suggest the negative effects on glucose metabolism caused by eRAPA on glucose metabolism is largely independent of genetic background.

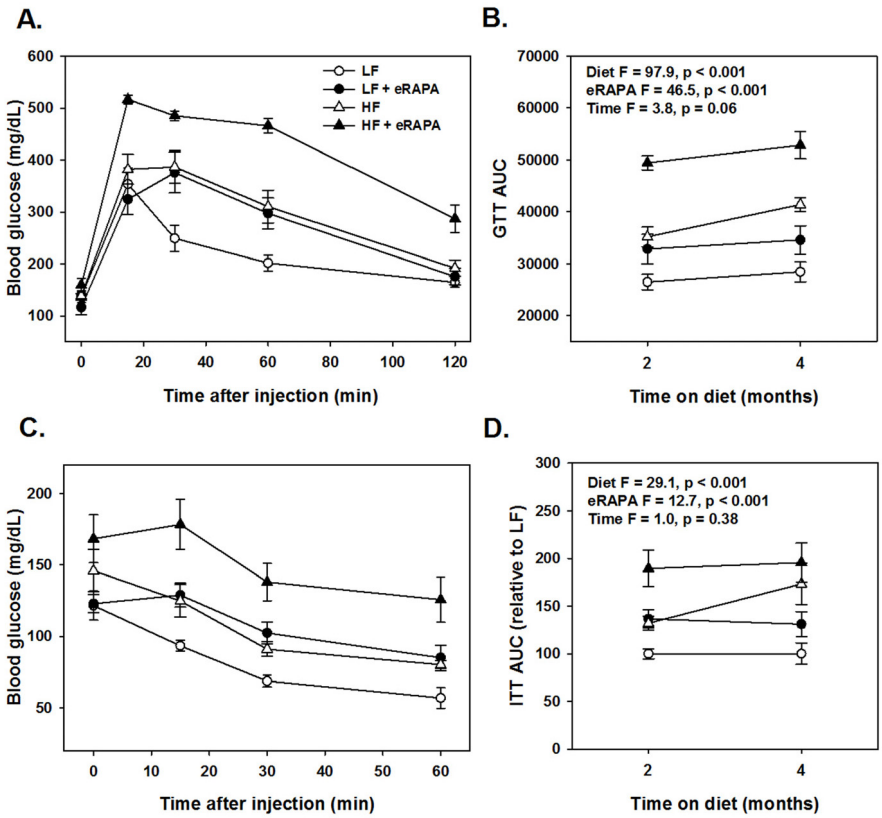


**Figure 1.** (**A**) Glucose tolerance tests for C57BL/6 males fed indicated diets for 2 months. (**B**) Area under curve (AUC) calculations for glucose tolerance tests following 2 or 4 months feeding indicated diets. (**C**) Insulin tolerance tests for C57BL/6 males fed indicated diets for 2 months. (**D**) AUC for insulin tolerance tests for insulin tolerance tests following 2 or 4 months feeding indicated diets. For all, symbols represent average (± SEM) values for n=6 mice at indicated time point for mice fed low fat (circle) or high fat (triangle) diets with (solid) or without (open) encapsulated rapamycin (eRAPA). F and p values are given for 3 way ANOVA testing indicated variables.

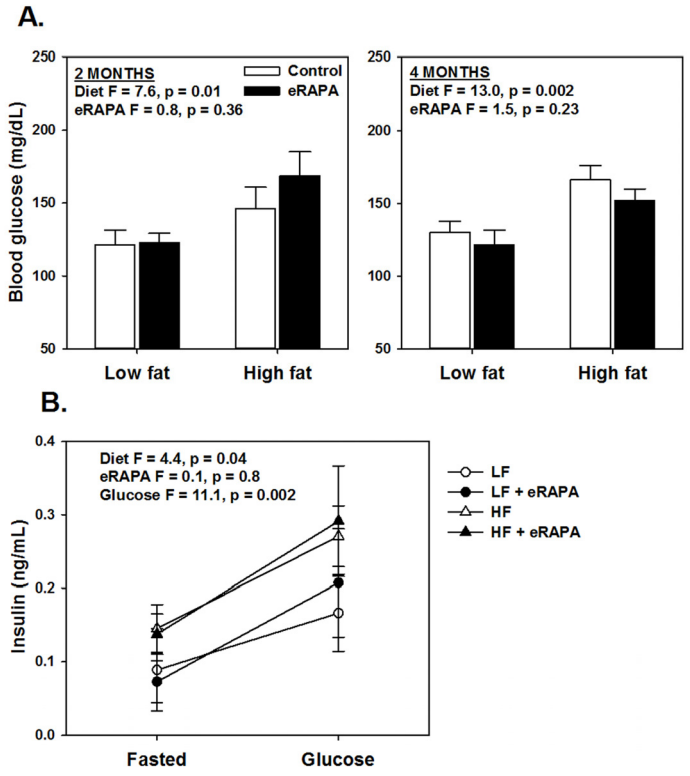


**Figure 2.** (**A**) Fasting blood glucose levels after feeding indicated diets for 2 and 4 months. (**B**) Fasting and glucose stimulated insulin levels in whole blood. For all, symbols represent average (± SEM) values for n=6 mice treated with (solid) or without (open) encapsulated rapamycin (eRAPA) for the indicated diet. F and p values are given for either 2 way (**A**) or 3 way (**B**) ANOVA testing indicated variables.

We found evidence that chronic eRAPA treatment significantly down-regulated both mTORC1 and mTORC2 signaling to a greater extent in high fat-fed mice compared to low fat fed mice (Figure 4). We assessed mTOR signaling in skeletal muscle and adipose tissue collected from UT-HET3 mice treated with eRAPA for 3 months. Both tissues showed significant reduction of phosphorylation of S6 in eRAPA - treated mice, indicative of inhibition of mTORC1 signaling. Chronic eRAPA treatment also inhibited mTORC2 signaling as measured by phosphorylation of Akt at Ser473; however, we only found this reduction of Akt phosphorylation in e-RAPA treated mice fed high fat diets. The interaction of mTOR with either raptor (mTORC1) or rictor (mTORC2) was reduced by eRAPA treatment with, again, a stronger inhibition in high fat-fed mice.

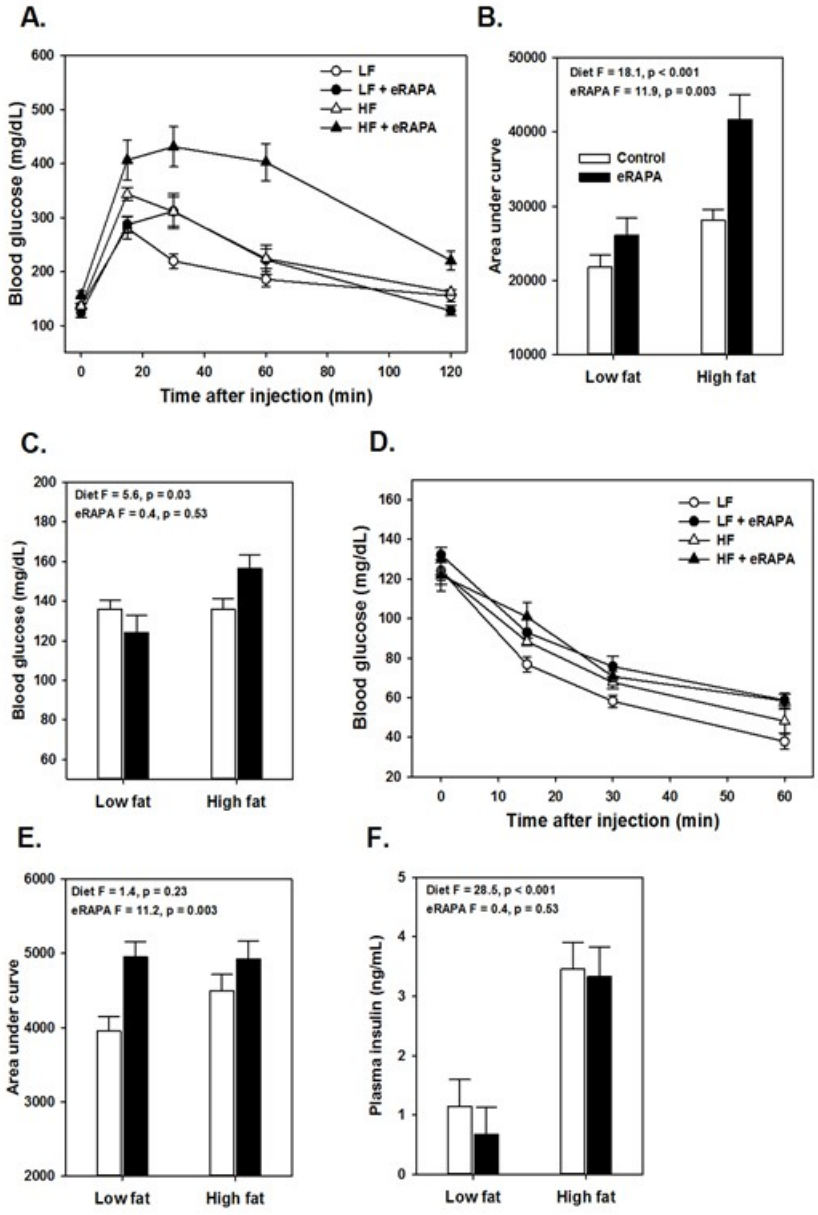


**Figure 3.** (**A**) Glucose tolerance tests for UT-HET3 males fed indicated diets for 3 months. (**B**) Area under curve (AUC) calculations for glucose tolerance tests shown in A. (**C**) Fasting blood glucose levels for mice in A. (**D**) Insulin tolerance tests for UT-HET3 males fed indicated diets for 3 months. (**E**) AUC for insulin tolerance tests for insulin tolerance tests. (**F**) Fasted plasma insulin levels. For all, symbols represent average (± SEM) values for n=6-10 mice at indicated time point for mice fed low fat (circle) or high fat (triangle) diets with (solid) or without (open) encapsulated rapamycin (eRAPA). F and p values are given for 2 way ANOVA testing indicated variables.

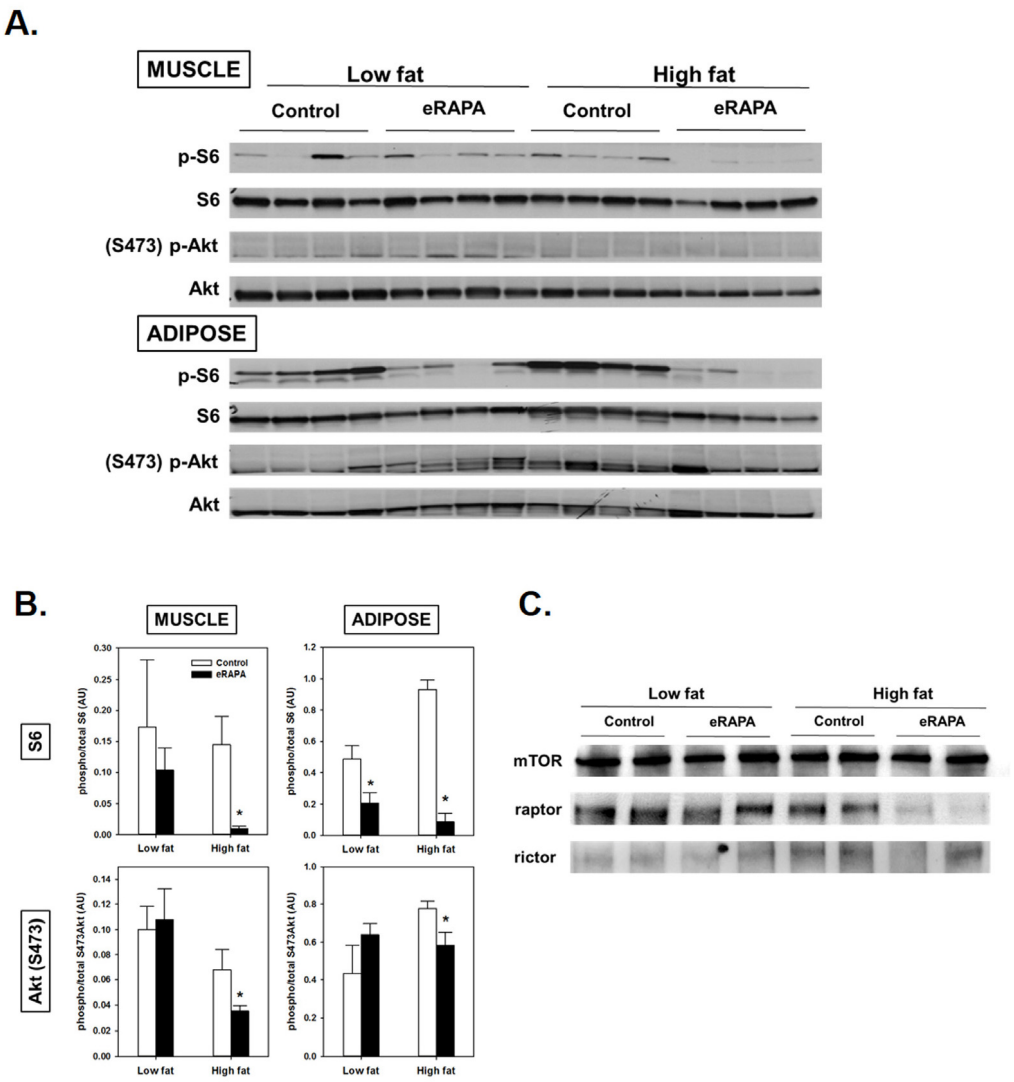


**Figure 4.** (**A**) Representative blots of p-S6, total S6, pAkt (Ser473) and total Akt in skeletal muscle and adipose of mice fed low fat or high fat diets with or without encapsulated rapamycin (eRAPA). (**B**) Quantification of relative phosphorylation of S6 or Akt (Ser473) of blots in A. Bars represent average (± SEM) values for n=4 mice treated with (solid) or without (open) eRAPA. Asterisks indicate significant difference between control and eRAPA group. (**C**) Representative blot of immunoprecipitation of mTOR from skeletal muscle protein homogenates and probed for the indicated proteins.

**Metabolism is normalized by ending rapamycin administration**

An important question heretofore unaddressed is whether chronic eRAPA treatment induces permanent changes *in vivo* to physiological processes altered by rapamycin. We addressed this question by shifting C57BL/6 mice that had been treated with eRAPA for 4 months to their equivalent diets without eRAPA (i.e, mice fed high fat diet with eRAPA were now fed high fat diet without eRAPA, etc.). While treated with eRAPA, both diet ($F = 56.3$, $p<0.001$) and eRAPA ($F = 19.7$, $p<0.001$) caused significant reduction in glucose tolerance in these mice (Figure 5A). Within 2 weeks of cessation of eRAPA treatment, glucose tolerance values were "normalized" to that of mice that had never been treated with eRAPA (Figure 5A). At this point, prior eRAPA treatment had no significant effect on glucose tolerance ($F = 2.6$, $p = 0.13$) while high fat diet still significantly impaired glucose tolerance ($F = 66.2$, $p<0.001$). Similarly, insulin sensitivity in these mice was relatively "normalized" within 2 weeks of cessation of eRAPA (Figure 5B). These data suggest that the impairment of glucose metabolism by eRAPA *in vivo* is reversible and can be mitigated by cessation of treatment.

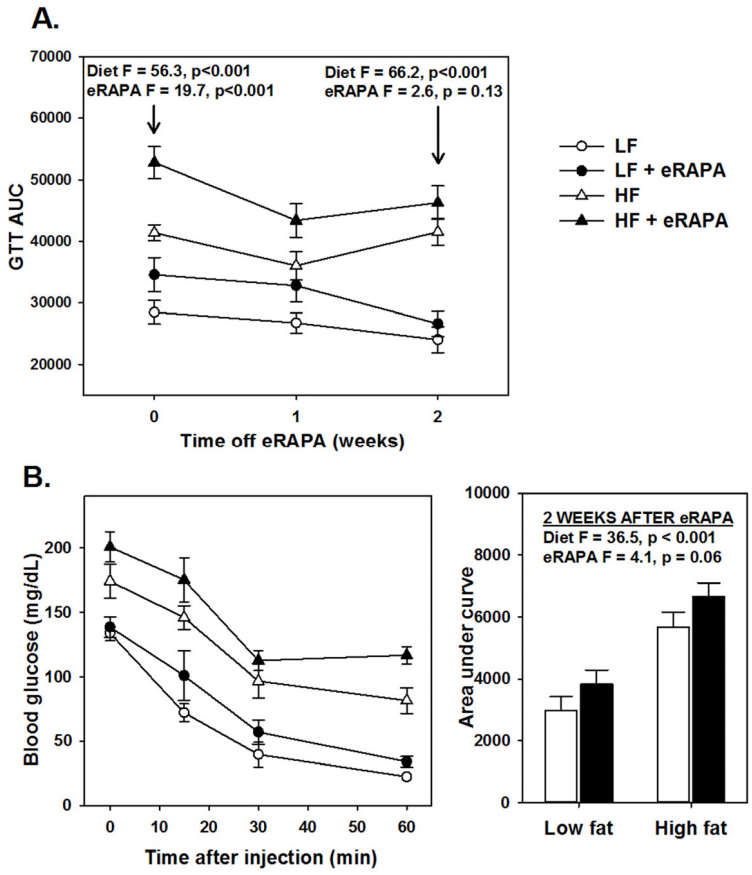


**Figure 5.** (**A**) Area under curve (AUC) calculations for glucose tolerance tests following 0, 1 and 2 weeks after cessation of encapsulated rapamycin (eRAPA) treatment. (**B**) Insulin tolerance tests (left) and AUC calculations (right) in mice 2 weeks after cessation of eRAPA treatment. For both, symbols represent average (± SEM) values for n=6 mice treated with (solid) or without (open) eRAPA for the indicated diet. F and p values are given for either 2 way ANOVA testing indicated variables.

**Chronic administration of encapsulated rapamycin promotes adiposity**

Under our experimental paradigm, eRAPA when treated in combination with low fat diet had no effect on body mass, fat mass or fat-free mass (Figures 6 and 7). Contrary to reports suggesting that rapamycin treatment reduces body mass and adiposity [16, 19, 20, 25], we found that eRAPA treatment actually promoted increased adiposity in high fat fed mice. In high fat-fed C57BL/6 mice, both body mass and fat mass were significantly increased with continuous eRAPA treatment (Figure 6). Fat-free mass, likely consisting largely of muscle and bone, was unchanged with eRAPA treatment. We also found a similar increase in fat mass in high fat-fed UT-HET3 mice treated with eRAPA (Figure 7). Surprisingly, the differences in body mass and fat mass caused by eRAPA treatment were not permanent in C57BL/6 mice. Within only a few weeks of removing eRAPA from the diet, body weight and fat mass declined in high fat-fed mice previously treated with eRAPA such that there was no significant difference between this group and the high fat-fed "control" group of mice (Figure 6, arrow represents time of diet switch).

These data suggest that the obesogenic effect of eRAPA in combination with high fat or high caloric intake may be through pathways of fat mobilization. In vitro, inhibition of mTOR blocks adipogenesis and stimulates

lipolysis [26-28]. In adipose tissue, we found that chronic eRAPA in combination with low fat diet reduces the phosphorylation of hormone sensitive lipase (HSL), the rate limiting step in lipolysis (Figure 8). In high fat-fed mice, there was almost no measurable phosphorylation of HSL in both control and eRAPA treated mice. These data suggest that lipolysis then is inhibited in vivo by chronic eRAPA treatment in mice fed the low fat fed mice. However, we also saw that adipose triglyceride lipase (ATGL), was elevated by eRAPA in both low fat and high fat diets suggesting increased breakdown of adipose resources (Figure 8). ATGL catalyzes the initial step in triacylglyceride hydrolysis, whereas HSL may have more specificity to the diacylglyceride form. These data then suggests a potentially complex effect of chronic eRAPA treatment that contributes to the adipose gain of these mice when also exposed to a high fat diet.

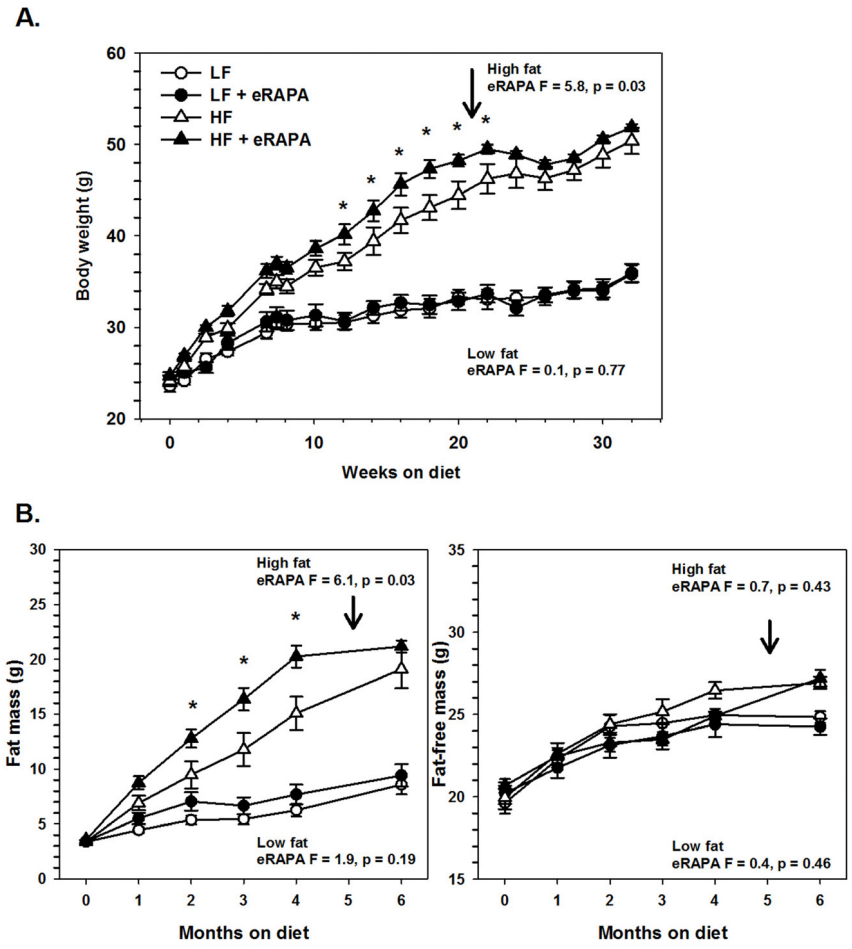


**Figure 6.** (**A**) Body weight of C57BL/6 males fed low fat (circles) or high fat (triangles) diets either with (solid) or without (open) encapsulated rapamycin (eRAPA). (**B**) Total fat content (left) and fat-free content (right) of mice in A. For all, symbols represent average (± SEM) values for n=6 mice. Downward arrow indicates time point of cessation of eRAPA treatment. F and p values given for repeated measures ANOVA testing the effect of eRAPA for the indicated diet. Asterisks indicate significant difference at time point between eRAPA and control for given diet from post-hoc analysis of ANOVA.

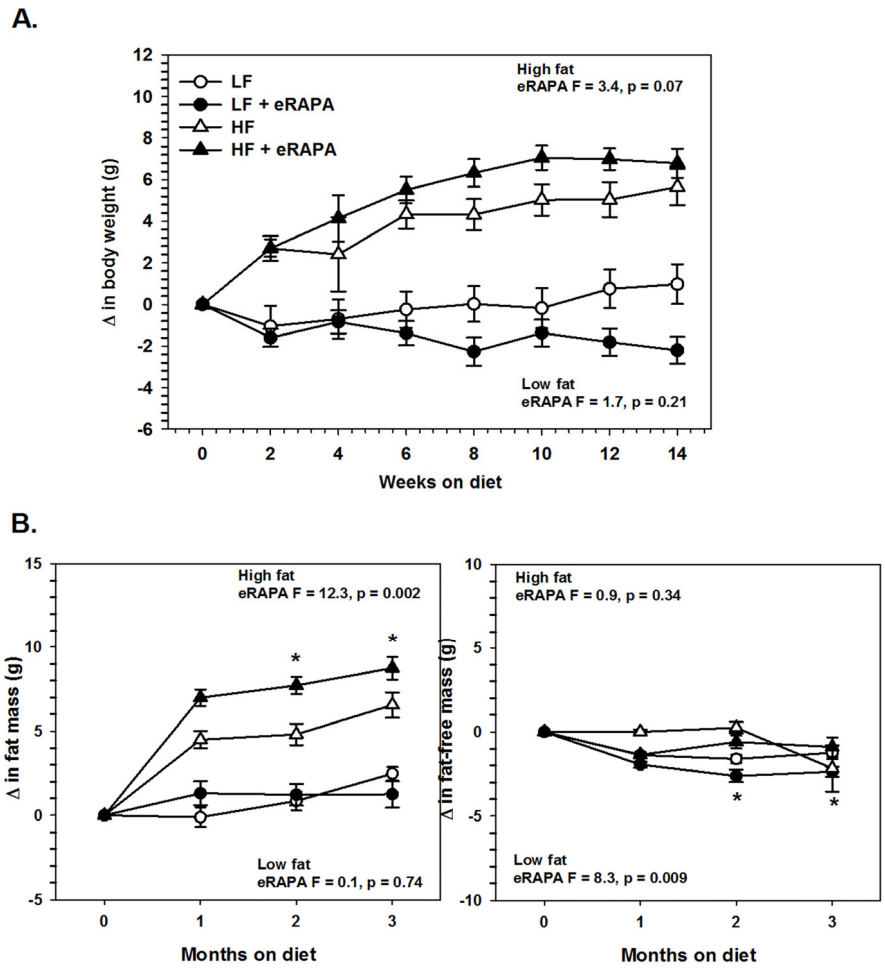


**Figure 7.** (**A**) Body weight of UT-HET3 males fed low fat (circles) or high fat (triangles) diets either with (solid) or without (open) encapsulated rapamycin (eRAPA). (**B**) Total fat content (left) and fat-free content (right) of mice in A. For all, symbols represent average (± SEM) values for n=6-10 mice. F and p values given for repeated measures ANOVA testing the effect of eRAPA for the indicated diet. Asterisks indicate significant difference at time point between eRAPA and control for given diet from post-hoc analysis of ANOVA.

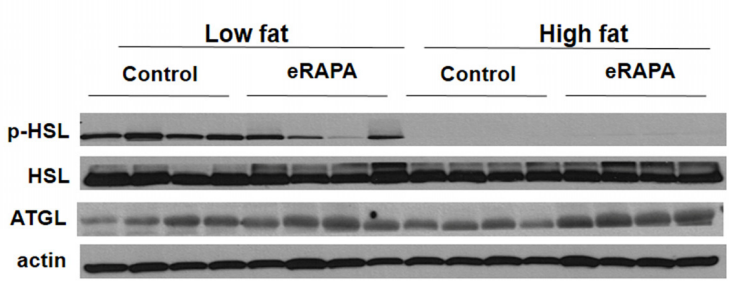


**Figure 8.** Representative western blot of p-HSL, HSL, and ATGL in adipose tissue from mice fed indicated diets.

## DISCUSSION

One important side-effect of long-term rapamycin treatment in both rodents and humans is the potential for significant impairment of the normal regulation of glucose metabolism [6, 15-22]. Despite the potential of rapamycin both clinically and as an anti-aging therapeutic, the risk of new onset type 2 diabetes or other metabolic diseases is a significant obstacle for chronic use of rapamycin in humans. Our results confirm that chronic delivery of encapsulated rapamycin (eRAPA) does cause insulin resistance and glucose intolerance in both inbred and genetically heterogeneous strains of mice. Further, mice these effects are exacerbated in mice fed a high fat diet, supporting the notion that rapamycin treatment may drive the onset of type 2 diabetes. However, the key finding of this study is that the impairment of glucose metabolism by eRAPA in both low fat (lean) and high fat-fed (obese) mice are completely reversed by ending eRAPA treatment. This suggests that the administration of rapamycin through this paradigm does not induce permanent changes to the gluco-regulatory system. These data raise the possibility that metabolic defects caused by long-term rapamycin use could be mitigated transient withdrawal from the treatment, or by concurrent or alternative therapies. Festuccia et al. recently showed that rosiglitazone reduces hyperglycemia, glucose intolerance and insulin resistance caused by short-term treatment of rapamycin in rats [29]. It remains to be seen whether preventing metabolic dysfunction by treatments like this will further potentiate the beneficial effects of rapamycin in cancer treatment and prevention or longevity extension. There has been some suggestion that rapamycin-mediated modulation of different markers of glucose metabolism may differ depending on the time course of treatment, though this may be dependent on the model used and method of treatment. For example, Ye et al. showed that rapamycin treatment of C2C12 muscle cell lines had a biphasic effect on insulin response in that short-term treatment with rapamycin caused insulin sensitivity whereas long-term treatment caused insulin resistance [30]. This insulin resistance was associated with rapamycin-mediated inhibition of mTORC2 signaling. Similar to our work here, Lamming et al. also showed that a chronic, 2 week treatment of rapamycin in C57BL/6 mice caused glucose intolerance and insulin resistance that was, at least in part, mediated by down-regulation of mTORC2 signaling [6]. Houde et al. also found that 15 days of rapamycin treatment caused similar metabolic impairments in Sprague-Dawley rats [19]. Likewise, data from Fang et al. suggest that chronic treatment with rapamycin in a mixed genetic background results in sustained glucose intolerance, but found that insulin sensitivity as assessed by an insulin tolerance test was dependent upon the length of treatment, with mice treated for 20 weeks actually showing increased insulin sensitivity (albeit glucose intolerance) relative to control mice [18]. In contrast, Lamming et al. found that both short (3 week) and long (3 month) term treatment of female UM-HET3 mice with diet-delivered eRAPA caused glucose intolerance but neither treatment, nor treatment for over a year resulted in dramatic changes in insulin sensitivity [17]. It is a challenge to integrate the results from all of these studies due to difference in the genetic backgrounds of the models used, diets used and whether rapamycin was administered by injection or in an encapsulated form in the diet. Our data show no evidence of any metabolic switch with long term oral eRAPA treatment (similar to Lamming et al. [17]) suggesting that this may not occur with chronic oral delivery of encapsulated rapamycin. Also similar to Lamming et al., we found that eRAPA did not dramatically affect insulin levels suggesting that encapsulated rapamycin may not impair pancreatic function at least in the time-frame of these studies [18, 31, 32]. It remains to be determined whether this means of administration is essential for the reversibility of rapamycin's effects on glucose metabolism.

We found it surprising that chronic eRAPA treatment tended to promote increased adiposity in both high fat fed C57BL/6 and genetically heterogeneous UT-HET3 mice. Chronic activation of mTORC1 by obesity and metabolic stress appears to play a primary role in the development of insulin resistance and type 2 diabetes [3, 4, 33]. Inhibition of mTOR by rapamycin impairs adipocyte differentiation in cell culture [34]. Reduced mTORC1 signaling either in adipose tissue (by adipose-specific Raptor deletion) or through genetic ablation of the mTORC1 effector S6K1 prevents high fat diet-induced metabolic dysfunction [3, 4, 35]. Furthermore, some, though not all, studies have suggested that rapamycin treatment may be sufficient to reduce adiposity both in high fat fed, obese animals and in normal fed, lean animals [8, 16, 20, 23, 25, 36, 37]. On the other hand, inhibition of mTORC2 has been shown to negatively affect glucose regulation; deletion of Rictor in adipose tissue leads to weight gain and insulin resistance [38, 39], deletion of Rictor in liver leads to impaired glucose tolerance and increased gluco-neogenesis [6], and ubiquitous deletion of Rictor leads to hyperglycemia and hepatic insulin resistance [6]. Our data do suggest that rapamycin treatment is associated with reduced mTORC2 in high fat-, but not low fat-, fed mice suggesting a plausible mechanism for the increased adiposity in this group of mice. It seems also likely too that the development of insulin resistance in adipose tissue could contribute to the expansion of this

tissue. It is also interesting to note that removal of eRAPA, and presumably the concurrent return of mTORC2 signaling and relative insulin sensitivity, completely reverses this phenotype.

The beneficial effects of rapamycin on lifespan, cancer and other diseases have largely been performed in models utilizing standard dietary conditions, i.e., normal, low fat rodent chow consisting of largely vegetable matter. In general, our results suggest that the physiological and cellular effects of chronic rapamycin are relatively similar even on diets containing differently levels of fat, and in fact may be actually exacerbated in conjunction with high fat diet. While more formal diet studies will be necessary, it seems likely that rapamycin may be effective under a variety of different conditions. In support of this, a recent study showed that the lifespan of C57BL/6 mice can be extended by rapamycin even when mice are fed a high fat diet [37]. However, the gluco-regulatory dysfunction experienced by mice treated with rapamycin while on a high fat diet highlights a potential concern with regard to the therapeutic use of rapamycin in humans, in which the dietary intake is much more varied in both content and composition.

The reversible nature of rapamycin's effects in this study also raises an important question about whether chronic treatment is required to reap the beneficial effects of rapamycin on longevity, cancer, etc.. It has been shown that rapamycin treatment extends longevity in mice to a similar degree whether it is started relatively early or late in life [8, 9]. Similarly, rapamycin impairs glucose metabolism in both young and old mice [17]. While these studies suggest that this compound may work equally well across ages, it is still not clear if short-term rapamycin treatments within a particular window(s) of time may cause persistent effects later in life. There is evidence that some physiological effects of dietary restriction, the most well-studied method to extend lifespan, are retained after this treatment is ended. For example, dietary restricted mice that are subsequently switched to an ad libitum diet retain significantly improved markers of glucose metabolism for months after this dietary modulation [40, 41]. In contrast though, the switch from dietary restricted to ad libitum feeding has also been shown to rapidly increase oxidative damage and alter the transcriptome to that of mice fed ad libitum their entire life [42, 43].

It has been suggested that the negative side effects of rapamycin treatment, including impaired glucose metabolism, will limit the use of rapamycin for the treatment of age-related diseases [44]. To our knowledge, the work presented here is the first to show that the metabolic defects caused by rapamycin are reversible after ending the treatment and suggests the possibility that the side effects of rapamycin could be minimized by short-term treatment with rapamycin. There is evidence that intermittent, rather than chronic, treatment with rapamycin is sufficient to extend lifespan in some mouse models [37, 45, 46]. Moreover, short-term or even single treatments with rapamycin have been shown to delay incidence or reduce prevalence in different mouse models of disease [46-48]. It will be of interest in the future to determine whether similar short-term treatments with rapamycin, or even treatment only at a few given points of life is also sufficient to extend lifespan and reduce disease burden in normally healthy mice without also causing potentially detrimental effects such as metabolic dysfunction.

## METHODS

Animals. Male C57BL/6J mice were purchased from Jackson Labs (Bar Harbor ME) at 2 months of age and were randomly assigned to cages in our animal facility at a density of 3 mice/cage. Genetically heterogeneous UT-HET3 mice were generated at UTHSCSA using a cross previously described [8, 9]. Male UT-HET3 mice were used at approximately 10-12 months of age and housed at a density of 3-4 mice/cage. For both groups of mice, cages were randomly assigned to one of four different defined diets based on commercially available formulations. Both low fat (10% kCal from fat, D12450B, Purina/Test Diet, St. Louis MO) and high fat diets (45% kCal from fat, D12451, Purina/Test Diet) were prepared containing either encapsulated (enteric-released) rapamycin (eRAPA) or the eudragit vehicle (control) at concentrations of 14 ppm (mg of drug per kg of diet). eRAPA was provided through the San Antonio Nathan Shock Center of Excellence in the Basic Biology of Aging and details on preparation of eRAPA have previously been described in detail [8, 9]. Diets were provided *ad libitum*, mice were checked daily and food consumption and body weight were monitored bi-weekly. Body composition of non-anesthetized mice was analyzed by Quantitative Magnetic Resonance imaging (QMRi) using an EchoMRI 3-in-1 composition analyzer (Echo Medical Systems, Houston TX). For diet shift experiments, mice fed eRAPA-containing diet were given eudragit-containing control diet of the equivalent dietary fat concentration at the indicated time point.

Glucose metabolism. Glucose and insulin tolerance tests were performed 2 and 4 months (C57BL/6) or 3 months (UT-HET3) after beginning dietary treatment. For glucose tolerance tests, mice were fasted 6 hours

(09:00-15:00) prior to each test and then injected intraperitoneally (IP) with glucose (1.5 g $kg^{-1}$) in saline. For insulin tolerance tests, mice were fasted 6 hours (09:00-15:00) prior to each test and then injected IP with insulin (1 U $kg^{-1}$) in saline. Blood glucose levels were measured at indicated time points from tail vein bleeding by hand-held glucometer (LifeScan, Milpitas CA). Area under curve (AUC) was calculated for each animal using the Trapezoid method. For glucose-stimulated insulin secretion, mice were fasted 6 hours (09:00-15:00) prior to each test and then injected intraperitoneally with glucose (1.5 g $kg^{-1}$) in saline. Whole blood was collected in EDTA-containing tubes from the tail vein prior to and 15 minutes after injection with glucose as previously described [6, 17]. Insulin levels in whole blood and plasma were measured using Crystal Chem ultra-sensitive mouse insulin ELISA (Downer's Grove IL).

Immunoblots. Total protein extracts were isolated from skeletal muscle (gastrocnemius) and visceral adipose (epigonadal) that was collected from mice, snap-frozen in liquid nitrogen, and stored at -80º C until use. Mice were fasted overnight and sacrificed 10 minutes after IP injection of insulin (1 U $kg^{-1}$). Protein extracts were made in RIPA buffer with added protease and phosphatase inhibitors (Thermo Scientific, Rockford IL), centrifuged at 13,000 g and 4º C for 15 min, then stored at -80ºC until use. Total protein content was measured by the Pierce BCA assay (Bio-Rad, Hercules CA). Proteins were separated by SDS-PAGE and transferred to PVDF membrane for immunoblotting. Phospho-S6, S6, Phospho-Akt (ser473), Akt, mTOR, raptor, rictor, phospho-HSL, HSL and ATGL antibodies were from Cell Signaling (Beverly MA). Actin antibody was from Sigma (St. Louis MO). For immuno-precipitation, muscle samples were lysed in cold 0.3% CHAPS lysis buffer [40 mM Hepes (pH 7.5), 120 mM NaCl, 1 mM EDTA, 0.3% CHAPS, 10 mM pyrophosphate, 10 mM β-glycerophosphate, 50 mM NaF, 0.5 mM orthovanadate, and protease inhibitors], then centrifuged 16,000 rpm for 15 min at 4°C. Protein A agarose beads were added to the supernatant and incubated with rotation for 1 h, centrifuged and mTOR antibodies were added to the cleared lysates. After overnight rotation at 4°C, protein A agarose beads were added incubated at 4°C for an additional hour. Immunoprecipitated complexes were washed in 0.3% CHAPS lysis buffer three times, boiled in SDS-sample buffer, separated by SDS-PAGE, and analyzed by immunoblotting. Protein bands were visualized by ECL and densitometry analyzed using Image J.

Statistical analysis. For glucose tolerance, insulin tolerance, and plasma glucose and insulin measurements, the effect of both diet (low fat vs. high fat) and rapamycin (control vs. eRAPA) were analyzed using two way ANOVA. Longitudinal studies in C57BL/6 mice were analyzed by three way ANOVA to determine effect of diet, rapamycin and time (2 mo. treatment vs. 4 mo. treatment). Glucose stimulated insulin secretion was assessed using three way ANOVA to determine effect of diet, rapamycin and glucose injection. Body weight, fat mass and fat-free mass were analyzed by repeated measures two-way ANOVA. Post-hoc multiple comparison tests were performed using the Holm-Sidak method. Immunoblots were analyzed by t-test comparing the effect of rapamycin within each diet treatment group.

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### Conflict of interest statement

A.R. (share-holder) and R.S. (uncompensated member of the scientific advisory board) have relationships with Rapamycin Holdings, Inc.

## REFERENCES

**1.** Laplante M and Sabatini David M. mTOR Signaling in Growth Control and Disease. Cell. 2012;149:274-293.

**2.** Khamzina L, Veilleux A, Bergeron S and Marette A. Increased Activation of the Mammalian Target of Rapamycin Pathway in Liver and Skeletal Muscle of Obese Rats: Possible Involvement in Obesity-Linked Insulin Resistance. Endocrinology. 2005; 146:1473-1481.

**3.** Um SH, D'Alessio D and Thomas G. Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. Cell Metab. 2006; 3:393-402.

**4.** Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, Fumagalli S, Allegrini PR, Kozma SC, Auwerx J and Thomas G. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature. 2004;431:200-205.

**5.** Sabatini DM, Erdjument-Bromage H, Lui M, Tempst P and Snyder SH. RAFT1: A mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell. 1994; 78:35-43.

**6.** Lamming DW, Ye L, Katajisto P, Goncalves MD, Saitoh M, Stevens DM, Davis JG, Salmon AB, Richardson A, Ahima RS, Guertin DA, Sabatini DM and Baur JA. Rapamycin-Induced Insulin Resistance Is Mediated by mTORC2 Loss and Uncoupled from Longevity. Science. 2012; 335:1638-1643.

**7.** Sarbassov DD, Ali SM, Sengupta S, Sheen J-H, Hsu PP, Bagley AF, Markhard AL and Sabatini DM. Prolonged Rapamycin Treatment Inhibits mTORC2 Assembly and Akt/PKB. Molecular Cell. 2006; 22:159-168.

**8.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009; 460:392-395.

**9.** Miller RA, Harrison DE, Astle CM, Baur JA, Boyd AR, de Cabo R, Fernandez E, Flurkey K, Javors MA, Nelson JF, Orihuela CJ, Pletcher S, Sharp ZD, et al. Rapamycin, But Not Resveratrol or Simvastatin, Extends Life Span of Genetically Heterogeneous Mice. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 2011; 66A:191-201.

**10.** Fok WC, Chen Y, Bokov A, Zhang Y, Salmon AB, Diaz V, Javors M, Wood WH, 3rd, Zhang Y, Becker KG, Pérez VI and Richardson A. Mice Fed Rapamycin Have an Increase in Lifespan Associated with Major Changes in the Liver Transcriptome. PLoS ONE. 2014; 9:e83988.

**11.** Zhang Y, Bokov A, Gelfond J, Soto V, Ikeno Y, Hubbard G, Diaz V, Sloane L, Maslin K, Treaster S, Réndon S, van Remmen H, Ward W, et al. Rapamycin Extends Life and Health in C57BL/6 Mice. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 2014; 69A:119-130.

**12.** Wilkinson JE, Burmeister L, Brooks SV, Chan C-C, Friedline S, Harrison DE, Hejtmancik JF, Nadon N, Strong R, Wood LK, Woodward MA and Miller RA. Rapamycin slows aging in mice. Aging Cell. 2012; 11:675-682.

**13.** Flynn JM, O'Leary MN, Zambataro CA, Academia EC, Presley MP, Garrett BJ, Zykovich A, Mooney SD, Strong R, Rosen CJ, Kapahi P, Nelson MD, Kennedy BK, et al. Late-life rapamycin treatment reverses age-related heart dysfunction. Aging Cell. 2013; 12:851-862.

**14.** Neff F, Flores-Dominguez D, Ryan DP, Horsch M, Schr, xF, der S, Adler T, Afonso LC, Aguilar-Pimentel JA, Becker L, Garrett L, Hans W, et al. Rapamycin extends murine lifespan but has limited effects on aging. The Journal of Clinical Investigation. 2013; 123:3272-3291.

**15.** Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK and Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. Nature. 2007; 450:736-740.

**16.** Chang G-R, Wu Y-Y, Chiu Y-S, Chen W-Y, Liao J-W, Hsu H-M, Chao T-H, Hung S-W and Mao FC. Long-term Administration of Rapamycin Reduces Adiposity, but Impairs Glucose Tolerance in High-Fat Diet-fed KK/HlJ Mice. Basic & Clinical Pharmacology & Toxicology. 2009; 105:188-198.

**17.** Lamming DW, Ye L, Astle CM, Baur JA, Sabatini DM and Harrison DE. Young and old genetically heterogeneous HET3 mice on a rapamycin diet are glucose intolerant but insulin sensitive. Aging Cell. 2013; 12:712-718.

**18.** Fang Y, Westbrook R, Hill C, Boparai Ravneet K, Arum O, Spong A, Wang F, Javors Martin A, Chen J, Sun Liou Y and Bartke A. Duration of Rapamycin Treatment Has Differential Effects on Metabolism in Mice. Cell Metabolism. 2013; 17:456-462.

**19.** Houde VP, Brûlé S, Festuccia WT, Blanchard P-G, Bellmann K, Deshaies Y and Marette A. Chronic Rapamycin Treatment Causes Glucose Intolerance and Hyperlipidemia by Upregulating Hepatic Gluconeogenesis and Impairing Lipid Deposition in Adipose Tissue. Diabetes. 2010; 59:1338-1348.

**20.** Deblon N, Bourgoin L, Veyrat-Durebex C, Peyrou M, Vinciguerra M, Caillon A, Maeder C, Fournier M, Montet X, Rohner-Jeanrenaud F and Foti M. Chronic mTOR inhibition by rapamycin induces muscle insulin resistance despite weight loss in rats. British Journal of Pharmacology. 2012; 165:2325-2340.

**21.** Teutonico A, Schena PF and Di Paolo S. Glucose Metabolism in Renal Transplant Recipients: Effect of Calcineurin Inhibitor Withdrawal and Conversion to Sirolimus. Journal of the American Society of Nephrology. 2005; 16:3128-3135.

**22.** Johnston O, Rose CL, Webster AC and Gill JS. Sirolimus Is Associated with New-Onset Diabetes in Kidney Transplant Recipients. Journal of the American Society of Nephrology. 2008; 19:1411-1418.

**23.** Miller RA, Harrison DE, Astle CM, Fernandez E, Flurkey K, Han M, Javors MA, Li X, Nadon NL, Nelson JF, Pletcher S, Salmon AB, Sharp ZD, et al. Rapamycin-mediated lifespan increase in mice is dose and sex dependent and metabolically distinct from dietary restriction. Aging Cell. 2014; 13:468-477.

**24.** Fok WC, Zhang Y, Salmon AB, Bhattacharya A, Gunda R, Jones D, Ward W, Fisher K, Richardson A and Pérez VI. Short-Term Treatment With Rapamycin and Dietary Restriction Have Overlapping and Distinctive Effects in Young Mice. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 2013; 68:108-116.

**25.** Chang G-R, Chiu Y-S, Wu Y-Y, Chen W-Y, Liao J-W, Chao T-H and Mao FC. Rapamycin Protects Against High Fat Diet-Induced Obesity in C57BL/6J Mice. Journal of Pharmacological Sciences. 2009; 109:496-503.

**26.** Pereira MJ, Palming J, Rizell M, Aureliano M, Carvalho E, Svensson MK and Eriksson JW. The immunosuppressive agents rapamycin, cyclosporin A and tacrolimus increase lipolysis, inhibit lipid storage and alter expression of genes involved in lipid metabolism in human adipose tissue. Molecular and Cellular Endocrinology. 2013; 365:260-269.

**27.** Soliman G, Acosta-Jaquez H and Fingar D. mTORC1 Inhibition via Rapamycin Promotes Triacylglycerol Lipolysis and Release of Free Fatty Acids in 3T3-L1 Adipocytes. Lipids. 2010; 45:1089-1100.

**28.** Chakrabarti P, English T, Shi J, Smas CM and Kandror KV. Mammalian target of rapamycin complex 1 suppresses lipolysis, stimulates lipogenesis, and promotes fat storage. Diabetes. 2010; 59:775-781.

**29.** Festuccia WT, Blanchard P-G, Belchior T, Chimin P, Paschoal VA, Magdalon J, Hirabara SM, Simões D, St-Pierre P, Carpinelli AR, Marette A and Deshaies Y. PPARγ activation attenuates glucose intolerance induced by mTOR inhibition with rapamycin in rats. 2014; 306:E1046-1054.

**30.** Ye L, Varamini B, Lamming DW, Sabatini DM and Baur JA. Rapamycin has a biphasic effect on insulin sensitivity in C2C12 myotubes due to sequential disruption of mTORC1 and mTORC2. Frontiers in genetics. 2012; 3:177.

**31.** Yang S-B, Lee H, Young D, Tien A-C, Rowson-Baldwin A, Shu Y, Jan Y and Jan L. Rapamycin induces glucose intolerance in mice by reducing islet mass, insulin content, and insulin sensitivity. J Mol Med. 2012; 90:575-585.

**32.** Fraenkel M, Ketzinel-Gilad M, Ariav Y, Pappo O, Karaca M, Castel J, Berthault MF, Magnan C, Cerasi E, Kaiser N and Leibowitz G. mTOR inhibition by rapamycin prevents beta-cell adaptation to hyperglycemia and exacerbates the metabolic state in type 2 diabetes. Diabetes. 2008; 57:945-957.

**33.** Cota D, Proulx K, Smith KA, Kozma SC, Thomas G, Woods SC and Seeley RJ. Hypothalamic mTOR signaling regulates food intake. Science. 2006; 312:927-930.

**34.** Bell A, Grunder L and Sorisky A. Rapamycin Inhibits Human Adipocyte Differentiation in Primary Culture. Obesity Research. 2000; 8:249-254.

**35.** Polak P, Cybulski N, Feige JN, Auwerx J, Rüegg MA and Hall MN. Adipose-Specific Knockout of raptor Results in Lean Mice with Enhanced Mitochondrial Respiration. Cell metabolism. 2008; 8:399-410.

**36.** Leontieva OV, Paszkiewicz G, Demidenko ZN and Blagosklonny MV. Resveratrol potentiates rapamycin to prevent hyperinsulinemia and obesity in male mice on high fat diet. Cell death & disease. 2013; 4:e472.

**37.** Leontieva OV, Paszkiewicz GM and Blagosklonny MV. Weekly administration of rapamycin improves survival and biomarkers in obese male mice on high-fat diet. Aging Cell. 2014; 13:616-622.

**38.** Cybulski N, Polak P, Auwerx J, Rüegg MA and Hall MN. mTOR complex 2 in adipose tissue negatively controls whole-body growth. Proceedings of the National Academy of Sciences. 2009; 106:9902-9907.

**39.** Kumar A, Lawrence JC, Jung DY, Ko HJ, Keller SR, Kim JK, Magnuson MA and Harris TE. Fat Cell–Specific Ablation of Rictor in Mice Impairs Insulin-Regulated Fat Cell and Whole-Body Glucose and Lipid Metabolism. Diabetes. 2010; 59:1397-1406.

**40.** Cameron K, Miwa S, Walker C and von Zglinicki T. Male mice retain a metabolic memory of improved glucose tolerance induced during adult onset, short-term dietary restriction. Longevity & Healthspan. 2012; 1:3.

**41.** Selman C and Hempenstall S. Evidence of a metabolic memory to early-life dietary restriction in male C57BL/6 mice. Longevity & Healthspan. 2012; 1:2.

**42.** Dhahbi JM, Kim H-J, Mote PL, Beaver RJ and Spindler SR. Temporal linkage between the phenotypic and genomic responses to caloric restriction. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101:5524-5529.

**43.** Forster MJ, Sohal BH and Sohal RS. Reversible Effects of Long-Term Caloric Restriction on Protein Oxidative Damage. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 2000; 55:B522-B529.

**44.** Lamming DW, Ye L, Sabatini DM and Baur JA. Rapalogs and mTOR inhibitors as anti-aging therapeutics. J Clin Invest. 2013; 123:980-989.

**45.** Chen C, Liu Y, Liu Y and Zheng P. mTOR Regulation and Therapeutic Rejuvenation of Aging Hematopoietic Stem Cells. Sci Signal. 2009; 2:ra75.

**46.** Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Antoch MP and Blagosklonny MV. Rapamycin extends maximal lifespan in cancer-prone mice. The American journal of pathology. 2010; 176:2092-2097.

**47.** Johnson SC, Yanos ME, Kayser EB, Quintana A, Sangesland M, Castanza A, Uhde L, Hui J, Wall VZ, Gagnidze A, Oh K, Wasko BM, Ramos FJ, et al. mTOR inhibition alleviates mitochondrial disease in a mouse model of Leigh syndrome. Science. 2013; 342:1524-1528.

**48.** Comas M, Toshkov I, Kuropatwinski KK, Chernova OB, Polinsky A, Blagosklonny MV, Gudkov AV and Antoch MP. New nanoformulation of rapamycin Rapatar extends lifespan in homozygous p53-/- mice by delaying carcinogenesis. Aging. 2012; 4:715-722.

Research Paper

# Target of rapamycin signalling mediates the lifespan-extending effects of dietary restriction by essential amino acid alteration

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**Abstract: Dietary restriction (DR), defined as a moderate reduction in food intake short of malnutrition, has been shown to extend healthy lifespan in a diverse range of organisms, from yeast to primates. Reduced signalling through the insulin/IGF-like (IIS) and Target of Rapamycin (TOR) signalling pathways also extend lifespan. In *Drosophila melanogaster* the lifespan benefits of DR can be reproduced by modulating only the essential amino acids in yeast based food. Here, we show that pharmacological downregulation of TOR signalling, but not reduced IIS, modulates the lifespan response to DR by amino acid alteration. Of the physiological responses flies exhibit upon DR, only increased body fat and decreased heat stress resistance phenotypes correlated with longevity via reduced TOR signalling. These data indicate that lowered dietary amino acids promote longevity via TOR, not by enhanced resistance to molecular damage, but through modified physiological conditions that favour fat accumulation.**

## INTRODUCTION

Dietary restriction (DR) is an intervention whereby a considerable reduction of food intake, just short of malnutrition, extends lifespan. This has been demonstrated to be effective in a wide range of evolutionarily diverse organisms, from yeast [1] to invertebrates [2] and mammals [3], and is considered one of the most robust environmental interventions to extend lifespan in laboratory organisms. Moreover, the longevity promoting effects of DR are accompanied by a range of health benefits. DR rodents had a delayed onset or a lesser severity of age-related diseases such as cancer, autoimmune diseases and motor dysfunction [4–6] and improved memory [7]. In *C. elegans*, DR was shown to reduce proteotoxicity [8]. DR rhesus monkeys were found to have improved triglyceride, cholesterol and fasting glucose profiles, and a reduced incidence of diabetes, cancer, cardiovascular disease and brain atrophy [9].

The molecular mechanisms underlying the physiological changes elicited by DR have yet to be elucidated, however, experimental data point towards nutrient signalling pathways as playing an important role. The evolutionarily conserved Target of Rapamycin Complex 1 (TORC1) pathway senses amino acid availability and signals to enhance translation via activation of S6 kinase-1 (S6K1) and inhibition of eIF4E binding protein-1(4E-BP1). TORC1 also regulates transcription and autophagy in response to a range of signals, including nutrient availability, cellular energy levels, and growth factors, in such a way that growth rates match resources [10]. Experimental validation of a role for TORC1 in determining lifespan has come from a range of laboratory organisms. Lifespan extension by inhibition of TORC1 pathway genes has been demonstrated in *S.cerevisiae* [11], *C.elegans* [12], *D. Melanogaster* [13] and in mice [14–19]. How TORC1 inhibition promotes longevity is unknown.

Another nutrient sensing pathway that is commonly associated with modified ageing is the insulin/insulin-like growth factor signalling (IIS) network. Mutations in components of the IIS pathway have extended lifespan in a host of model organisms [20]. Because the IIS pathway senses nutrients, considerable effort has been made to assess the role for IIS in modulating the longevity responses to DR. While IIS does not seem to be solely accountable for DR, some experimental data suggest overlapping mechanisms for IIS- and DR-mediated lifespan extension [21].

Recent work has shown that adjustments to the dietary amino acid balance can mimic the benefits to lifespan by DR in *D. melanogaster* [22]. Supplementing a DR diet with the ten essential amino acids (EAA) phenocopy the effects of full feeding (FF) on lifespan and fecundity, indicating that the beneficial effects of DR are a consequence of improved amino acid balance. Experimentally, the addition of EAAs to DR (DR+EAA) offers a sharper instrument with which to dissect the potential causes of lifespan change in response to nutritional balance than the FF condition, which is achieved by increasing the concentration of dietary yeast. Here we characterize physiological and metabolic parameters that define DR and fully fed flies with the aim of identifying candidate factors for causation of the lifespan response to DR.

## RESULTS

### TORC1 signalling but not IIS signalling is required for the effect of EAA on lifespan and fecundity

Dietary restricted (DR) flies are longer-lived than fully fed flies, but produce fewer eggs. The effect of full feeding to shorten lifespan and increase egg laying can be mimicked by the addition of the 10 essential amino acids (EAA) to DR food (Figures 1a-1c).

To assess the role of the longevity-associated nutrient signalling pathways as potential mediators of the effect of EAA on lifespan, we tested the response to DR of flies that are long lived due to deletion for genes encoding three of the *Drosophila* insulin-like peptides, (DILPs) *ilp*2, *ilp*3 and *ilp*5. We found no difference between the responses of wild type and DILP mutant flies to the addition of EAA to DR food, indicating that IIS is not required for the lifespan extension by DR (data not shown). In contrast, addition of the TORC1 inhibitor rapamycin extended the lifespan of flies on DR+EAA such that their lifespan was not shorter than those subjected to DR (Figure 2a). Rapamycin treatment also prevented the increase in egg laying seen for EAA addition to DR food, in fact egg laying was effectively blocked by rapamycin treatment. We also found that

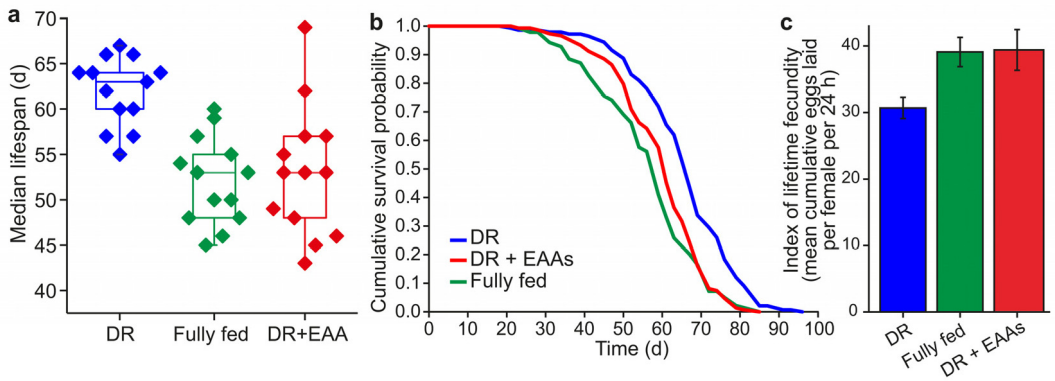


**Figure 1. Amino acids mediate lifespan and fecundity changes under DR. (a)** Summary of *Drosophila* median lifespans under dietary restriction (DR), full feeding (FF) and essential amino acid supplementation of DR (DR+EAA) ($n$=13 biological replicates; DR vs FF, $P$<0.001; FF vs DR+EAA, $P$=0.9383; DR vs DR+EAA, $P$=0.002; Wilcoxon rank-sum test) **(b)** A representative lifespan experiment: adding EAAs to DR food shortened lifespan ($P$< 0.001) to that of FF flies ($P$=0.194); $n$=150 per treatment; compared using the log-rank test. **(c)** Adding EAAs to DR food increased egg-laying ($P$<0.001) to that of FF flies ($P$<0.936). Fecundity: mean±s.e.m.; $n$=15; compared using the Wilcoxon rank-sum test.

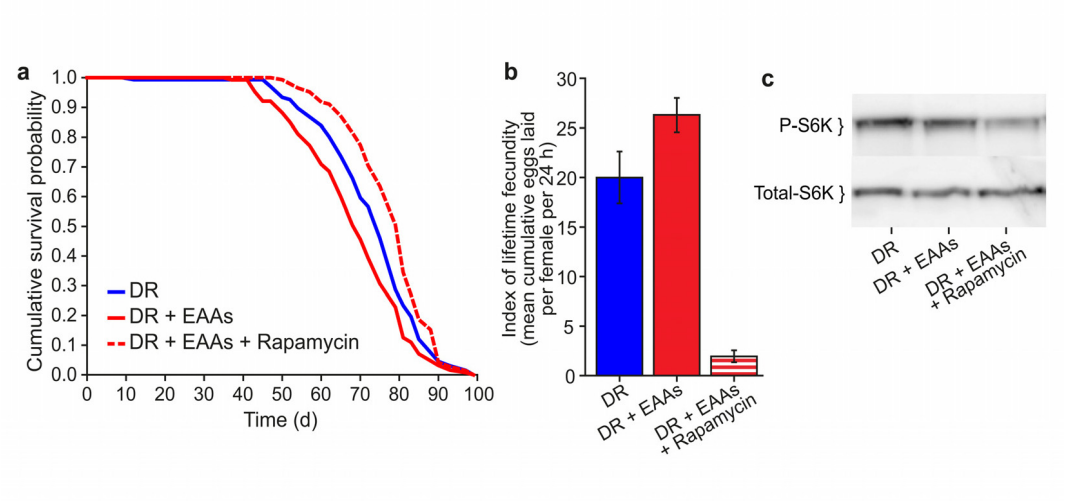


**Figure 2. Effect of Rapamycin treatment on EAA-supplemented flies. (a)** Rapamycin treatment extended the lifespan of DR+EAA flies beyond that of DR (DR+EAA vs DR+EAA+Rapamycin, $P<0.001$; DR vs DR+EAA, $P<0.012$). $n=150$ per treatment; log-rank test. **(b)** Rapamycin treatment decreased the lifetime fecundity of DR+EAA flies ($P<0.001$). Fecundity: mean±s.e.m.; $n=10$; Wilcoxon rank-sum test. **(c)** Levels of phospho-T398-S6K were measured from whole-fly protein extracts. Treatment with rapamycin for 7 days decreased phospho-T398-S6K levels in DR+EAA+Rapamycin flies relative to DR+EAA flies.

phosphorylation of the TORC1 target S6K was reduced by the addition of rapamycin (Figures 2b, 2c). Together, these data are consistent with TORC1 signalling playing a role in mediating the change in lifespan upon DR.

### EAA supplementation alters responses of DR flies to $H_2O_2$ stress, heat stress, starvation stress, and TAG levels

We set out to identify phenotypic correlates of lifespan change under our dietary conditions in order to understand the causal mechanisms of increased lifespan under DR. Long-lived animal models often have an associated increase in the ability to resist environmental stresses and this is assumed to reflect a general increase in their health. Long-lived insulin/IGF-like signalling (IIS) mutant flies have been shown to be resistant to acute toxic doses of DDT, paraquat and hydrogen peroxide ($H_2O_2$) [23–25]. We tested whether long-lived DR flies are protected from the harmful effects of these compounds. We found that DR flies were significantly more resistant than DR+EAA flies to a toxic dose of $H_2O_2$, whereas no difference was apparent for paraquat (Figures 3a, 3b). Surprisingly, DR flies were more sensitive to a toxic dose of DDT than DR+EAA flies (Figure 3c), indicating that, at least for DDT resistance, DR does not protect against this toxin in the same way that lowered insulin signalling does.

Long-lived DR *C. elegans* have increased resistance to heat stress [26, 27]. Upon testing the response of flies to heat shock stress, we found that DR flies were significantly less resistant than DR+EAA flies (Figure 3d), indicating that longevity associated with amino acid reduction comes at a cost to heat stress resistance.

Finally, we found that DR flies showed greater resistance to starvation than DR+EAA flies (Figure 3e), suggesting a possible mechanistic relationship between longevity and starvation resistance. Resistance to starvation stress could depend on the availability of enhanced energy stores within the fly. While we found no difference between groups in the levels of the storage carbohydrates glycogen or trehalose (Figure 3f, 3g) we did find that DR flies had significantly higher levels of triacylglycerides (TAG) than DR+EAA flies (Figure 3h). It is possible that this difference in TAG levels is causative of the longevity differences between DR and DR+EAA flies such that increased TAG confers some benefit to survival.

### Increased TAG and decreased heat-stress resistance correlate with increased lifespan with DR

If the above phenotypes induced by DR are causally linked to longevity through reduced TORC1 signalling, it should be possible to reproduce the same

physiological outcomes by treating flies with rapamycin. We therefore tested the effect of rapamycin on DR+EAA flies for $H_2O_2$ stress resistance, starvation sensitivity, heat shock stress resistance and TAG levels (Figures 4a-d). Of these, heat stress resistance and TAG levels changed upon rapamycin treatment of DR+EAA flies, such that the responses became more similar to DR flies; Like DR, rapamycin treatment increased the sensitivity of EAA-treated DR flies to a 39ºC heat stress, and increased their TAG content. There was no effect of rapamycin on the response of EAA-treated flies to $H_2O_2$ stress or to starvation stress.

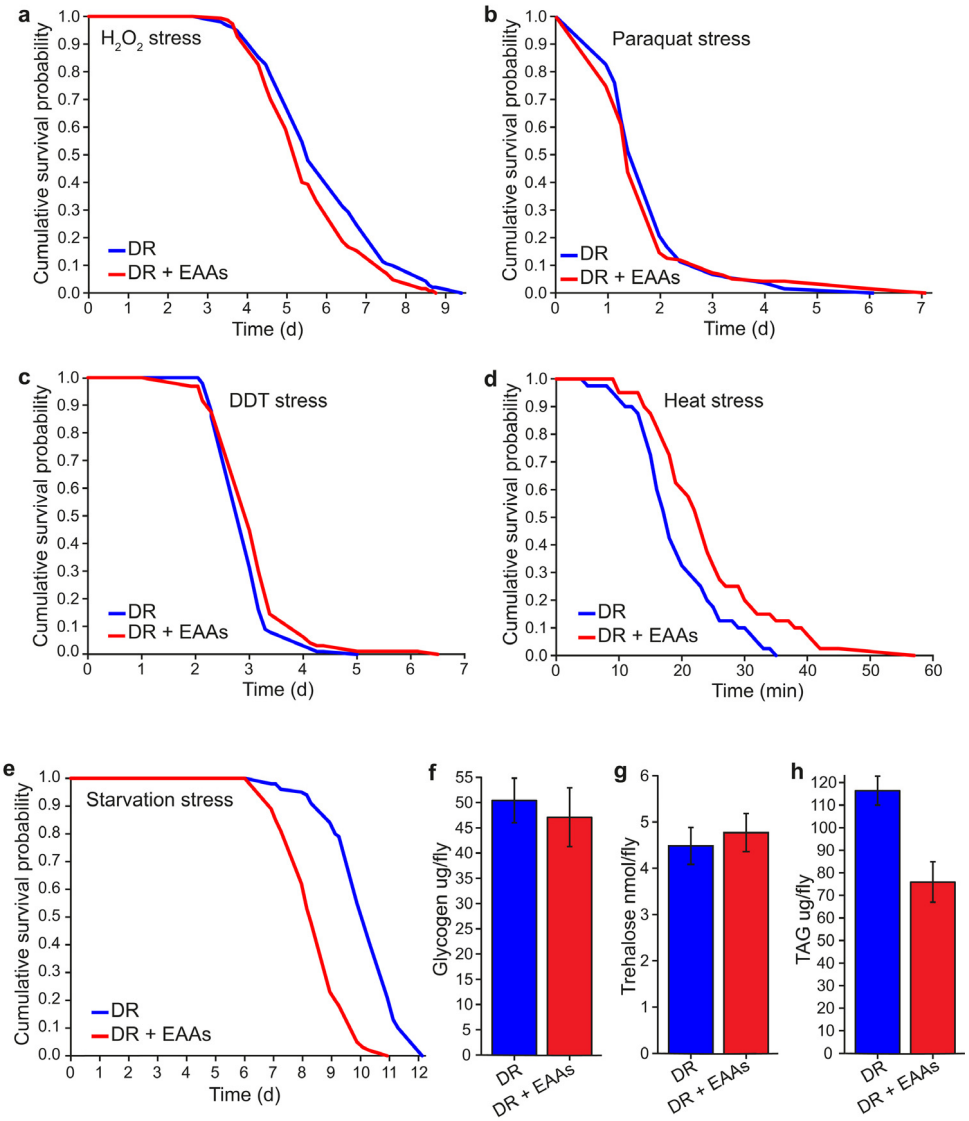


**Figure 3. Phenotype comparisons between dietary restricted flies and those supplemented with EAAs. (a)** DR+EAA flies showed a decreased resistance to hydrogen peroxide toxicity compared to DR flies ($P$=0.013; $n$=150 flies per condition). **(b)** There was no difference between DR and DR+EAA flies in their sensitivity to paraquat stress ($P$=0.517; $n$=150 flies per condition). **(c)** DR+EAA flies showed only a marginal, but significantly improved tolerance to DDT compared to that of DR flies ($P$=0.042, $n$=100 flies per condition). **(d)** DR+EAA flies were significantly more resistant to a 39ºC heat stress compared to DR flies ($P$<0.001; $n$=40 flies per condition). **(e)** DR+EAA flies were significantly more sensitive to starvation than DR flies ($P$<0.001; $n$=100 flies per condition). **(f)** After 7 days of treatment there was no difference in the amounts of glycogen measured for DR+EAA flies compared to DR flies ($P$=0.656; $n$= 6). **(g)** There was no difference in the levels of trehalose measured for DR+EAA flies compared to DR flies ($P$=0.630; $n$=6 flies per condition). **(h)** DR+EAA flies had significantly reduced levels of TAG compared to DR flies ($P$<0.001; $n$=6 flies per condition). For figures a-e, $P$ values were calculated using the log-rank test. For figures f-h, $P$ values were calculated by T-test, and error bars represent the s.e.m.

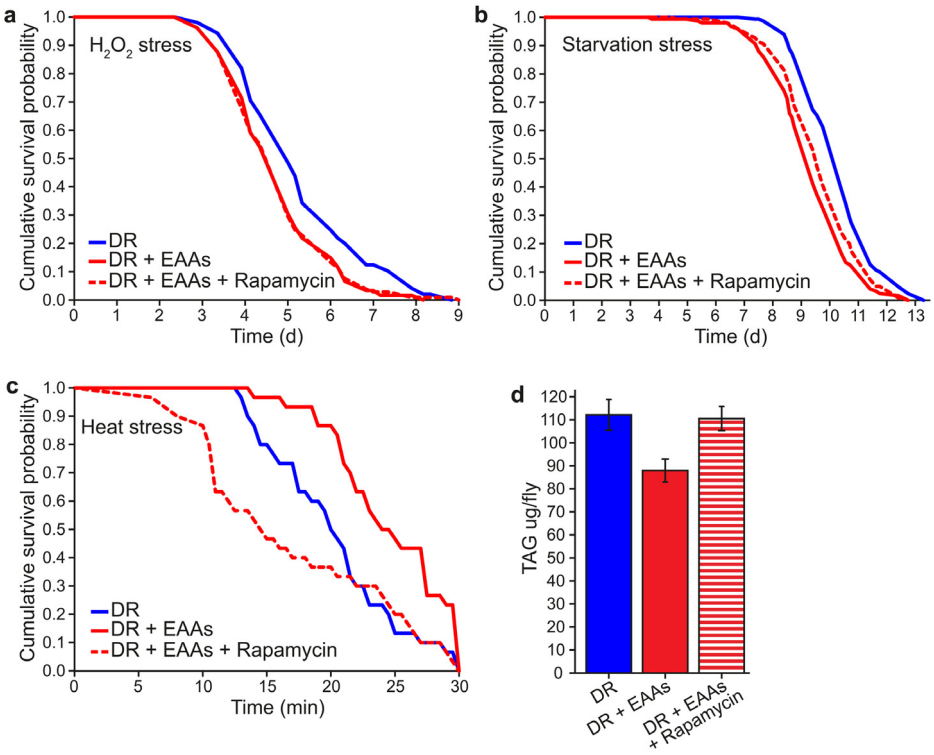


**Figure 4. The effect of rapamycin to alter phenotypic differences between DR and DR+EAA flies.** **(a)** Rapamycin had no effect on the sensitivity of DR+EAA flies to $H_2O_2$ stress (*P*=0.963; *n*=105 flies per condition). **(b)** Rapamycin had no effect on the sensitivity of DR+EAA flies to starvation stress (*P*=0.071; *n*=150 flies per condition). **(c)** Rapamycin, like DR, increased the sensitivity of DR+EAA flies to a 39ºC heat stress (*P*<0.001; *n*=30 flies per condition). For figures a-c, *P* values were calculated using the log-rank test. **(d)** Rapamycin treatment increased the triacyglyceride (TAG) levels of DR+EAA flies to the level of DR (*P*=0.011; *n*=6; T-test; error bars represent the s.e.m).

## DISCUSSION

We have described the physiological and metabolic features that define long-lived DR flies in order to understand the mechanisms by which longevity is achieved. Our data indicate that dietary amino acids modify TORC1 signalling, which in turn alters lifespan outcomes. We also found that both dietary amino acid manipulation and TORC1 modification in flies alter TAG levels, such that higher body fat may play a causal role in enhancing fly lifespan in response to dietary restriction.

We found that the lifespan of insulin-mutant flies responded in a similar way to DR as wild-types, indicating that reduced IIS is not required for the lifespan-extending effects of DR. This appears to contrast previous studies that have reported interacting effects of IIS on DR, such that lifespan modification in response to yeast dilution is abolished in some IIS mutants [22,23,28]. These differences could be due to the fact that in the current study we modulated lifespan by adjusting EAA alone, rather than yeast. In doing so, we report a markedly different sampling of nutritional space than for yeast dilution, since we change the ratio of EAAs to all other dietary components, such as lipids, carbohydrates, non-essential amino acids, vitamins and trace elements. This may also explain why the phenotypes of our long-lived flies are somewhat different from those of other organisms subjected to DR. Interestingly, our experiments also showed that long-lived DR flies had decreased resistance to DDT, which is the opposite phenotype seen for IIS mutant flies, in which longevity is accompanied by

dFOXO-dependent DDT resistance [23,29]. Together, these data suggest that the beneficial effects on lifespan of DR can be achieved independently of IIS, similar to that reported by Tatar [21]. Moreover, it has been suggested that the effects of IIS on longevity are dependent on the status of TOR activity [30].

One of the strategies taken to understand the mechanisms by which dietary or genetic treatments enhance longevity is to seek out correlated physiological changes that may provide insights into the treatment's mode of action. A common mechanistic explanation for longevity requires enhancing systems to protect against the damaging side-effects of aerobic metabolism, such as that caused by oxidative stress or endogenous lipophilic toxins [31,32]. In our analyses, we found no evidence for broad-spectrum enhanced protection against stressors under DR. Thus, the mechanism for increased longevity under DR may not involve enhanced resistance to stress. Similar observations in studies on worms [33,34] has led to an alternative hypothesis that "hypertrophy" caused by inappropriate continuation of early-life growth programmes into later life is detrimental to an organism and causes ageing [35–38]. This explanation also implicates high levels of TOR signalling as its mechanism.

We found increased TAG levels correlated with longer life in our flies subjected to DR or rapamycin treatment. DR by yeast restriction in *Drosophila* has also been shown to increase lipid content [39–41], and several rodent studies show that higher fat levels correlate with increased lifespan [42–44]. In a recent study, Kapahi and colleagues showed that DR flies have increased TAG, and demonstrated an increased requirement for muscle-specific fatty-acid synthesis and breakdown in extending lifespan under DR [45]. Moreover, some long-lived TOR and IIS pathway mutants have increased fat levels [46–49]. Given that not all fat mutants are long-lived [50], it is likely that if fat levels are causally involved in extending life, the quality of fat accumulated is important. It would be interesting in future work to determine how lipid profiles change under different dietary conditions, to identify the specific types of lipids that are altered, and whether experimental manipulation can enhance lifespan.

## EXPERIMENTAL PROCEDURES

### General Methods

Standard laboratory food . Dietary restriction medium (1xSYA) contained100 g/l yeast (1x; MP Biomedicals, OH, USA), 50 g/l sucrose (Tate & Lyle, London, UK), 15 g/l agar (Sigma-Aldrich, Dorset, UK), and 30ml/l nipagin (Chemlink Specialities, Manchester, UK) and 3ml/l propionic acid (Sigma-Aldrich, Dorset, UK).This diet and its method of preparation is described in Bass et al., 2007 [51]. The fully fed medium (2xSYA) was prepared in the same way, except that it contained 200g/l yeast.

Experimental food. Rapamycin (LC Laboratories, MA, USA) was dissolved in ethanol and added to 1xSYA food at a final concentration of 200μM. Essential amino acids (Sigma-Aldrich, Dorset, UK) were dissolved in MiliQ water, and added to 1xSYA food at concentrations shown in Table 1. As control measures, ethanol alone was added to the food conditions that did not contain rapamycin, and water was added to food conditions that did not contain essential amino acids.

**Table 1. Quantities of each of the essential amino acids added to 1l of 1xSYA food medium**

| Essential amino acid (Sigma-Aldrich) | Concentration in 1xSYA medium (g/l) |
|---|---|
| L-arginine | 0.43 |
| L-histidine | 0.21 |
| L-isoleucine | 0.34 |
| L-leucine | 0.48 |
| L-lysine | 0.52 |
| L-methionine | 0.10 |
| L-phenylalanine | 0.26 |
| L-threonine | 0.37 |
| L-tryptophan | 0.09 |
| L-valine | 0.40 |

Fly stocks and husbandry. The wild-type Dahomey strain was originally collected in 1970 from Dahomey (now known as the Republic of Benin) and since maintained as a large outbred stock with overlapping generations at 25°C on a 12h light:12h dark cycle. These conditions allow for inter-generational breeding and the life expectancy of flies remain similar to that of newly caught wild flies [52]. Flies used for experimentation came from parental flies of the same age at egg laying, thereby controlling for the effects of parental age on lifespan [53].

Insulin-signalling mutant flies lacking the *Drosophila* insulin-like peptides (DILPs) *ilp*2, *ilp*3 and *ilp*5 were generated as described in Gronke et al., 2010 [23]. These flies were backcrossed into a control $white^{Dahomey}$ background stock, which was derived by backcrossing $w^{1118}$ into the outbred wild-type Dahomey background [24]. All mutations were back-crossed into their control backgrounds for a minimum of 6 generations.

Lifespan. All experiments were conducted at 25°C on a 12h light:12h dark cycle, at a constant humidity of 65%. Flies were reared at a standard larval density of ~300 flies per bottle, and all experimental adults were collected within a 12 hour period after eclosion. Flies were allowed to mate for 48 hours after eclosion before the experimental females were separated out under $CO_2$ anaesthesia. Females were then randomly allocated to the experimental food treatments and housed in plastic vials containing food at a density of 10 flies per vial, with 15 vials per condition ($n$=150). Flies were transferred to a fresh food source 3 times per week, during which any deaths and censors were recorded.

Fecundity. Lifetime fecundity was measured as the cumulative total for days 7, 14, 21 and 28 of the mean number of eggs laid per female fly over each 24-hour period. Eggs in each vial were counted by eye using a light microscope after 18-24 hours exposure to flies.

Western blots. Protein extracts for western blot analysis were made from whole flies, sampled after 7 days of food treatment, using a TCA-based extraction protocol. 10µl of each sample was loaded into a 12% SDS-PAGE gel and blots were probed with anti-phospho-Thr398- S6K antibody (#9209, Cell Signaling Technologies, MA, USA), and total-S6K (re-made using a peptide sequence previously used to generate the total S6K antibody in Stewart et al., 1996 [54]). Both antibodies were used at a dilution of 1:12000 and normalised by probing with an anti-actin antibody at a dilution of 1:5000. Secondary antibodies conjugated to HRP (AbCam, Cambridge, UK) were used at a dilution of 1:5000, and the signals were detected by chemiluminescence.

**Stress Experiments**

Experimental flies were reared and housed as described for the lifespan experiment. Mated female flies were kept on the experimental food types for 7 days before being transferred to the stress conditions.

Paraquat, DDT and $H_2O_2$ stress. The orally administered stressors were as made up follows: 1xSYA containing 20mM paraquat (Sigma-Aldrich, Dorset, UK), 1xSYA containing 0.03% w/v DDT (Supelco Sigma-Aldrich, Dorset, UK), 1.5% agar medium containing 5% $H_2O_2$ (Sigma-Aldrich, Dorset, UK) and 50g/l sucrose, or plain 1.5% agar medium for the starvation experiment.

Heat shock. Experimental flies were transferred singly into dry empty 2ml glass vials, plugged with cotton wool and placed into a water bath set at 39°C. The time taken for each fly to fall onto its back and stop twitching (knockout) was recorded.

**Metabolic measurements**

Experimental flies were reared and housed as described for the lifespan experiment. Mated female flies were kept on the experimental food types for 7 days before being frozen in liquid nitrogen. 6 replicas of 5 flies per condition were used for all metabolic measurements.

Triacylglyceride measurement. Flies per condition were homogenised in 0.05% Tween 20 (Sigma-Aldrich, Dorset, UK) according to Gronke et al., 2003 [55]. TAG content was quantified using the Triglyceride Infinity Reagent (Thermo Fisher Scientific, Surrey, UK).

Glycogen measurement. Flies were homogenised in 200µl saturated $Na_2SO_4$ solution and centrifuged for 1 min. 80µl of each sample was transferred to new Eppendorf tubes and 800µl chloroform:methanol (1:1) solution was added. Samples were centrifuged for 5 minutes and the supernatant was removed. The remaining pellet, containing precipitated glycogen, was resuspended in 1ml anthrone solution (anthrone in 50 ml 70% $H_2SO_4$) and incubated at 90°C for 20 minutes. 200µl of each sample was dispensed into the wells of a flat-bottomed 96-well plate, and the absorbance in each well was measured at 620nm and compared against a set of glycogen standards ranging from 0-2µg/µl (protocol adapted from Van Handel, 1965 [56]).

Trehalose measurement. Trehalose levels were measured using the Glucose Infinity Reagent (Thermo Fisher Scientific, Surrey, UK), as described in Broughton et al., 2005 [24].

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### Conflict of interest statement

The authors of this manuscript declare no conflict of interests.

## REFERENCES


**1.** Jiang J, Jaruga E, Repnevskaya M, Jazwinski S. An intervention resembling caloric restriction prolongs life span and retards aging in yeast. The FASEB Journal. 2000; 14:2135–2137.

**2.** Klass M. Aging in the nematode Caenorhabditis elegans: major biological and environmental factors influencing life span. Mechanisms of ageing and development 1977; 6:413–429.

**3.** McCay C. The effect of retarded growth upon the length of life span and upon the ultimate body size. J Nutr. 1935; 10:63–79.

**4.** Blackwell BN, Bucci TJ, Hart RW, Turturro A. Longevity, Body Weight, and Neoplasia in Ad Libitum-Fed and Diet-Restricted C57BL6 Mice Fed NIH-31 Open Formula Diet. Toxicologic Pathology. 1995; 23:570–582.

**5.** Weindruch RH, Kristie JA, Cheney KE, Walford RL. Influence of controlled dietary restriction on immunologic function and aging. Federation Proceedings. 1979; 38:2007–2016.

**6.** Ingram DK, Weindruch R, Spangler EL, Freeman JR, Walford RL. Dietary restriction benefits learning and motor performance of aged mice. J Gerontol. 1987; 42:78–81.

**7.** Stewart J, Mitchell J, Kalant N. The effects of life-long food restriction on spatial memory in young and aged Fischer 344 rats measured in the eight-arm radial and the Morris water mazes. Neurobiology of aging. 1989; 10:669–675.

**8.** Steinkraus K, Smith ED, Davis C, Carr D, Pendergrass WR, Sutphin GL, Kennedy BK, Kaeberlein M. Dietary restriction suppresses proteotoxicity and enhances longevity by an hsf-1-dependent mechanism in Caenorhabditis elegans. Aging Cell. 2008; 7:394–404.

**9.** Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, Allison DB, Cruzen C, Simmons HA, Kemnitz JW, Weindruch R. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science. 2009; 325:201–204.

**10.** Martin DE, Hall MN. The expanding TOR signaling network. Current Opinion in Cell Biology. 2005; 17:158–166.

**11.** Kaeberlein M, Powers RW, Steffen KK, Westman EA, Hu D, Dang N, Kerr EO, Kirkland KT, Fields S, Kennedy BK. Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. Science. 2005; 310:1193–1196.

**12.** Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L, Müller F. Genetics: influence of TOR kinase on lifespan in C. elegans. Nature. 2003; 426:620.

**13.** Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S. Regulation of Lifespan in Drosophila by Modulation of Genes in the TOR Signaling Pathway. Curr Biol. 2004; 14:885–890.

**14.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2010; 460:392–395.

**15.** Khapre RV, Kondratova AA, Patel S, Dubrovsky Y, Wrobel M, Antoch MP, Kondratov RV. BMAL1-dependent regulation of the mTOR signaling pathway delays aging. Aging. 2014; 6:48–57.

**16.** Johnson SC, Yanos ME, Kayser E-B, Quintana A, Sangesland M, Castanza A, Uhde L, Hui J, Wall VZ, Gagnidze A, Oh K, Wasko BM, Ramos FJ et al. mTOR inhibition alleviates mitochondrial disease in a mouse model of Leigh syndrome. Science. 2013; 342:1524–1524.

**17.** Ye L, Widlund AL, Sims CA, Lamming DW, Guan Y, Davis JG, Sabatini DM, Harrison DE, Vang O, Baur JA. Rapamycin doses sufficient to extend lifespan do not compromise muscle mitochondrial content or endurance. Aging. 2013; 5:539–550.

**18.** Komarova E, Antoch M, Novototskaya L, Chernova O, Paszkiewicz G, Leontieva O, Blagosklonny MV, Gudkov A. Rapamycin extends lifespan and delays tumorigenesis in heterozygous p53+/- mice. Aging. 2014; 4:709–714.

**19.** Popovich I, Anisimov V, Zabezhinski M, Semenchenko A, Tyndyk M, Yurova M, Blagosklonny MV. Lifespan extension and cancer prevention in HER-2/neu transgenic mice treated with low intermittent doses of rapamycin. Cancer Biology & Therapy. 2014; 15:586–592.

**20.** Fontana L, Partridge L, Longo VD. Extending healthy life span--from yeast to humans. Science. 2010; 328:321–326.

**21.** Min K-J, Yamamoto R, Buch S, Pankratz M, Tatar M. Drosophila lifespan control by dietary restriction independent of insulin-like signaling. Aging Cell. 2008; 7:199–206.

**22.** Grandison RC, Piper MDW, Partridge L. Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. Nature. 2009; 462:1061–1064.

**23.** Grönke S, Clarke D-F, Broughton S, Andrews TD, Partridge L. Molecular evolution and functional characterization of Drosophila insulin-like peptides. PLoS Genetics. 2010; 6:e1000857.

**24.** Broughton SJ, Piper MDW, Ikeya T, Bass TM, Jacobson J, Driege Y, Martinez P, Hafen E, Withers DJ, Leevers SJ, Partridge L. Longer lifespan, altered metabolism, and stress resistance in Drosophila from ablation of cells making insulin-like ligands. Proc Natl Acad Sci U S A. 2005; 102:3105–3110.

**25.** Slack C, Werz C, Wieser D, Alic N, Foley A, Stocker H, Withers DJ, Thornton JM, Hafen E, Partridge L. Regulation of lifespan, metabolism, and stress responses by the Drosophila SH2B protein, Lnk. PLoS Genetics. 2010; 6:e1000881.

**26.** Chen D, Thomas EL, Kapahi P. HIF-1 modulates dietary restriction-mediated lifespan extension via IRE-1 in Caenorhabditis elegans. PLoS Genetics. 2009; 5:e1000486.

**27.** Kaeberlein TL, Smith ED, Tsuchiya M, Welton KL, Thomas JH, Fields S, Kennedy BK, Kaeberlein M. Lifespan extension in Caenorhabditis elegans by complete removal of food. Aging Cell. 2006; 5:487–494.

**28.** Broughton SJ, Slack C, Alic N, Metaxakis A, Bass TM, Driege Y, Partridge L. DILP-producing median neurosecretory cells in the Drosophila brain mediate the response of lifespan to nutrition. Aging Cell. 2010; 9:336–346.

**29.** Slack C, Giannakou ME, Foley A, Goss M, Partridge L. dFOXO-independent effects of reduced insulin-like signaling in Drosophila. Aging Cell. 2011; 10:735–748.

**30.** Blagosklonny MV. Once again on rapamycin-induced insulin resistance and longevity: despite of or owing to. Aging. 2012; 4:350–358.

**31.** McElwee JJ, Schuster E, Blanc E, Thomas JH, Gems D. Shared transcriptional signature in Caenorhabditis elegans Dauer larvae and long-lived daf-2 mutants implicates detoxification system in longevity assurance. The Journal of Biological Chemistry. 2004; 279:44533–44543.

**32.** McElwee JJ, Schuster E, Blanc E, Piper MDW, Thomas JH, Patel DS, Selman C, Withers DJ, Thornton JM, Partridge L, Gems D. Evolutionary conservation of regulated longevity assurance mechanisms. Genome Biology. 2007; 8:R132.

**33.** Gems D, Doonan R. Antioxidant defense and aging in C. elegans. Cell Cycle. 2009; 8:1–7.

**34.** Doonan R, McElwee JJ, Matthijssens F, Walker GA, Houthoofd K, Back P, Matscheski A, Vanfleteren JR, Gems D. Against the oxidative damage theory of aging: superoxide dismutases protect against oxidative stress but have little or no effect on life span in Caenorhabditis elegans. Genes & Development. 2008; 22:3236–3241.

**35.** Blagosklonny MV. Ageing ROS or TOR. Cell Cycle. 2008; 7:3344–3354.

**36.** Blagosklonny MV. Cell senescence: hypertrophic arrest beyond the restriction point. Journal of Cellular Physiology. 2006; 209:592–597.

**37.** Gems D, de la Guardia Y. Alternative Perspectives on Aging in Caenorhabditis elegans: Reactive Oxygen Species or Hyperfunction? Antioxidants & Redox Signaling. 2013; 19:321–329.

**38.** Blagosklonny MV. Aging and Immortality: Quasi-programmed senescence and its pharmacologic inhibition. Cell Cycle. 2006; 5:2087–2102.

**39.** Skorupa DA, Dervisefendic A, Zwiener J, Pletcher SD. Dietary composition specifies consumption, obesity, and lifespan in Drosophila melanogaster. Aging Cell. 2008; 7:478–490.

**40.** Chippindale AK, Leroi M, Kim SB, Rose MR. Phenotypic plasticity and selection in Drosophila life-history evolution . I . Nutrition and the cost of reproduction. J Evol Biol. 1993; 6:171–193.

**41.** Bradley TJ, Simmons FH. An analysis of resource allocation in response to dietary yeast in Drosophila melanogaster. Journal of Insect Physiology. 1997; 43:779–788.

**42.** Liao C-Y, Rikke BA, Johnson TE, Gelfond JAL, Diaz V, Nelson JF. Fat maintenance is a predictor of the murine lifespan response to dietary restriction. Aging Cell. 2011; 10:629–639.

**43.** Harrison DE, Archer JR, Astle CM. Effects of food restriction on aging: separation of food intake and adiposity. Proc Natl Acad Sci U S A. 1984; 81:1835–1838.

**44.** Miller RA, Buehner G, Chang Y, Harper JM, Sigler R, Smith-Wheelock M. Methionine-deficient diet extends mouse lifespan, slows immune and lens aging, alters glucose, T4, IGF-I and insulin levels, and increases hepatocyte MIF levels and stress resistance. Aging Cell. 2005; 4:119–125.

**45.** Katewa SD, Demontis F, Kolipinski M, Hubbard A, Gill MS, Perrimon N, Melov S, Kapahi P. Intramyocellular Fatty-Acid Metabolism Plays a Critical Role in Mediating Responses to Dietary Restriction in Drosophila melanogaster. Cell Metabolism. 2012; 16:97–103.

**46.** Böhni R, Riesgo-Escovar J, Oldham S, Brogiolo W, Stocker H, Andruss BF, Beckingham K, Hafen E. Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. Cell. 1999; 97:865–875.

**47.** Zhang H, Stallock JP, Ng JC, Reinhard C, Neufeld TP. Regulation of cellular growth by the Drosophila target of rapamycin dTOR. Genes & Development. 2000; 14:2712–2724.

**48.** Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, Partridge L. Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. Cell Metabolism. 2010; 11:35–46.

**49.** Teleman AA, Chen Y, Cohen SM. 4E-BP functions as a metabolic brake used under stress conditions but not during normal growth. Genes & Development. 2005; 19:1844–1848.

**50.** Grönke S, Mildner A, Fellert S, Tennagels N, Petry S, Müller G, Jäckle H, Kühnlein RP. Brummer lipase is an evolutionary conserved fat storage regulator in Drosophila. Cell Metabolism. 2005; 1:323–330.

**51.** Bass TM, Grandison RC, Wong R, Martinez P, Partridge L, Piper MDW. Optimization of dietary restriction protocols in Drosophila. J Gerontol A Biol Sci Med Sci. 2007; 62:1071–1081.

**52.** Sgrò CM, Partridge L. Laboratory adaptation of life history in Drosophila. The American Naturalist. 2001; 158:657–658.

**53.** Priest N, Mackowiak B, Promislow D. The role of parental age effects on the evolution of aging. Evolution. 2002; 56:927–935.

**54.** Stewart MJ, Berry CO, Zilberman F, Thomas G, Kozma SC. The Drosophila p70s6k homolog exhibits conserved regulatory elements and rapamycin sensitivity. Proc Natl Acad Sci U S A. 1996; 93:10791–10796.

**55.** Grönke S, Beller M, Fellert S, Ramakrishnan H, Jäckle H, Kühnlein RP. Control of fat storage by a Drosophila PAT domain protein. Current Biology. 2003; 13:603–606.

**56.** Van Handel E. Microseparation of Glycogen, Sugars, and Lipids. Analytical Biochemistry. 1965; 11:266–271.

Editorial

# Rapamycin in preventive (very low) doses

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In the recent paper of Popovich et al. published in Cancer Biology and Therapy [1], positive effects of low doses of rapamycin on survival of caner-prone HER-2/neu mice have been reported. Inhibitors of the mTOR signaling pathway are already widely employed for anticancer therapy in humans. At the same time, over several last years a substantial body of evidence suggesting an anti-aging effect of at least one mTOR inhibitor, rapamycin, was accumulated. For example, rapamycin delayed death without changing the distribution of presumptive causes of death in genetically heterogeneous mice [2] and extended lifespan of cancer-prone mice, such as transgenic HER-2/neu mice [3]. Thus, a question was raised whether lifespan extension by rapamycin is a consequence of the anti-cancer effect of rapamycin (such as suppression of tumor initiation or slowing down tumor progression) or a direct result of its anti-aging properties (or both). Several studies supported effectiveness of rapamycin in delaying of aging independently from its anti-cancer activities. For example, Wilkinson et al. [4] demonstrated that rapamycin treatment significantly decreased numerous manifestations of aging and alleviated age-dependent decline in spontaneous activity in 20-22 month genetically heterogeneous mice (the age when the majority of mice survived). In the study of Anisimov et. al. [5] conducted with inbred female mice, rapamycin treatment extended lifespan, inhibited age-related weight gain, and increased the percentage of mice having regular estrous cycle at 18 months. However, the question whether lifespan extension was in part a result of suppression of cancer was not answered in these studies. A recent work from our laboratory, conducted on *Bmal1-/-* mice suffering from premature aging, provides an example of lifespan extension induced by rapamycin which is not due to inhibition of neoplastic diseases [6]. Strikingly, these mice, in spite of having many prominent aging phenotypes, virtually never develop cancer, and die from systemic failure due to progressive degeneration of nervous and cardiovascular and muscle systems. We found that rapamycin treatment extended lifespan of *Bmal1-/-* mice from 8 to 12 months. Activity of mTORC1 in tissues of *Bmal1-/-* was highly elevated, thus, treatment with rapamycin extended their lifespan mostly likely through suppression of mTOR signaling. Therefore, this study helps to further separate the life-extending effect of rapamycin from its cancer-preventing properties. Taken together, these findings indicate that rapamycin can be considered as a good candidate for a preventive anti-aging medicine.

Using a substance as a preventive medicine immediately raises questions about its overall safety and potential side effects; these questions are even more principle here compared with the situation of disease treatment. For example, in cancer treatment the drugs are used under conditions when the disease has already been developed and is deadly dangerous. Therefore, administration of high doses of drugs (even in spite of particular side effects) is justified; additionally, drugs are often administered during relatively short periods of time. On the contrary, prevention suggests chronic exposure to a medicine before the actual disease is developed, which may not happen whatsoever even without taking the preventive medication (considered as a disease, aging is an apparent exception). Thus, an ideal chemopreventive medicine should not have any side effects. Rapamycin, like any other existing medicine, does have side effects. In addition to well-known side effects of high doses of rapamycin in humans [7], life-long chronic exposure to rapamycin, while preventing most age-related diseases and extending healthspan, was shown to increase incidence of cataracts and some other alterations in mice [4]. This type of known and potential side effects makes consideration of rapamycin as a chemopreventor much less attractive for a healthy individual. Several groups noticed another side effect, a general decrease in animal robustness, when treatment with rapamycin was started in very young mice [1, 8]; this underscores the importance of mTOR pathway activity during development and points out to the significance of another variable, the onset of treatment, in diminishing of side effects. Therefore, the question about safe dosage, timing and mechanisms of delivery of rapamycin is extremely important.

Popovich and colleagues reported the lifespan extension activity of low doses of rapamycin administered in the intermittent manner (with two-week breaks between two-week-long bi-daily treatments) in a mouse model of breast cancer. In this model, mammary carcinoma develops in female mice overexpressing oncogene

HER2; cancer advances very fast with average lifespan of about 9 months. Different regimes of treatment with low doses of rapamycin allowed increasing lifespan with or without significant cancer prevention (correlating with the age the treatment started). The present study is an extension of the previous work by this team of collaborators on anticancer/longevity effects of rapamycin given in doses corresponding to the therapeutic oral dose in humans in the same mouse model [3]. There, rapamycin reduced cancer incidents, suppressed development of tumors, and extended lifespan by almost two fold. The positive effect of low doses of rapamycin on longevity in the follow-up study supports previous observations. The most important advantage of the new study derives from the significant reduction of the dose used. The above-discussed side effects are generally a matter of dose: indeed, at particular doses any substance, even water, turns out to be toxic; thus, the ability of rapamycin to work at low doses makes it substantially more attractive as a candidate for a preventive medicine.

In conclusion, we would like to accentuate that indeed anti-cancer and anti-aging activities of rapamycin can be separated. In the case of HER2-overexpressing mice, whether rapamycin works through the delay of aging or other mechanisms are involved still has to be found. At the same time, it also supports the strategy to use rapamycin in combination with other anticancer drugs. Indeed, even if rapamycin on its own would not affect tumor, it still might improve survival and therapeutic outcome. It is worth testing at least in other animal models.

## REFERENCES


**1.** Popovich IG et al. Cancer Biol Ther. 2014; 15 PMID: 24556924
**2.** Harrison DE et al. Nature. 2009; 460: 392-395.
**3.** Anisimov VN et al. Am J Pathol. 2010; 176: 2092-2097.
**4.** Wilkinson JE et al. Aging Cell. 2012; 11: 675-682.
**5.** Anisimov VN et al. Cell Cycle. 2011; 10: 4230-6.
**6.** Khapre RV et al. Aging (Albany NY). 2014; 6: 48-57.
**7.** Blagosklonny MV. Oncoimmunology. 2013;2:e26961.
**8.** Johnson SC et al. Science. 2013; 342: 1524-1528.



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Review

# Immunostimulatory activity of lifespan-extending agents

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**Abstract: During the past two decades, several interventions have been shown to increase the healthy lifespan of model organisms as evolutionarily distant from each other as yeast, worms, flies and mammals. These anti-aging maneuvers include (but are not limited to) cycles of caloric restriction, physical exercise as well as the administration of multiple, chemically unrelated agents, such as resveratrol, spermidine and various rapamycin-like compounds collectively known as rapalogs. Most, if not all, lifespan-extending agents promote macroautophagy (hereafter referred to as autophagy), an evolutionarily old mechanism that contributes to the maintenance of intracellular homeostasis and plays a critical role in the adaptive response of cells to stress. In line with this notion, the activation of autophagy appears to mediate significant anti-ageing effects in several organisms, including mice. Here, we focus on rapalogs to discuss the possibility that part of the beneficial activity of lifespan-extending agents stems from their ability to exert immunostimulatory effects. Accumulating evidence indicates indeed that the immune system can recognize and eliminate not only cells that are prone to undergo malignant transformation, but also senescent cells, thus playing a significant role in the control of organismal aging. In addition, it has recently become clear that rapamycin and other rapalogs, which for a long time have been viewed (and used in the clinic) as pure immunosuppressants, can mediate robust immunostimulatory functions, at least in some circumstances.**

The hypothesis that organismal aging might be slowed down, and hence the appearance of aging-associated disorders delayed, has been the subject of intense investigation throughout the past two decades [1, 2]. In this context, several interventions have been demonstrated to significantly extend the healthy lifespan of model organisms as distant from each other on the evolution scale as yeast, worms, flies and mammals [3-6]. For illustrative purposes, such interventions can be classified into two large groups: (1) lifestyle modifications and (2) pharmacological/genetic maneuvers. The former include cycles of caloric restriction as well as a regular physical activity. These are actually known to extend the healthy lifespan of humans since a long time, although the molecular mechanisms underlying this phenomenon have only recently begun to emerge [7-10]. The latter encompass the administration of an increasingly wide panel of chemically unrelated molecules, including (but not limited to) resveratrol (a polyphenol found in grapes and red wine), spermidine (a polyamine that is abundant in grapefruits and soybeans), rapamycin (a macrolide originally isolated from the Easter Island micro-organism *Streptomyces hygroscopicus*) and multiple rapamycin-like compounds that are collectively known as rapalogs [11-15]. Most, if not all, these interventions share the ability to promote macroautophagy (hereafter referred to as autophagy), a mechanism for the lysosomal degradation of super-fluous, damaged or ectopic intracellular constituents [16, 17]. Moreover, the

beneficial effects of both lifestyle modifications and pharmacological/genetic maneuvers have been shown to depend on an intact autophagic machinery, at least in some models [18-21]. In line with this notion, the moderate overexpression of one essential mediator of autophagy (i.e., ATG5) at the whole body level has recently been shown to extend the median lifespan of mice by approximately 17% [22]. Conversely, the genetic inhibition of autophagy-relevant proteins such as Beclin 1 (ATG6) , ATG7 and ATG12 reportedly mediates a negative effect on the healthy lifespan of model organisms including *Caenorhabditis elegans* [23]. As a matter of fact, baseline levels of autophagy play a major role in the maintenance of intracellular (and hence organismal) homeostasis, hence mediating a robust oncosuppressive activity [24-26]. In addition, autophagy orchestrates the adaptive response of cells to multiple adverse conditions, including nutritional, physical and chemical cues [27]. It is therefore not surprising that autophagy might increase the organismal fitness and hence delay aging [28].

Nonetheless, the precise mechanisms whereby specific changes in lifestyle as well as selected chemicals or genetic manipulations delay aging (at least in model organisms) have not yet been fully elucidated. Thus, the efficacy of some anti-aging interventions may rely on mechanisms other than the upregulation of the autophagic flux. The immune system stands out as a good candidate for a part in this process, based on at least two lines of evidence: (1) autophagy plays a major role not only in the activation of innate responses against intracellular pathogens at the cell-autonomous level [29, 30], but also in the elicitation of adaptive immune responses based on the interaction between antigen-presenting cells and antigen-specific $CD4^+$ and $CD8^+$ T lymphocytes [31-33]; and (2) the immune system has been shown to recognize and eliminate not only cells that are prone to undergoing malignant transformation, but also senescent cells, thus contributing to the control of organismal aging [34, 35]. Interestingly, however, rapamycin and other rapalogs have long been known (and currently employed in the clinic) for their capacity to mediate robust immunosuppressive effects [36-38]. Indeed, rapamycin (which is also known as sirolimus) has first been approved by the US Food and Drug Administration (FDA) in 1999 for use in combination with ciclosporin and corticosteroids to prevent acute organ rejection in patients receiving kidney transplants [39, 40]. As it stands, however, the immunosuppressive potential of rapamycin and multiple rapalogs in humans has never been properly tested, as the clinical trials performed to date invariably employed as a control condition the gold-standard immunosuppressive regimens available [41]. In addition, accumulating preclinical and clinical evidence indicates that, at odds with immunosuppressants that operate by inhibiting calcineurin, such as tacrolimus, rapamycin and other rapalogs might exert a significant immunostimulatory activity, at least under some circumstances.

This hypothesis first originated from the observation that the recipients of solid organs maintained on rapamycin-based regimens manifested a reduced incidence of various tumors, notably lymphoma, as compared to patients subjected to organ transplantation and treated with conventional immunosuppressants such as corticosteroids, ciclosporin, azathioprine or tacrolimus [42-48]. Transplant recipients are indeed known to exhibit an increased incidence of multiple malignancies, encompassing lymphoma as well as hepatocellular carcinoma, Kaposi's sarcoma, and other cutaneous cancers, presumably owing to the state of systemic immunosuppression that is required to avoid rejection [49]. In transplanted patients, rapamycin was associated not only with robust oncosuppressive effects, but also with a *bona fide* anticancer activity against pre-existent tumors, in particular Kaposi's sarcomas [50-54]. Moreover, local or systemic inflammatory responses have been detected in a fraction of transplanted patients on rapamycin-based maintenance regimens [55, 56]. Often, such responses and the consequent toxicity (be it systemic or selectively affecting the transplant) could be promptly reversed by the reintroduction of calcineurin inhibitors [55, 56].

Recently, cancer-preventive and antineoplastic effects have also been attributed to everolimus (also known as RAD001), a rapalog approved by the US FDA for use in patients affected by various malignancies, including renal cell carcinoma (upon the failure of sunitinib- or sorafenib-based chemotherapeutic regimen) [57], subependymal giant cell astrocytoma [58], progressive neuroendocrine tumors of pancreatic origin [59], and advanced estrogen receptor $(ER)^+$, *v-erb-b2* avian erythroblastic leukemia viral oncogene homolog 2 $(ERBB2)^+$ breast carcinoma (in combination with the aromatase inhibitor exemestane [60]. Although such a beneficial (and completely unsuspected) activity of rapalogs was initially ascribed to their capacity to robustly inhibit the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) in cancer cells, accumulating preclinical evidence indicates that the therapeutic and oncopreventive effects of rapamycin-like compounds originates, at least in part, from cancer cell-extrinsic mechanisms that involve the immune system [61]. In further support of this notion, transplant recipients treated with rapamycin- or everolimus-based

maintenance regimens appear to be significantly less redisposed to cytomegalovirus infections than their counterparts receiving conventional immunosuppressants [62, 63].

**Table 1. Preclinical evidence in support of the immunostimulatory activity of rapalogs**

| Rapalog | Model | Stimulus | Observation(s) | Ref. |
|---|---|---|---|---|
| Everolimus (RAD001) | Breast cancer-bearing mice | IL-15-coding plasmid | Improved inhibition of tumor growth | [95] |
| | HTLV1-infected T cells and patient-derived ATLL cells | IKK inhibitor | Decreased the secretion of IL-10 | [77] |
| | Sprague-Dawley rats | Remnant kidney model | Worsened disease progression correlating with several markers of inflammation | [105] |
| Sirolimus (Rapamycin) | Human PBMCs and DCs | LPS from *Escherichia coli* | Increased NF-κB activation and pro-inflammatory cytokine secretion; decreased STAT3 activation and IL-10 release | [78] |
| | Murine DCs | LPS from *Escherichia coli* | Increased secretion of IL-12 | [79] |
| | Monocytes, macrophages and primary DCs | LPS from *Escherichia coli* | Increased NF-κB activation and pro-inflammatory cytokine secretion; decreased STAT3 activation and IL-10 release | [80] |
| | Murine DCs and C57Bl/10, C3H/HeJ, *Il4ra*$^{-/-}$ mice | LPS from *Escherichia coli* | Increased cytokine secretion and improved T-cell co-stimulation | [82] |
| | Human whole blood | LPS, LTA or peptidoglycan | Inhibition of IL-10 secretion | [81] |
| | HEK293 cells stably expressing TLR2 of TLR4 | *Mycobacterium tuberculosis* | Increased IL-23 secretion at both the mRNA and protein level | [69] |
| | Murine macrophages, DCs and C57Bl/6 mice | *Mycobacterium tuberculosis* | Enhanced $T_H1$ responses in mice vaccinated with sirolimus-treated DCs | [73] |
| | THP1 cells, primary human PBMCs and DCs | *Staphylococcus aureus* | Increased IL-12 secretion at both the mRNA and protein level | [68] |
| | Wild-type and transgenic C57Bl/6 mice | *Listeria monocytogenes* | Improved antigen-specific T-cell responses in the course of infection | [70] |
| | *Traf6*$^{-/-}$ mice | Attenuated *Listeria monocytogenes* strain | Improved long-lived $CD8^+$ memory T-cell responses | [71] |
| | DCs from wild-type and PI3K-deficient mice | *Leishmania major* | Improved IL-12 secretion by DCs, robust $T_H1$ responses *in vivo* | [72] |
| | Wild-type and transgenic *Rag1*$^{-/-}$ mice | Myxoma virus | Increased anticancer activity of adoptively transferred T lymphocytes | [75] |
| | Old C57Bl/6 mice | Influenza virus | Improve production of B lymphocytes and optimal responses to vaccination | [76] |
| | Wild-type and transgenic C57Bl/6 mice | LCMV and engineered vaccinia virus | Increased amounts of antigen-specific T cells | [74] |
| | HUVECs | Thrombin | Increased NF-κB activation | [106] |
| | Tumor-bearing transgenic C57Bl/6 mice | Anti-CD3/anti-CD8 antibodies Antigen-derived peptides plus CD80 | Generated OT-I cells that were more effective than IL-12-conditioned effector OT-I cells after adoptive transfer | [85] |

| | | | | |
|---|---|---|---|---|
| | Human PBMCs and TU167 cells | IL-2 and isopentenyl pyrophosphate | Increased the yield and effector function of human γδ T cells *in vitro* | [90] |
| Temsirolimus (CCI-779) | RCC and melanoma-bearing mice | HSP-based anticancer vaccine | Improved $CD8^+$ T-cell memory responses and effector functions | [96] |

**Abbreviations:** ATLL, adult T-cell leukemia-lymphoma; CAR, chimeric antigen receptor; DC, dendritic cell; HSP, heat-shock protein; HTLV-1, human T-cell lymphotropic virus type 1; HUVEC, human umbilical vein endothelial cell; IKK, IκB kinase; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; IL, interleukin; LCMV, lymphocytic choriomeningitis virus; LPS, lipopolysaccharide; LTA, peptidoglycan; PBMC, peripheral blood mononuclear cell; PI3K, phosphoinositide-3-kinase; STAT3, signal transducer and activator of transcription 3; TLR, Toll-like receptor.

**Table 2. Clinical evidence in support of the immunostimulatory activity of rapalogs**

| Rapalog | Setting | Observation(s) | Ref. |
|---|---|---|---|
| Everolimus (RAD001) | Cardiac transplantation | Decreased incidence of CMV infection among everolimus-treated patients | [62] |
| Sirolimus (Rapamycin) | Liver transplantation | Limited rate of HCV progression and associated hepatic fibrosis | [107] |
| | Renal transplantation | Anemia correlating with biochemical evidence of a chronic inflammatory state | [56] |
| | Renal transplantation | Development of glomerulonephritis upon conversion from a calcineurin inhibitor-based immunosuppression to rapamycin | [55] |
| | Solid organ transplantation | Decreased incidence of CMV infection among sirolimus-treated patients | [63] |
| | Solid organ transplantation | Decreased incidence of multiple tumors among sirolimus-treated patients | [42-48] |
| | Solid organ transplantation | Consistent antitumor responses in patients with post-transplantation neoplasms treated with sirolimus | [50-54] |
| Temsirolimus (CCI-779) | Advanced cancer | No signs of immunosuppression among everolimus-treated patients | [108] |

**Abbreviations:** CMV, cytomegalovirus; HCV, hepatitis C virus.

Rapamycin and other rapalogs have been shown to exert robust antineoplastic and oncopreventive effects in both transplantable and oncogene-driven tumor models. In immunocompromised mice xenografted with human tumors, this activity obviously reflects cancer cell-intrinsic (or stromal) mechanisms. As a matter of fact, mTORC1 is hyperactivated (hence delivering critical pro-survival signals) in a large number of malignancies, most often due to genetic or epigenetic alterations that result in constitutive signaling via upstream tyrosine kinase receptors (e.g., the epidermal growth factor receptor, EGFR) [64-67]. Conversely, the anticancer effects of rapalogs in immunocompetent settings appear to rely, at least in part, on the elicitation of tumor-targeting immune responses. Rapamycin appears to enhance multiple facets of immune and inflammatory responses elicited in mice by stimuli encompassing replication-competent bacteria [68-73] and viruses [74-77], as well as purified components thereof [74, 78-82] and synthetic immunomodulatory agents [83, 84]. Ovalbumin-specific αβ T lymphocytes exposed to microspheres coated with an ovalbumin-derived peptide

plus co-stimulatory CD80 molecules (or with anti-CD3/anti-CD28 antibodies) in the presence of rapamycin exhibited improved memory and antitumor functions *in vivo* than T cells of the same type activated in the presence of interleukin (IL)-12 [85-87]. Along similar lines, rapamycin has been shown to enhance tumor-targeting $CD8^+$ T-cell memory responses elicited by a poxviral anticancer vaccine in mice [88, 89]. Such an immunostimulatory activity was observed only when rapamycin was administered in a high-dose short therapeutic course, as opposed to both a single, low-dose course as well as prolonged treatment schedules [88, 89]. Of note, rapamycin has also been shown to increase the yield and effector functions of human γδ T cells activated *in vitro* with isopentenyl pyrophosphate plus recombinant IL-2 [90]. In particular, γδ T cells subjected to antigen stimulation in the presence of rapamycin expressed increased levels of the activation marker CD69, the anti-apoptotic protein BCL-2 and IL-2 receptor α (IL2RA, best known as CD25) [90, 91]. These findings suggest that rapamycin may potentiate purely adaptive immune responses, such as those mediated by αβ T lymphocytes, as well as immune responses with mixed adaptive/innate features, such as those orchestrated by γδ T cells [92, 93]. Other rapalogs, including everolimus and temsirolimus (CCI-779, which has originally been approved by the US FDA for the treatment of advanced renal cell carcinoma in 2007) [94], have been demonstrated to exert immunostimulatory effects, *in vitro* and *in vivo* [77, 95, 96]. As a standalone example, temsirolimus was shown significantly improve the therapeutic potential of a peptide-based anticancer vaccine against established renal cell carcinomas and melanomas in mice [96, 97]. Thus, the potential immunostimulatory activity of rapalogs appears to stem from an on-target effect, i.e., the inhibition of mTORC1 [61, 98, 99].

Taken together, these observations suggest that rapamycin and other rapalogs are capable of stimulating, rather than inhibiting, immune responses, at least under selected circumstances. Whether such an immunostimulatory function truly underlies the anti-aging effects of rapamycin remains to be formally demonstrated. Nonetheless, accumulating preclinical data (Table **1**) as well as a large amount of circumstantial clinical evidence (Table 2) suggests that these lifespan-extending chemicals can be harnessed to promote therapeutically relevant antitumor immune responses. Properly designed trials that evaluate the actual immunotherapeutic potential of rapamycin-like compounds are urgently awaited. Alternatively, it will be interesting to see whether circulating or intratumoral biomarkers of pre-existing or therapy-elicited immune responses are capable of identifying a subset of cancer patients that obtain full-blown clinical benefits from the administration of rapalogs. The standardized immuno-monitoring procedures that are required in this context have just begun to be defined and implemented into clinical trials [100, 101]. Altogether, these studies will cast new light on whether rapamycin and other rapalogs should still be considered as immuno-suppressants or whether their immunomodulatory activity, similar that of other drugs like cyclophosphamide [102, 103], rather depends on a large panel of factors, including dose and administration schedule. In this latter scenario, rapalogs may turn out to constitute good candidates for the development of novel immunochemotherapeutic regimens [104].

## Conflicts of Interest Statement

The authors have no conflict of interests to declare.

## REFERENCES


**1.** Campisi J and d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nature reviews Molecular cell biology. 2007; 8:729-740.

**2.** Fossel M. Cell senescence in human aging and disease. Annals of the New York Academy of Sciences. 2002; 959:14-23.

**3.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009; 460:392-395.

**4.** Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A and Partridge L. Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. Cell metabolism. 2010; 11:35-46.

**5.** Robida-Stubbs S, Glover-Cutter K, Lamming DW, Mizunuma M, Narasimhan SD, Neumann-Haefelin E, Sabatini DM and Blackwell TK. TOR signaling and rapamycin influence longevity by regulating SKN-1/Nrf and DAF-16/FoxO. Cell metabolism. 2012; 15:713-724.

**6.** Powers RW, 3rd, Kaeberlein M, Caldwell SD, Kennedy BK and Fields S. Extension of chronological life span in yeast by decreased TOR pathway signaling. Genes & development. 2006; 20:174-184.

**7.** He C, Bassik MC, Moresi V, Sun K, Wei Y, Zou Z, An Z, Loh J, Fisher J, Sun Q, Korsmeyer S, Packer M, May HI, et al. Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. Nature. 2012; 481:511-515.

**8.** Galluzzi L and Kroemer G. Autophagy mediates the metabolic benefits of endurance training. Circulation research. 2012; 110:1276-1278.

**9.** Blagosklonny MV. Linking calorie restriction to longevity through sirtuins and autophagy: any role for TOR. Cell death & disease. 2010; 1:e12.

**10.** Warr MR, Binnewies M, Flach J, Reynaud D, Garg T, Malhotra R, Debnath J and Passegue E. FOXO3A directs a protective autophagy program in haematopoietic stem cells. Nature. 2013; 494:323-327.

**11.** Eisenberg T, Knauer H, Schauer A, Buttner S, Ruckenstuhl C, Carmona-Gutierrez D, Ring J, Schroeder S, Magnes C, Antonacci L, Fussi H, Deszcz L, Hartl R, et al. Induction of autophagy by spermidine promotes longevity. Nature cell biology. 2009; 11:1305-1314.

**12.** Morselli E, Maiuri MC, Markaki M, Megalou E, Pasparaki A, Palikaras K, Criollo A, Galluzzi L, Malik SA, Vitale I, Michaud M, Madeo F, Tavernarakis N, et al. Caloric restriction and resveratrol promote longevity through the Sirtuin-1-dependent induction of autophagy. Cell death & disease. 2010; 1:e10.

**13.** Morselli E, Galluzzi L, Kepp O, Criollo A, Maiuri MC, Tavernarakis N, Madeo F and Kroemer G. Autophagy mediates pharmacological lifespan extension by spermidine and resveratrol. Aging. 2009; 1:961-970.

**14.** Benjamin D, Colombi M, Moroni C and Hall MN. Rapamycin passes the torch: a new generation of mTOR inhibitors. Nature reviews Drug discovery. 2011; 10:868-880.

**15.** Lamming DW, Ye L, Sabatini DM and Baur JA. Rapalogs and mTOR inhibitors as anti-aging therapeutics. The Journal of clinical investigation. 2013; 123:980-989.

**16.** Choi AM, Ryter SW and Levine B. Autophagy in human health and disease. The New England journal of medicine. 2013; 368:1845-1846.

**17.** Klionsky DJ. Autophagy: from phenomenology to molecular understanding in less than a decade. Nature reviews Molecular cell biology. 2007; 8:931-937.

**18.** Jia K and Levine B. Autophagy is required for dietary restriction-mediated life span extension in C. elegans. Autophagy. 2007; 3:597-599.

**19.** Hansen M, Chandra A, Mitic LL, Onken B, Driscoll M and Kenyon C. A role for autophagy in the extension of lifespan by dietary restriction in C. elegans. PLoS genetics. 2008; 4:e24.

**20.** Dwivedi M, Song HO and Ahnn J. Autophagy genes mediate the effect of calcineurin on life span in C. elegans. Autophagy. 2009; 5:604-607.

**21.** Minina EA, Sanchez-Vera V, Moschou PN, Suarez MF, Sundberg E, Weih M and Bozhkov PV. Autophagy mediates caloric restriction-induced lifespan extension in Arabidopsis. Aging cell. 2013; 12:327-329.

**22.** Pyo JO, Yoo SM, Ahn HH, Nah J, Hong SH, Kam TI, Jung S and Jung YK. Overexpression of Atg5 in mice activates autophagy and extends lifespan. Nature communications. 2013; 4:2300.

**23.** Hars ES, Qi H, Ryazanov AG, Jin S, Cai L, Hu C and Liu LF. Autophagy regulates ageing in C. elegans. Autophagy. 2007; 3:93-95.

**24.** Green DR, Galluzzi L and Kroemer G. Mitochondria and the autophagy-inflammation-cell death axis in organismal aging. Science. 2011; 333:1109-1112.

**25.** Morselli E, Galluzzi L, Kepp O, Vicencio JM, Criollo A, Maiuri MC and Kroemer G. Anti- and pro-tumor functions of autophagy. Biochimica et biophysica acta. 2009; 1793(9):1524-1532.

**26.** White E. Deconvoluting the context-dependent role for autophagy in cancer. Nature reviews Cancer. 2012; 12:401-410.

**27.** Kroemer G, Marino G and Levine B. Autophagy and the integrated stress response. Molecular cell. 2010; 40:280-293.

**28.** Martins I, Galluzzi L and Kroemer G. Hormesis, cell death and aging. Aging. 2011; 3:821-828.

**29.** Levine B, Mizushima N and Virgin HW. Autophagy in immunity and inflammation. Nature. 2011; 469:323-335.

**30.** Benjamin JL, Sumpter R, Jr., Levine B and Hooper LV. Intestinal epithelial autophagy is essential for host defense against invasive bacteria. Cell host & microbe. 2013; 13:723-734.

**31.** Yi Y, Zhou Z, Shu S, Fang Y, Twitty C, Hilton TL, Aung S, Urba WJ, Fox BA, Hu HM and Li Y. Autophagy-assisted antigen cross-presentation: Autophagosome as the argo of shared tumor-specific antigens and DAMPs. Oncoimmunology. 2012; 1:976-978.

**32.** Ma Y, Galluzzi L, Zitvogel L and Kroemer G. Autophagy and cellular immune responses. Immunity. 2013; 39:211-227.

**33.** Michaud M, Sukkurwala AQ, Martins I, Shen S, Zitvogel L and Kroemer G. Subversion of the chemotherapy-induced anticancer immune response by the ecto-ATPase CD39. Oncoimmunology. 2012; 1:393-395.

**34.** Senovilla L, Galluzzi L, Zitvogel L and Kroemer G. Immunosurveillance as a regulator of tissue homeostasis. Trends in immunology. 2013; 34:471-481.

**35.** Prendergast GC and Metz R. A perspective on new immune adjuvant principles: Reprogramming inflammatory states to permit clearance of cancer cells and other age-associated cellular pathologies. Oncoimmunology. 2012; 1:924-929.

**36.** Law BK. Rapamycin: an anti-cancer immunosuppressant? Critical reviews in oncology/hematology. 2005; 56:47-60.

**37.** Morelon E, Mamzer-Bruneel MF, Peraldi MN and Kreis H. Sirolimus: a new promising immunosuppressive drug. Towards a rationale for its use in renal transplantation. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2001; 16:18-20.

**38.** Sehgal SN, Molnar-Kimber K, Ocain TD and Weichman BM. Rapamycin: a novel immunosuppressive macrolide. Medicinal research reviews. 1994; 14:1-22.

**39.** Kahan BD, Podbielski J, Napoli KL, Katz SM, Meier-Kriesche HU and Van Buren CT. Immunosuppressive effects and safety of a sirolimus/cyclosporine combination regimen for renal transplantation. Transplantation. 1998; 66:1040-1046.

**40.** Brattstrom C, Sawe J, Tyden G, Herlenius G, Claesson K, Zimmerman J and Groth CG. Kinetics and dynamics of single oral doses of sirolimus in sixteen renal transplant recipients. Therapeutic drug monitoring. 1997; 19:397-406.

**41.** Blagosklonny MV. Immunosuppressants in cancer prevention and therapy Oncoimmunology. 2013; 2:e26961.

**42.** Mathew T, Kreis H and Friend P. Two-year incidence of malignancy in sirolimus-treated renal transplant recipients: results from five multicenter studies. Clinical transplantation. 2004; 18:446-449.

**43.** Yakupoglu YK, Buell JF, Woodle S and Kahan BD. Individualization of immunosuppressive therapy. III. Sirolimus associated with a reduced incidence of malignancy. Transplantation proceedings. 2006; 38:358-361.

**44.** Campistol JM, Eris J, Oberbauer R, Friend P, Hutchison B, Morales JM, Claesson K, Stallone G, Russ G, Rostaing L, Kreis H, Burke JT, Brault Y, et al. Sirolimus therapy after early cyclosporine withdrawal reduces the risk for cancer in adult renal transplantation. Journal of the American Society of Nephrology : JASN. 2006; 17:581-589.

**45.** Alberu J, Pascoe MD, Campistol JM, Schena FP, Rial Mdel C, Polinsky M, Neylan JF, Korth-Bradley J, Goldberg-Alberts R, Maller ES and Sirolimus CTSG. Lower malignancy rates in renal allograft recipients converted to sirolimus-based, calcineurin

inhibitor-free immunotherapy: 24-month results from the CONVERT trial. Transplantation. 2011; 92:303-310.

**46.** Kauffman HM, Cherikh WS, Cheng Y, Hanto DW and Kahan BD. Maintenance immunosuppression with target-of-rapamycin inhibitors is associated with a reduced incidence of de novo malignancies. Transplantation. 2005; 80:883-889.

**47.** Salgo R, Gossmann J, Schofer H, Kachel HG, Kuck J, Geiger H, Kaufmann R and Scheuermann EH. Switch to a sirolimus-based immunosuppression in long-term renal transplant recipients: reduced rate of (pre-)malignancies and nonmelanoma skin cancer in a prospective, randomized, assessor-blinded, controlled clinical trial. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2010; 10:1385-1393.

**48.** Campbell SB, Walker R, Tai SS, Jiang Q and Russ GR. Randomized controlled trial of sirolimus for renal transplant recipients at high risk for nonmelanoma skin cancer. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2012; 12:1146-1156.

**49.** London NJ, Farmery SM, Will EJ, Davison AM and Lodge JP. Risk of neoplasia in renal transplant patients. Lancet. 1995; 346:403-406.

**50.** Mohsin N, Budruddin M, Pakkyara A, Darweesh A, Nayyer M, Amitabh J and Daar AS. Complete regression of visceral Kaposi's sarcoma after conversion to sirolimus. Experimental and clinical transplantation : official journal of the Middle East Society for Organ Transplantation. 2005; 3:366-369.

**51.** Zmonarski SC, Boratynska M, Puziewicz-Zmonarska A, Kazimierczak K and Klinger M. Kaposi's sarcoma in renal transplant recipients. Annals of transplantation : quarterly of the Polish Transplantation Society. 2005; 10:59-65.

**52.** Zmonarski SC, Boratynska M, Rabczynski J, Kazimierczak K and Klinger M. Regression of Kaposi's sarcoma in renal graft recipients after conversion to sirolimus treatment. Transplantation proceedings. 2005; 37:964-966.

**53.** Cullis B, D'Souza R, McCullagh P, Harries S, Nicholls A, Lee R and Bingham C. Sirolimus-induced remission of posttransplantation lymphoproliferative disorder. American journal of kidney diseases : the official journal of the National Kidney Foundation. 2006; 47:e67-72.

**54.** Stallone G, Schena A, Infante B, Di Paolo S, Loverre A, Maggio G, Ranieri E, Gesualdo L, Schena FP and Grandaliano G. Sirolimus for Kaposi's sarcoma in renal-transplant recipients. The New England journal of medicine. 2005; 352:1317-1323.

**55.** Dittrich E, Schmaldienst S, Soleiman A, Horl WH and Pohanka E.Rapamycin-associated post-transplantation glomerulonephritis and its remission after reintroduction of calcineurin-inhibitor therapy. Transplant international : official journal of the European Society for Organ Transplantation. 2004; 17:215-220.

**56.** Thaunat O, Beaumont C, Chatenoud L, Lechaton S, Mamzer-Bruneel MF, Varet B, Kreis H and Morelon E. Anemia after late introduction of sirolimus may correlate with biochemical evidence of a chronic inflammatory state. Transplantation. 2005; 80:1212-1219.

**57.** Motzer RJ, Escudier B, Oudard S, Hutson TE, Porta C, Bracarda S, Grunwald V, Thompson JA, Figlin RA, Hollaender N, Urbanowitz G, Berg WJ, Kay A, et al. Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. Lancet. 2008; 372:449-456.

**58.** Krueger DA, Care MM, Holland K, Agricola K, Tudor C, Mangeshkar P, Wilson KA, Byars A, Sahmoud T and Franz DN. Everolimus for subependymal giant-cell astrocytomas in tuberous sclerosis. The New England journal of medicine. 2010; 363:1801-1811.

**59.** Yao JC, Shah MH, Ito T, Bohas CL, Wolin EM, Van Cutsem E, Hobday TJ, Okusaka T, Capdevila J, de Vries EG, Tomassetti P, Pavel ME, Hoosen S, et al. Everolimus for advanced pancreatic neuroendocrine tumors. The New England journal of medicine. 2011; 364:514-523.

**60.** Baselga J, Campone M, Piccart M, Burris HA, 3rd, Rugo HS, Sahmoud T, Noguchi S, Gnant M, Pritchard KI, Lebrun F, Beck JT, Ito Y, Yardley D, et al. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. The New England journal of medicine. 2012; 366:520-529.

**61.** Araki K, Ellebedy AH and Ahmed R. TOR in the immune system. Current opinion in cell biology. 2011; 23:707-715.

**62.** Hill JA, Hummel M, Starling RC, Kobashigawa JA, Perrone SV, Arizon JM, Simonsen S, Abeywickrama KH and Bara C. A lower incidence of cytomegalovirus infection in de novo heart transplant recipients randomized to everolimus. Transplantation. 2007; 84:1436-1442.

**63.** Demopoulos L, Polinsky M, Steele G, Mines D, Blum M, Caulfield M, Adamkovic A, Liu Q, Harler MB, Hahn C and Singh A. Reduced risk of cytomegalovirus infection in solid organ transplant recipients treated with sirolimus: a pooled analysis of clinical trials. Transplantation proceedings. 2008; 40:1407-1410.

**64.** Pollack BP. EGFR inhibitors, MHC expression and immune responses : Can EGFR inhibitors be used as immune response modifiers? Oncoimmunology. 2012; 1:71-74.

**65.** Zoncu R, Efeyan A and Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. Nature reviews Molecular cell biology. 2011; 12:21-35.

**66.** Wouters BG and Koritzinsky M. Hypoxia signalling through mTOR and the unfolded protein response in cancer. Nature reviews Cancer. 2008; 8:851-864.

**67.** Shackelford DB and Shaw RJ. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. Nature reviews Cancer. 2009; 9:563-575.

**68.** Uthaisangsook S, Day NK, Hitchcock R, Lerner A, James-Yarish M, Good RA and Haraguchi S. Negative regulation of interleukin-12 production by a rapamycin-sensitive signaling pathway: a brief communication. Experimental biology and medicine. 2003; 228:1023-1027.

**69.** Yang CS, Song CH, Lee JS, Jung SB, Oh JH, Park J, Kim HJ, Park JK, Paik TH and Jo EK. Intracellular network of phosphatidylinositol 3-kinase, mammalian target of the rapamycin/70 kDa ribosomal S6 kinase 1, and mitogen-activated protein kinases pathways for regulating mycobacteria-induced IL-23 expression in human macrophages. Cellular microbiology. 2006; 8:1158-1171.

**70.** Ferrer IR, Wagener ME, Robertson JM, Turner AP, Araki K, Ahmed R, Kirk AD, Larsen CP and Ford ML. Cutting edge: Rapamycin augments pathogen-specific but not graft-reactive CD8+ T cell responses. Journal of immunology. 2010; 185:2004-2008.

**71.** Pearce EL, Walsh MC, Cejas PJ, Harms GM, Shen H, Wang LS, Jones RG and Choi Y. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. Nature. 2009; 460:103-107.

**72.** Fukao T, Tanabe M, Terauchi Y, Ota T, Matsuda S, Asano T, Kadowaki T, Takeuchi T and Koyasu S. PI3K-mediated negative

feedback regulation of IL-12 production in DCs. Nature immunology. 2002; 3:875-881.

**73.** Jagannath C, Lindsey DR, Dhandayuthapani S, Xu Y, Hunter RL, Jr. and Eissa NT. Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. Nature medicine. 2009; 15:267-276.

**74.** Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, Larsen CP and Ahmed R. mTOR regulates memory CD8 T-cell differentiation. Nature. 2009; 460:108-112.

**75.** Thomas DL, Doty R, Tosic V, Liu J, Kranz DM, McFadden G, Macneill AL and Roy EJ. Myxoma virus combined with rapamycin treatment enhances adoptive T cell therapy for murine melanoma brain tumors. Cancer immunology, immunotherapy : CII. 2011; 60:1461-1472.

**76.** Chen C, Liu Y, Liu Y and Zheng P. mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells. Science signaling. 2009; 2:ra75.

**77.** Nishioka C, Ikezoe T, Yang J, Udaka K and Yokoyama A. The combination of IkappaB kinase beta inhibitor and everolimus modulates expression of interleukin-10 in human T-cell lymphotropic virus type-1-infected T cells. Immunology. 2013; 138:216-227.

**78.** Haidinger M, Poglitsch M, Geyeregger R, Kasturi S, Zeyda M, Zlabinger GJ, Pulendran B, Horl WH, Saemann MD and Weichhart T. A versatile role of mammalian target of rapamycin in human dendritic cell function and differentiation. Journal of immunology. 2010; 185:3919-3931.

**79.** Ohtani M, Nagai S, Kondo S, Mizuno S, Nakamura K, Tanabe M, Takeuchi T, Matsuda S and Koyasu S. Mammalian target of rapamycin and glycogen synthase kinase 3 differentially regulate lipopolysaccharide-induced interleukin-12 production in dendritic cells. Blood. 2008; 112:635-643.

**80.** Weichhart T, Costantino G, Poglitsch M, Rosner M, Zeyda M, Stuhlmeier KM, Kolbe T, Stulnig TM, Horl WH, Hengstschlager M, Muller M and Saemann MD. The TSC-mTOR signaling pathway regulates the innate inflammatory response. Immunity. 2008; 29:565-577.

**81.** Jorgensen PF, Wang JE, Almlof M, Solberg R, Okkenhaug C, Scholz T, Thiemermann C, Foster SJ and Aasen AO. Sirolimus interferes with the innate response to bacterial products in human whole blood by attenuation of IL-10 production. Scandinavian journal of immunology. 2001; 53:184-191.

**82.** Hackstein H, Taner T, Zahorchak AF, Morelli AE, Logar AJ, Gessner A and Thomson AW. Rapamycin inhibits IL-4--induced dendritic cell maturation in vitro and dendritic cell mobilization and function in vivo. Blood. 2003; 101:4457-4463.

**83.** Vacchelli E, Galluzzi L, Eggermont A, Fridman WH, Galon J, Sautes-Fridman C, Tartour E, Zitvogel L and Kroemer G. Trial watch: FDA-approved Toll-like receptor agonists for cancer therapy. Oncoimmunology. 2012; 1:894-907.

**84.** Galluzzi L, Vacchelli E, Eggermont A, Fridman WH, Galon J, Sautes-Fridman C, Tartour E, Zitvogel L and Kroemer G. Trial Watch: Experimental Toll-like receptor agonists for cancer therapy. Oncoimmunology. 2012; 1:699-716.

**85.** Rao RR, Li Q, Odunsi K and Shrikant PA. The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin. Immunity. 2010; 32:67-78.

**86.** You CX, Shi M, Liu Y, Cao M, Luo R and Hermonat PL. AAV2/IL-12 gene delivery into dendritic cells (DC) enhances CTL stimulation above other IL-12 applications: Evidence for IL-12 intracrine activity in DC. Oncoimmunology. 2012; 1:847-855.

**87.** Song DG and Powell DJ. Pro-survival signaling via CD27 costimulation drives effective CAR T-cell therapy. Oncoimmunology. 2012; 1:547-549.

**88.** Li Q, Rao R, Vazzana J, Goedegebuure P, Odunsi K, Gillanders W and Shrikant PA. Regulating mammalian target of rapamycin to tune vaccination-induced CD8(+) T cell responses for tumor immunity. Journal of immunology. 2012; 188:3080-3087.

**89.** Srivastava RK, Utley A and Shrikant PA. Rapamycin: A rheostat for CD8(+) T-cell-mediated tumor therapy. Oncoimmunology. 2012; 1:1189-1190.

**90.** Li H and Pauza CD. Rapamycin increases the yield and effector function of human gammadelta T cells stimulated in vitro. Cancer immunology, immunotherapy : CII. 2011; 60:361-370.

**91.** Putz EM, Schuster C and Sexl V. Bcl-2: Live and let die. Oncoimmunology. 2012; 1:749-750.

**92.** Anderson J, Gustafsson K, Himoudi N, Yan M and Heuijerjans J. Licensing of gammadeltaT cells for professional antigen presentation: A new role for antibodies in regulation of antitumor immune responses. Oncoimmunology. 2012; 1:1652-1654.

**93.** Gertner-Dardenne J, Fauriat C, Vey N and Olive D. Immunotherapy of acute myeloid leukemia based on gammadelta T cells. Oncoimmunology. 2012; 1:1614-1616.

**94.** Hudes G, Carducci M, Tomczak P, Dutcher J, Figlin R, Kapoor A, Staroslawska E, Sosman J, McDermott D, Bodrogi I, Kovacevic Z, Lesovoy V, Schmidt-Wolf IG, et al. Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma. The New England journal of medicine. 2007; 356:2271-2281.

**95.** Zhao N, Li X, He X, Qiu Y, Zhu L and Qi F. Interleukin-15 gene therapy and the mammalian target of rapamycin inhibitor everolimus inhibit the growth of metastatic breast cancer. The journal of gene medicine. 2013; 15:366-374.

**96.** Wang Y, Wang XY, Subjeck JR, Shrikant PA and Kim HL. Temsirolimus, an mTOR inhibitor, enhances anti-tumour effects of heat shock protein cancer vaccines. British journal of cancer. 2011; 104:643-652.

**97.** Vacchelli E, Martins I, Eggermont A, Fridman WH, Galon J, Sautes-Fridman C, Tartour E, Zitvogel L, Kroemer G and Galluzzi L. Trial watch: Peptide vaccines in cancer therapy. Oncoimmunology. 2012; 1:1557-1576.

**98.** Metz R, Rust S, Duhadaway JB, Mautino MR, Munn DH, Vahanian NN, Link CJ and Prendergast GC. IDO inhibits a tryptophan sufficiency signal that stimulates mTOR: A novel IDO effector pathway targeted by D-1-methyl-tryptophan. Oncoimmunology. 2012; 1:1460-1468.

**99.** Araki K, Youngblood B and Ahmed R. The role of mTOR in memory CD8 T-cell differentiation. Immunological reviews. 2010; 235:234-243.

**100.** Hoos A, Janetzki S and Britten CM. Advancing the field of cancer immunotherapy: MIATA consensus guidelines become available to improve data reporting and interpretation for T-cell immune monitoring. Oncoimmunology. 2012; 1:1457-1459.

**101.** Senovilla L, Vacchelli E, Galon J, Adjemian S, Eggermont A, Fridman WH, Sautes-Fridman C, Ma Y, Tartour E, Zitvogel L, Kroemer G and Galluzzi L. Trial watch: Prognostic and predictive value of the immune infiltrate in cancer. Oncoimmunology. 2012; 1:1323-1343.

**102.** Malvicini M, Alaniz L, Bayo J, Garcia M, Piccioni F, Fiore E, Atorrasagasti C, Aquino JB, Matar P and Mazzolini G. Single low-dose cyclophosphamide combined with interleukin-12 gene

therapy is superior to a metronomic schedule in inducing immunity against colorectal carcinoma in mice. Oncoimmunology. 2012; 1:1038-1047.

**103.** Galluzzi L, Senovilla L, Zitvogel L and Kroemer G. The secret ally: immunostimulation by anticancer drugs. Nature reviews Drug discovery. 2012; 11:215-233.

**104.** Egilmez NK, Harden JL and Rowswell-Turner RB. Chemoimmunotherapy as long-term maintenance therapy for cancer. Oncoimmunology. 2012; 1:563-565.

**105.** Vogelbacher R, Wittmann S, Braun A, Daniel C and Hugo C. The mTOR inhibitor everolimus induces proteinuria and renal deterioration in the remnant kidney model in the rat. Transplantation. 2007; 84:1492-1499.

**106.** Minhajuddin M, Fazal F, Bijli KM, Amin MR and Rahman A. Inhibition of mammalian target of rapamycin potentiates thrombin-induced intercellular adhesion molecule-1 expression by accelerating and stabilizing NF-kappa B activation in endothelial cells. Journal of immunology. 2005; 174:5823-5829.

**107.** McKenna GJ, Trotter JF, Klintmalm E, Onaca N, Ruiz R, Jennings LW, Neri M, O'Leary JG, Davis GL, Levy MF, Goldstein RM and Klintmalm GB. Limiting hepatitis C virus progression in liver transplant recipients using sirolimus-based immunosuppression. American journal of transplantation: official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2011; 11:2379-2387.

**108.** Raymond E, Alexandre J, Faivre S, Vera K, Materman E, Boni J, Leister C, Korth-Bradley J, Hanauske A and Armand JP. Safety and pharmacokinetics of escalated doses of weekly intravenous infusion of CCI-779, a novel mTOR inhibitor, in patients with cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2004; 22:2336-2347.

Research Perspective

# Rapamycin extends life- and health span because it slows aging

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**Abstract: Making headlines, a thought-provocative paper by Neff, Ehninger and coworkers claims that rapamycin extends life span but has limited effects on aging. How is that possibly possible? And what is aging if not an increase of the probability of death with age. I discuss that the JCI paper actually shows that rapamycin slows aging and also extends lifespan regardless of its direct anti-cancer activities. Aging is, in part, MTOR-driven: a purposeless continuation of developmental growth. Rapamycin affects the same processes in young and old animals: young animals' traits and phenotypes, which continuations become hyperfunctional, harmful and lethal later in life.**

Found by chance on the mystical Easter island [1], the anti-aging drug rapamycin gave birth to numerous myths. This time, it is claimed that rapamycin prolongs lifespan and prevents aging-associated changes by aging-independent mechanisms, not by affecting aging itself [2]. But what is then aging itself.

## What is aging?

Aging is an exponential increase of the probability of death with age [3]. No one has died from health or without a cause. Most elderly humans die from age-related diseases [4-10], which are also called "natural causes", if a precise diagnosis is unnecessary. In mammals, death from aging means death from age-related diseases. Not only humans and other mammals but also aging worms and flies die from pathologies [11-27].

## Diseases are biomarkers of aging

Age-related diseases are biomarkers of aging [8]. The most common are cardiovascular diseases (associated with atherosclerosis, hypertension and cardiac hypertrophy), cancer, diabetes (and other complications of metabolic syndrome), Alzheimer and Parkinson diseases, macular degeneration and so on. Many manifestations of aging are not considered as diseases because they develop in everyone (e.g. female menopause). The distinction is arbitrarily. For example, cancer-prone transgenic mice can exclusively die from cancer but still cancer is a disease. But many alterations, although associated with age, are not involved in aging. And these phenotypes are not affected by rapamycin.

## Cellular aging

Unless we believe in vitalism, organismal aging should be linked to cellular aging. Cellular aging is, in part, MTOR-dependent process. The MTOR (mechanistic or mammalian target of rapamycin) pathway is activated by growth factors, hormones (such as insulin and testosterone), nutrients, oxygen and some conditions such as obesity [28-38]. Figuratively, MTOR is a "molecular hypothalamus", a sensing pathway in every cell [39]. In turn, MTOR stimulates specific functions of differentiated cells and cellular mass growth. In proliferating cells, growth is balanced by division. In resting cells, active MTOR causes cellular hypertrophy, hyperfunctions (such as hypersecretion). MTOR-driven geroconversion is a conversion from quiescence to senescence [40-59]. And cellular senescence is characterized by increased cell-type-specific cellular functions (hyperfunctions), altering homeostasis and leading to age-related diseases [9].

## Systemic hyperfunctions and aging

Except of terminal stages of age-related diseases, aging

is associated with systemic hyperfunctions: increased blood pressure (hypertension), increased platelet aggregation (hyper-aggregation), hyper-contractility of arterial smooth muscle cells, hyper-coagulation, hyperlipidemia, hyperglycemia, hyperinsulinemia, increased resistance to hormones, pro-inflammatory conditions, organ hypertrophy, fibrosis and hyperplasia. These hyperfunctions are damaging to the organs and, when damage occurs, then some functions are lost. So only late stages of aging are decline and loss of functions. Terminal stages are MTOR-independent and will not be reversed by rapamycin. For example, hyperfunctional osteoclasts cause osteoporosis, leading to a broken bone and a sequence of events (immobilization, pneumonia, etc), which require standard medical interventions, not anti-aging drugs [60]. Not only in mammals, but also in *C elegans* and *Drosophila*, life-limiting pathologies are caused of exacerbated and intensified normal processes and functions [19, 25, 61, 62].

**Aging processes do not spring from nothing. They are continuations of normal cellular, tissue, organ and system functions in young animals. Unless miracle is possible, rapamycin must affect the same processes in old and young animals. And it does.**

### Aging is a quasi-program (not a program)

Why systemic hyperfunctions arise? Aging is an unintended continuation of organismal growth, like cellular senescence is a continuation of cellular growth [63]. In other words, aging is a quasi-program (not a program): an unintended and purposeless continuation of developmental programs, which are not switched off upon their completion [64-67], causing age-related diseases. For example, blood pressure is increased from birth to adulthood and continuation of this trend leads to hypertension. Menopause is a hyperfunctional continuation of reproductive program [68]. Aging-associated pathologies are continuation of normal functions of the young organism. Therefore, rapamycin must affect the same processes in young and old animals, because aging is a continuation of normal functions. Aging processes do not spring from nothing. They are continuations of normal cellular, tissue, organ and system functions in young animals.

**Rapamycin extends life span independently of its anti-cancer effect and prevents cancer by slowing down aging.**

### Cancer and aging

Cancer is an aging-related disease and interventions that slow aging (e.g. calorie restriction) delay cancer [69-78]. Furthermore, compared with calorie restriction, rapamycin stronger inhibits MTOR. It is predictable that if rapamycin slows aging, it should delay cancer [79, 80]. Studies support these predictions [81-84] and rapamycin extended lifespan and delayed cancer, even when calorie restriction did not [85]. Although rapamycin is a potent cancer-preventive agent, it is only modestly effective for cancer treatment. Rapalogs are most effective in drug combinations [86-93]. They also may decrease side effects by suppressing senescence of normal cells [51, 58, 59, 94, 95]. Also, senescence of normal cells creates cancer-promoting micro-environment [96-103]. If rapamycin indeed prevents cancer by slowing aging (not by killing cancer cells), the prevention must be started *before* cancer is initiated. In other words, if rapamycin treatment is started too late in life, then its anti-cancer effect will be blunted. This was shown in cancer-prone p53+/- mice [104]. The same was shown by Neff et al: rapamycin rapamycin did not prevent cancer when the treatment was started at middle and old age [2]. Thus, the JCI study confirms the notion that rapamycin delays cancer by slowing aging (see also discussion here in the last section). Anti-cancer effects simply cannot be responsible for life extension by rapamycin. First, effective anti-cancer drugs that are curative in lymphomas, testicular and ovarian cancers (methotrexate, cisplatin, paclitaxel) would greatly shorten murine lifespan, especially when started in young age. Even further, typical anti-cancer drugs accelerate cancer. For example, radiation (a classic anti-cancer intervention) dramatically accelerates cancer in p53+/- mice and shortens life span [105-109]. And anti-cancer drugs cause secondary cancers in patients. In contrast, not only rapamycin extends lifespan, it is the only known drug that extends life span consistently. Second, apart from cancer-prone strains of mice, cancer is not the main cause of death in most animals. MTOR is involved in most age-related diseases and rapamycin prevents them in mammals [64, 110-123] and slows down aging [81, 124-127]. Finally, yeast, worm and flies do not die from cancer and still inhibition of the MTOR pathway extends lifespan [128-137].

### Inhibition of TOR slows aging: converging evidence [124]

1. Rapamycin suppresses geroconversion: conversion from cellular quiescence to senescence. Geroconversion is cellular basis of organismal aging
2. Genetic manipulations that inhibit the TOR pathway extend life-span in diverse species from yeast to mammals

**3.** Rapamycin extends lifespan in all species tested
**4.** Calorie restriction, which inhibits MTOR, extends lifespan
**5.** MTOR is involved in diseases of aging and rapamycin prevents these diseases in animal models

### Rapamycin slows aging: the JCI paper [2]

How does the Neff et al study support the model of quasi-programmed aging?

**1.** As shown by Neff *et al*, chronic administration of rapamycin extends lifespan in male C57BL/6J mice, when started at both young and old age. Note:
This extension is impressive given that (a) effects of rapamycin in male mice are blunted compared with female mice in previous studies, (b) C57BL/6J mice are intrinsically long-lived and (c) rapamycin was administrated in everyday schedule (chronic or immunosuppressive schedule) instead of intermittent or pulse administration (anti-aging schedule).
**2.** C57BL/6J mice are refractory to many tumors
http://jaxmice.jax.org/strain/000664.html
Therefore, life extension is difficult to explain by anti-cancer effects of rapamycin.
**3.** In fact, rapamycin did not prevent cancer when the treatment was started at middle and old age, but still extended life span. As stated by Neff *et al* [2]: "Rapamycin … had no measurable effect in the 25-month cohort (vehicle, 1 of 5; rapamycin, 2 of 8; P = 1.0, Fisher exact test) or the 34-month cohort (vehicle, 1 of 5; rapamycin, 3 of 10; P = 1.0, Fisher exact test)." As we discussed here, this indicates that effects of rapamycin are probably due to suppression of aging. Rapamycin treatment decreased cancer incidence only when it was started in young mice.
**4.** Rapamycin counteracted certain aging-related alterations in both young and old mice. This suggests that aging is a continuation of normal traits in young organisms. Aging is driven by intensified and exacerbated normal cellular functions.
**5.** Rapamycin did not affect many parameters that are not aging-specific such as alterations in plasma sodium, calcium and chloride concentrations. This is expectable. Aging is not associated with alterations of electrolyte homeostasis. These alterations are terminal phases of medical conditions due to organ (e.g. renal) failure.
**6.** Some age-related alterations actually counteract aging. For example, although RNA/protein synthesis is decreased with aging in model organisms, yet its further inhibition prolongs life span further [138-141]. As shown by Neff et al, rapamycin did not prevent *anti-aging* alterations such as a decrease in testosterone levels. Noteworthy, testosterone activates mTOR.
**7.** Some trends reported by Neff et al are not typical for aging. For example, while Neff reported a decrease in blood glucose and lipids with age, these parameters tend to increase with age, especially when age-related diseases develop. Perhaps mice with hyperglycemia and hyperlipidemia died during the study, while only surviving (the healthiest) mice were examined at the end of the study.

## REFERENCES


**1.** Stipp D. A new path to longevity. Sci Am. 2012; 306: 32-39.
**2.** Neff F, Flores-Dominguez D, Ryan DP, Horsch M, Schroder S, Adler T, Afonso LC, Aguilar-Pimentel JA, Becker L, Garrett L, Hans W, Hettich MM, Holtmeier R, Holter SM, Moreth K, Prehn C *et al*. Rapamycin extends murine lifespan but has limited effects on aging. J Clin Invest. 2013.
**3.** Olshansky SJ, Passaro DJ, Hershow RC, Layden J, Carnes BA, Brody J, Hayflick L, Butler RN, Allison DB, Ludwig DS. A potential decline in life expectancy in the United States in the 21st century. N Engl J Med. 2005; 352: 1138-1145.
**4.** Horiuchi S, Wilmoth JR. Age patterns of the life table aging rate for major causes of death in Japan, 1951-1990. J Gerontol A Biol Sci Med Sci. 1997; 52: B67-77.
**5.** Sonnenschein E, Brody JA. Effect of population aging on proportionate mortality from heart disease and cancer, U.S. 2000-2050. J Gerontol B Psychol Sci Soc Sci. 2005; 60: S110-112.
**6.** Jemal A, Ward E, Hao Y, Thun M. Trends in the leading causes of death in the United States, 1970-2002. Jama. 2005; 294: 1255-1259.
**7.** Gorina Y, Lentzner H. Multiple causes of death in old age. Aging Trends. 2008: 1-9.
**8.** Blagosklonny MV. Validation of anti-aging drugs by treating age-related diseases. Aging (Albany NY). 2009; 1: 281-288.
**9.** Blagosklonny MV. Prospective treatment of age-related diseases by slowing down aging. Am J Pathol. 2012; 181: 1142-1146.
**10.** Fontana L, Partridge L, Longo VD. Extending healthy life span--from yeast to humans. Science. 2010; 328: 321-326.
**11.** Partridge L, Gems D. Mechanisms of ageing: public or private? Nat Rev Genet. 2002; 3: 165-175.
**12.** Michno K, van de Hoef D, Wu H, Boulianne GL. Modeling age-related diseases in Drosophila: can this fly? Curr Top Dev Biol. 2005; 71: 199-223.
**13.** Birse RT, Choi J, Reardon K, Rodriguez J, Graham S, Diop S, Ocorr K, Bodmer R, Oldham S. High-fat-diet-induced obesity and heart dysfunction are regulated by the TOR pathway in Drosophila. Cell Metab. 2010; 12: 533-544.
**14.** Musselman LP, Fink JL, Narzinski K, Ramachandran PV, Hathiramani SS, Cagan RL, Baranski TJ. A high-sugar diet produces obesity and insulin resistance in wild-type Drosophila. Dis Model Mech. 2011; 4: 842-849.
**15.** Teleman AA, Ratzenbock I, Oldham S. Drosophila: a model for understanding obesity and diabetic complications. Exp Clin Endocrinol Diabetes. 2012; 120: 184-185.
**16.** Kumar S, Walter J. Phosphorylation of amyloid beta (Abeta) peptides - a trigger for formation of toxic aggregates in Alzheimer's disease. Aging (Albany NY). 2011; 3: 803-812.
**17.** Sarkar S, Ravikumar B, Floto RA, Rubinsztein DC. Rapamycin and mTOR-independent autophagy inducers ameliorate toxicity of polyglutamine-expanded huntingtin and related

proteinopathies. Cell Death Differ. 2009; 16: 46-56.
**18.** Brignull HR, Morley JF, Garcia SM, Morimoto RI. Modeling polyglutamine pathogenesis in C. elegans. Methods Enzymol. 2006; 412: 256-282.
**19.** Rera M, Clark RI, Walker DW. Intestinal barrier dysfunction links metabolic and inflammatory markers of aging to death in Drosophila. Proc Natl Acad Sci U S A. 2012; 109: 21528-21533.
**20.** Wessells RJ, Fitzgerald E, Cypser JR, Tatar M, Bodmer R. Insulin regulation of heart function in aging fruit flies. Nat Genet. 2004; 36: 1275-1281.
**21.** Stefanatos R, Sriram A, Kiviranta E, Mohan A, Ayala V, Jacobs HT, Pamplona R, Sanz A. dj-1beta regulates oxidative stress, insulin-like signaling and development in Drosophila melanogaster. Cell Cycle. 2012; 11: 3876-3886.
**22.** Shmookler Reis RJ, Xu L, Lee H, Chae M, Thaden JJ, Bharill P, Tazearslan C, Siegel E, Alla R, Zimniak P, Ayyadevara S. Modulation of lipid biosynthesis contributes to stress resistance and longevity of C. elegans mutants. Aging (Albany NY). 2011; 3: 125-147.
**23.** Walker JA, Bernards A. Drosophila melanogaster neurofibromatosis-1: ROS, not Ras? Nat Genet. 2007; 39: 443-445.
**24.** Lee JH, Budanov AV, Park EJ, Birse R, Kim TE, Perkins GA, Ocorr K, Ellisman MH, Bodmer R, Bier E, Karin M. Sestrin as a feedback inhibitor of TOR that prevents age-related pathologies. Science. 2010; 327: 1223-1228.
**25.** Biteau B, Hochmuth CE, Jasper H. JNK activity in somatic stem cells causes loss of tissue homeostasis in the aging Drosophila gut. Cell Stem Cell. 2008; 3: 442-455.
**26.** McGee MD, Day N, Graham J, Melov S. cep-1/p53-dependent dysplastic pathology of the aging C. elegans gonad. Aging (Albany NY). 2012; 4: 256-269.
**27.** Banerjee KK, Ayyub C, Sengupta S, Kolthur-Seetharam U. dSir2 deficiency in the fatbody, but not muscles, affects systemic insulin signaling, fat mobilization and starvation survival in flies. Aging (Albany NY). 2012; 4: 206-223.
**28.** Loewith R, Hall MN. Target of rapamycin (TOR) in nutrient signaling and growth control. Genetics. 2011; 189: 1177-1201.
**29.** Sengupta S, Peterson TR, Sabatini DM. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Mol Cell. 2010; 40: 310-322.
**30.** Oh WJ, Jacinto E. mTOR complex 2 signaling and functions. Cell Cycle. 2011; 10: 2305-2316.
**31.** Duran RV, Hall MN. Glutaminolysis feeds mTORC1. Cell Cycle. 2012; 11: 4107-4108.
**32.** Conn CS, Qian SB. mTOR signaling in protein homeostasis: less is more? Cell Cycle. 2011; 10: 1940-1947.
**33.** Cornu M, Albert V, Hall MN. mTOR in aging, metabolism, and cancer. Curr Opin Genet Dev. 2013; 23: 53-62.
**34.** Kim S, Snyder SH. Nutrient amino acids signal to mTOR via inositol polyphosphate multikinase. Cell Cycle. 2011; 10: 1708-1710.
**35.** Feng Z, Levine AJ. The regulation of energy metabolism and the IGF-1/mTOR pathways by the p53 protein. Trends Cell Biol. 2010; 20: 427-434.
**36.** Leontieva OV, Blagosklonny MV. Hypoxia and gerosuppression: the mTOR saga continues. Cell Cycle. 2012; 11: 3926-3931.
**37.** Wang CY, Kim HH, Hiroi Y, Sawada N, Salomone S, Benjamin LE, Walsh K, Moskowitz MA, Liao JK. Obesity increases vascular senescence and susceptibility to ischemic injury through chronic activation of Akt and mTOR. Sci Signal. 2009; 2: ra11.
**38.** Weber JD, Gutmann DH. Deconvoluting mTOR biology. Cell Cycle. 2012; 11: 236-248.
**39.** Blagosklonny MV. M(o)TOR of aging: MTOR as a universal molecular hypothalamus. Aging (Albany NY). 2013; 5: 490-494.
**40.** Blagosklonny MV. Cell senescence and hypermitogenic arrest. EMBO Rep. 2003; 4: 358-362.
**41.** Demidenko ZN, Blagosklonny MV. Growth stimulation leads to cellular senescence when the cell cycle is blocked. Cell Cycle. 2008; 7: 3355-3361.
**42.** Demidenko ZN, Zubova SG, Bukreeva EI, Pospelov VA, Pospelova TV, Blagosklonny MV. Rapamycin decelerates cellular senescence. Cell Cycle. 2009; 8: 1888-1895.
**43.** Demidenko ZN, Korotchkina LG, Gudkov AV, Blagosklonny MV. Paradoxical suppression of cellular senescence by p53. Proc Natl Acad Sci U S A. 2010; 107: 9660-9664.
**44.** Korotchkina LG, Leontieva OV, Bukreeva EI, Demidenko ZN, Gudkov AV, Blagosklonny MV. The choice between p53-induced senescence and quiescence is determined in part by the mTOR pathway. Aging (Albany NY). 2010; 2: 344-352.
**45.** Leontieva OV, Blagosklonny MV. DNA damaging agents and p53 do not cause senescence in quiescent cells, while consecutive re-activation of mTOR is associated with conversion to senescence. Aging (Albany NY). 2010; 2: 924-935.
**46.** Leontieva OV, Natarajan V, Demidenko ZN, Burdelya LG, Gudkov AV, Blagosklonny MV. Hypoxia suppresses conversion from proliferative arrest to cellular senescence. Proc Natl Acad Sci U S A. 2012; 109: 13314-13318.
**47.** Leontieva OV, Lenzo F, Demidenko ZN, Blagosklonny MV. Hyper-mitogenic drive coexists with mitotic incompetence in senescent cells. Cell Cycle. 2012; 11: 4642 - 4649.
**48.** Cho S, Hwang ES. Status of mTOR activity may phenotypically differentiate senescence and quiescence. Mol Cells. 2012; 33: 597-604.
**49.** Pani G. From growing to secreting: new roles for mTOR in aging cells. Cell Cycle. 2011; 10: 2450-2453.
**50.** Kolesnichenko M, Hong L, Liao R, Vogt PK, Sun P. Attenuation of TORC1 signaling delays replicative and oncogenic RAS-induced senescence. Cell Cycle. 2012; 11: 2391-2401.
**51.** Mercier I, Camacho J, Titchen K, Gonzales DM, Quann K, Bryant KG, Molchansky A, Milliman JN, Whitaker-Menezes D, Sotgia F, Jasmin JF, Schwarting R, Pestell RG, Blagosklonny MV, Lisanti MP. Caveolin-1 and accelerated host aging in the breast tumor microenvironment: chemoprevention with rapamycin, an mTOR inhibitor and anti-aging drug. Am J Pathol. 2012; 181: 278-293.
**52.** Halicka HD, Zhao H, Li J, Lee YS, Hsieh TC, Wu JM, Darzynkiewicz Z. Potential anti-aging agents suppress the level of constitutive mTOR- and DNA damage- signaling. Aging (Albany NY). 2012; 4: 952-965.
**53.** Denchi EL. A critical role for TORC1 in cellular senescence. Cell Cycle. 2012; 11: 2976.
**54.** Menendez JA, Vellon L, Oliveras-Ferraros C, Cufi S, Vazquez-Martin A. mTOR-regulated senescence and autophagy during reprogramming of somatic cells to pluripotency: a roadmap from energy metabolism to stem cell renewal and aging. Cell Cycle. 2011; 10: 3658-3677.
**55.** Serrano M. Dissecting the role of mTOR complexes in cellular senescence. Cell Cycle. 2012; 11: 2231-2232.
**56.** Gan B, DePinho RA. mTORC1 signaling governs hematopoietic stem cell quiescence. Cell Cycle. 2009; 8: 1003-

1006.

**57.** Chen C, Liu Y, Zheng P. mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells. Sci Signal. 2009; 2: ra75.

**58.** Iglesias-Bartolome R, Patel V, Cotrim A, Leelahavanichkul K, Molinolo AA, Mitchell JB, Gutkind JS. mTOR inhibition prevents epithelial stem cell senescence and protects from radiation-induced mucositis. Cell Stem Cell. 2012; 11: 401-414.

**59.** Iglesias-Bartolome R, Gutkind SJ. Exploiting the mTOR paradox for disease prevention. Oncotarget. 2012; 3: 1061-1063.

**60.** Blagosklonny MV. How to save Medicare: the anti-aging remedy. Aging (Albany NY). 2012; 4: 547-552.

**61.** Gems D, Partridge L. Genetics of Longevity in Model Organisms: Debates and Paradigm Shifts. Annu Rev Physiol. 2013; 75: 621-644

**62.** Gems DH, de la Guardia YI. Alternative Perspectives on Aging in C. elegans: Reactive Oxygen Species or Hyperfunction? Antioxid Redox Signal. 2013; 19: 321-329

**63.** Blagosklonny MV, Hall MN. Growth and aging: a common molecular mechanism. Aging (Albany NY). 2009; 1: 357-362.

**64.** Blagosklonny MV. Aging and immortality: quasi-programmed senescence and its pharmacologic inhibition. Cell Cycle. 2006; 5: 2087-2102.

**65.** Blagosklonny MV. mTOR-driven aging: speeding car without brakes. Cell Cycle. 2009; 8: 4055-4059.

**66.** Blagosklonny MV. Revisiting the antagonistic pleiotropy theory of aging: TOR-driven program and quasi-program. Cell Cycle. 2010; 9: 3151-3156.

**67.** Blagosklonny M. Answering the ultimate question "What is the Proximal Cause of Aging?" Aging (Albany NY). 2012; 4: 861-877.

**68.** Blagosklonny MV. Why men age faster but reproduce longer than women: mTOR and evolutionary perspectives. Aging (Albany NY). 2010; 2: 265-273.

**69.** Yancik R. Population aging and cancer: a cross-national concern. Cancer J. 2005; 11: 437-441.

**70.** Ikeno Y, Bronson RT, Hubbard GB, Lee S, Bartke A. Delayed occurrence of fatal neoplastic diseases in ames dwarf mice: correlation to extended longevity. J Gerontol A Biol Sci Med Sci. 2003; 58: 291-296.

**71.** Berrigan D, Perkins SN, Haines DC, Hursting SD. Adult-onset calorie restriction and fasting delay spontaneous tumorigenesis in p53-deficient mice. Carcinogenesis. 2002; 23: 817-822.

**72.** Ikeno Y, Hubbard GB, Lee S, Cortez LA, Lew CM, Webb CR, Berryman DE, List EO, Kopchick JJ, Bartke A. Reduced incidence and delayed occurrence of fatal neoplastic diseases in growth hormone receptor/binding protein knockout mice. J Gerontol A Biol Sci Med Sci. 2009; 64: 522-529.

**73.** Anisimov VN, Berstein LM, Popovich IG, Zabezhinski MA, Egormin PA, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Kovalenko IG, Poroshina TE. If started early in life, metformin treatment increases life span and postpones tumors in female SHR mice. Aging (Albany NY). 2011; 3: 148-157.

**74.** Pinkston JM, Garigan D, Hansen M, Kenyon C. Mutations that increase the life span of C. elegans inhibit tumor growth. Science. 2006; 313: 971-975.

**75.** Buffenstein R. The naked mole-rat: a new long-living model for human aging research. J Gerontol A Biol Sci Med Sci. 2005; 60: 1369-1377.

**76.** Kim EB, Fang X, Fushan AA, Huang Z, Lobanov AV, Han L, Marino SM, Sun X, Turanov AA, Yang P, Yim SH, Zhao X, Kasaikina MV, Stoletzki N, Peng C, Polak P *et al*. Genome sequencing reveals insights into physiology and longevity of the naked mole rat. Nature. 2011; 479: 223-227.

**77.** Berman AE, Leontieva OV, Natarajan V, McCubrey JA, Demidenko ZN, Nikiforov MA. Recent progress in genetics of aging, senescence and longevity: focusing on cancer-related genes. Oncotarget. 2012; 3: 1522-1532.

**78.** Borras C, Monleon D, Lopez-Grueso R, Gambini J, Orlando L, Pallardo FV, Santos E, Vina J, Font de Mora J. RasGrf1 deficiency delays aging in mice. Aging (Albany NY). 2011; 3: 262-276.

**79.** Blagosklonny MV. Prevention of cancer by inhibiting aging. Cancer Biol Ther. 2008; 7: 1520-1524.

**80.** Blagosklonny MV. Rapalogs in cancer prevention: Anti-aging or anticancer? Cancer Biol Ther. 2012; 13: 1349-1354.

**81.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandezr E, Miller RA. Rapamycin fed late in life extends lifespan in genetically heterogenous mice. Nature. 2009; 460: 392-396.

**82.** Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Rosenfeld SV, Blagosklonny MV. Rapamycin increases lifespan and inhibits spontaneous tumorigenesis in inbred female mice. Cell Cycle. 2011; 10: 4230-4236.

**83.** Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Antoch MP, Blagosklonny MV. Rapamycin extends maximal lifespan in cancer-prone mice. Am J Pathol. 2010; 176: 2092-2097.

**84.** Selman C, Partridge L. A double whammy for aging? Rapamycin extends lifespan and inhibits cancer in inbred female mice. Cell Cycle. 2012; 11: 17-18.

**85.** Livi CB, Hardman RL, Christy BA, Dodds SG, Jones D, Williams C, Strong R, Bokov A, Javors MA, Ikeno Y, Hubbard G, Hasty P, Sharp ZD. Rapamycin extends life span of Rb1+/- mice by inhibiting neuroendocrine tumors. Aging (Albany NY). 2013; 5: 100-110.

**86.** Chiarini F, Lonetti A, Teti G, Orsini E, Bressanin D, Cappellini A, Ricci F, Tazzari PL, Ognibene A, Falconi M, Pagliaro P, Iacobucci I, Martinelli G, Amadori S, McCubrey JA, Martelli AM. A combination of temsirolimus, an allosteric mTOR inhibitor, with clofarabine as a new therapeutic option for patients with acute myeloid leukemia. Oncotarget. 2012; 3: 1615-1628.

**87.** Garrett JT, Chakrabarty A, Arteaga CL. Will PI3K pathway inhibitors be effective as single agents in patients with cancer? Oncotarget. 2011; 2: 1314-1321.

**88.** Zhang Y, Zheng XF. mTOR-independent 4E-BP1 phosphorylation is associated with cancer resistance to mTOR kinase inhibitors. Cell Cycle. 2012; 11: 594-603.

**89.** Kurmasheva RT, Dudkin L, Billups C, Debelenko LV, Morton CL, Houghton PJ. The insulin-like growth factor-1 receptor-targeting antibody, CP-751,871, suppresses tumor-derived VEGF and synergizes with rapamycin in models of childhood sarcoma. Cancer Res. 2009; 69: 7662-7671.

**90.** Martelli AM, Chiarini F, Evangelisti C, Cappellini A, Buontempo F, Bressanin D, Fini M, McCubrey JA. Two hits are better than one: targeting both phosphatidylinositol 3-kinase and mammalian target of rapamycin as a therapeutic strategy for acute leukemia treatment. Oncotarget. 2012; 3: 371-394.

**91.** McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Franklin RA, Montalto G, Cervello M, Libra M, Candido S, Malaponte G, Mazzarino MC, Fagone P, Nicoletti F, Basecke J, Mijatovic S,

Maksimovic-Ivanic D *et al*. Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR cascade inhibitors: how mutations can result in therapy resistance and how to overcome resistance. Oncotarget. 2012; 3: 1068-1111.

**92.** Steelman LS, Navolanic P, Chappell WH, Abrams SL, Wong EW, Martelli AM, Cocco L, Stivala F, Libra M, Nicoletti F, Drobot LB, Franklin RA, McCubrey JA. Involvement of Akt and mTOR in chemotherapeutic- and hormonal-based drug resistance and response to radiation in breast cancer cells. Cell Cycle. 2011; 10: 3003-3015.

**93.** Bressanin D, Evangelisti C, Ricci F, Tabellini G, Chiarini F, Tazzari PL, Melchionda F, Buontempo F, Pagliaro P, Pession A, McCubrey JA, Martelli AM. Harnessing the PI3K/Akt/mTOR pathway in T-cell acute lymphoblastic leukemia: eliminating activity by targeting at different levels. Oncotarget. 2012; 3: 811-823.

**94.** Blagosklonny MV. The power of chemotherapeutic engineering: arresting cell cycle and suppressing senescence to protect from mitotic inhibitors. Cell Cycle. 2011; 10: 2295-2298.

**95.** Apontes P, Leontieva OV, Demidenko ZN, Li F, Blagosklonny MV. Exploring long-term protection of normal human fibroblasts and epithelial cells from chemotherapy in cell culture. Oncotarget. 2011; 2: 222-233.

**96.** Liu D, Hornsby PJ. Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. Cancer Res. 2007; 67: 3117-3126.

**97.** Pasciu D, Montisci S, Greco M, Doratiotto S, Pitzalis S, Pani P, Laconi S, Laconi E. Aging is associated with increased clonogenic potential in rat liver in vivo. Aging Cell. 2006; 5: 373-377.

**98.** Caruso C, Lio D, Cavallone L, Franceschi C. Aging, longevity, inflammation, and cancer. Ann N Y Acad Sci. 2004; 1028: 1-13.

**99.** Bavik C, Coleman I, Dean JP, Knudsen B, Plymate S, Nelson PS. The gene expression program of prostate fibroblast senescence modulates neoplastic epithelial cell proliferation through paracrine mechanisms. Cancer Res. 2006; 66: 794-802.

**100.** Campisi J. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell. 2005; 120: 513-522.

**101.** Krtolica A, Parrinello S, Lockett S, Desprez PY, Campisi J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. Proc Natl Acad Sci U S A. 2001; 98: 12072-12077.

**102.** Parrinello S, Coppe JP, Krtolica A, Campisi J. Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. J Cell Sci. 2005; 118: 485-496.

**103.** Henry CJ, Marusyk A, DeGregori J. Aging-associated changes in hematopoiesis and leukemogenesis: what's the connection? Aging (Albany NY). 2011; 3: 643-656.

**104.** Komarova EA, Antoch MP, Novototskaya LR, Chernova OB, Paszkiewicz G, Leontieva OV, Blagosklonny MV, Gudkov AV. Rapamycin extends lifespan and delays tumorigenesis in heterozygous p53+/- mice. Aging (Albany NY). 2012; 4: 709-714.

**105.** Kemp CJ, Wheldon T, Balmain A. p53-deficient mice are extremely susceptible to radiation-induced tumorigenesis. Nat Genet. 1994; 8: 66-69.

**106.** Kony SJ, de Vathaire F, Chompret A, Shamsaldim A, Grimaud E, Raquin MA, Oberlin O, Brugieres L, Feunteun J, Eschwege F, Chavaudra J, Lemerle J, Bonaiti-Pellie C. Radiation and genetic factors in the risk of second malignant neoplasms after a first cancer in childhood. Lancet. 1997; 350: 91-95.

**107.** Venkatachalam S, Shi YP, Jones SN, Vogel H, Bradley A, Pinkel D, Donehower LA. Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. Embo J. 1998; 17: 4657-4667.

**108.** Lee JM, Abrahamson JL, Kandel R, Donehower LA, Bernstein A. Susceptibility to radiation-carcinogenesis and accumulation of chromosomal breakage in p53 deficient mice. Oncogene. 1994; 9: 3731-3736.

**109.** Leonova KI, Shneyder J, Antoch MP, Toshkov IA, Novototskaya LR, Komarov PG, Komarova EA, Gudkov AV. A small molecule inhibitor of p53 stimulates amplification of hematopoietic stem cells but does not promote tumor development in mice. Cell Cycle. 2010; 9: 1434-1443.

**110.** Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, Scaravilli F, Easton DF, Duden R, O'Kane CJ, Rubinsztein DC. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat Genet. 2004; 36: 585-595.

**111.** Mueller MA, Beutner F, Teupser D, Ceglarek U, Thiery J. Prevention of atherosclerosis by the mTOR inhibitor everolimus in LDLR-/- mice despite severe hypercholesterolemia. Atherosclerosis. 2008; 198: 39-48.

**112.** Tsang CK, Qi H, Liu LF, Zheng XFS. Targeting mammalian target of rapamycin (mTOR) for health and diseases. Drug Disc Today. 2007; 12: 112-124.

**113.** Blagosklonny MV. An anti-aging drug today: from senescence-promoting genes to anti-aging pill. Drug Disc Today. 2007; 12: 218-224.

**114.** Neef M, Ledermann M, Saegesser H, Schneider V, Reichen J. Low-dose oral rapamycin treatment reduces fibrogenesis, improves liver function, and prolongs survival in rats with established liver cirrhosis. J Hepatol. 2006; 45: 786-796.

**115.** Nistala R, Sowers JR, Whaley-Connell A. Over-nutrition contributes to tubulointerstitial fibrosis by targeting nutrient-sensing kinases: role for the mTOR/S6K pathway. Cell Cycle. 2012; 11: 831-832.

**116.** Dazert E, Hall MN. mTOR signaling in disease. Curr Opin Cell Biol. 2011.

**117.** Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol. 2011; 12: 21-35.

**118.** Bove J, Martinez-Vicente M, Vila M. Fighting neurodegeneration with rapamycin: mechanistic insights. Nat Rev Neurosci. 2011; 12: 437-452.

**119.** Flynn JM, O'Leary MN, Zambataro CA, Academia EC, Presley MP, Garrett BJ, Zykovich A, Mooney SD, Strong R, Rosen CJ, Kapahi P, Nelson MD, Kennedy BK, Melov S. Late life rapamycin treatment reverses age-related heart dysfunction. Aging Cell. 2013.

**120.** Kolosova NG, Muraleva NA, Zhdankina AA, Stefanova NA, Fursova AZ, Blagosklonny MV. Prevention of age-related macular degeneration-like retinopathy by rapamycin in rats. Am J Pathol. 2012; 181: 472-477.

**121.** Blagosklonny MV. Once again on rapamycin-induced insulin resistance and longevity: despite of or owing to. Aging (Albany NY). 2012; 4: 350-358.

**122.** Ramos FJ, Chen SC, Garelick MG, Dai DF, Liao CY, Schreiber KH, MacKay VL, An EH, Strong R, Ladiges WC, Rabinovitch PS, Kaeberlein M, Kennedy BK. Rapamycin reverses elevated mTORC1 signaling in lamin A/C-deficient mice, rescues cardiac and skeletal muscle function, and extends survival. Sci Transl Med. 4: 144ra103.

**123.** Passtoors WM, Beekman M, Deelen J, van der Breggen R, Maier AB, Guigas B, Derhovanessian E, van Heemst D, de Craen AJ, Gunn DA, Pawelec G, Slagboom PE. Gene expression analysis of mTOR pathway: association with human longevity. Aging Cell. 2013; 12: 24-31.

**124.** Blagosklonny MV. Rapamycin and quasi-programmed aging: Four years later. Cell Cycle. 2010; 9: 1859-1862.

**125.** Spong A, Bartke A. Rapamycin slows aging in mice. Cell Cycle. 2012; 11: 845.

**126.** Wilkinson JE, Burmeister L, Brooks SV, Chan CC, Friedline S, Harrison DE, Hejtmancik JF, Nadon N, Strong R, Wood LK, Woodward MA, Miller RA. Rapamycin slows aging in mice. Aging Cell. 2012; 11: 675-682.

**127.** Zhang Y, Bokov A, Gelfond J, Soto V, Ikeno Y, Hubbard G, Diaz V, Sloane L, Maslin K, Treaster S, Rendon S, van Remmen H, Ward W, Javors M, Richardson A, Austad SN *et al*. Rapamycin Extends Life and Health in C57BL/6 Mice. J Gerontol A Biol Sci Med Sci. 2013.

**128.** Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S. Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. Curr Biol. 2004; 14: 885-890.

**129.** Kapahi P, Chen D, Rogers AN, Katewa SD, Li PW, Thomas EL, Kockel L. With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. Cell Metab. 2010; 11: 453-465.

**130.** Katewa SD, Kapahi P. Role of TOR signaling in aging and related biological processes in Drosophila melanogaster. Exp Gerontol. 2011; 46: 382-390.

**131.** Moskalev AA, Shaposhnikov MV. Pharmacological Inhibition of Phosphoinositide 3 and TOR Kinases Improves Survival of Drosophila melanogaster. Rejuvenation Res. 2010; 13: 246-247.

**132.** Partridge L, Alic N, Bjedov I, Piper MD. Ageing in Drosophila: the role of the insulin/Igf and TOR signalling network. Exp Gerontol. 2011; 46: 376-381.

**133.** Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, Partridge L. Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. Cell Metab. 2010; 11: 35-46.

**134.** Bjedov I, Partridge L. A longer and healthier life with TOR down-regulation: genetics and drugs. Biochem Soc Trans. 2011; 39: 460-465.

**135.** Johnson SC, Rabinovitch PS, Kaeberlein M. mTOR is a key modulator of ageing and age-related disease. Nature. 2013; 493: 338-345.

**136.** Kaeberlein M. Longevity and aging. F1000Prime Rep. 2013; 5: 5.

**137.** Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell. 153: 1194-1217.

**138.** Pan KZ, Palter JE, Rogers AN, Olsen A, Chen D, Lithgow GJ, Kapahi P. Inhibition of mRNA translation extends lifespan in Caenorhabditis elegans. Aging Cell. 2007; 6: 111-119.

**139.** Syntichaki P, Troulinaki K, Tavernarakis N. eIF4E function in somatic cells modulates ageing in Caenorhabditis elegans. Nature. 2007; 445: 922-926.

**140.** Hansen M, Taubert S, Crawford D, Libina N, Lee SJ, Kenyon C. Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans. Aging Cell. 2007; 6: 95-110.

**141.** Mehta R, Chandler-Brown D, Ramos FJ, Shamieh LS, Kaeberlein M. Regulation of mRNA translation as a conserved mechanism of longevity control. Adv Exp Med Biol. 2011; 694: 14-29.

Editorial

# Prolonged Rapamycin treatment led to beneficial metabolicswitch

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The evolutionarily conserved TOR (Target of Rapamycin) signaling controls growth and metabolism from yeast to mammals [1]. Mammalian or mechanistic TOR (mTOR) plays the key role in aging and age-related disease [2]. Rapamycin, a drug used clinically for organ transplants, coronary stent coating and certain forms of cancer treatments, is an inhibitor for mTOR. In the first robust demonstration of pharmacologically-induced life extension in a mammal, rapamycin increased longevity of mice via either feeding or injection [2]. However, rapamycin treatment also showed the detrimental metabolic effects, including hyperinsulinemia, hyperlipidemia, glucose intolerance and insulin resistance. Those observations present a paradox of improved survival despite metabolic impairments. How rapamycin extended lifespan with such paradoxical metabolic effects remains to be elucidated [3]. In the various studies of rapamycin treatment, length of rapamycin treatment varied from two weeks to two years. With short-term rapamycin treatment, mice showed the detrimental metabolic effects, while a much longer length (up to 1.5 to 2 years) of rapamycin treatment led to increased longevity. Duration of rapamycin treatment may be one of the key factors that determine outcomes of the treatment. Longer-term rapamycin treatment may cause beneficial metabolic "switch" that is associated with enhanced insulin signaling and extended longevity.

In the issue of Cell Metabolism (Volume 17, Issue 3, 456-462, 5 March 2013), we reported that duration of rapamycin treatment indeed has differential effects on metabolism. In our study, rapamycin was given to mice for two, six or 20 weeks. Consistently with the previous reports, mice with two weeks of rapamycin treatment had characteristics of metabolic syndrome. Mice with six weeks of rapamycin treatment were in the metabolic transition status. When rapamycin treatment continued for 20 weeks, the detrimental metabolic effects were reversed or diminished. Insulin signaling is important in the control of longevity in both mice and humans. Lower insulin levels and higher insulin sensitivity are associated with extended longevity in long-lived mutants, such as Ames dwarf or GHR-KO (Growth Hormone Receptor Knock-out) mice [4]. In our study, alterations in insulin sensitivity induced by different durations of rapamycin treatment were closely associated with changes of glucose and lipid homeostasis and metabolism, as well as body composition. Short-term rapamycin treatment increased insulin levels drastically, but reduced insulin sensitivity with lower insulin signaling represented by lower phosphorylation of AKT at Ser473 (a key phosphorylation site to activate AKT), lower insulin tolerance and higher HOMA-IR (Homeostatic Model for Assessment of Insulin Resistance) scores. Additionally, pancreas mass was decreased and liver mass was increased, two body characteristics associated with metabolic syndrome. It is worth mentioning in a recent study, which has shown that "prolonged" rapamycin treatment caused insulin resistance, the mice were treated with rapamycin for 2 to 4 weeks [5]. This could reflect the effects of "short"-term rapamycin treatment in our study. Therefore, it is not surprising to observe insulin resistance after 2 to 4 weeks of rapamycin treatment in liver Rictor knock-out mice [5]. 20 weeks of rapamycin treatment decreased insulin levels, but enhanced insulin sensitivity significantly. Most likely due to hypoinsulinemia, mice with 20 weeks of rapamycin treatment had higher fed glucose and a certain degree of glucose intolerance in the early stage of GTT (Glucose Tolerance Test), however, those mice had normal glucose levels during fasting, suggesting hypersensitivity to insulin, and ITT (Insulin Tolerance Test) results showed enhanced insulin sensitivity as well. Additionally, levels of Grb10, a newly-identified insulin signaling inhibitor which lies downstream of mTOR [6] were decreased in the muscle from mice with 20 weeks of rapamycin treatment (unpublished data). Consequently, mice eventually were able to clear glucose, albeit at a slower pace due to lower basal levels of insulin and higher insulin sensitivity, and HOMA-IR was much lower than in the controls. Lipid metabolism was also altered in relation to the length of rapamycin treatment. Mice with 20 weeks of rapamycin treatment had reduced adiposity and better lipid profiles with increased oxygen consumption (one of indicators of more lipid usage as fuel), and enhanced ketogenesis (a process that is involved in fatty acid breakdown and linked to

modulation of aging). Interestingly, similar to the findings in human renal transplant patients, who received rapamycin as an immunosuppressant for 12 months, hypertriglyceridemia detected after short rapamycin treatment was normalized. mTOR inhibits insulin-induced stimulation of lipogenesis and enhances insulin-regulated lipolysis [7]. Higher insulin levels after short-term rapamycin treatment may cause impairment of normal flux of lipid metabolism by enhancing lipogenesis and inhibiting lipolysis, indicated by higher levels of triglycerides and lower levels of glycerol. After 20 weeks of rapamycin treatment, insulin levels dropped substantially. Theoretically, lipogenesis should be lower and lipolysis should be higher, generating more glycerol and NEFA (Non-Esterified Fatty Acids). However, instead of increasing, both glycerol and NEFA, especially NEFA, were decreased. These changes suggest that some lipid metabolic processes, such as using more fatty acids as fuel, could be triggered and enhanced. Consequently, energy metabolism in mice with 20 weeks of rapamycin treatment switched from low (using more carbohydrates as metabolic substrates) to high (expending more energy to burn more fatty acids), most likely via enhancing uncoupled energy generation processes (unpublished data). Taken together, prolonged rapamycin treatment caused beneficial metabolic switch, possibly by increasing metabolic flexibility [8] triggered by mTOR controlled insulin-induced lipid metabolism, which in turn may enhance insulin sensitivity in glucose metabolism.

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## REFERENCES

**1.** Laplante M and Sabatini DM. Cell. 2012; 149: 274-293.
**2.** Johnson SC et al. Nature. 2013; 493: 338-345.
**3.** Blagosklonny MV. Aging (Albany NY). 2012; 4: 350-358
**4.** Bartke A. Gerontology. 2012; 58: 337-343.
**5.** Lamming DW et al. Science. 2012; 335: 1638-1643.
**6.** Yu Y et al. Science. 2011; 332: 1322-1326.
**7.** Soliman GA. Cell Cycle. 2011; 10: 861–862.
**8.** Galgani JE et al. Am J Physiol Endocrinol Metab. 2008; 295: E1009-E1017.

Research Paper

# Rapamycin extends life span of Rb1$^{+/-}$ mice by inhibiting neuroendocrine tumors

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**Abstract: Chronic treatment of mice with an enterically released formulation of rapamycin (eRapa) extends median and maximum life span, partly by attenuating cancer. The mechanistic basis of this response is not known. To gain a better understanding of these *in vivo* effects, we used a defined preclinical model of neuroendocrine cancer, *Rb1*$^{+/-}$ mice. Previous results showed that diet restriction (DR) had minimal or no effect on the lifespan of *Rb1*$^{+/-}$ mice, suggesting that the beneficial response to DR is dependent on pRb1. Since long-term eRapa treatment may at least partially mimic chronic DR in lifespan extension, we predicted that it would have a minimal effect in *Rb1*$^{+/-}$ mice. Beginning at 9 weeks of age until death, we fed *Rb1*$^{+/-}$ mice a diet without or with eRapa at 14 mg/kg food, which results in an approximate dose of 2.24 mg/kg body weight per day, and yielded rapamycin blood levels of about 4 ng/ml. Surprisingly, we found that eRapa dramatically extended life span of both female and male *Rb1*$^{+/-}$ mice, and slowed the appearance and growth of pituitary and decreased the incidence of thyroid tumors commonly observed in these mice. In this model, eRapa appears to act differently than DR, suggesting diverse mechanisms of action on survival and anti-tumor effects. In particular the beneficial effects of rapamycin did not depend on the dose of *Rb1*.**

## INTRODUCTION

Age is by far the biggest independent risk factor for a wide range of intrinsic diseases [1], including most types of cancer [2]. The age-adjusted cancer mortality rate for persons over 65 years of age is 15-times greater than for younger individuals [3]. Numerous studies demonstrate that age retarding interventions reduce cancer by decreasing incidence and/or severity (Reviewed in [4]). Diet restriction (DR) has a long history of retarding cancer [5] and most of the other

age-associated diseases [6], consistent with life span extension in a wide range of organisms [7]. Genetic interventions resulting in pituitary dwarfism in mice, which causes growth factor reduction (GFR) and a reduction in associated signaling, also result in maximum lifespan extension [8], with a concomitant reduction in cancer severity [9, 10]. Thus, factors that inhibit growth appear to extend life span and reduce cancer.

mTORC1 (mechanistic Target of Rapamycin Complex 1) is central to cell growth by integrating upstream signals that include nutrients, growth factors and energy levels with stress responses for regulated cell growth. Thus, chronic mTORC1 inhibition could act similarly to DR and GFR. Supporting this possibility, the mTOR inhibitor rapamycin, increases life span in a variety of organisms including yeast [11], nematodes [12] and flies [13]. Using a chow containing a novel formulation of enterically delivered rapamycin (eRapa [14]), the NIA Intervention Testing Program [15] reported that long-term treatment extends both median and maximum lifespan of genetically heterogeneous mice (UM-HET3), even when started in late adulthood (20 months of age) [16], or at 9 months of age [17]. eRapa is the first drug reported to be capable of extending both median and maximum lifespan.

One explanation for the lifespan enhancement by eRapa is that chronic mTOR inhibition delays the onset and growth of neoplasms. Indeed, chronic eRapa (2.24 mg/kg/day diet) treatment reduced the incidence of lymphoma and hemangiosarcoma (two major cancers in the genetically heterogeneous mice studied by the ITP), and increased the mean age at death due to liver, lung and mammary tumors [16, 17]. Alternate possibilities are that the immune systems of treated mice better defend against their cancers or that the mice simply tolerate them longer. What is the basis of eRapa's ability to reduce cancer, and how does it compare to DR?

To gain an understanding of how chronic eRapa treatment compares with DR and affects cancer development, growth and progression, we used a mouse model deficient in the prototypical tumor suppressor, *Rb1*. Rb1 regulates cell cycle checkpoints for differentiation and in response to stress and is important for genome maintenance [18]. $Rb1^{+/-}$ mice are highly predisposed to cancers of neuroendocrine origin [19] including pituitary (intermediate and anterior lobe), thyroid C-cell (which can metastasize to lung), and adrenal. Tumorigenic cells form after losing the remaining functional copy of the Rb1 tumor suppressor gene. The complete penetrance of tumor formation, growth and progression results in a short lifespan for $Rb1^{+/-}$ mice, which, unlike wild type mice, is minimally affected by diet restriction [20]. If eRapa acts in a similar manner to DR [16], we predicted that chronic eRapa treatment of $Rb1^{+/-}$ mice would also have minimal effects on tumor development, growth, progression and life span. Surprisingly we find that eRapa treatment has a dramatic and positive effect on life span in both sexes of $Rb1^{+/-}$ mice, which is associated with slower tumor development and growth.

## RESULTS

To address the question of whether eRapa mimics DR in mice deficient for a prototypical tumor suppressor gene function, we initiated chronic treatment by feeding randomly grouped males and females chow that included either eRapa at the concentration previously shown to extend life span (14 mg/kg food), [16, 17] or Eudragit (empty capsule control). Treatment was begun at approximately 9 weeks of age (>80% of animals started between 8-10 weeks (minimum at 7 weeks and maximum at 12 weeks, Table S1). Mice continued on these diets for the remainder of their lives. Based on the average amount of chow consumed per day, this delivers an approximate rapamycin dose of 2.24 mg/kg body weight/day [16]. Blood levels of rapamycin (determined by a mass spectrometry) averaged 3.9 ng/ml for males, 3.8 ng/ml for females for $Rb1^{+/-}$ mice and 3.4 for males and 4.6 ng/ml for females for $Rb1^{+/+}$ mice (Figure S1). Hematocrits were performed on blood from $Rb1^{+/+}$ mice between 18 and 24 months of age and readings indicated normal values for mice (between 40 and 49%), indicating that long-term eRapa treatment does not adversely effect red blood cell production (data not included).

**Table 1. eRapa effects on survival of $Rb1^{+/-}$ mice**

| | Coefficient | Hazard Ratio | SE | z | P |
|---|---|---|---|---|---|
| **eRapa** | -1.3177 | 0.2678 | 0.2400 | -5.4909 | 0.00000004 |
| **Sex** | 0.1693 | 1.1844 | 0.2144 | 0.8005 | 0.42344718 |

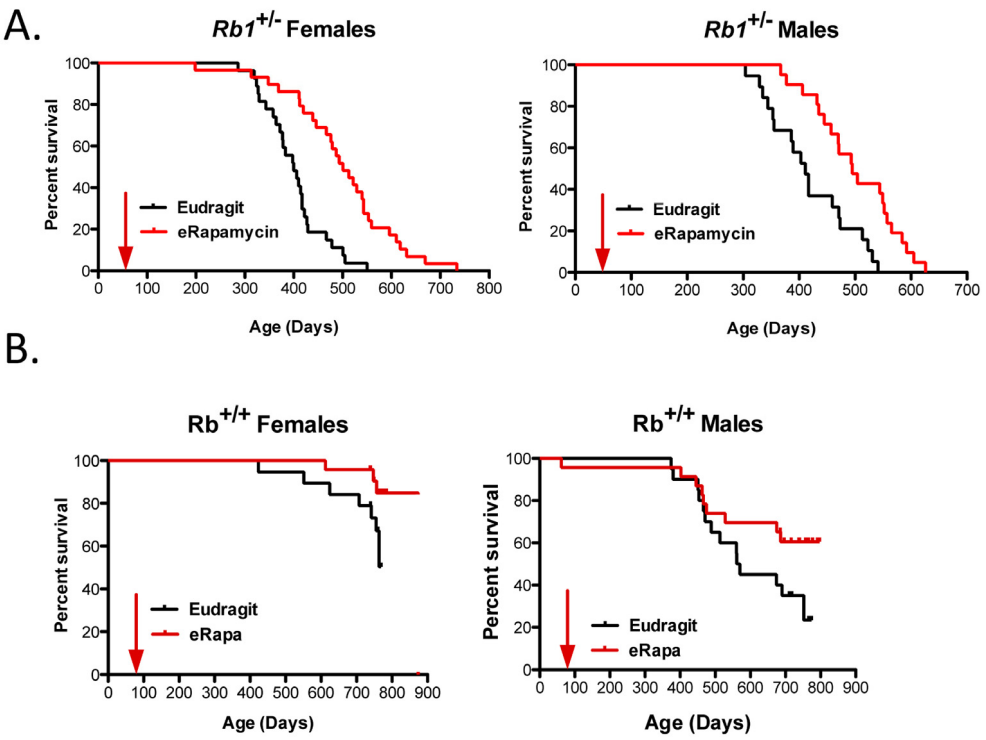


**Figure 1.** Survival plots for male and female *Rb1*$^{+/-}$ (**A**) and *Rb1*$^{+/+}$ (**B**) mice, comparing control-fed mice to those fed eRapa in the diet starting at approximately 9 weeks of age (indicated by arrow). Control (black line) and eRapa (red line) survival curves are shown. The horizontal axes represent life span in days and the vertical axes represent survivorship. *Rb1*$^{+/-}$ mice obtained from the NCI Mouse Repository were bred by the Nathan Shock animal core to obtain the cohorts of male and female mice used in this study. Genotype was confirmed as previously described [20]. eRapa mice were fed microencapsulated rapamycin-containing food (14mg/kg food designed to deliver approximately 2.24mg of rapamycin per kg body weight/day that achieved about 4 ng/ml blood [14]. Diets were prepared by TestDiet, Inc., Richmond, IN using Purina 5LG6 as the base [14]. Control diet was the same but with empty capsules. P values in (**B**) were calculated by the log-rank test.

### eRapa extended life span of Rb1$^{+/-}$ mice

Unlike most mouse models of cancer [5], 50% DR had little (if any) effect on the development, growth and progression of neuroendocrine tumors or on life span of *Rb1*$^{+/-}$ mice [20]. Since rapamycin has been predicted to act in a similar way to DR [16], we investigated if eRapa would also have little effect in this model. In stark contrast to DR, Figure 1A shows that *Rb1*$^{+/-}$ males and females derive a significant longevity benefit from chronic treatment with eRapa. The Eudragit control-fed mice had a shorter mean life span than the eRapa-fed cohort for both females (377.5 versus 411 days) and males (mean age is 368.8 versus 419.8 days). Sex did not modulate the effect of eRapa on *Rb1*$^{+/-}$ animals (Table 1).

Male and female *Rb1*$^{+/+}$ littermates of the *Rb1*$^{+/-}$ mice were also fed eRapa or control diets to ensure that this particular mutant strain (with a C57BL/6 background) is responsive to rapamycin. Once all *Rb1*$^{+/-}$ mice had died and the effects of eRapa were evident, the *Rb1*$^{+/+}$ littermates were euthanized. At this time, as expected, eRapa improved survival for both male and female *Rb1*$^{+/+}$ mice as well (Fig. 1B). Similar to the previous results from the Intervention Testing Program eRapa experiments [16, 17], lifespan was extended more in females than in males (Table 2) in wild type (WT) littermates.

| Table 2. eRapa effects on survival of *Rb1*$^{+/+}$ mice | | | | | |
|---|---|---|---|---|---|
| | **Coefficient** | **Hazard Ratio** | **SE** | **z** | **P** |
| **eRapa** | -0.9305 | 0.3943 | 0.3631 | -2.5625 | 0.01039082 |
| **Sex** | -1.2818 | 0.2775 | 0.3840 | -3.3382 | 0.00084312 |

| Table 3. Pathology of *Rb1*$^{+/-}$ mice at necropsy | | |
|---|---|---|
| | Eudragit | eRapa |
| **Tumor Incidence** | | |
| Pituitary | 97.5% (40) | 100%[a] (39) |
| Thyroid | 90.0% (40) | 66.7%[b] (39) |
| Thyroid with lung metastases | 37.5% (40) | 28.2%[c] (39) |
| Thyroid with adrenal metastases | 2.5% (40) | 7.7%[d] (39) |
| Adrenal | 30.0% (40) | 23.1%[e] (39) |

a, p = 0.9858, b, p = 0.0112; c, p = 0.3859; d, p = 0.5472, e, p = 0.4925
Two tailed, unpaired t test, GraphPad Prism.

**eRapa effects on tumor incidence at the end of life**

At necropsy, *Rb1*$^{+/-}$ mice were evaluated for the presence of neuroendocrine tumors and lung metastases. As shown in Table 3**,** there were no differences in the eRapa and Eudragit control groups in terms of presence of pituitary tumors (although we did observe a delay in their detection and reduction in size by magnetic resonance imaging (MRI), discussed below). We did observe a decreased incidence of thyroid C-cell carcinomas in the eRapa treated group of *Rb1*$^{+/-}$ mice (p = 0.0112). Except for the modest decrease in thyroid tumors, this tumor spectrum is similar to *Rb1* heterozygotes treated with DR compared to those fed *ad libitum* [20]. Along with the decrease in thyroid C-cell tumors, eRapa also tended to reduce the incidence and severity of C-cell lung metastases (Table 4). Thus mice have a decreased cancer burden and live with tumors longer.

**eRapa delayed tumor development and slowed growth**

Is delayed and/or reduced tumor growth the basis of life span extension by eRapa in this model? To address this question, we took advantage of the synchronous (spatial and temporal) development of tumors in this model Nikitin et al. [19, 21]. *Rb1*-deficient cells are first identified as atypical proliferates in the intermediate and anterior lobes of the pituitary, thyroid and parathyroid glands and the adrenal medulla at about 12 weeks of postnatal development. Atypical proliferates eventually form gross tumors with varying degrees of malignancy by postnatal day 350. Since we started treatment at around 8 weeks of age, eRapa might have an effect on the initiating events leading to loss of heterozygosity and/or subsequent formation of atypically proliferating cells. Perhaps more likely, eRapa slows growth and development of proliferates to gross tumors, which had probably begun at or around the time treatment was started. To test this latter possibility, we used MRI to follow pituitary and thyroid tumor development and growth in a subset of eRapa-treated *Rb1*$^{+/-}$ mice (8 mice per treatment group were imaged between 1 and 4 times up to twice a month). MRI is well suited for following head and neck tumors that correspond to the primary tumor types *Rb1*$^{+/-}$ mice develop. An initial cohort was used to identify the best timeframe for MRI scans. For this, 6 female *Rb1*$^{+/+}$ mice (3 per group) and 10 *Rb1*$^{+/-}$ mice (3 per group in males and 2 per group in females) were imaged in a single session or with 2 serial scans. This study indicated the ideal timeframe to image pituitary tumors was a window between 9 and 12 months of age, which covers the time from initial detection through monitoring tumor growth.

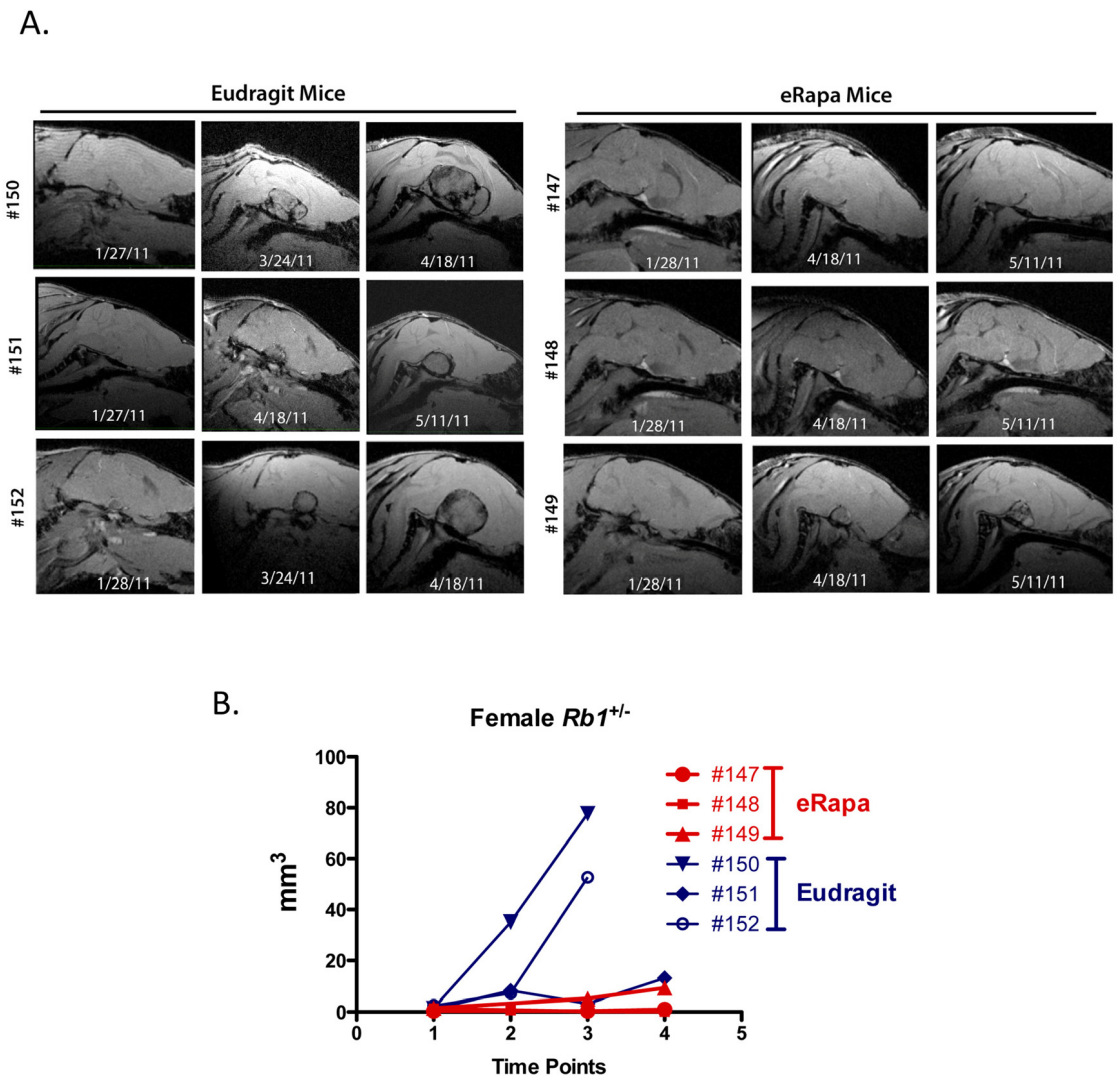


**Figure 2**. Effects of eRapa on pituitary and thyroid tumor development and growth. To identify effects on tumors, we used MRI as a non-invasive method to longitudinally monitor individual $Rb^{+/-}$ mice. High-resolution images were obtained on a very high field strength Bruker Pharmascan 7.0T animal MRI scanner using a coil to focus on pituitary and thyroid tumors. Images were acquired using a spoiled gradient echo named Fast low angle shot MRI (FLASH) on the scanner. Images were acquired to yield predominantly T1 weighted contrast with TE (echo time) 4.5 msec, TR (repetition time) 450 msec, FA (Flip angle) 40 degrees, FOV (field of view) 20 x 20 mm, in plane spatial resolution 0.078 x 0.078 mm. Tumor volume was determined for each time point. (**A**) Serially acquired MRI images from eRapa and Eudragit-fed control mice at 9, 11 and 12 months of age. (**B**) Tumor volumes calculated from MRI image stacks at each time point comparing individual mice at multiple ages. Tumors in two of the Eudragit-fed (control) mice are detected earlier and grow faster than the 3 eRapa-fed mice.

Age matched $Rb1^{+/-}$ females (3 per group) were scanned using MRI at 9, 11 and 12 months of age (Figure 2A shows sagittal plane sections of the serially acquired MRI images through the pituitary of eRapa and Eudragit treated mice). Calculated volumes based on the MRI image stacks (analyzed blind by a single radiologist, RLH) were plotted versus age at the date of imaging. In concert with extended longevity, the detection of pituitary tumors was delayed with a decrease in their growth in the eRapa-treated mice. Figure 2B shows that eRapa delayed development and/or reduced tumor growth at each time point when mice were imaged. More $Rb1^{+/-}$ mice had detectable tumors identified during two separate MRI imaging

sessions from the Eudragit control cohort (4 pituitary and 2 thyroid tumors out of 8 mice in March 2011 scan and 7 pituitary and 4 thyroid tumors out of 8 mice in April 2011 scan) compared to the mice eRapa-fed cohort (1 pituitary and 0 thyroid tumors out of 8 mice in March 2011 scan and 2 pituitary and 3 thyroid tumors out of 8 mice in April 2011 scan). Longitudinal monitoring allowed us to conclude that chronic rapamycin delays both the development of visible tumors and inhibited the growth of tumors once they were present.

**Table 4. Incidence and pathology of *Rb1*$^{+/-}$ lung metastases**

| | Eudragit | | eRapa | |
|---|---|---|---|---|
| | Males | Females | Males | Females |
| **Grade** | | | | |
| 0 | 6 | 6 | 5 | 11 |
| 1 | 1 | 1 | 1 | 3 |
| 2 | 3 | 7 | 1 | 2 |
| 3 | 1 | 1 | 1 | 2 |
| 4 | 0 | 1 | 0 | 0 |
| Total (Gr 1-4) | 5 | 10 | 4 | 7 |

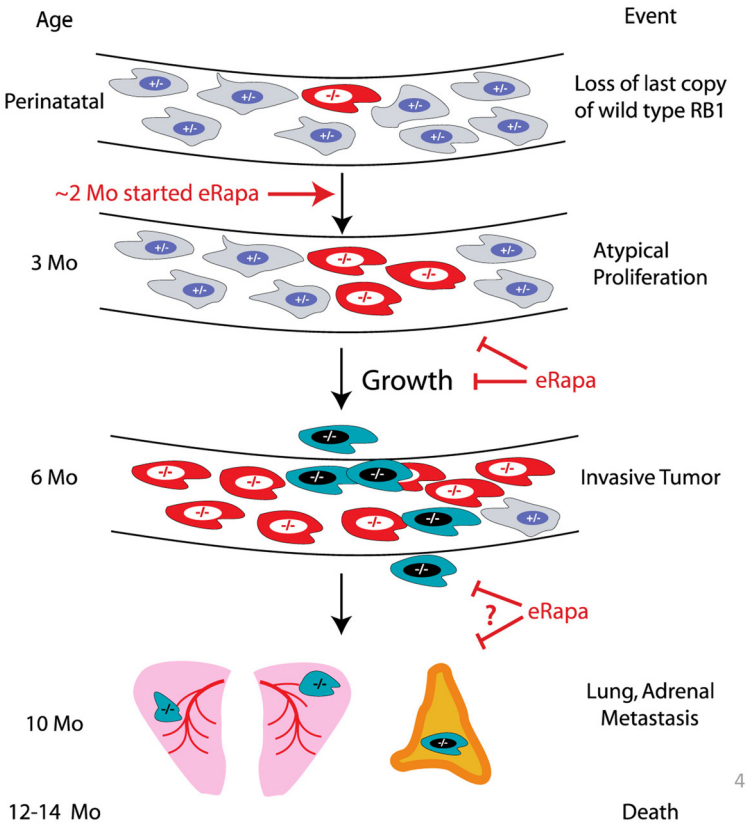


**Figure 3.** Summary of eRapa effects in the *Rb1*$^{+/-}$ model of neuroendocrine tumorigenesis**.** Our MRI data are consistent with a delay of tumor development perhaps by inhibition of atypical proliferates and reduction in tumor growth. eRapa may inhibit lung metastasis and slow their growth.

## DISCUSSION

In mice, pRb1 is critical for DR-mediated lifespan extension [20], but not rapamycin-mediated life span extension. It is unclear why this is the case, since both of these interventions chronically inhibit mTORC1 [22]. However, differences in the downstream *in vivo* effects of DR and rapamycin have been previously reported [22]. As previously described by Harrison et al. [16], a distinguishing feature of eRapa is its ability to extend median and maximum life when the intervention starts at a relatively old age (600 days) in mice. By comparison, DR in most [23] but not all [24] reports shows little if any longevity benefit when started after 550 days of age (equivalent to 60 human years). DR started at 6 weeks of age reduced body growth for $Rb1^{+/-}$ mice but did not affect growth of $Rb1^{-/-}$ tumors [20]. In contrast to DR, chronic eRapa treatment did not affect body weight of $Rb1^{+/-}$ mice (Livi et al., in preparation), but did reduce tumor growth. Previous studies in fruit flies show that rapamycin extends life span through a mechanism that is at least partly independent of TOR [13]. Consistent with those results, we find that eRapa, but not DR, extended life span and reduced the growth of neuroendocrine tumors in the $Rb1^{+/-}$ model. It will be interesting to determine if pRb1 might be at least partially involved in those settings where responses to chronic eRapa and DR diverge.

Based on the longitudinal imaging data acquired by MRI (Figure 2), eRapa appears to inhibit $Rb1^{-/-}$ pituitary tumor development and growth in $Rb1^{+/-}$ mice (summarized in Figure 3), which is likely a major factor in its ability to extend lifespan in this model. Since we started eRapa at between 2 and 3 months of age, it would be interesting to know if it affects loss of heterozygosity (LOH) (Figure 3) in neuroendocrine tissues. The significant reduction in the incidence of thyroid C-cell carcinoma at necropsy in eRapa treated $Rb1^{+/-}$ mice (Table 3) also likely contributes to extended longevity. We also observed an apparent lessening of severity in lung metastases (Table 4), but this may be due to overall reduction of C-cell carcinomas. Metastasis of these to tumors to the adrenal (Table 3) has, to our knowledge, not been previously reported. A recent report linked an increase in metastasis with RAD001 treatment in a rat model of transplanted neuroendocrine tumors, which the authors attributed to alternations in tissue immune microenvironment [25]. Since RAD001 treatment was started subsequent to tumor implantation, it might be interesting to test this model in a prevention rather than treatment setting.

Two reports have linked pRb1 and mTOR. A genetic study in *D. melanogaster* established synergy between deletion of mTOR and pRb1 using an *in vivo* synthetic lethality screen of *Rb*-negative cells [26]. These authors found that inactivation of *gig* (fly TSC2) and *rbf* (fly Rb) is synergistically responsible for oxidative stress leading to lethality. In a separate study, El-Naggar et al., [27] found that loss of the *Rb1* family (Rb1, Rbl1 and Rbl2) in primary cells derived from triple-knockout mice led to overexpression of mTOR and constitutive phosphorylation of Ser473 on Akt , which is oncogenic. The inhibition of tumor development and growth in $Rb1^{+/-}$ mice by eRapa is also consistent with a recent report showing that mTOR inhibition partially alleviated tumor development in an $Rb^{F/F}$;K14creER$^{TM}$ ;$p107^{-/-}$ model of squamous cell carcinoma [28], and with several reports demonstrating the effectiveness of rapamycin in mouse cancer models for tumor reduction and life span extension [29-31]. Potential mechanism may be by way of indirect effects or rapamycin on the tumor microenvironment [32] and/or senescent cells [33].

The reduction in lung metastases is consistent with ribosome profiling that revealed transcript-specific translational control mediated by oncogenic mTOR signaling, including a distinct set of pro-invasion and metastasis genes [34]. It will be interesting to determine whether chronic eRapa treatment affects these genes in thyroid C-cell neoplasms. We also observed metastasis of thyroid tumors to adrenal glands, albeit at a low frequency but eRapa treatment did not effect.

Neuroendocrine tumors are unique in their ability to secrete hormones or deleterious bioactive products [35]. It was previously reported that the rapalog Everolimus (RAD-001) in combination with ocreotide lanreotide (compared to placebo) improved the clinical picture of carcinoid patients by reducing circulating chromogranin A and 5-hydroxyindoleacetic acid, two tumor-secreted bioactive products responsible for some of the symptoms [36]. Thus, another potential mechanism for life span extension in $Rb1^{+/-}$ mice by eRapa could be due the prevention of the production and/or secretion of hormones or deleterious bioactive factors.

*Rb1* is known to have an important role in somatic growth regulation, since increased *RB1* dose reduced animal size [37]. Determining if there is a link between *Rb1* (a negative regulator of growth) and mTORC1 (a positive regulator of growth) in growth of tumors could suggest new therapeutic and prevention targets for drug development. One prediction is that mice over expressing pRb1 will have decreased mTOR activity and be long lived through prevention, delay or a reduction in severity of age-related diseases.

Here we show that eRapa extends the life span for *Rb1*$^{+/-}$ mice. We find eRapa-fed mice exhibit a delay in the onset and/or progression of neuroendocrine tumors. These results are in direct contrast with DR. Thus, mTORC1 inhibition and DR likely use different modes for life span extension.

## METHODS

Mice and life span. Mice (strain B6.129S2(Cg)-Rb1$^{tm1Tyj}$ ) for breeding were obtained from the NCI MMHCC Repository. Although they have similar phenotypes, the strain used in the diet restriction study by Sharp et al., [20] was different having been generated by Lee et al [38]. The procedures and experiments involving use of mice were approved by the Institutional Animal Care and Use Committee and are consistent with the NIH Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Education, the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act (National Academy Press, Washington, DC). Genotyping was done as described previously [20]. Cohorts of mice were fed microencapsulated rapamycin-containing food (14 mg/kg food designed to deliver ~2.24 mg of rapamycin per kg body weight/day to achieve about 4 ng/ml of rapamycin per kg body weight/day) prepared by TestDiet, Inc., Richmond, IN using Purina 5LG6 as the base [14]. Control diet was the same but with empty capsules.

Rapamycin food concentration. Rapamycin was quantified in food using HPLC with tandem mass spectrometry detection. Briefly, 100 mg of food for spiked calibrators and unknown samples were crushed with a mortar and pestle, then vortexed vigorously with 10 µL of 250 µg/mL ASCO (internal standard) and 4.0 ml of mobile phase A. The samples were then mechanically shaken for 10 min, centrifuged for 10 min, and then centrifuged in microfilterfuge tubes for 1 minute. Ten µL of the final extracts was injected into the LC/MS/MS. 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, 5, 10, 30, and 60 ng/mg of food to quantify rapamycin. The concentration of rapamycin in food was expressed as ng/mg food (parts per million).

Rapamycin blood measurements. Measurement of rapamycin used HPLC-tandem MS. RAPA and Ascomycin (ASCO) were obtained from LC Laboratories (Woburn, MA). HPLC grade methanol and acetonitrile were purchased from Fisher (Fair Lawn, NJ). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO). Milli-Q water was used for preparation of all solutions. RAPA and ASCO super stock solutions were prepared in methanol at a concentration of 1 mg/ml and stored in aliquots at -80$^{o}$C. A working stock solution prepared each day from the super stock solutions at a concentration of 10 µg/ml was used to spike the calibrators.

Calibrator and unknown whole blood samples (100 µL) were mixed with 10 µL of 0.5 µg/mL ASCO (internal standard), and 300 µL of a solution containing 0.1% formic acid and 10 mM ammonium formate dissolved in 95% HPLC grade methanol. The samples were vortexed vigorously for 2 min, and then centrifuged at 15,000 *g* for 5 min at 23$^{o}$C (subsequent centrifugations were performed under the same conditions). Supernatants were transferred to 1.5 ml microfilterfuge tubes and centrifuged at 15,000 *g* for 1 min and then 40 µL of the final extracts were injected into the LC/MS/MS. The ratio of the peak area of rapamycin to that of the internal standard ASCO (response ratio) for each unknown sample was compared against a linear regression of calibrator response ratios at 0, 1.25, 3.13, 6.25, 12.5, 50, and 100 ng/ml to quantify rapamycin.

The HPLC system consisted of a Shimadzu SCL-10A Controller, LC-10AD pump with a FCV-10AL mixing chamber (quarternary gradient), SIL-10AD autosampler, and an AB Sciex API 3200 tandem mass spectrometer with turbo ion spray. The analytical column was a Grace Alltima C18 (4.6 x 150 mm, 5 µ) purchased from Alltech (Deerfield, IL) and was maintained at 60$^{o}$C during the chromatographic runs using a Shimadzu CTO-10A column oven. Mobile phase A contained 10 mM ammonium formate and 0.1% formic acid dissolved in HPLC grade methanol. Mobil phase B contained 10 mM ammonium formate and 0.1% formic acid dissolved in 90% HPLC grade methanol. The flow rate of the mobile phase was 0.5 ml/min. Rapamycin was eluted with a step gradient. The column was equilibrated with 100% mobile phase B. At 6.10 minutes after injection, the system was switched to 100% mobile phase A. Finally, at 15.1 min, the system was switched back to 100% mobile phase B in preparation for the next injection. The rapamycin transition was detected at 931.6 Da (precursor ion) and the daughter ion was detected at 864.5 Da. ASCO was detected at 809.574 Da and the daughter ion was 756.34 Da.

Survival analysis methods. An entry for each mouse in the study was created in a database used by the Nathan Shock Animal core. The age at which each animal died was recorded. Survival durations for animals that either lived past the end-date of the study, were terminated, or

died accidentally were treated as right-censored events. Cox proportional hazard models [39] were fitted to the wild type and *Rb1*$^{+/-}$ subsets of the data, with eRapa and gender as additive predictor variables. Some animals were transferred to a different facility part-way through their life spans so the final facility at which they were housed was also added to the Cox models, as a stratifying variable. The R statistical language was used for the analysis [40, 41]. The mice in the life span studies were allowed to live out their life span, i.e., there was no censoring due to morbidity in the groups of mice used to measure lifespan of *Rb1*$^{+/-}$ mice. Mice were euthanized only if they were either (1) unable to eat or drink, (2) bleeding from a tumor or other condition, or (3) when they were laterally recumbent, i.e., they fail to move when prodded or are unable to right themselves.

MRI methods. Images were acquired on a Bruker Pharmascan 7.0T MRI scanner. Images were obtained in the sagittal plane through the brain and coronal plain through the neck (focused on the thyroid gland) using 2D spoiled gradient echo technique to quickly obtain high-resolution images (fast low angle shot magnetic resonance imaging - FLASH on our scanner). FLASH protocol was TE/TR 5 msec/450msec, Averages 1, Flip angle 40 deg, Field of view 20 mm x 20 mm, matrix size 256x256, In plane resolution was 0.078 x 0.078 mm, slice thickness 0.5 mm. The FLASH sequence shows predominantly T1 weighted image contrast. A single blinded radiologist (RLH) evaluated images for the presence and tumor volume used to plot detection and growth data. Images were analyzed using an open source image processing software, OsiriX, version 2.7.5. The pituitary gland was identified on all images and volume was calculated by measuring the greatest anterior-posterior, cranial-caudal, and right-left length. Volumes were then determined using prolate ellipse formula. Data were then parsed by treatment group and plotted in Prism (GraphPad).

Procedures for examination of pathology in mice. Fixed tissues (in 10% neutralized formalin) were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin. Diagnosis of each histopathological change was made using histological classifications for aging mice as previously described [9, 20, 42, 43].

Pathology assessments. A list of lesions was compiled for each mouse. The severity of neoplastic lesions was assessed using the grading system previously described [9, 20, 42, 43]. Two pathologists separately examined all of the samples without knowledge of their genotype or age. Briefly, lung pathology grade is based on the area of the lung section infiltrated by metastatic tumor tissue with 0 being no tumor cells observed and 4 being the largest area taken by tumor.

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### Conflict of Interest Statement

ZDS and RS were unpaid consultants to Rapamycin Holdings, Inc. Other authors declare no conflicts.

## REFERENCES

**1.** Kirkwood TB. Gerontology: Healthy old age. Nature. 2008; 455:739-740.

**2.** Blagosklonny MV, Campisi J. Cancer and aging: more puzzles, more promises? Cell Cycle. 2008; 7:2615-2618.

**3.** Altekruse SF, Kosary CL, Krapcho M, Neyman N, Aminou R, Waldron W, et al. SEER Cancer Statistics Review 1975-2007 Bethesday, MD: National Cance Institute; 2010. Available from: http://seer.cancer.gov/csr/1975_2007, based on November 2009 SEER data submission, posted to the SEER web site, 2010.

**4.** Sharp Z, Richardson A. Aging and cancer: can mTOR inhibitors kill two birds with one drug? Targeted Oncology. 2011;6:41-51.

**5.** Hursting SD, Smith SM, Lashinger LM, Harvey AE, Perkins SN. Calories and carcinogenesis: lessons learned from 30 years of calorie restriction research. Carcinogenesis. 2010;31:83-89.

**6.** Fontana L, Partridge L, Longo VD. Extending Healthy Life Span--From Yeast to Humans. Science. 2010; 328:321-326.

**7.** Weindruch R, Colman RJ, Pérez V, Richardson AG. How Does Calorie Restriction Increase the Longevity of Mammals? In: Guarente LP, Partridge L, Wallace DC, editors. Molecular Biology of Aging. Cold Spring Harbor, NY 11724: Cold Spring Harbor Laboratory Press; 2008; 409-426.

**8.** Bartke A. Growth hormone, insulin and aging: The benefits of endocrine defects. Exp Gerontol. 2011;46:108-111.

**9.** Ikeno Y, Bronson RT, Hubbard GB, Lee S, Bartke A. Delayed Occurrence of Fatal Neoplastic Diseases in Ames Dwarf Mice: Correlation to Extended Longevity. J Gerontol A Biol Sci Med Sci. 2003; 58:B291-B296.

**10.** Ikeno Y, Hubbard GB, Lee S, Cortez LA, Lew CM, Webb CR, et al. Reduced Incidence and Delayed Occurrence of Fatal Neoplastic Diseases in Growth Hormone Receptor/Binding Protein Knockout Mice. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 2009; 64A:522-529.

**11.** Powers RW, 3rd, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S. Extension of chronological life span in yeast by decreased TOR pathway signaling. Genes Dev. 2006; 20:174-184.

**12.** Robida-Stubbs S, Glover-Cutter K, Lamming DW, Mizunuma M, Narasimhan SD, Neumann-Haefelin E, et al. TOR Signaling and Rapamycin Influence Longevity by Regulating SKN-1/Nrf and DAF-16/FoxO. Cell Metabolism. 2012;15:713-724.

**13.** Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, et al. Mechanisms of Life Span Extension by Rapamycin in the Fruit Fly Drosophila melanogaster. Cell Metabolism. 2010; 11:35-46.

**14.** Nadon NL, Strong R, Miller RA, Nelson J, Javors M, Sharp ZD, et al. Design of aging intervention studies: the NIA interventions testing program. AGE. 2008; 30:187-199.

**15.** Nadon NL, Strong R, Miller RA, Nelson J, Javors M, Sharp ZD, et al. Design of aging intervention studies: the NIA interventions testing program. Age (Dordr). 2008; 30:187-199.

**16.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009; 460:392-395.

**17.** Miller RA, Harrison DE, Astle CM, Baur JA, Boyd AR, de Cabo R, et al. Rapamycin, But Not Resveratrol or Simvastatin, Extends Life Span of Genetically Heterogeneous Mice. J Gerontol A Biol Sci Med Sci. 2011; 66:191-201.

**18.** Zheng L, Lee WH. The retinoblastoma gene: a prototypic and multifunctional tumor suppressor. Exp Cell Res. 2001; 264:2-18.

**19.** Nikitin AY, Juarez-Perez MI, Li S, Huang L, Lee WH. RB-mediated suppression of spontaneous multiple neuroendocrine neoplasia and lung metastases in Rb+/- mice. Proc Natl Acad Sci U S A. 1999 Mar 30; 96:3916-3921.

**20.** Sharp ZD, Lee WH, Nikitin AY, Flesken-Nikitin A, Ikeno Y, Reddick R, et al. Minimal effects of dietary restriction on neuroendocrine carcinogenesis in Rb+/- mice. Carcinogenesis. 2003; 24:179-183.

**21.** Nikitin A, Lee WH. Early loss of the retinoblastoma gene is associated with impaired growth inhibitory innervation during melanotroph carcinogenesis in Rb+/- mice. Genes Dev. 1996; 10:1870-1879.

**22.** Laplante M, Sabatini DM. mTOR Signaling in Growth Control and Disease. Cell. 2012; 149:274-293.

**23.** Masoro EJ. Overview of caloric restriction and ageing. Mech Ageing Dev. 2005;126:913-922.

**24.** Dhahbi JM, Kim HJ, Mote PL, Beaver RJ, Spindler SR. Temporal linkage between the phenotypic and genomic responses to caloric restriction. Proc Natl Acad Sci U S A. 2004; 101:5524-5529.

**25.** Pool SE, Bison S, Koelewijn SJ, van der Graaf LM, Melis M, Krenning EP, et al. mTOR inhibitor RAD001 promotes metastasis in a rat model of pancreaticneuroendocrine cancer. Cancer Res. 2013; 73:12-18.

**26.** Li B, Gordon GM, Du CH, Xu J, Du W. Specific killing of Rb mutant cancer cells by inactivating TSC2. Cancer Cell. 2010; 17:469-480.

**27.** El-Naggar S, Liu Y, Dean DC. Mutation of the Rb1 Pathway Leads to Overexpression of mTor, Constitutive Phosphorylation of Akt on Serine 473, Resistance to Anoikis and a Block in c-Raf Activation. Mol Cell Biol. 2009; 29:5710-5717.

**28.** Costa C, Santos M, Segrelles C, Duenas M, Lara MF, Agirre X, et al. A Novel Tumor suppressor network in squamous malignancies. Sci Rep. 2012; 2:828.

**29.** Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, et al. Rapamycin Extends Maximal Lifespan in Cancer-Prone Mice. Am J Pathol. 2010; 176:2092-2097.

**30.** Comas M, Toshkov I, Kuropatwinski KK, Chernova OB, Polinsky A, Blagosklonny MV, et al. New nanoformulation of rapamycin Rapatar extends lifespan in homozygous p53-/- mice by delaying carcinogenesis. Aging (Albany NY). 2012; 4:715-722.

**31.** Komarova EA, Antoch MP, Novototskaya LR, Chernova OB, Paszkiewicz G, Leontieva OV, et al. Rapamycin extends lifespan and delays tumorigenesis in heterozygous p53+/- mice. Aging (Albany NY). 2012; 4:709-714.

**32.** Mercier I, Camacho J, Titchen K, Gonzales DM, Quann K, Bryant KG, et al. Caveolin-1 and accelerated host aging in the breast tumor microenvironment: chemoprevention with rapamycin, an mTOR inhibitor and anti-aging drug. Am J Pathol. 2012; 181:278-293.

**33.** Blagosklonny MV. Prevention of cancer by inhibiting aging. Cancer Biol Ther. 2008; 7:1520-1524.

**34.** Hsieh AC, Liu Y, Edlind MP, Ingolia NT, Janes MR, Sher A, et al. The translational landscape of mTOR signalling steers cancer initiation and metastasis. Nature. 2012; 485:55-61

**35.** Dogliotti L, Tampellini M, Stivanello M, Gorzegno G, Fabiani L. The clinical management of neuroendocrine tumors with long-acting repeatable (LAR) octreotide: comparison with standard subcutaneous octreotide therapy. Ann Oncol. 2001; 12:Suppl 2:S105-109.

**36.** Pavel ME, Hainsworth JD, Baudin E, Peeters M, Horsch D, Winkler RE, et al. Everolimus plus octreotide long-acting repeatable for the treatment of advanced neuroendocrine tumours associated with carcinoid syndrome (RADIANT-2): a randomised, placebo-controlled, phase 3 study. Lancet. 2011; 378:2005-2012.

**37.** Nikitin A, Shan B, Flesken-Nikitin A, Chang KH, Lee WH. The retinoblastoma gene regulates somatic growth during mouse development. Cancer Res. 2001; 61:3110-3118.

**38.** Lee EY, Chang CY, Hu N, Wang YC, Lai CC, Herrup K, et al. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature. 1992; 359:288-294.

**39.** Therneau TM. Modeling Survival Data: Extending the Cox Model. New York: Springer; 2000.

**40.** Therneau TM. A package for Survival in S. Rochester, MN: Mayo Foundation; 2012.

**41.** Team RDC. R: A Language and Environment for Statistical Computing 2012. Available from: http://www.R-project.org.

**42.** Bronson RT, Lipman RD. Reduction in rate of occurrence of age related lesions in dietary restricted laboratory mice. Growth Dev Aging. 1991; 55:169-184.

**43.** Ikeno Y, Hubbard GB, Lee S, Richardson A, Strong R, Diaz V, et al. Housing Density Does Not Influence the Longevity Effect of Calorie Restriction. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 2005; 60:1510-1517.

## SUPPLEMENTARY DATA

| Table S1. Age (weeks) at treatment initiation | | | | |
|---|---|---|---|---|
| | *Rb1*$^{+/-}$ | | *Rb1*$^{+/+}$ | |
| **Diet Start Range** | **#Mice** | **%** | **# Mice** | **%** |
| 7-8 | 7 | 7.2 | 11 | 11.2 |
| 8-9 | 49 | 50.5 | 47 | 48.0 |
| 9-10 | 40 | 40.3 | 39 | 48.0 |
| 12 | 1 | 1.0 | 1 | 1.0 |
| **Average** | 8.9 | | 8.8 | |
| **Youngest** | 7.0 | | 7.0 | |
| **Oldest** | 12.0 | | 12.0 | |

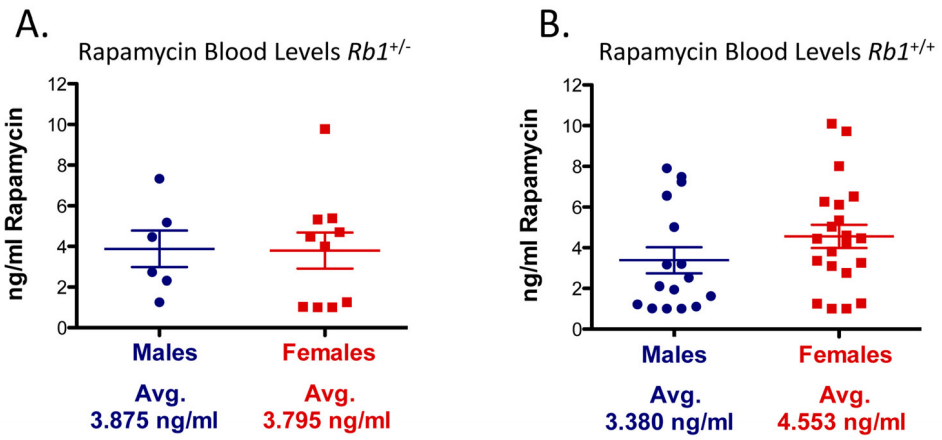


**Figure S1.** Rapamycin levels were quantified as described in Methods. The concentration of rapamycin was expressed as ng/ml of whole blood.

Editorial

# Rapamycin as longevity enhancer and cancer preventative agent in the context of p53 deficiency

**Lawrence A. Donehower** DOI: 10.18632/aging.100494

Since initial rodent studies in the mid-1930s, caloric restriction (CR) has been known to be an effective non-pharmacological intervention that can extend longevity in many species. Over the last 15-20 years, studies in yeast, worms, and flies have defined many of the signaling pathways mediating these CR-driven longevity effects. A prominent mediator of CR is the target of rapamycin (TOR) signaling pathway, which functions to monitor nutrient levels in the cell and modulate protein synthesis and cell growth in response. Dysfunctional regulation of TOR in humans has been associated with a number of aging-associated diseases such as diabetes, obesity, cardiovascular disease, and cancer [1]. On the other hand, downregulation of the TOR pathway in yeast, worms and flies by the inhibitory molecule rapamycin has been shown to significantly increase lifespan in each of those species. Moreover, in 2009, Miller, Harrison and colleagues showed that mixed inbred mice treated with rapamycin at a relatively late age (600 days) exhibited extended lifespans [2]. Recently, this group also showed that aging phenotypes were significantly delayed in the rapamycin-treated group, though testicular degeneration and cataracts increased [3].

Despite potential side effects, the rapamycin-induced longevity enhancement in a mammalian species has generated much excitement, and further studies in animal models have now indicated that cancer incidence is delayed by rapamycin treatment. This should not be too surprising, since rapamycin integrates signals initiated from a number of growth factor receptors, is upregulated in numerous cancers, and has been used as a cancer therapeutic drug in some contexts [1]. One such study by Anisomov et al. [4] showed that rapamycin treatment of HER-2 transgenic mammary cancer prone mice not only resulted in significantly extended lifespans, but also dramatically delayed tumor appearance and decreased tumor number and size. Thus, rapamycin may be a highly effective cancer preventative drug in addition to its many other beneficial effects.

To follow up and extend this initial exciting result, Gudkov, Blagosklonny, Antoch, and their colleagues have investigated the effects of rapamycin on tumor incidence and longevity in p53-deficient mice. The results, appearing in two papers in this issue of *Aging*, confirm that the cancer preventative effects of rapamycin are significant and broad in scope [5,6]. The p53 tumor suppressor protects against an array of different tumors and p53-deficient mice succumb to lymphomas and many different types of sarcomas [7]. In the paper by Komarova et al. [5], mice heterozygous for a germline p53 null allele (p53+/-) that were continuously treated with rapamycin in the drinking water beginning at a young age (<5 months) had a mean lifespan of 480 days compared to that of the control group's 373 day mean lifespan (a 28% increase). Importantly, these rapamycin-treated mice developed only half as many tumors as the control mice, a dramatic and significant anti-cancer effect. They also show direct inhibition of mTOR kinase activity in several tissues of the rapamycin-treated mice, an indicator that the rapamycin effects continuously inhibit mTOR signaling. In their discussion, the authors acknowledge that the anti-cancer effects of rapamycin are likely to be indirect, but don't speculate further. However, because mTOR integrates signals from so many growth signaling pathways, intersects with so many key growth signal transducers (such as AKT, PI-3 kinase, and Ras), and drives so many cell growth outputs, it's easy to argue that reduction of mTOR activity by rapamycin acts as a major brake on transformation. The authors suggest that the dramatic effects of rapamycin on p53+/- mice could lead to use of this agent as a cancer preventative drug in Li-Fraumeni syndrome patients. Li-Fraumeni patients are analogous to p53+/- mice, as they carry germline p53 mutations and are highly cancer prone at a young age [8]. This may be a good place to start in considering patients for rapamycin in clinical trials, though some of the side effects of rapamycin in mice indicated above [3] certainly need further evaluation.

In the second paper by Comas et al. [6], the authors treat p53 null (p53-/-) mice with rapamycin from the age of 8 weeks. These mice are profoundly tumor prone and succumb to lymphomas by 4-6 months of age. In this paper, however, the bioavailability of the relatively insoluble rapamycin was enhanced by a novel rapamycin formulation called Rapatar that improved

water solubility. The authors showed that blood rapamycin levels were significantly increased in animals treated with Rapatar compared to the standard form of rapamycin. As with the p53+/- mice, Rapatar treatment of the p53-/- mice resulted in significant longevity extension and delayed cancer formation relative to untreated p53-/- mice. Mean tumor latencies for the control p53-/- mice and the Rapatar-treated p53-/- were 161 and 261 days, respectively, a very significant effect. The authors argue that improvement of rapamycin bioavailabity through improved formulations is a necessity for clinical applications. They are uncertain whether the rapamycin effects are direct or indirect, but believe it to delay tumorigenesis by slowing aging. However, because the p53-/- mice are relatively young when they develop tumors, this interpretation seems less likely. Nevertheless, both papers represent exciting new advances that could lead us closer to pharmaceuticals that both enhance lifespan and delay cancer.

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## REFERENCES

**1.** Zoncu R, Efeyan A, Sabatini DM. Nat Rev Mol Cell Biol. 2011; 12:21-35.
**2.** Harrison DE, Strong R, Sharp ZD et al. Nature 2009; 460:392-395.
**3.** Wilkinson JE, Burmeister L, Brooks SV et al. Aging Cell. 2012; 11:675-682.
**4.** Anisimov VN, Zabezhinski MA, Popovich IG et al. Am J Pathol. 2010; 176:2092-2097.
**5.** Komarova EA, Antoch MP, Novototskaya LR et al. Aging. 2012; 4: this issue
**6.** Comas M, Toshkov I, Kuropatwinski KK et al. Aging. 2012; 4
**7.** Donehower LA, Lozano G. Nat Rev Cancer 2009; 9:831-841.
**8.** Malkin D. Genes Cancer. 2011; 2:475-484.

Research Paper

# Rapamycin extends lifespan and delays tumorigenesis in heterozygous p53+/- mice

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**Abstract: The TOR (Target of Rapamycin) pathway accelerates cellular and organismal aging. Similar to rapamycin, p53 can inhibit the mTOR pathway in some mammalian cells. Mice lacking one copy of p53 (p53+/- mice) have an increased cancer incidence and a shorter lifespan. We hypothesize that rapamycin can delay cancer in heterozygous p53+/- mice. Here we show that rapamycin (given in a drinking water) extended the mean lifespan of p53+/- mice by 10% and when treatment started early in life (at the age less than 5 months) by 28%. In addition, rapamycin decreased the incidence of spontaneous tumors. This observation may have applications in management of Li-Fraumeni syndrome patients characterized by heterozygous mutations in the *p53* gene.**

## INTRODUCTION

The mTOR (mammalian Target of Rapamycin) pathway plays a crucial role in the geroconversion from cell cycle arrest to senescence (geroconversion) [1]. Rapamycin suppresses or decelerates geroconversion, maintaining quiescence instead [2-8]. Furthemore, inhibition of the TOR pathway prolongs lifespan in model organisms, including mice [9-13]. In an organism, nutrients activate mTOR [14-16], whereas fasting or calorie restriction deactivates mTOR [17-19]. Calorie restriction slows down aging [20] and postpones tumorigenesis in several animal models [21, 22], including p53-deficient mice [23-25].

Similar to other tumor suppressors, p53 can inhibit mTOR in mammalian cells [26-31]. While causing cell cycle arrest, p53 can suppress geroconversion, thus preventing a senescent phenotype in the arrested cells [30, 31]. Therefore, it is not suprising that p53 inhibits hyper-secretory phenotype, a hallmark of senescence [32] whereas p53-deficiency resulted in pro-inflammatory phenotype [33, 34]. Noteworthy, the activity of p53 is decreased with aging [35]. Lack of one p53-allele (p53+/- ) accelerates carcinogenesis and shortens lifespan [36-41]. We propose that rapamycin can decelerate cancer development in p53+/- mice. Here we show experimental evidence supporting this hypothesis.

## RESULTS

Rapamycin (approximate dose, 1.5 mg/kg/day) was given in drinking water. 75 mice were divided into two groups: control (n=38) and rapamycin-treated (n=37). The mean lifespan of animals in control group was 373 days and the last 10% of survivals lived as long as 520 days (Fig. 1 A). In rapamycin-treated mice, the mean lifespan was 410 days and lifespan of the last 10% of survivals could not be determined (Fig. 1 A). Mice in both groups were also monitored for tumor development. The data presented in Fig. 1B

demonstrate that carcinogenesis was significantly delayed in rapamycin-treated mice compared to control mice.

Since in our experiments animals started to receive rapamycin at different age, we sought to test whether this affected the outcome of the treatment.

For this, we further subdivided all mice used into two groups: "young" (receiving rapamycin from the age of 5 months or earlier) and "old" (receiving rapamycin starting at 5 months of age or older). Results of the data analysis for the "young" group are shown in Figure 1C and D. The mean lifespan in control group was 373 days, whereas in rapamycin-treated "young" mice the mean lifespan reached 480 days, 3.5 months increase over the control group. Furthermore, 40% of rapamycin-treated "young" mice survived 550 days (Fig. 1C) and by this age developed 2 times less tumors than control mice (Fig. 1D). In the "old" group the difference between control and treated group was blunted (data not shown).

Thus, the life-extending effect of rapamycin is more pronounced when treatment starts earlier in life. In order to confirm that rapamycin administered with drinking water has biological activity in vivo, we measured levels of phosphorylated ribosomal protein S6 (pS6), a marker of the mTOR activity in tissues of control and rapamycin-treated mice. After receiving rapamycin in drinking water for 2 days, mice were sacrificed and the levels of total S6 and pS6 were estimated by Western blot analysis and immuno-cytochemistry (Fig. 2).

As shown in Fig. 2A, levels of pS6 were reduced in the heart, kidney and liver of rapamycin-treated mice. Also, pS6/S6 ratios were lower in rapamycin-treated mice (Fig. S1).

These results were confirmed by immunohistochemical staining showing lower levels of pS6 in tissues of rapamycin-treated mice (Fig. 2B). The variability of pS6 levels among mice may explain the variability of biological effects of rapamycin.

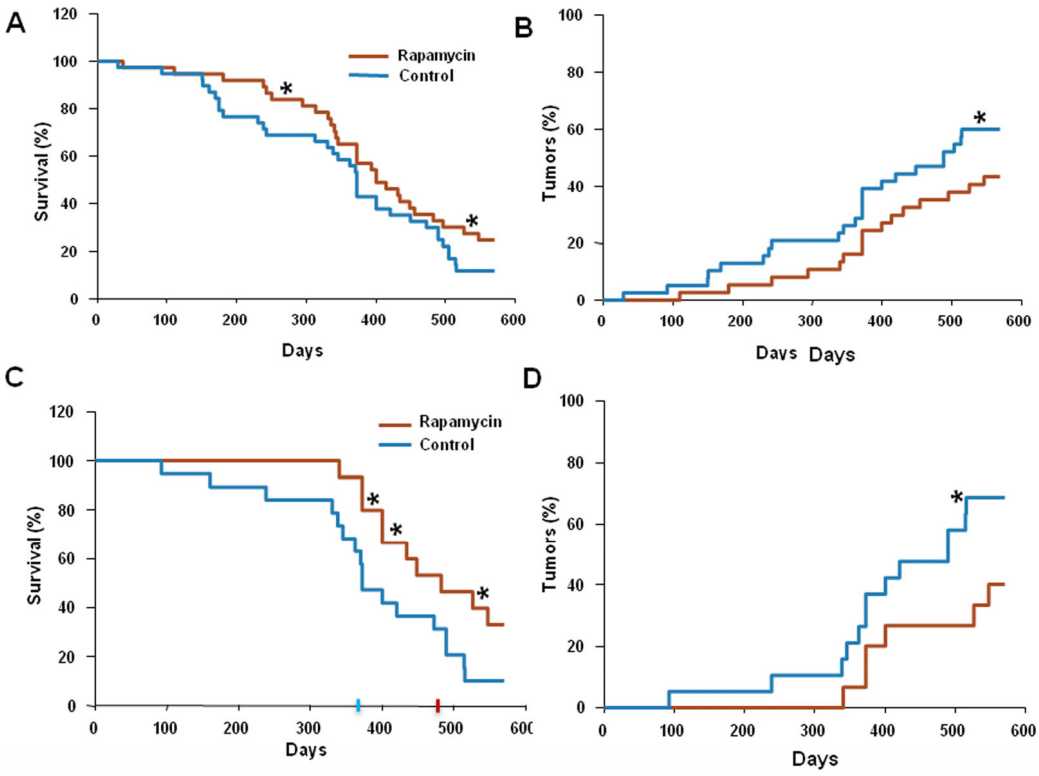



**Figure 1. Administration of rapamycin extends lifespan and delays carcinogenesis in p53+/- male mice.** (**A**) Kaplan Meier survival curve of rapamycin-treated (red line) and control (blue line) mice. (**B**) Incidence of tumors in rapamycin-treated (red) and control (blue) mice. Animals received rapamycin starting at various ages at 1.5 mg/kg per day in drinking water throughout entire life. * p<0.05. (**C**) Kaplan Meier survival curve of rapamycin-treated (red line) and control (blue line) mice that start receiving rapamycin early in life (<5 months). (**D**) Incidence of tumors in rapamycin-treated (red) and control (blue) mice that start receiving rapamycin early in life (<5 months). * p<0.05 toph

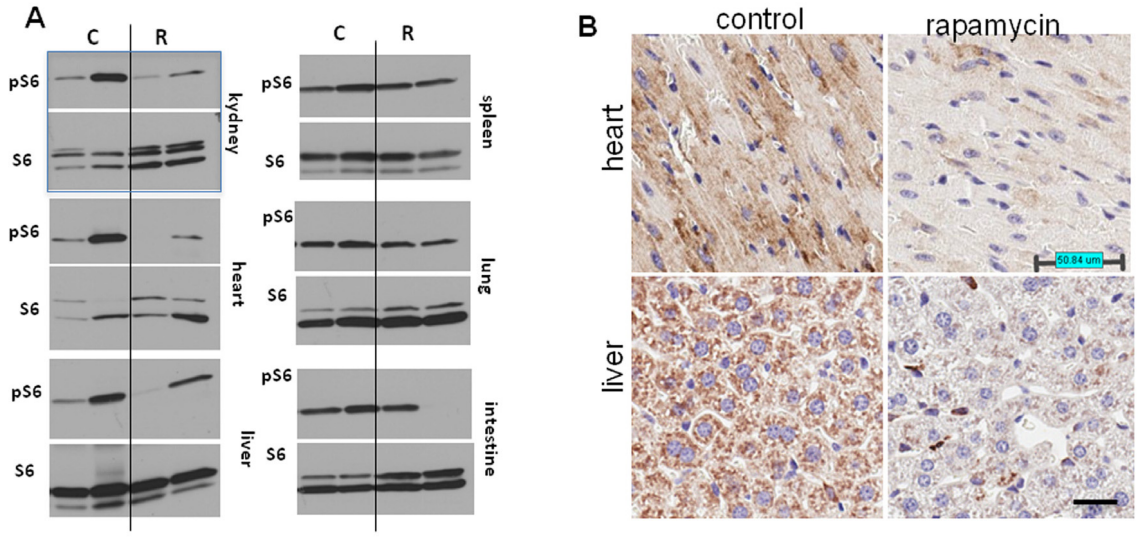



**Figure 2. Administration of rapamycin in drinking water inhibits the mTOR pathway in p53+/- male mice.** (A) Western blot analysis of whole cell lysates of 6 organs of rapamycin-treated and control mice probed with antibodies specific to S6 and phospho-S6 (Ser240/244). Mice received rapamycin in drinking water for 2 days. (**B**) Immunohistochemistry. pS6 in the heart and the liver. Mice received rapamycin in drinking water for 2 days.

## DISCUSSION

Previously it was shown that rapamycin prolongs lifespan in genetically heterogeneous mice [11], [12], inbred mice [42] and Her2-expressing mice [13]. In normal genetically heterogeneous mice, rapamycin extended life span even when its administration was started later in life [11]. Our data in p53+/- mice show that the effect of rapamycin was blunted when treatment started at the age of 5 months or older.

This indicates that the anti-cancer effect of rapamycin is likely to be indirect and is imposed via its systemic effect at the level of an organism rather than through direct inhibition of tumor growth. To further address this question we plan to test the effect of rapamycin on animals with established tumors (by measuring tumor growth) along with evaluating the functional status of mTOR and the ability of rapamycin to suppress it in tumors and normal tissues. As we report here, administration of rapamycin starting early in life increased mean lifespan in p53+/- male mice by 28%. Previous work has demonstrated that the life-extending effects of rapamycin [11, 12] as well as metformin [43, 44], calorie restriction [45] and genetic inhibition of the IGF-I/mTOR/S6K pathway [46, 47] were less pronounced in male mice compared with female mice. Moreover, in some cases, life span extension was achieved in female mice only [43, 47]. Therefore, the observed increase in the median lifespan is dramatic, taking into account that it was achieved in male mice. However, because of low bioavailability of rapamycin, it was given constantly (in drinking water) without interruptions, whereas intermittent schedules may be more appropriate for future clinical developments as cancer-preventive interventions. In fact, a novel formulation of rapamycin (Rapatar) may be given intermittently, which still reveal even more pronounced extension of life span in p53-deficient mice (Comas et al, Aging 2012; this issue).

Our study suggests that rapamycin can be considered for cancer prevention in patients with Li-Fraumeni syndrome. Li-Fraumeni syndrome is an autosomal dominant disorder with a germline p53 mutation [48]. The incidence of cancer in carriers of mutation reaches 50% at the age of 40 and 90% at the age 60. Children of affected parents have an approximate 50% risk of inheriting the familial mutation [48]. Although functional assays have been established allowing for easy genetic testing for TP53 mutation, no effective chemopreventive therapy is currently available. The p53 rescue compounds may hold some promise in the future [48-50]; however these are not clinically approved drugs. In contrast, rapamycin has been used in the clinic for over a decade mostly in renal transplant patients. It was reported that rapamycin significantly decreased cancer incidence in renal transplant patients [51-53].

Our data suggest that rapamycin or its analogs can be considered for cancer prevention in Li-Fraumeni syndrome.

## METHODS

Mice. All animal studies were conducted in accordance with the regulations of the Committee of Animal Care and Use at Roswell Park Cancer Institute. The colony of p53-knockout mice on a C57B1/6 background (originally obtained from Jackson Laboratories, Bar Habor, ME) was maintained by crossing p53+/- females with p53-/- males followed by genotyping of the progeny (PCR) as described previously [54]. Heterozygous p53+/- mice were generated by crossing p53-/- males with wild type p53 females. Male mice were kept in polypropelene cages (30x21x10 cm) under standard light/dark regimen (12 hours light : 12 hours darkness) at 22 ± 2 °C, And received standard laboratory chow and water ad libitum.

Rapamycin treatment. Rapamycin (LC Laboratories, USA) was diluted in ethanol at concentration 15 mg/ml. Then the stock was diluted 1:1000 in drinking water. Drinking water was changed every week. Male mice were randomly divided into two groups. Mice of the first group (n=37) were given rapamycin in drinking water (approximately 1.5 mg/kg per day), whereas mice of the second group (n=38) were given tap water without rapamycin and served as control. Once a week all mice were palpated for detection of tumor mass appearance.

Pathomorphological examination. All animals were autopsied. Site, number and size of tumors were checked. All tumors, as well as the tissues and organs with suspected tumor development were excised and fixed in 10% neutral formalin. After the routine histological processing the tissues were embedded into paraffin. 5-7 μm thin histological sections were stained with haematoxylin and eosine and were microscopically examined. Tumors were classified according to International Agency for Research on Cancer recommendations.

Western blot analysis. Tissues were homogenized in Bullet blender using stainless steel 0.5 mm diameter beads (Next Advantage, Inc. NY, USA) and RIPA lysis buffer supplemented with protease and phosphatase inhibitors tablets (Roche Diagnostics, Indianopolis, IN, USA). Lysates were cleared by centrifugation at 4°C at 13000 rpm. Equal amounts of protein were separated on gradient Criterion gels (BioRad) and immunoblotting was performed with rabbit anti-phospho S6 (Ser 240/244) and mouse anti-S6 antibodies from Cell Signaling Biotechnology as described previously [55], [56].

Immunochemistry. Dissected tissue samples were fixed in 10% buffered formalin, embedded into paraffin. 5-7 μm thin histological sections were stained with anti-phospho S6 (Ser240/244) antibody (Cell Signaling) and counterstained with Hematoxylin.

Statistical analyses. The SigmaStat software package was used for analysis. The P values were calculated using Fisher's Exact Test (2-tail). $P<0.05$ was considered as statistically significant.

### Conflict of Interest Statement

The authors of this manuscript have no conflict of interests to declare.

## REFERENCES


**1.** Blagosklonny MV. Cell cycle arrest is not yet senescence, which is not just cell cycle arrest: terminology for TOR-driven aging. Aging (Albany NY). 2012; 4: 159-165.

**2.** Demidenko ZN, Zubova SG, Bukreeva EI, Pospelov VA, Pospelova TV, Blagosklonny MV. Rapamycin decelerates cellular senescence. Cell Cycle. 2009; 8: 1888-1895.

**3.** Gan B, Sahin E, Jiang S, Sanchez-Aguilera A, Scott KL, Chin L, Williams DA, Kwiatkowski DJ, DePinho RA. mTORC1-dependent and -independent regulation of stem cell renewal, differentiation, and mobilization. Proc Natl Acad Sci U S A. 2008; 105: 19384-19389.

**4.** Gan B, DePinho RA. mTORC1 signaling governs hematopoietic stem cell quiescence. Cell Cycle. 2009; 8: 1003-1006.

**5.** Chen C, Liu Y, Liu R, Ikenoue T, Guan KL, Zheng P. TSC-mTOR maintains quiescence and function of hematopoietic stem cells by repressing mitochondrial biogenesis and reactive oxygen species. J Exp Med. 2008; 205: 2397-2408.

**6.** Chen C, Liu Y, Zheng P. mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells. Sci Signal. 2009; 2: ra75.

**7.** Adhikari D, Zheng W, Shen Y, Gorre N, Hamalainen T, Cooney AJ, Huhtaniemi I, Lan ZJ, Liu K. Tsc/mTORC1 signaling in oocytes governs the quiescence and activation of primordial follicles. Human Molecular Genet. 2010; 19:397-410.

**8.** Kolesnichenko M, Hong L, Liao R, Vogt PK, Sun P. Attenuation of TORC1 signaling delays replicative and oncogenic RAS-induced senescence. Cell Cycle. 2012; 11: 2391-2401.

**9.** Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S. Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. Curr Biol. 2004; 14: 885-890.

**10.** Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, Partridge L. Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. Cell Metab. 2010; 11: 35-46.

**11.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandezr E, Miller RA. Rapamycin fed late in life extends

lifespan in genetically heterogenous mice. Nature. 2009; 460: 392-396.

**12.** Miller RA, Harrison DE, Astle CM, Baur JA, Boyd AR, de Cabo R, Fernandez E, Flurkey K, Javors MA, Nelson JF, Orihuela CJ, Pletcher S, Sharp ZD, Sinclair D, Starnes JW, Wilkinson JE et al. Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice. J Gerontol A Biol Sci Med Sci. 2011; 66: 191-201.

**13.** Anisimov VN, al. e, Antoch M, Blagosklonny MV. Am J Pathol. 2010: in press.

**14.** Khamzina L, Veilleux A, Bergeron S, Marette A. Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: possible involvement in obesity-linked insulin resistance. Endocrinology. 2005; 146: 1473-1481.

**15.** Tremblay F, Krebs M, Dombrowski L, Brehm A, Bernroider E, Roth E, Nowotny P, WaldhŠusl W, Marette A, Roden M. Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability. Diabetes. 2005; 54: 2674-2684.

**16.** Krebs M, Brunmair B, Brehm A, Artwohl M, Szendroedi J, Nowotny P, Roth E, FŸrnsinn C, Promintzer M, Anderwald C, Bischof M, Roden M. The Mammalian target of rapamycin pathway regulates nutrient-sensitive glucose uptake in man. Diabetes. 2007; 56: 1600-1607.

**17.** Jiang W, Zhu Z, Thompson HJ. Dietary energy restriction modulates the activity of AMP-activated protein kinase, Akt, and mammalian target of rapamycin in mammary carcinomas, mammary gland, and liver. Cancer Res. 2008; 68: 5492-5499.

**18.** Estep PWr, Warner JB, Bulyk ML. Short-term calorie restriction in male mice feminizes gene expression and alters key regulators of conserved aging regulatory pathways. PLoS One. 2009; 4: e5242.

**19.** Masternak MM, Panici JA, Bonkowski MS, Hughes LF, Bartke A. Insulin sensitivity as a key mediator of growth hormone actions on longevity. J Gerontol A Biol Sci Med Sci. 2009; 64: 516-521.

**20.** Wang C, Maddick M, Miwa S, Jurk D, Czapiewski R, Saretzki G, Langie SA, Godschalk RW, Cameron K, von Zglinicki T. Adult-onset, short-term dietary restriction reduces cell senescence in mice. Aging (Albany NY). 2010; 2: 555-566.

**21.** Dirx MJ, Zeegers MP, Dagnelie PC, van den Bogaard T, van den Brandt PA. Energy restriction and the risk of spontaneous mammary tumors in mice: a meta-analysis. Int J Cancer. 2003; 106: 766-770.

**22.** Sarkar NH, Fernandes G, Telang NT, Kourides IA, Good RA. Low-calorie diet prevents the development of mammary tumors in C3H mice and reduces circulating prolactin level, murine mammary tumor virus expression, and proliferation of mammary alveolar cells. Proc Natl Acad Sci U S A. 1982; 79: 7758-7762.

**23.** Berrigan D, Perkins SN, Haines DC, Hursting SD. Adult-onset calorie restriction and fasting delay spontaneous tumorigenesis in p53-deficient mice. Carcinogenesis. 2002; 23: 817-822.

**24.** Hursting SD, Perkins SN, Phang JM. Calorie restriction delays spontaneous tumorigenesis in p53-knockout transgenic mice. Proc Natl Acad Sci U S A. 1994; 91: 7036-7040.

**25.** Hursting SD, Perkins SN, Brown CC, Haines DC, Phang JM. Calorie restriction induces a p53-independent delay of spontaneous carcinogenesis in p53-deficient and wild-type mice. Cancer Res. 1997; 57: 2843-2846.

**26.** Feng Z, Hu W, de Stanchina E, Teresky AK, Jin S, Lowe S, Levine AJ. The regulation of AMPK beta1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways. Cancer Res. 2007; 67: 3043-3053.

**27.** Feng Z, Zhang H, Levine AJ, Jin S. The coordinate regulation of the p53 and mTOR pathways in cells. Proc Natl Acad Sci U S A. 2005; 102: 8204-8209.

**28.** Levine AJ, Feng Z, Mak TW, You H, Jin S. Coordination and communication between the p53 and IGF-1-AKT-TOR signal transduction pathways. Genes Dev. 2006; 20: 267-275.

**29.** Budanov AV, Karin M. p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. Cell. 2008; 134: 451-460.

**30.** Demidenko ZN, Korotchkina LG, Gudkov AV, Blagosklonny MV. Paradoxical suppression of cellular senescence by p53. Proc Natl Acad Sci U S A. 2010; 9660-4: 9660-9664.

**31.** Korotchkina LG, Leontieva OV, Bukreeva EI, Demidenko ZN, Gudkov AV, Blagosklonny MV. The choice between p53-induced senescence and quiescence is determined in part by the mTOR pathway. Aging (Albany NY). 2010; 2: 344-352.

**32.** CoppŽ JP, Patil CK, Rodier F, Sun Y, Mu–oz DP, Goldstein J, Nelson PS, Desprez PY, Campisi J. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol. 2008; 6: 2853-2868.

**33.** Komarova EA, Krivokrysenko V, Wang K, Neznanov N, Chernov MV, Komarov PG, Brennan ML, Golovkina TV, Rokhlin OW, Kuprash DV, Nedospasov SA, Hazen SL, Feinstein E, Gudkov AV. p53 is a suppressor of inflammatory response in mice. FASEB J. 2005; 19: 1030-1032.

**34.** Gudkov AV, Gurova KV, Komarova EA. Inflammation and p53: A Tale of Two Stresses. Genes Cancer. 2011; 2: 503-516.

**35.** Feng Z, Hu W, Teresky AK, Hernando E, Cordon-Cardo C, Levine AJ. Declining p53 function in the aging process: a possible mechanism for the increased tumor incidence in older populations. Proc Natl Acad Sci U S A. 2007; 104: 16633-16638.

**36.** Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Jr., Butel JS, Bradley A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature. 1992; 356: 215-221.

**37.** Harvey M, McArthur MJ, Montgomery CA, Jr., Butel JS, Bradley A, Donehower LA. Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. Nat Genet. 1993; 5: 225-229.

**38.** Jacks T, Remington L, Williams BO, Schmitt EM, Halachmi S, Bronson RT, Weinberg RA. Tumor spectrum analysis in p53-mutant mice. Curr Biol. 1994; 4: 1-7.

**39.** Donehower LA, Harvey M, Vogel H, McArthur MJ, Montgomery CA, Jr., Park SH, Thompson T, Ford RJ, Bradley A. Effects of genetic background on tumorigenesis in p53-deficient mice. Mol Carcinog. 1995; 14: 16-22.

**40.** Venkatachalam S, Shi YP, Jones SN, Vogel H, Bradley A, Pinkel D, Donehower LA. Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. Embo J. 1998; 17: 4657-4667.

**41.** Hinkal G, Parikh N, Donehower LA. Timed somatic deletion of p53 in mice reveals age-associated differences in tumor progression. PLoS One. 2009; 4: e6654.

**42.** Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Rosenfeld SV,

Blagosklonny MV. Rapamycin increases lifespan and inhibits spontaneous tumorigenesis in inbred female mice. Cell Cycle. 2011; 10: 4230-4236.

**43.** Anisimov VN, Piskunova TS, Popovich IG, Zabezhinski MA, Tyndyk ML, Egormin PA, Yurova MV, Rosenfeld SV, Semenchenko AV, Kovalenko IG, Poroshina TE, Berstein LM. Gender differences in metformin effect on aging, life span and spontaneous tumorigenesis in 129/Sv mice. Aging (Albany NY). 2010; 2: 945-958.

**44.** Blagosklonny MV. Metformin and sex: Why suppression of aging may be harmful to young male mice. Aging (Albany NY). 2010; 2: 897-899.

**45.** Turturro A, Duffy P, Hass B, Kodell R, Hart R. Survival characteristics and age-adjusted disease incidences in C57BL/6 mice fed a commonly used cereal-based diet modulated by dietary restriction. J Gerontol A Biol Sci Med Sci. 2002; 57: B379-389.

**46.** Holzenberger M, Dupont J, Ducos B, Leneuve P, Geloen A, Even PC, Cervera P, Le Bouc Y. IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature. 2003; 421: 182-187.

**47.** Selman C, Tullet JM, Wieser D, Irvine E, Lingard SJ, Choudhury AI, Claret M, Al-Qassab H, Carmignac D, Ramadani F, Woods A, Robinson IC, Schuster E, Batterham RL, Kozma SC, Thomas G et al. Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. Science. 2009; 326: 140-144.

**48.** Upton B, Chu Q, Li BD. Li-Fraumeni syndrome: the genetics and treatment considerations for the sarcoma and associated neoplasms. Surg Oncol Clin N Am. 2009; 18: 145-156, ix.

**49.** Glazer RI. A new therapeutic basis for treating Li-Fraumeni Syndrome breast tumors expressing mutated TP53. Oncotarget. 2010; 1: 470-471.

**50.** Herbert BS, Chanoux RA, Liu Y, Baenziger PH, Goswami CP, McClintick JN, Edenberg HJ, Pennington RE, Lipkin SM, Kopelovich L. A molecular signature of normal breast epithelial and stromal cells from Li-Fraumeni syndrome mutation carriers. Oncotarget. 2010; 1: 405-422.

**51.** Mathew T, Kreis H, Friend P. Two-year incidence of malignancy in sirolimus-treated renal transplant recipients: results from five multicenter studies. Clin Transplant. 2004; 18: 446-449.

**52.** Campistol JM, Eris J, Oberbauer R, Friend P, Hutchison B, Morales JM, Claesson K, Stallone G, Russ G, Rostaing L, Kreis H, Burke JT, Brault Y, Scarola JA, Neylan JF. Sirolimus Therapy after Early Cyclosporine Withdrawal Reduces the Risk for Cancer in Adult Renal Transplantation. J Am Soc Nephrol. 2006; 17: 581-589.

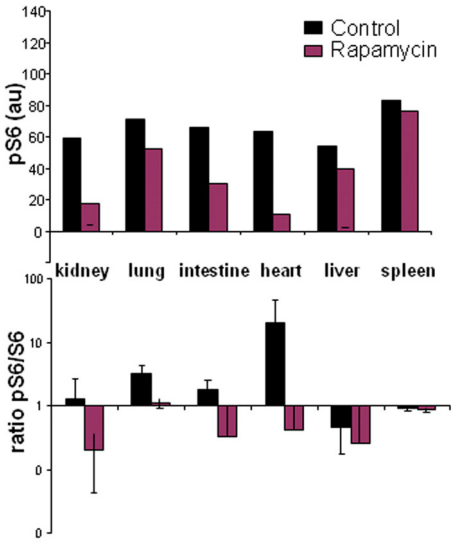
**53.** Blagosklonny MV. Prevention of cancer by inhibiting aging. Cancer Biol Ther. 2008; 7: 1520-1524.

**54.** Leonova KI, Shneyder J, Antoch MP, Toshkov IA, Novototskaya LR, Komarov PG, Komarova EA, Gudkov AV. A small molecule inhibitor of p53 stimulates amplification of hematopoietic stem cells but does not promote tumor development in mice. Cell Cycle. 2010; 9: 1434-1443.

**55.** Leontieva O, Gudkov A, Blagosklonny M. Weak p53 permits senescence during cell cycle arrest. Cell Cycle. 2010; 9: 4323-4327.

**56.** Leontieva OV, Blagosklonny MV. DNA damaging agents and p53 do not cause senescence in quiescent cells, while consecutive re-activation of mTOR is associated with conversion to senescence. Aging (Albany NY). 2010; 2: 924-935.

## SUPPLEMENTAL FIGURE




**Supplemental Figure S1. Quantitative analysis of data shown in Figure 2A.** Top panel - Intensity of phosphorylated S6 (pS6) signal was quantified using ImageJ program (intensity units, IU). Bottom panel – Intensity of pS6 and S6 signals were quantified and the ratio pS6/S6 was calculated.

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Research Paper

# New nanoformulation of rapamycin Rapatar extends lifespan in homozygous *p53*$^{-/-}$ mice by delaying carcinogenesis

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**Abstract: The nutrient-sensing mTOR (mammalian Target of Rapamycin) pathway regulates cellular metabolism, growth functions, and proliferation and is involved in age-related diseases including cancer, type 2 diabetes, neurodegeneration and cardiovascular disease. The inhibition of mTOR by rapamycin, or calorie restriction, has been shown to extend lifespan and delays tumorigenesis in several experimental models suggesting that rapamycin may be used for cancer prevention. This requires continuous long-term treatment making oral formulations the preferred choice of administration route. However, rapamycin by itself has very poor water solubility and low absorption rate. Here we describe pharmacokinetic and biological properties of novel nanoformulated micelles of rapamycin, Rapatar. Micelles of Rapatar were rationally designed to increase water solubility of rapamycin to facilitate oral administration and to enhance its absorption. As a result, bioavailability of Rapatar was significantly increased (up to 12%) compared to unformulated rapamycin, which concentration in the blood following oral administration remained below level of detection. We also demonstrated that the new formulation does not induce toxicity during lifetime administration. Most importantly, Rapatar extended the mean lifespan by 30% and delayed tumor development in highly tumor-prone *p53*$^{-/-}$ mice. Our data demonstrate that water soluble Rapatar micelles represent safe, convenient and efficient form of rapamycin suitable for a long-term treatment and that Rapatar may be considered for tumor prevention.**

## INTRODUCTION

Rapamycin (or Sirolimus) is a macrolide antibiotic that was first isolated from *Streptomyces hydroscopicus* and was initially utilized as an antifungal agent [1, 2]. Under the name of Rapamune, it is now used as an immunosuppressant to prevent organ rejection after transplantation. Rapamycin inhibits the nutrient-sensing mTOR (mammalian Target of Rapamycin), a conserved protein kinase that controls cellular growth and metabolism. The mTOR signaling pathway is activated by nutrients, growth factors, hormones, cytokines, and cellular energy status. When nutrients and growth factors are abundant, mTOR promotes protein synthesis, ribosome biogenesis, angiogenesis, cell cycle progression and cytoskeleton re-organization (reviewed in [3]-5]).

Recent data demonstrated that rapamycin extends life span in various model organisms including mammals [4-6]. The life-long administration of rapamycin inhibits age-related weight gain, decreases aging rate and increases lifespan of inbred [7] and genetically heterogeneous [6] mice. Previous data has

demonstrated that rapamycin significantly delayed the onset of spontaneous carcinogenesis both in normal (129/Sv [7]) and cancer-prone (HER-2/neu transgenic [8] and $p53^{+/-}$ [9]) mice. Importantly, the anti-cancer effect of rapamycin in $p53^{+/-}$ mice was blunted when treatment started at the age of 5 months [9] suggesting that rapamycin does not directly inhibit tumor growth but rather has an indirect effect.

Since rapamycin exhibits poor water solubility and instability in aqueous solutions, its clinical use through oral administration requires development of special drug design such as complex nanoparticle formulation to facilitate increased bioavailability and efficacy. Therefore, various oral formulations, such as inclusion complexes [10, 11], liposomes [12], nanocrystals [13], and solid dispersion [14] have been developed and tested in pre-clinical and clinical studies. In this study, we tested the biological activity of a novel formulation of rapamycin, Rapatar. This formulation is based on Pluronic block copolymers as nanocarriers, which serves to improve water solubility of the drug, and to enhance various biological responses favorable for therapeutics, such as activity of drug efflux transporters (reviewed in [15]). We show that Rapatar has significantly higher bioavailability after oral administration when compared to unformulated rapamycin. We also show that Rapatar effectively blocks mTOR in mouse tissues. Moreover, life-long administration of Rapatar increases lifespan and delays carcinogenesis in highly tumor-prone $p53^{-/-}$ mice.

## RESULTS

### Rapatar is efficiently absorbed and systemically distributed and effectively inhibits mTOR *in vivo*

To compare the absolute and relative bioavailability and other pharmacokinetic properties of Rapatar with those of an unformulated rapamycin, we administered both compounds as a single dose to female ICR mice. Rapatar was administered intravenously (IV) or orally (PO) at a dose of 0.4 mg/kg and 4 mg/kg respectively, while rapamycin was administered PO at 4 mg/kg. Blood samples were collected at different times after administration and analyzed for rapamycin by mass spectrometry (LC/MS/MS). Pharmacokinetic values of the area under the curve (AUC), the maximum drug concentration ($C_{max}$), the time of peak concentration ($T_{max}$), and the absolute bioavailability (F) were calculated from whole blood drug concentration-time data (Fig. 1A). Importantly, following oral administration, rapamycin could only be detected in whole blood samples of mice that received Rapatar whereas its concentration in blood of rapamycin-treated mice was beyond the level of detection. As shown in Table 1, when compared to unformulated rapamycin, Rapatar demonstrated very fast absorption ($T_{max}$ 15 min) and significant increase in AUC value with mean $T_{1/2}$ extending to 6.4 hours. Consequently, a single oral administration of Rapatar resulted in 12% bioavailability, which is comparable with commercially available formulations used in clinical practice (14% when administered orally in combination with cyclosporine A).

Ribosomal protein S6 is a substrate of mTOR, and therefore phospho-ribosomal protein S6 is a marker of mTOR activity [16-19]. To test whether Rapatar inhibits mTOR activity in vivo, we compared levels of phosphorylated S6 (pS6) in livers of wild type C57Bl/6J mice, in which mTOR was suppressed by a period of food deprivation. Rapatar (0.5mg/kg or PBS were given by gavage at a time when animals were allowed access to food. Fig. 1B shows that S6 is highly phosphorylated in livers of control animals indicating mTOR activation in response to food. In contrast, in animals that received Rapatar, S6 phosphorylation was reduced ~10- fold. Thus, Rapatar successfully inhibits mTOR activity in the liver in vivo.

**Table 1.** Pharmacokinetic parameters of unformulated rapamycin and Rapatar in C57Bl/6J mice. Abbreviations: $C_{max}$ – the peak concentration; $T_{max}$ – time taken to reach peak concentration; AUC – area under the curve; F – absolute bioavailability.

| | Units | Rapamycin, IV 0.4mg/kg | Rapatar, PO 4mg/kg |
|---|---|---|---|
| Dose amount | ng | 10.4 | 104 |
| Dosage | ng/kg | 400 | 4000 |
| Cmax | ng/ml | 958 | 656 |
| Tmax | hr | 0.04 | 0.25 |
| AUC | ng-hr/ml | 2634.6 | 3161.5 |
| Half-life | hr | 6.4 | N/A |
| F | % | 100 | 12 |

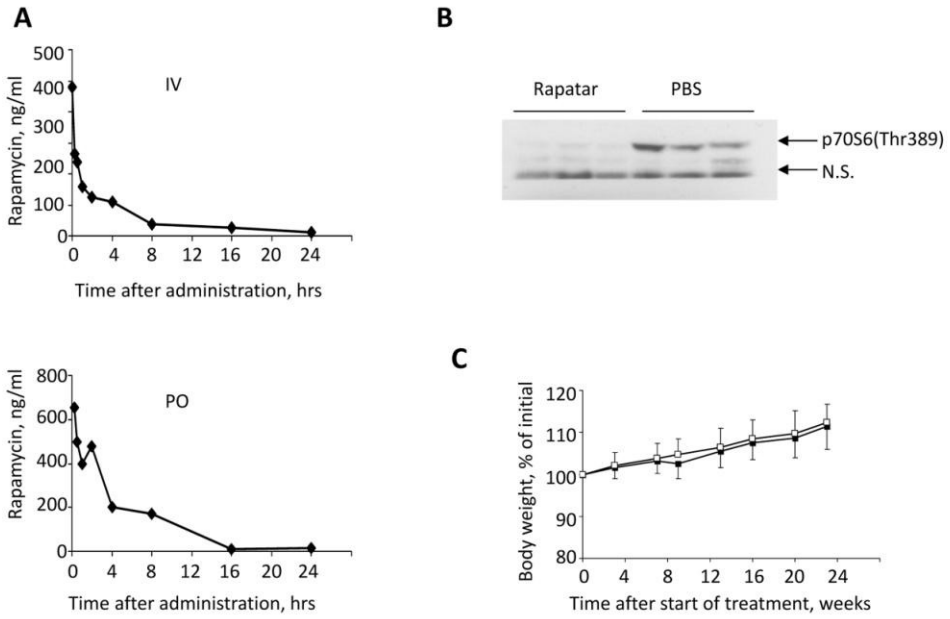


**Figure 1**. Pharmacokinetic and biological characteristics of Rapatar. (**A**) Rapamycin concentration–time profile in blood after intravenous (IV, top) and oral (PO, bottom) administration of Rapatar to mice (mean values, n = 3). A single dose of Rapatar was administered either IV (0.4mg/kg) or PO (4mg/kg). Blood samples were collected at designated times and analyzed for rapamycin by **LC/MS/MS.** (**B**) Rapatar blocks mTOR activation in vivo. Six C57/Bl/6J mice were food-deprived for 18 hrs. At the end of fasting period animals received either Rapatar (0.5mg/kg) or PBS via gavage and were allowed access to food. One hour later animals were sacrificed, livers were dissected and protein lysates were analyzed for mTOR activity by probing with p70S6(Thr389) antibody. (**C**) No acute or long-term toxicity are associated with PO administration of Rapatar. C57Bl/6J male mice received either Rapatar or PBS starting 8 weeks of age (10 mice/group) for 24 weeks according to the protocol described above. No loss in body weight was detected in experimental group throughout the treatment period. Both experimental and control groups showed similar gain in body weight with age.

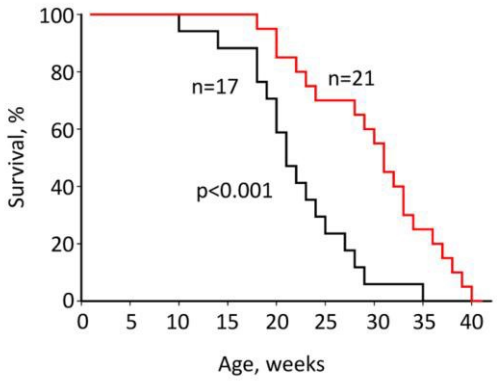


**Figure 2**. Rapatar increases lifespan in *p53*$^{-/-}$ mice. Mice received Rapatar at 0.5 mg/kg via gavage according to the schedule described in Materials and Methods. Rapatar increased lifespan from 23 to 31 weeks (p<0.001, Mantel-Cox log-rank test).

To test whether life-long administration of Rapatar causes in vivo toxicity, we administered it to wild type C57Bl/6J mice at 0.5 mg/kg via gavage according to protocol described in Materials and Methods section. Rapatar- and PBS-treated animals were monitored for any signs of toxicity by visual inspection and body weight measurements. Mice receiving Rapatar maintained a healthy appearance with physical activities and body weights comparable to the control mice (Fig. 1C).

### Rapatar increases lifespan of *p53*$^{-/-}$ mice

Our data showed that Rapatar effectively inhibits mTOR *in vivo*. Suppression of mTOR by rapamycin has been shown to increase lifespan in various model organisms including mice [6-8, 20-25]. To test whether Rapatar can extend lifespan, we administered it to mice with targeted disruption of tumor suppressor p53. *p53*$^{-/-}$

mice are characterized by increased carcinogenesis and reduced lifespan (reviewed in [26]. Twenty $p53^{-/-}$ mice received Rapatar starting 8 weeks of age at a dose of 0.5mg/kg according to the schedule described in Material and Methods. Another group of 17 $p53^{-/-}$ mice received PBS as control. Throughout the experiment, animals were monitored for tumor development by visual inspection and total body weight measurements. Both Rapatar- and PBS-treated $p53^{-/-}$ mice die early in life due to a high rate of spontaneous carcinogenesis, which is characteristic for this mouse model. However, treatment with Rapatar resulted in an overall significant increase in median survival of $p53^{-/-}$ mice from 23 (±10) weeks in the control group to 31 (±1.5) weeks in the experimental group (Fig. 2A).

To gain insight into the potential mechanism of increase in survival of Rapatar-treated animals, we performed a detailed histological analysis of all tissues collected from each individual animal in the course of the experiment (summarized in Table 2). Based on this analysis, 82% of mice in the control group (14 out of 17) developed lymphomas whereas 12% (2 out of 17) developed sarcomas. One animal showed the presence of both sarcoma and lymphoma and one animal developed myeloid leukemia. This spectrum of tumors is characteristic to $p53^{-/-}$ mice and comparable to previous reports [27]. The mice developed these spontaneous neoplasms from 2 to over 8 months of age with an average latency time of 161 days. When compared to the control group, Rapatar-treated mice showed later appearance and delayed progression of spontaneous tumors. They arose from 4.5 to over 9.5 months, with average latency of 261 days; one animal remained tumor-free until the end of the experiment. Interestingly, the incidence of sarcomas in Rapatar-treated mice was increased to 30% compared to 17% in control group (Table 2); however the number of animals used in the experiment was not enough to obtain a statistically significant difference.

**Table 2.** Summary of histological analysis. Tissues of 17 control and 20 Rapatar-treated $p53^{-/-}$ mice were evaluated for the presence of tumor cells. The type of tumors and the stage of their development were determined as described in Materials and Methods. The incidence of sarcomas in Rapatar-treated $p53^{-/-}$ mice was higher than in control group (30% and 17% respectively); however, due to a relatively small group size, statistical significance was not achieved (p=0.2; Fisher's exact test).

| | Initial Lymphoma | Advanced Lymphoma | Sarcoma | Leukemia | TumorΔfree |
|---|---|---|---|---|---|
| Rapatar | 7 (35%) | 6 (30%) | 6 (30%) | 1 (5%) | 1 (5%) |
| PBS | 4 (23%) | 10 (58%) | 3 (17%) | 1 (6%) | 0 |

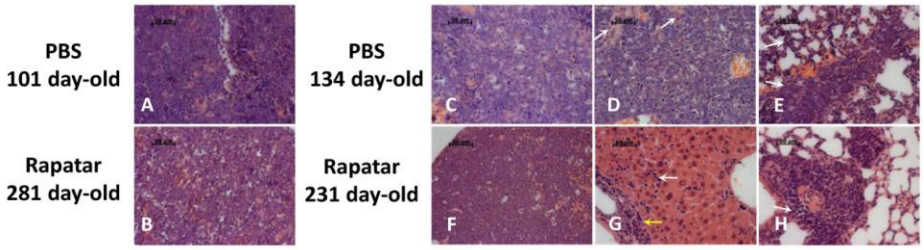


**Figure 3**. Rapatar delays development of lymphomas in $p53^{-/-}$ mice. (**A)** Representative initial lymphoma developed in control mouse at the age of 101 days. (**B**) Similar appearance of lymphoma in Rapatar-treated mouse at 281 days of age. Both **A** and **B** show monotonous infiltrate of medium-sized neoplastic cells with round nuclei, fine chromatin, indistinct nucleoli, and numerous mitotic figures and apoptotic cells. (**C**) Advanced lymphoma in 134-day old control mouse with metastases in liver (**D**) and lung (**E**). (**D**) Metastasis in liver showing the extensive spread of neoplastic cells effaces the normal structure and only minimal remnants of hepatocytes (marked by arrows). (**E**) Metastasis in the lung showing neoplastic infiltrates in perivascular area and in the alveolar walls (arrows) (**F**) Advanced lymphoma with pathological changes similar to shown in C in the thymus of 241day-old Rapatar-treated animal with metastasis in liver (**G**) and lung (**H**). (**G**) Metastasis in liver showing neoplastic infiltrates in portal tract (yellow arrow) and sinusoids (white arrow). (**H**) Metastasis in the lung showing perivascular neoplastic infiltrate (arrow).

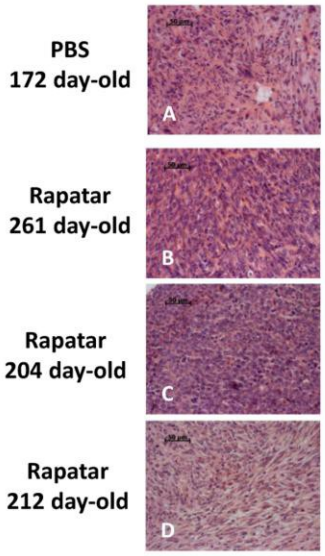


**Figure 4.** Rapatar delays development of sarcomas in *p53*$^{-/-}$ mice. (**A**) Liver sarcoma in 172-old control mouse. (**B,C**) Sarcoma developed in 261 day- and 204 day-old Rapatar-treated mice. No metastases are detected. D. Sarcoma in 212-day old Rapatar-treated mouse with metastases in the lung.

Since lymphomas represented the major type of tumor in both groups, we performed a detailed pathological evaluation of individual tumors. Based on the severity of pathological changes, the developmental stage, and involvement of non-lymphoid tissues, all lymphomas were graded as initial or advanced. Initial lymphomas mainly involved thymus and were presented macroscopically as enlarged masses. Under the microscope they were seen to be composed of broad sheets of densely packed rather uniform large lymphoblastic cells, with little or sparse cytoplasm that completely obliterated the normal thymus structure and cortical and medullary zones. In most cases, neoplastic lymphoid cells expanded through the thymic capsule and spread through the mediastinal fat, lymph nodes, along peritracheal and periaortal spaces, even infiltrating lungs and pericardium with limited penetration of the myocardium. Such lymphomas with predominantly local involvement were designated provisionally as initial. Tumors were graded as advanced when the rise of the malignancy and aggressiveness of the lymphoma cells resulted in metastases and infiltration into spleen, liver, lung, kidney, mesentery lymph nodes, testis, and bone marrow. Based on this designation, the proportion of the initial lymphomas in Rapatar-treated group was larger compared to controls (35% and 23% for experimental and control groups respectively) suggesting that Rapatar slows down tumorigenesis. Consistently, the proportion of the advanced disseminated lymphomas, spreading to other organs in Rapatar-treated group was smaller than in control (30% and 58% respectively). Although histopathological appearance of lymphomas and sarcomas were very similar in control and experimental groups, Rapatar-treated mice develop tumors significantly later in life (Fig. 3 and 4). Based on these data we concluded that Rapatar increased lifespan of *p53*$^{-/-}$ mice by delaying tumorigenesis.

## DISCUSSION

The mTOR signaling pathway is a key coordinator of cell growth and cell proliferation in response to a variety of environmental conditions. Its deregulation has been implicated in many pathological conditions, including those that are associated with aging, such as cancer, type 2 diabetes, neurological and cardiovascular disorders (reviewed in [28, 29]). Furthermore, the activation of the mTOR pathway is the most universal alteration in cancer [30]. Several analogs of rapamycin (rapalogs) have been approved for cancer therapy [31-35] and numerous clinical trials are underway. However, as anti-cancer drugs rapamycin and other rapalogs showed modest efficacy. There are several reasons that can explain relatively low therapeutic effect. First, rapamycin itself is not cytotoxic. Additionally, mTOR inhibition activates several feedback loops that drive mitogenic signaling (reviewed in [28, 36]). Therefore, it is still not quite clear whether rapamycin exhibits direct antitumor activity or whether it acts in a more indirect systemic way. Our previous data [9] and data presented here show that rapamycin delays carcinogenesis in tumor-prone *p53*$^{+/-}$ and *p53*$^{-/-}$ mice, most likely by slowing down the process of aging. If this is the case, than rapamycin can be considered as a tumor-preventive agent (i.e. administration is required before tumor initiation). This necessitates the development of efficacious nontoxic rapamycin-formulations that could be taken orally for extended periods of time. Here we show that oral administration of Rapatar results in high systemic bioavailability and does not induce toxicity during life-long administration. Importantly, biological effects of Rapatar were prominent at low doses (0.5 mg/kg) and intermittent schedules. Taken together, our data suggest that Rapatar is a promising candidate for clinical use as an effective cancer prevention drug.

## MATERIALS AND METHODS

Materials. Rapamycin was purchased from LC Laboratories (Woburn, MA). Polymeric formulation of

rapamycin (Rapatar) was developed by Tartis Aging, Inc. using Pluronic block co-polymers [15] according to the following protocol. One gram of rapamycin was dissolved in 25 ml of ethanol. The resulting solution was mixed with 5 grams of Pluronic L-92 (BASF) and 2 grams of citric acid dissolved in 200 ml of 20% Pluronic F-127 (BASF) solution in ethanol and water mixture (97:3 v:v). The solution was then incubated at 20-25ºC for 30 minutes with constant stirring. The ethanol was removed using Speedvac and the formulation was further dried using high vacuum.

Animals. ICR female mice were obtained from Charles River. C57Bl/6J mice were obtained from Jackson Laboratory. p53-/- mice on C57Bl/6J background originally obtained from Jackson Laboratory, were housed and bred at the Department of Experimental Animal Resources of Roswell Park Cancer Institute. For pharmacokinetic analysis, three groups of 8 weeks old ICR female mice received a single dose of either Rapatar (2 groups) or rapamycin. Rapatar was administered via gavage at 4mg/kg in 0.5% methyl cellulose or IV at 0.4mg/kg in PBS. Rapamycin was administered via gavage at 4mg/kg in 0.5% methyl cellulose.

For estimating potential long-term toxic effects of Rapatar, two groups of C57BL/6J mice received the drug at a dose of 0.5 mg/kg via gavage once a day for 5 consecutive days, followed by 9-day interval without treatment. Mice were maintained on this treatment schedule for 24 weeks and were weighed weekly. Control mice receive PBS according to the same schedule.

For longevity studies, 38 *p53*$^{-/-}$ male mice were randomly divided into two groups. Twenty one experimental animals received 0.5 mg/kg Rapatar and 17 animals received PBS according to the above described schedule. Treatment started at 8 weeks of age and continued until tumor appearance was visually observed or dramatic loss of weight, indicative of tumor appearance, was detected. At this point, mice were sacrificed and examined for gross pathological changes. Tumors, heart, kidney, liver, lungs, thymus and spleen were collected for histological evaluation. All procedures were approved by the Institutional Animal Care and Use Committee of Roswell Park Cancer Institute.

Pharmacokinetic study. Whole blood was collected into EDTA-blood tubes 0.5, 1, 2, 4, 8, 16 and 24 hours after administration of either Rapatar or unformulated rapamycin. Tubes were inverted a few times, and stored on ice in dark container during the experiment. At the end of the experiment, all samples were placed for storage at -70$^{0}$C in a light-protected container. Frozen blood samples were submitted to the Rocky Mountain Instrumental Laboratory (Fort Collins, Co) for LC/MS/MS analysis of rapamycin. Pharmacokinetic analysis was performed using data from individual mice for which the mean and standard error of the mean (SEM) were calculated for each group using PK Solutions software (Version 2.0).

Western blot analysis**.** In order to maximize and be able to detect p70S6 phosphorylation [37, 38], six C57Bl/6J mice were food-deprived for 18 hrs. At the end of the fasting period, animals received either Rapatar (0.5 mg/kg) or PBS via gavage and 15 minutes later were allowed access to food. One hour later animals were sacrificed; livers were dissected, lysed in RIPA buffer and loaded on a 8% SDS-PAGE gel. After separation and transfer to a PVDF membrane, protein lysates were analyzed for mTOR activation by probing with an antibody against phospho-*p70 S6* Kinase (*Thr389*) (1:1000; Cell Signaling) and Actin (1:10000 Cell Signaling). After incubation with HRP conjugated secondary antibodies (Santa Cruz Biotechnologies), transferred proteins were visualized with the ECL detection kit (Jackson Research Laboratories).

Histopathology. The mice were visually inspected for tumor development and weighed weekly. Animals showing deteriorating clinical status manifested by constant weight loss or visual tumor appearance were sacrificed and evaluated for gross pathological changes by complete necropsy. For histological evaluation, all tissues were fixed in 10% neutral formalin for 24 hours, and then transferred to 70% ethanol. Samples were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Histopathological examination was performed on tumors, gross lesions and target tissues using Zeiss AxioImager A1 with Axiocam MRc digital camera. The guidelines of Bethesda classification was used in determining the diagnosis [39].

Statistical analyses. Differences in survival and tumor incidence were evaluated by the Mantel-Cox log-rank test.

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### Conflict of Interest Statement

The authors of this manuscript have no conflict of interests to declare.

## REFERENCES


**1.** Vézina C, Kudelski A and Sehgal S. Rapamycin (AY-22,989), a new antifungal antibiotic I. Taxonomy of the producing streptomycete and isolation of the active principle. Journal of antibiotics. 1975; 28:721-726.

**2.** Sehgal SN, Baker H and Vezina C. Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. Journal of antibiotics. 1975; 28:727–732.

**3.** Wullschleger S, Loewith R and Hall MN. TOR Signaling in Growth and Metabolism. Cell. 2006; 124:471-484.

**4.** Kaeberlein M, Powers RW, 3rd, Steffen KK, Westman EA, Hu D, Dang N, Kerr EO, Kirkland KT, Fields S and Kennedy BK. Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. Science. 2005; 310:1193-1196.

**5.** Wilkinson JE, Burmeister L, Brooks SV, Chan CC, Friedline S, Harrison DE, Hejtmancik JF, Nadon N, Strong R, Wood LK, Woodward MA and Miller RA. Rapamycin slows aging in mice. Aging Cell.

**6.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009; 460:392-395.

**7.** Spong A and Bartke A. Rapamycin slows aging in mice. Cell Cycle. 2012; 11:845.

**8.** Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Antoch MP and Blagosklonny MV. Rapamycin extends maximal lifespan in cancer-prone mice. Am J Pathol. 2010; 176:2092-2097.

**9.** Komarova E, Antoch M, Novototskaya L, Chernova O, Paszkiewicz G, Leontieva O, Blagosklonny M and Gudkov A. Rapamycin extends lifespan and delays tumorigenesis in heterozygous p53+/- mice. Aging (Albany NY). 2012.

**10.** Baspinar Y, Bertelmann E, Pleyer U, Buech G, Siebenbrodt I and Borchert HH. Corneal permeation studies of everolimus microemulsion. J Ocul Pharmacol Ther. 2008; 24:399-402.

**11.** Buech G, Bertelmann E, Pleyer U, Siebenbrodt I and Borchert HH. Formulation of sirolimus eye drops and corneal permeation studies. J Ocul Pharmacol Ther. 2007; 23:292-303.

**12.** Alemdar AY, Sadi D, McAlister V and Mendez I. Intracerebral co-transplantation of liposomal tacrolimus improves xenograft survival and reduces graft rejection in the hemiparkinsonian rat. Neuroscience. 2007; 146:213-224.

**13.** Junghanns JU and Muller RH. Nanocrystal technology, drug delivery and clinical applications. International journal of nanomedicine. 2008; 3:295-309.

**14.** Kim MS, Kim JS, Park HJ, Cho WK, Cha KH and Hwang SJ. Enhanced bioavailability of sirolimus via preparation of solid dispersion nanoparticles using a supercritical antisolvent process. International journal of nanomedicine. 2011; 6:2997-3009.

**15.** Batrakova EV and Kabanov AV. Pluronic block copolymers: evolution of drug delivery concept from inert nanocarriers to biological response modifiers. J Control Release. 2008; 130:98-106.

**16.** Brown EJ, Beal PA, Keith CT, Chen J, Bum Shin T and Schreiber SL. Control of p70 S6 kinase by kinase activity of FRAP in vivo. Nature. 1995; 377:441-446.

**17.** Brunn GJ, Fadden P, Haystead TAJ and Lawrence JC. The Mammalian Target of Rapamycin Phosphorylates Sites Having a (Ser/Thr)-Pro Motif and Is Activated by Antibodies to a Region near Its COOH Terminus. Journal of Biological Chemistry. 1997; 272:32547-32550.

**18.** Brunn GJ, Hudson CC, SekuliÄ‡ A, Williams JM, Hosoi H, Houghton PJ, Lawrence JC and Abraham RT. Phosphorylation of the Translational Repressor PHAS-I by the Mammalian Target of Rapamycin. Science. 1997; 277:99-101.

**19.** Burnett PE, Barrow RK, Cohen NA, Snyder SH and Sabatini DM. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. Proceedings of the National Academy of Sciences. 1998; 95:1432-1437.

**20.** Miller RA, Harrison DE, Astle CM, Baur JA, Boyd AR, de Cabo R, Fernandez E, Flurkey K, Javors MA, Nelson JF, Orihuela CJ, Pletcher S, Sharp ZD, et al. Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice. J Gerontol A Biol Sci Med Sci. 2011; 66:191-201.

**21.** Moskalev AA and Shaposhnikov MV. Pharmacological inhibition of phosphoinositide 3 and TOR kinases improves survival of Drosophila melanogaster. Rejuvenation Res. 13:246-247.

**22.** Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A and Partridge L. Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. Cell Metab. 11:35-46.

**23.** Kapahi P, Chen D, Rogers AN, Katewa SD, Li PW, Thomas EL and Kockel L. With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. Cell Metab. 2010; 11:453-465.

**24.** Khanna A and Kapahi P. Rapamycin: killing two birds with one stone. Aging (Albany NY). 2011; 3:1043-1044.

**25.** Ramos FJ, Chen SC, Garelick MG, Dai DF, Liao CY, Schreiber KH, Mackay VL, An EH, Strong R, Ladiges WC, Rabinovitch PS, Kaeberlein M and Kennedy BK. Rapamycin Reverses Elevated mTORC1 Signaling in Lamin A/C-Deficient Mice, Rescues Cardiac and Skeletal Muscle Function, and Extends Survival. Sci Transl Med. 2012; 4:144ra103.

**26.** Donehower LA. Using mice to examine p53 functions in cancer, aging, and longevity. Cold Spring Harb Perspect Biol. 2009; 1:a001081.

**27.** Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Jr., Butel JS and Bradley A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature. 1992; 356:215-221.

**28.** Laplante M and Sabatini DM. mTOR signaling in growth control and disease. Cell. 2012; 149:274-293.

**29.** Dazert E and Hall MN. mTOR signaling in disease. Curr Opin Cell Biol. 2011; 23:744-755.

**30.** Guertin DA and Sabatini DM. Defining the role of mTOR in cancer. Cancer cell. 2007; 12:9-22.

**31.** Hudes G, Carducci M, Tomczak P, Dutcher J, Figlin R, Kapoor A, Staroslawska E, Sosman J, McDermott D, Bodrogi I, Kovacevic Z, Lesovoy V, Schmidt-Wolf IG, et al. Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma. The New England journal of medicine. 2007; 356:2271-2281.

**32.** Motzer RJ, Hudes GR, Curti BD, McDermott DF, Escudier BJ, Negrier S, Duclos B, Moore L, O'Toole T, Boni JP and Dutcher JP. Phase I/II trial of temsirolimus combined with interferon alfa for advanced renal cell carcinoma. J Clin Oncol. 2007; 25:3958-3964.

**33.** Wander SA, Hennessy BT and Slingerland JM. Next-generation mTOR inhibitors in clinical oncology: how pathway complexity informs therapeutic strategy. The Journal of clinical investigation. 121:1231-1241.

**34.** Chappell WH, Steelman LS, Long JM, Kempf RC, Abrams SL, Franklin RA, Basecke J, Stivala F, Donia M, Fagone P, Malaponte G, Mazzarino MC, Nicoletti F, et al. Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR inhibitors: rationale and importance to inhibiting these pathways in human health. Oncotarget. 2011; 2:135-164.

**35.** Markman B, Dienstmann R and Tabernero J. Targeting the PI3K/Akt/mTOR pathway--beyond rapalogs. Oncotarget. 2010; 1:530-543.

**36.** Garrett JT, Chakrabarty A and Arteaga CL. Will PI3K pathway inhibitors be effective as single agents in patients with cancer? Oncotarget. 2011; 2:1314-1321.

**37.** Anand P and Gruppuso PA. The Regulation of Hepatic Protein Synthesis during Fasting in the Rat. Journal of Biological Chemistry. 2005; 280:16427-16436.

**38.** Demirkan G, Yu K, Boylan JM, Salomon AR and Gruppuso PA. Phosphoproteomic Profiling of In Vivo Signaling in Liver by the Mammalian Target of Rapamycin Complex 1 (mTORC1). PLoS ONE. 6:e21729.

**39.** Morse HC, 3rd, Anver MR, Fredrickson TN, Haines DC, Harris AW, Harris NL, Jaffe ES, Kogan SC, MacLennan IC, Pattengale PK and Ward JM. Bethesda proposals for classification of lymphoid neoplasms in mice. Blood. 2002; 100:246-258.

Opinions in Science and Society

# How to save Medicare: the anti-aging remedy

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**Abstract: The unprecedented progress in aging research has revealed that rapamycin, a clinically approved drug, is actually an anti-aging agent, which potentially could be employed to delay age-related diseases, thus extending healthy life span. The possibility of preventing diseases by staying young is remarkable in itself. At the same time this advance could save Medicare as we know it. Here I discuss how anti-aging interventions could solve otherwise intractable political problems without tax increases or curtailment of health care benefits.**

## Health care crisis

Social Security and Medicare accounted for 36% of federal spending in 2011, and as baby boomers age, those costs are projected to keep rising. As recently forecasted, the Social Security trust fund will be exhausted in 2033, three years sooner than projected last year. And Medicare's hospital insurance trust fund will be depleted in 2024 [1]. One solution is to cut Medicare and other health care benefits, to narrow treatment options, to slow the growth in benefits somewhat for wealthier recipients. Another solution is to increase taxes (in whatever form) and/or to increase the federal budget deficit. These solutions are political. Here I will discuss a biomedical solution, which can be easily incorporated into their political program by both Democrats and Republicans. Then there will be no dilemma either to increase taxes or to decrease benefits. But first we will discuss what is the cause of the forthcoming crisis.

## Crisis as a side effect of improved health care

As recently noted "Republicans and Democrats are noisily blaming each other for the problems of the popular programs, which provide benefits to more than 55 million people. [1]" Yet, the health care crisis is not the fault of either Republicans or Democrats. The crisis is a "side-effect" of the ever-increasing effectiveness of medicine. That is, the crisis is indirectly due to the marvelous achievements of the modern medicine such as organ transplantation, coronary stents, intensive and emergency care, antibiotics against resistant bacteria, MRI and sophisticated tests, all of which decrease human suffering and allow patients with deadly conditions to live for many years. But this life-saving medicine is also responsible, in part, for increasing health care costs.

First, obviously but not most importantly, these medical options are expensive. For example, organ transplantation may cost hundreds of thousands of dollars. The development of a new antibiotic against drug resistant bacteria requires substantial spending for research. Second, and most importantly, precisely because medicine is becoming so effective in saving lives, this increases a number of elderly patients with chronic and multiple diseases (Figure 1 from A to B), which necessitates multiple treatments all of which cost money. No one dies from aging itself, all humans die from age-related diseases such as cancer, atherosclerosis, hypertension, diabetes, osteoporosis or actually from their complications. So every old person becomes a patient at some point. Medical interventions delay death from age-related diseases, often without curing them. For example, saved by defibrillation from sudden death due to coronary atherosclerosis, a patient can live for many decades (with treatment) and may even die from another age-related disease. With treatment and nursing, patients with macular degeneration, Alzheimer and Parkinson diseases, type II diabetes, hypertension, coronary atherosclerosis,

sarcopenia and osteoporosis can live for decades. Cancer is also becoming a chronic disease. Of course this is a great medical and social success. As "a side effect," however, this increases a number of elderly people with chronic age-related diseases in constant need of health care (who would otherwise have died). Since diseases of aging tend to gradually develop with age, such a patient suffers from several and sometimes many diseases. A combination of obesity, diabetes, atherosclerosis, hypertension, retinopathy, osteoporosis is very common. So there is simultaneously an increase of the number of diseases afflicting each elderly person and an increase in the number of such patients.

In summary, current medicine is effective in preventingdeath from age-related diseases without delaying their onset, thus increasing the number of people with age-related diseases and the number of diseases afflicting each elderly person. In addition, each disease of aging is now treated separately, which is costly and can lead to unavaoidable adverse effects. For example, chemotherapy, used for cancer treatment, has a negative impact on normal tissues and organs. And vice versa, insulin, which is used for treatment of diabetes, is a pro-aging factor [2] and may accelerate some pathologies such as cancer [3], [4]. (Note: In contrast, due to some anti-aging activities, the anti-diabetic drug metformin prevents cancer [5]). One solution is to delay age-related diseases, thus extending healthy life span. But is it possible?

## Slow aging is manifested as healthy aging

There is a misconception that an anti-aging medicine would increase the number of chronically ill people because they are old. On the contrary, it would decrease the ratio of unhealthy to healthy population (Figure 1C) because an anti-aging medicine will delay the onset of aging, diseases and their complications at older age [6-8]. Fast-aging animals (mice) develop diseases of aging fast, whereas slowly aging organisms such as humans acquire these diseases at 40 times older age than mice. Centenarians, people who live more than 100 years, age slowly and generally experience good health until very old ages, when diseases that kill them finally develop [8-12]. Furthermore, the period of morbidity is not only delayed but also shortened [8]. (Perhaps, extremely old (chronologically) patients are nor treated vigorously compared with younger patients).

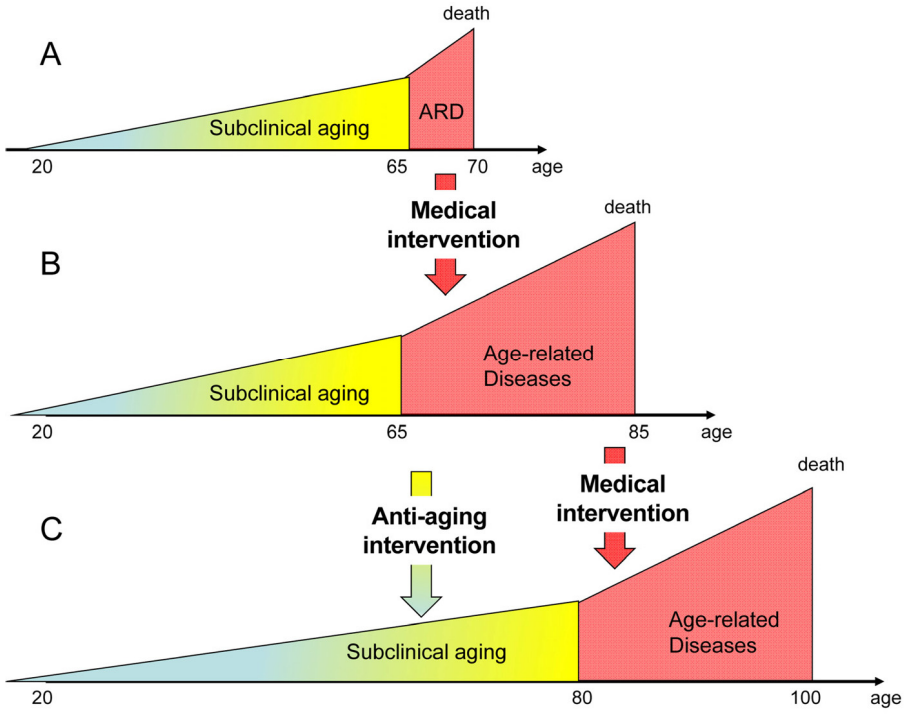


**Figure 1. From longer life span to longer health span (and life span).** From **A** to **B**: Standard medicine increases lifespan by preventing death from age-related diseases. It simultaneously increases a number of old people suffering from age-related diseases. A ratio healthspan to lifespan is decreased. From **B** to **C**: Anti-aging intervention will slow down aging and delay the onset of age-related diseases. This in theory will restore a ratio of healthspan to lifespan.

In the past, most people died (from incidents, infections, malnutrition and homicide) before they achieved the age of age-related diseases. For example, in the 17th century in London, only 25% of people survived until the age of 26 (see for references [7]). By the mid of 20th century civilization and medicine allowed most people to live long enough to die from aging or strictly speaking from age-related diseases (Figure 1A). Still until very recently people died soon after they reached the age of age-related diseases from the complications of these age-related diseases (Figure 1 A). Now effective medical interventions can keep a patient alive despite age-related diseases (Figure 1B). This increases the ratio of unhealthy to healthy population (Figure 1B). What is needed is to delay age-related diseases (Figure 1C). And by lucky co-incidence, this could be done right now, potentially preventing the health care crisis (see text).

Slowing down aging both increases lifespan and postpones diseases. One may even say that **anti-aging interventions increase lifespan by postponing diseases**. Thus, calorie restriction slows aging and delays the development of all age-related diseases in mammals including non-human and human primates [13-18]. And vice versa excessive nutrition that causes obesity and accelerates aging also accelerates development of all age-related diseases from type II diabetes to atherosclerosis to cancer. A mere reduction of visceral fat decreases mortality [19]. Yet, severe calorie restriction may cause malnutrition. It may be possible to use a calorie-restriction mimetic such as rapamycin instead of calorie restriction. There is evidence that rapamycin can slow aging and delay onset of age-related diseases.

## Aging and Target of Rapamycin (TOR)

It was long thought that aging is caused by accumulation of random molecular damage and wear and tear. Accordingly, it was assumed that diseases can be treated but aging cannot. A rapidly increasing number of studies has convincingly established that inhibition of certain signal-transduction molecules extends life span in diverse species [20-26]. These proteins form signaling pathways, which sense nutrients (glucose, fatty acids, amino acids), insulin and other hormones, oxygen, cytokines and growth factors. Activation of such nutrient-sensing pathways promotes growth and, when growth is completed, aging [27]. The nutrient-sensing and growth-promoting TOR (Target of Rapamycin) stands out for four important reasons. First, most of pro-aging and anti-aging molecules can be diagrammed as part of the TOR pathway [28, 29]. Second, mammalian TOR (mTOR) links cellular and organismal aging [28, 30]. Thus, mTOR is involved in cellular aging [31-33]. Inhibition of mTOR suppresses conversion of post-mitotic cells into senescent cells [34, 35]. In resting cells, re-activation of mTOR causes senescence (geroconversion) [36]. Rapamycin prevents hyperactivation and exhaustion of stem cells in the organism [37, 38]. Third, numerous preclinical studies revealed that mTOR is involved in most age-related diseases including including cancer, atherosclerosis, neurodegeneration and age-related macular degeneration [28, 29, 39-43]. Fourth, and most importantly, rapamycin and its analogs (rapalogs) are clinically approved drugs.

## Rapamycin and other rapalogs

For a decade, rapamycin (Sirolimus) and its analogs have been used in high doses in transplant patients. At high and chronic doses, in combinations with immunosuppressants (in order to prevent transplant organ rejection), rapamycin has some reversible side effects. As a "side effect" rapamycin prevents cancer in renal transplant patients [44-46]. There are some "therapeutic side effects" such as lipolysis [40]. There is a misconception that rapamycin may increase risk of cancer and lymphomas. Instead, rapamycin and other rapalogs prevent and treat cancer and lymphomas. Rapamycin prevents many age-related diseases in animal models. In patients, oral rapamycin decreases atherosclerotic re-stenosis [47-49]. Finally, it slows aging and extends life span in flies [50, 51] and mice [52-57].

As an anti-aging drug, however, rapamycin should be used at low doses and intermittent schedules [58, 59] ("Intermittent rapamycin" in preparation). In fact, intermittent therapy with rapamycin still extends lifespan in mice [54, 55]. Similarly, intermittent calorie restriction prolongs life span in rodents. Low doses, intermittent administration and rational combinations with such drugs as metformin (in contrast to immunossuprressants) would distinguish anti-aging schedules of rapamyicin from its use to prevent organ rejection. Doses and schedules always make the difference. Consider arsenic, the most famous poison, used for millennia by murderers. In different doses and schedules, arsenic is now used as the most effective treatment for acute promyelocytic leukemia. Potassium chloride is one of the most useful drugs widely used in medicine. In different administration, potassium chloride is also used in lethal injections for capital punishment. In comparison with other drugs, rapamycin is exceptionally non-toxic, and it cannot be possibly

used in lethal injections. The oral dose that is lethal to mice cannot be actually achieved since LD50> 2500 mg/kg. (And this is thousand times more than even a high therapeutic dose). A single dose of rapamycin is not lethal at any dose and furthermore has no side effects in healthy volunteers, providing one of rationales for intermittent schedules. Of course, additional clinical trials of low doses of rapamycin will be necessary to demonstrate that it decreases incidence of age-related diseases. I need to emphasize that there is no evidence yet that rapamycin increases human lifespan. (This would take a human life span to demonstrate). But such evidence is not needed. For practical purposes, to safe Medicare, it is important to delay age-related diseases. As discussed in detail [40], such a clinical trial would take just a couple of years to conduct. And if all diseases will be delayed, then both health span and life span will be increased.

### How to implement and future developments?

Government and public efforts have been very effective in reducing smoking in the U.S. This was achieved despite the fact that smoking is highly addictive! In comparison, wide introduction of low/intermittent doses of the anti-aging drug rapamycin, which by the way decreases incidence of smoke-related cancer in mice on 90% (!)[60], seems less challenging. In general, this would be comparable to the introduction of vaccination, which government and other programs have very effectively carried out. And introduction of rapamycin is just a first step in the development of anti-aging interventions. A program of how to extend life span in our lifetime was recently discussed [61].

### Closing remarks: this seems to be the most civilized solution

Everyone agrees that the ever-rising costs of Medicare must be slowed._Of course the costs can be reduced in part by making the health care system more effective, eliminating any kind of abuse of the health care system by insurance companies, the industry and care providers. These issues are well addressed by politicians. Yet, the costs can be slowed but cannot be frozen. Unfortunately, the only way to stop the rising costs of Medicare completely is to prevent the use of more effective (and expensive) medical options and to stop further biomedical research. This draconian option would accelerate the mortality of the sickest elderly, further decreasing Medicare costs. Of course this is unacceptable. So costs must continue to rise. But this wouldn't necessarily lead to a fiscal crisis, given that anti-aging medicine could increase health span and therefore the ratio of healthy to unhealthy individuals in the elderly population. In conjunction with the increase in health span, the age of retirement could be increased. This would increase federal revenues and provide a means to cover increasing costs of Medicare. In any case, the eligibility age for full benefits is now gradually increasing. For those born after 1960, it will be 67. Some politicians would increase it further, allowing it to rise along with increases in longevity. Yet, although longevity is increasing, the rapid rise in Medicare costs is due to prevention of death from age-related diseases, not to prevention of diseases themselves. Anti-aging interventions may postpone diseases, thus naturally increasing the age of retirement, because at 87 a person would be biologically 67 and feel as healthy and energetic as he or she currently does at 67.

### Summary

1. Anti-aging interventions may increase health span, increase the age of disability allowing chronologically older people to be biologically younger. This naturally increases the age of retirement, increasing revenue without increasing taxes.
2. Currently, there is no other sensible solution. The alternatives, both untenable, are either to let elderly (unhealthy) people die by drastically limiting medical benefits, or perennially to increase taxes.

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### Conflict of Interest Statement

The author of this opinion article is a co-editor of the journal Aging

## REFERENCES

**1.** Pear R. Social Security and Financial Health Worsens. The New Yourk Times. 2012: http://www.nytimes.com/2012/2004/2024/us/politics/financial-outlook-dims-for-social-security.html.
**2.** Bartke A. Insulin and aging. Cell Cycle. 2008; 7: 3338-3343.
**3.** Bowker SL, Majumdar SR, Veugelers P, Johnson JA. Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. Diabetes Care. 2006; 29: 254-258.
**4.** Hammarsten J, Hogstedt B. Hyperinsulinaemia: a prospective risk factor for lethal clinical prostate cancer. Eur J Cancer. 2005; 41: 2887-2895.
**5.** Anisimov VN. Metformin for aging and cancer prevention. Aging. 2010; 2: 760-774.
**6.** Gems D. Tragedy and delight: the ethics of decelerated ageing. Philos Trans R Soc Lond B Biol Sci. 2011; 366: 108-112.
**7.** Blagosklonny MV. Why human lifespan is rapidly increasing:

solving "longevity riddle" with "revealed-slow-aging" hypothesis. Aging. 2010; 2: 177-182.

**8.** Andersen SL, Sebastiani P, Dworkis DA, Feldman L, Perls TT. Health span approximates life span among many supercentenarians: compression of morbidity at the approximate limit of life span. J Gerontol A Biol Sci Med Sci. 2012; 67: 395-405.

**9.** Hitt R, Young-Xu Y, Silver M, Perls T. Centenarians: the older you get, the healthier you have been. Lancet. 1999; 354: 652.

**10.** Newson RS, Witteman JC, Franco OH, Stricker BH, Breteler MM, Hofman A, Tiemeier H. Predicting survival and morbidity-free survival to very old age. Age (Dordr). 2010; 32: 521-534.

**11.** Willcox DC, Willcox BJ, Wang NC, He Q, Rosenbaum M, Suzuki M. Life at the extreme limit: phenotypic characteristics of supercentenarians in Okinawa. J Gerontol A Biol Sci Med Sci. 2008; 63: 1201-1208.

**12.** Evert J, Lawler E, Bogan H, Perls T. Morbidity profiles of centenarians: survivors, delayers, and escapers. J Gerontol A Biol Sci Med Sci. 2003; 58: 232-237.

**13.** Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, Allison DB, Cruzen C, Simmons HA, Kemnitz JW, Weindruch R. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science. 2009; 325: 201-204.

**14.** McKiernan SH, Colman RJ, Lopez M, Beasley TM, Aiken JM, Anderson RM, Weindruch R. Caloric restriction delays aging-induced cellular phenotypes in rhesus monkey skeletal muscle. Exp Gerontol. 2011; 46: 23-29.

**15.** Weiss EP, Racette SB, Villareal DT, Fontana L, Steger-May K, Schechtman KB, Klein S, Holloszy JO, Group. WUSoMC. Improvements in glucose tolerance and insulin action induced by increasing energy expenditure or decreasing energy intake: a randomized controlled trial. Am J Clin Nutr. 2006; 84: 1033-1042.

**16.** Fontana L. The scientific basis of caloric restriction leading to longer life. Curr Opin Gastroenterol. 2009; 25: 144-150.

**17.** Fontana L, Partridge L, Longo VD. Extending healthy life span--from yeast to humans. Science. 2010; 328: 321-326.

**18.** Blagosklonny MV. Calorie restriction: Decelerating mTOR-driven aging from cells to organisms (including humans). Cell Cycle. 2010; 9: 683-688.

**19.** Muzumdar R, Allison DB, Huffman DM, Ma X, Atzmon G, Einstein FH, Fishman S, Poduval AD, McVei T, Keith SW, Barzilai N. Visceral adipose tissue modulates mammalian longevity. Aging Cell. 2008; 7: 438-440.

**20.** Guarente L, Kenyon C. Genetic pathways that regulate ageing in model organisms. Nature. 2000; 408: 255-262.

**21.** Sinclair DA, Guarente L. Unlocking the secrets of longevity genes. Sci Am. 2006; 294: 48-51.

**22.** Stanfel MN, Shamieh LS, Kaeberlein M, Kennedy BK. The TOR pathway comes of age. Biochim Biophys Acta. 2009; 1790: 1067-1074.

**23.** Kenyon CJ. The genetics of ageing. Nature. 2010; 464: 504-512.

**24.** Selman C, Tullet JM, Wieser D, Irvine E, Lingard SJ, Choudhury AI, Claret M, Al-Qassab H, Carmignac D, Ramadani F, Woods A, Robinson IC, Schuster E, Batterham RL, Kozma SC, Thomas G et al. Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. Science. 2009; 326: 140-144.

**25.** Kapahi P, Chen D, Rogers AN, Katewa SD, Li PW, Thomas EL, Kockel L. With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. Cell Metab. 2010; 11: 453-465.

**26.** Gems DH, de la Guardia YI. Alternative Perspectives on Aging in C. elegans: Reactive Oxygen Species or Hyperfunction? Antioxid Redox Signal. 2012.in press

**27.** Blagosklonny MV, Hall MN. Growth and aging: a common molecular mechanism. Aging. 2009; 1: 357-362.

**28.** Blagosklonny MV. Aging and immortality: quasi-programmed senescence and its pharmacologic inhibition. Cell Cycle. 2006; 5: 2087-2102.

**29.** Blagosklonny MV. An anti-aging drug today: from senescence-promoting genes to anti-aging pill. Drug Disc Today. 2007; 12: 218-224.

**30.** Blagosklonny MV. Prospective Treatment of Age-Related Diseases by Slowing Down Aging. Am J Pathol. 2012.

**31.** Demidenko ZN, Blagosklonny MV. Growth stimulation leads to cellular senescence when the cell cycle is blocked. Cell Cycle. 2008; 7: 3355-3361.

**32.** Cao K, Graziotto JJ, Blair CD, Mazzulli JR, Erdos MR, Krainc D, Collins FS. Rapamycin reverses cellular phenotypes and enhances mutant protein clearance in Hutchinson-Gilford progeria syndrome cells. Sci Transl Med. 2011; 3: 89ra58.

**33.** Kolesnichenko M, Hong L, Liao R, Vogt PK, Sun P. Attenuation of TORC1 signaling delays replicative and oncogenic RAS-induced senescence. Cell Cycle. 2012; 11: 2391-2401.

**34.** Demidenko ZN, Zubova SG, Bukreeva EI, Pospelov VA, Pospelova TV, Blagosklonny MV. Rapamycin decelerates cellular senescence. Cell Cycle. 2009; 8: 1888-1895.

**35.** Blagosklonny MV. Cell cycle arrest is not yet senescence, which is not just cell cycle arrest: terminology for TOR-driven aging. Aging. 2012; 4: 159-165.

**36.** Leontieva OV, Blagosklonny MV. DNA damaging agents and p53 do not cause senescence in quiescent cells, while consecutive re-activation of mTOR is associated with conversion to senescence. Aging. 2010; 2: 924-935.

**37.** Gan B, Sahin E, Jiang S, Sanchez-Aguilera A, Scott KL, Chin L, Williams DA, Kwiatkowski DJ, DePinho RA. mTORC1-dependent and -independent regulation of stem cell renewal, differentiation, and mobilization. Proc Natl Acad Sci U S A. 2008; 105: 19384-19389.

**38.** Chen C, Liu Y, Zheng P. mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells. Sci Signal. 2009; 2: ra75.

**39.** Tsang CK, Qi H, Liu LF, Zheng XFS. Targeting mammalian target of rapamycin (mTOR) for health and diseases. Drug Disc Today. 2007; 12: 112-124.

**40.** Blagosklonny MV. Validation of anti-aging drugs by treating age-related diseases. Aging. 2009; 1: 281-288.

**41.** Dazert E, Hall MN. mTOR signaling in disease. Curr Opin Cell Biol. 2011; 23: 744-755.

**42.** Bjedov I, Partridge L. A longer and healthier life with TOR down-regulation: genetics and drugs. Biochem Soc Trans. 2011; 39: 460-465.

**43.** Zheng XF. Chemoprevention of age-related macular regeneration (AMD) with rapamycin. Aging. 2012; 4: 375-376.

**44.** Mathew T, Kreis H, Friend P. Two-year incidence of malignancy in sirolimus-treated renal transplant recipients: results from five multicenter studies. Clin Transplant. 2004; 18: 446-449.

**45.** Campistol JM, Eris J, Oberbauer R, Friend P, Hutchison B, Morales JM, Claesson K, Stallone G, Russ G, Rostaing L, Kreis H,

Burke JT, Brault Y, Scarola JA, Neylan JF. Sirolimus Therapy after Early Cyclosporine Withdrawal Reduces the Risk for Cancer in Adult Renal Transplantation. J Am Soc Nephrol. 2006; 17: 581-589.

**46.** Blagosklonny MV. Prevention of cancer by inhibiting aging. Cancer Biol Ther. 2008; 7: 1520-1524.

**47.** Hausleiter J, Kastrati A, Mehilli J, Vogeser M, Zohlnhofer D, Schuhlen H, Goos C, Pache J, Dotzer F, Pogatsa-Murray G, Dirschinger J, Heemann U, Schomig A. Randomized, double-blind, placebo-controlled trial of oral sirolimus for restenosis prevention in patients with in-stent restenosis: the Oral Sirolimus to Inhibit Recurrent In-stent Stenosis (OSIRIS) trial. Circulation. 2004; 110: 790-795.

**48.** Waksman R, Ajani AE, Pichard AD, Torguson R, Pinnow E, Canos D, Satler LF, Kent KM, Kuchulakanti P, Pappas C, Gambone L, Weissman N, Abbott MC, Lindsay J. Oral rapamycin to inhibit restenosis after stenting of de novo coronary lesions: the Oral Rapamune to Inhibit Restenosis (ORBIT) study. J Am Coll Cardiol. 2004; 44: 1386-1392.

**49.** Brito FS, Jr., Rosa WC, Arruda JA, Tedesco H, Pestana JO, Lima VC. Efficacy and safety of oral sirolimus to inhibit in-stent intimal hyperplasia. Catheter Cardiovasc Interv. 2005; 64: 413-418.

**50.** Moskalev AA, Shaposhnikov MV. Pharmacological Inhibition of Phosphoinositide 3 and TOR Kinases Improves Survival of Drosophila melanogaster. Rejuvenation Res. 2010; 13: 246-247.

**51.** Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, Partridge L. Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. Cell Metab. 2010; 11: 35-46.

**52.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandezr E, Miller RA. Rapamycin fed late in life extends lifespan in genetically heterogenous mice. Nature. 2009; 460: 392-396.

**53.** Miller RA, Harrison DE, Astle CM, Baur JA, Boyd AR, de Cabo R, Fernandez E, Flurkey K, Javors MA, Nelson JF, Orihuela CJ, Pletcher S, Sharp ZD, Sinclair D, Starnes JW, Wilkinson JE *et al* . Rapamycin, But Not Resveratrol or Simvastatin, Extends Life Span of Genetically Heterogeneous Mice. J Gerontol A Biol Sci Med Sci. 2011; 66: 191-201.

**54.** Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Rosenfeld SV, Blagosklonny MV. Rapamycin increases lifespan and inhibits spontaneous tumorigenesis in inbred female mice. Cell Cycle. 2011; 10: 4230-4236.

**55.** Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Antoch MP, Blagosklonny MV. Rapamycin extends maximal lifespan in cancer-prone mice. Am J Pathol. 2010; 176: 2092-2097.

**56.** Wilkinson JE, Burmeister L, Brooks SV, Chan CC, Friedline S, Harrison DE, Hejtmancik JF, Nadon N, Strong R, Wood LK, Woodward MA, Miller RA. Rapamycin slows aging in mice. Aging Cell. 2012; 11: 675-682.

**57.** Spong A, Bartke A. Rapamycin slows aging in mice. Cell Cycle. 2012; 11: 845.

**58.** Blagosklonny MV. Aging, stem cells, and mammalian target of rapamycin: a prospect of pharmacologic rejuvenation of aging stem cells. Rejuvenation Res. 2008; 11: 801-808.

**59.** Blagosklonny MV. Once again on rapamycin-induced insulin resistance and longevity: despite of or owing to. Aging. 2012; 4: 350-358.

**60.** Granville CA, Warfel N, Tsurutani J, Hollander MC, Robertson M, Fox SD, Veenstra TD, Issaq HJ, Linnoila RI, Dennis PA. Identification of a highly effective rapamycin schedule that markedly reduces the size, multiplicity, and phenotypic progression of tobacco carcinogen-induced murine lung tumors. Clin Cancer Res. 2007; 13: 2281-2289.

**61.** Blagosklonny MV. Increasing healthy lifespan by suppressing aging in our lifetime: Preliminary proposal. Cell Cycle. 2010; 9: 4788-4794.

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Research Paper

# Once again on rapamycin-induced insulin resistance and longevity: despite of or owing to

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**Abstract: Calorie restriction (CR), which deactivates the nutrient-sensing mTOR pathway, slows down aging and prevents age-related diseases such as type II diabetes. Compared with CR, rapamycin more efficiently inhibits mTOR. Noteworthy, severe CR and starvation cause a reversible condition known as "starvation diabetes." As was already discussed, chronic administration of rapamycin can cause a similar condition in some animal models. A recent paper published in Science reported that chronic treatment with rapamycin causes a diabetes-like condition in mice by indirectly inhibiting mTOR complex 2. Here I introduce the notion of benevolent diabetes and discuss whether starvation-like effects of chronic high dose treatment with rapamycin are an obstacle for its use as an anti-aging drug.**

## Starvation diabetes-like condition with low mTOR activity

If you read the Abstract, you might wonder whether rapamycin extends lifespan despite or because of "starvation-like diabetes". As described by Lamming et al [1, 2] extending several previous observations [3-6], chronic administration of high doses of rapamycin causes insulin resistance in mice. Yet, at similar doses, rapamycin prolongs life span in mice [7, 8]. Moreover, in several studies, rapamycin prevented complications of diabetes such as nephropathy [9-14]. Also, theoretical considerations indicate rapamycin for retinopathy [15], which was recently confirmed in an animal model [16]. Rapamycin prevents atherosclerosis in rodents [17-20] and coronary re-stenosis in humans [21, 22]. In contrast, diabetes promotes nephropathy, retinopathy, atherosclerosis and coronary disease. How could this be reconciled? mTOR is a part of a nutrient-sensing pathway [23-27]. Nutrients and insulin activate mTOR. Rapamycin, which inhibits mTOR, is a "starvation-mimetic", making the organism "think" that food is in a short supply. The most starvation-sensitive organ is the brain. The brain consumes only glucose and ketones. Therefore, to feed the brain during starvation, the liver produces glucose from amino acids (gluconeogenesis) and ketones from fatty acids (ketogenesis). Since insulin blocks both processes, the liver needs to become resistant to insulin. Also secretion of insulin by beta-cells is decreased. And adipocytes release fatty acids (lipolysis) to fuel ketogenesis by the liver. Thus, there are five noticeable metabolic alterations of starvation: gluconeogenesis, ketogenesis, insulin resistance, low insulin levels and increased lipolysis. This metabolic switch is known as starvation diabetes, a reversible condition, described 160 years ago (see for references [28]). Starvation diabetes could be explained by deactivation of mTOR, which otherwise is activated by nutrients. In theory, rapamycin can cause similar symptoms in the presence of nutrients.

## Type II diabetes: insulin-resistance due to active mTOR

Starvation-diabetes is not a true type II diabetes. Type II diabetes is a consequence of insulin-resistance in part due to excessive nutrients and obesity. Even brief overfeeding may induce insulin resistance [29]. Nutrients and insulin activate mTOR. In turn, over-activated mTOR causes insulin resistance [30-42]. This feedback loop is shown in figure 1A. mTOR activates S6 kinase (S6K), which causes degradation of insulin-

receptor substrates (IRS), thus impairing insulin signaling. Also, mTOR causes insulin resistance by an additional feedback mechanism [43, 44].

In high fat-fed obese rats, the mTOR pathway is activated in the liver and muscle, leading to insulin resistance [35]. In mice, sustained activation (by high fat feeding) of mTOR is associated with hepatic insulin resistance [45]. Chronic increase of insulin levels (hyperinsulinemia) causes insulin resistance, preventable by rapamycin [46]. In some animal models, removal of visceral fat prevents insulin resistance [47-49]. In humans, infusion of amino acids activate mTOR/S6K1, causing insulin resistance [38, 40]. In healthy men, rapamycin prevented activation of mTOR and insulin resistance caused by amino acid mixture [50]. Insulin stimulates glucose uptake and also activates mTOR. By a feedback loop, mTORC1 promotes insulin-resistance, decreasing glucose uptake by the cell. And most detrimentally, mTOR is involved in diabetic complications and age-related diseases [24-27, 51, 52-54].

## The two opposite conditions?

Type II diabetes and starvation diabetes seem to be the two opposite conditions: the first is associated with activation of nutrient-sensing pathways, whereas the second is associated with deactivation of nutrient sensing pathways such as mTOR. Type II diabetes is dangerous by its complications such as retinopathy, neuropathy and accelerated atherosclerosis and cancer. Long-term effects of prolonged "starvation diabetes" is not known of course: it could not last for a long time, otherwise an animal (or human) would die from starvation. Or would not? An outstanding study by Fontana et al provides some answers [55]. Among individuals who had been practicing sever CR for an average of 7 years, 40% of CR individuals exhibited "diabetic-like" glucose intolerance, despite low levels of fasting glucose, insulin and inflammatory cytokines as well as excellent other metabolic profiles. In comparison with the rest CR individuals, they had lower BMI, leptin, circulating IGF-I, testosterone, and high levels of adiponectin, which are key adoptations to CR in rodents, suggesting severe CR [55]. The authors speculated that the "insulin resistance" in this severe CR group might have the effect of slowing aging, also based on the finding that a number of insulin-resistant strains of mice are long-lived [55]. The same conclusion could be reached from the mTOR perspective (Appendix 1).

"The paradox of the insu-lin/IGF-1 signaling pathway in longevity" was first discussed by Nir Barzilai and co-workers, who precisely noticed that insulin-resistance, which is so detrimental in obese and aging mammals, can be associated with genetic manipulations that extend life span in model organisms [56]. Later Barzilai et al suggested that insulin-resistance might serve as an adaptive mechanism in some tissues by preventing excess uptake of nutrients by cells [57]. This very interesting idea implies that insulin resistance is partially beneficial and partially hazardous in the same condition such as type II diabetes. But still insulin resistance in type II diabetes is overall harmful (leading to retinopathy and other complications), whereas insulin resistance during severe CR is benevolent. These are clearly different conditions. In fact, they are the opposite conditions. So insulin resistance may be harmful or beneficial depending on the underlying condition.

The model of TOR-driven hyper-functional aging almost automatically solves paradoxes of aging, including the insulin paradox (see paradox 7 and figure 4 in "Paradoxes of aging" [58]). From the TOR perspective, insulin resistance is beneficial or harmful when it is associated with ether low or high TOR activity, respectively (Appendix, Fig. 1 and 3). And this should not be surprising. Consider insulin resistance as a symptom. The assessment of symptoms depends on the underlying cause. For example, weight loss due to calorie restriction is good, whereas weight loss in terminal cancer is bad. Positive Tuberculosis Skin (PPD) Test due to vaccination indicates protection from tuberculosis, whereas positive test due to tuberculosis is a symptom of tuberculosis. Similarly, hyperlipidemia in obesity is bad, whereas hyperlipidemia due to rapamycin-induced lipolysis is good (see figure 2 in reference [53]). The list of examples is endless. Similarly, insulin resistance, associated with TOR overactivation, is bad (Fig. 1 B-C). But either insulin sensitivity (Fig. 2) or insulin resistance (Fig. 3), associated with inactive TOR, is good.

## Type zero or benevolent diabetes

There are two types of diabetes, which at advanced stages may become similar. Insulin resistance may develop in type I diabetes (due to high glucose), whereas insulin insufficiency in type II diabetes (due to loss of beta-cells). Both types of diabetes lead to complications. In comparison, starvation diabetes [28] is only superficially resembles either type of diabetes. Also, diabetes-like symptoms may occur in rapamycin-treated mice and animals with genetically inhibited insulin/IGFI signaling (Fig. 3). To encompass all these

cases, I suggest the term type 0 (zero) or benevolent diabetes. It is possible that some patients with diabetes have inactivating mutations in the insulin/IGFI pathway and thus "suffer" from benevolent diabetes. Furthermore, the condition can be imitated by chronic administration of rapamycin at least in some strains of mice. Both calorie restriction and rapamycin extend life span in mice. Rapamycin prevents retinopathy and nephropathy. Also CR prevents type II diabetes and other diseases [59], [60], [61], [62]. One can suggest that type 0 diabetes should prevent type 2 diabetes. Should type 0 diabetes be treated? Perhaps CR-associated type 0 diabetes should not. What about rapamycin-associated diabetes? Definitely, it should not be treated with insulin. It was discussed that in theory the most rational combinations with rapamycin are mild calorie and fat restriction, physical exercise and metformin [52]. Metformin may in theory counteract rapamycin-induced gluconeogenesis in the liver. And this rational drug combination may be also considered as treatment of type 0 diabetes.

## Inconsistencies in the literature on rapamycin-induced insulin resistance

As demonstrated by Lamming et al, chronic administration of rapamycin caused insulin-resistance due to deactivation of mTORC2 and Akt [1]. This is consistent with previous data that IRS signaling and AKT activation was impaired in patients treated with rapamycin [63]. However, there are some inconsistencies. In another clinical study, rapamycin therapy in contrast caused activation of Akt [64]. Second, whereas Lamming et al found that rapamycin increased insulin levels after feeding [1], other studies reported that rapamycin in contrast inhibited insulin secretion [3], 4, 65]. Furthermore, inhibition of beta-cell adaptation and insulin production by rapamycin was considered as the main mechanism of rapamycin-induced diabetes in mice [6, 66-69]. On the other hand, selective inactivation of mTORC2 in the liver can cause hyperinsulinemia [70].

Finally, diabetic-like symptoms were not observed in numerous studies in mice. And rapamycin-induced diabetes is rare in human patients, even though most of them are prone to diabetes for other reasons.

## Diabetes in patients receiving rapamycin

In renal transplant patients, who are prone to diabetes (due to several reasons), chronic administration of rapamycin modestly increases incidence of diabetes [71, 72]. Although the increase is statistically significant, it took many years to detect it. For many years it was thought that, unlike other agents used in these patients, rapamycin either do not increase the incidence of diabetes or increases it in combinations with tacrolimus [73-79]. In the study involving 20124 recipients of kidney transplant sirolimus (rapamycin) was independently associated with new onset diabetes [72]. And although it statistically significantly increases the incidence of diabetes in renal transplant patient, we do not know whether this is true diabetes, which is dangerous by its complications, or starvation-like diabetes, that prevents the complications of true diabetes . Will chronic high doses of rapamycin cause or prevent diabetes in humans without organ transplantation? More investigations are needed.

## Intermittent administration of rapamycin

Is glucose intolerance a part of therapeutic effects of starvation-like drugs such as rapamycin? And may such condition be not only benign but also prevent true diabetes and its complications? Although these questions are very intriguing, the answers are not immediately crucial. Simply, the most rational anti-aging schedule is an intermittent (rather than chronic) administration of rapamycin [53, 80]. First, this will eliminate potential side effects. Second, intermittent administration of rapamycin may in theory rejuvenate stem and wound-healing cells and (in contrast to chronic treatment) improve wound healing [80]. And intermittent administration of rapamycin extended life span in mice [81-86]. Also, brief treatment with rapamycin does not affect mTORC2 [87].

Rapalogs (rapamycin and its analogs such evirolimus and temsirolimus) inhibit only one target (mTORC1). That was considered as a disadvantage of rapalogs for cancer therapy. Inhibitors of both mTORC1 and mTORC2 are under development [88, 89]. But if inhibition of mTORC2 is not needed for the longevity effect, then mTORC1 selectivity is an advantage for anti-aging therapy. Rapalogs (rapamycin and its analogs) are selective inhibitors of TORC1 and inhibitors of mTORC1 will have the same side effects as rapalogs. Yet, these (non-rapalog) inhibitors of the TOR kinase also have off-target effects and side effects. Therefore, rapamycin will remain the least toxic anti-aging drug in the near future [90].

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## Appendix 1: Paradoxes of diabetes

Previously I discussed that the mTOR-centered model can solve so called insulin paradox [58], which was exploited by Tom Kirkwood to undermine the notion that aging is genetically regulated: "it seems paradoxical that reduced insulin/ IGF-1 signaling extends life span but insulin resistance leads to type II diabetes. The real paradox is why, in mammals, low insulin levels are associated with good health, but low insulin responsiveness with bad health" [91]. In other words, (a) low insulin signaling are associated with good health and longevity and (b) insulin resistance is associated with poor health. In both cases (A and B), the insulin signaling is decreased. So why? From the mTOR point of view, the cases are opposites. In case A (Fig. 2), low insulin signaling is insufficient to activate mTOR (and this is good). In case B (Fig. 1 B-C), insulin signaling is low because of the active mTOR (this is bad), which blocks insulin signaling. In case B, active mTOR is a cause of insulin resistance and low insulin signaling (Fig. 1 B-C). In case A, low insulin signaling keeps mTOR inactive (Fig. 2).

Noteworthy, dwarf (GH-/-) (Fig. 2) and Klotho (Fig. 3) mice have an extended longevity. But Klotho induces IGF-1 and insulin resistance, whereas dwarf mice with reduced IGF-1 and insulin levels have enhanced insulin sensitivity. Bartke et al suggested that signaling downstream from IGF-1 and insulin receptors is reduced in both Klotho and dwarf mice [92, 93]. This is in agreement with the mTOR-centric model [58], given that the mTOR pathway is downstream from insulin/IGF receptors.

The same mTOR-centered point of view is applicable to the diabetes paradox. When diabetes is caused by high mTOR activity, then it is associated with complications, diseases and shortened life span (Fig. 1). This is type 2 diabetes. But when diabetic-like condition is caused by either starvation or rapamycin, then it might be benevolent (Fig. 3).

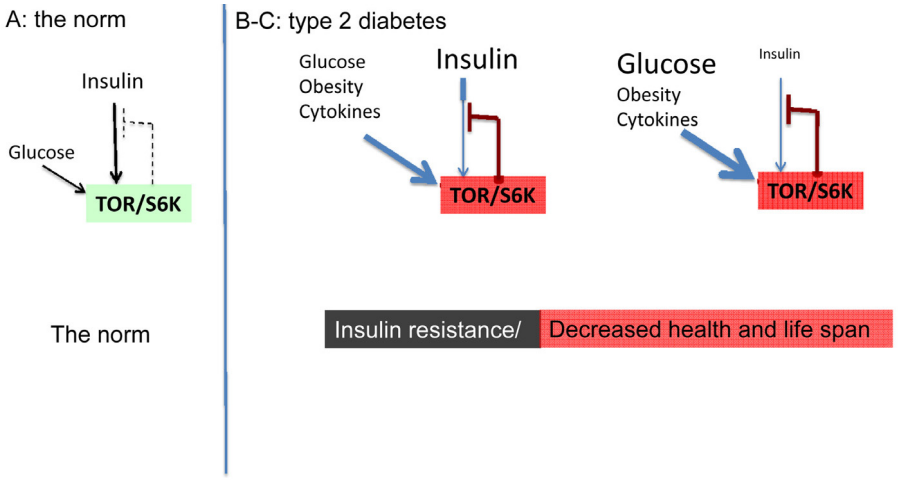


**Figure 1. The norm and type 2 diabetes (simplified schema).**
**(A) The norm.** Insulin and nutrients such as glucose stimulate mTOR, which blocks insulin signaling (feedback loop).
(**B-C**) **High mTOR/S6K activity: insulin resistance plus decreased lifespan.** (**B**) Overactivated by nutrients, cytokins, insulin and other hormones, mTOR blocks insulin signaling causing insulin resistance. Nutrients overstimulate beta-cells and insulin is increased. (**C**) In type II diabetes, beta-cells eventually fail and levels of insulin may be decreased.

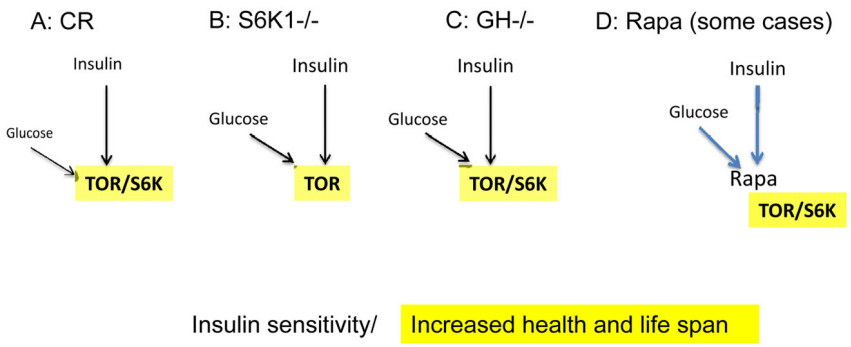


**Figure 2. Low mTOR/S6K activity: insulin sensitivity plus longevity.**
**(A) Calorie restriction.** Deactivation of the nutrient-sensing mTOR pathway results in insulin sensitivity.
**(B) Knockout of S6K1** in mice abolishes feedback block of insulin signaling, resulting in insulin sensitivity [94].
**(C) Decreased levels of growth hormone (GH).** In mice, absence of GH or GH receptor leads to a remarkable extension of longevity [95]. GH receptor deficiency is associated with a reduction in pro-aging signaling, cancer, and diabetes in humans [96]. Growth hormone signaling accelerates aging in mammals [97]. Remarkably, growth stimulation promotes cellular aging, when cells cannot proliferate [98, 99]. Thus, the growth promoting pathways such as mTOR are involved in both organismal and cellular aging.
**(D) Acute treatment with rapamycin**. Deactivation of the nutrient-sensing mTOR pathway abolishes a feedback block of insulin signaling, resulting in insulin sensitivity [50].

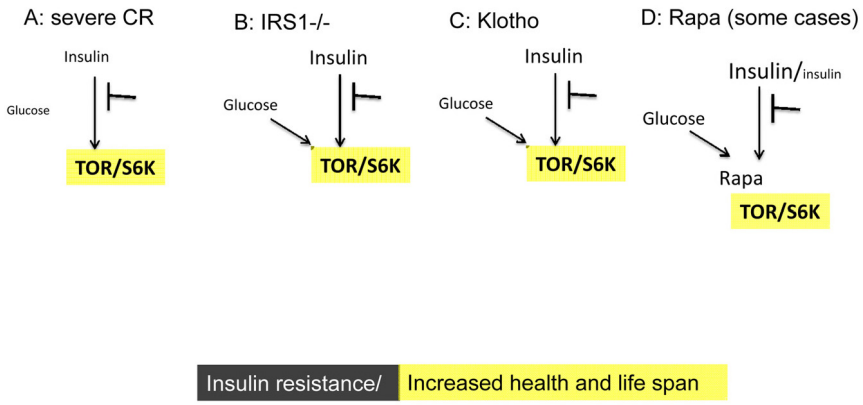


**Figure 3. Low TOR/S6K activity: insulin resistance plus longevity (type 0 diabetes).**
**(A) Severe CR and starvation.** Insulin resistance and symptoms of diabetes are observed during starvation [28] and prolong severe CR [55]. Furthermore, CR may reduce rather than enhance insulin effects in the insulin-sensitive dwarf mice [100].
**(B) IRS1 knockout.** Insulin receptor substrate 1 null mice live longer despite insulin resistance [101].
**(C) Klotho mice.** Overexpression of Klotho in mice extends life span. Klotho protein represses intracellular signals of insulin and insulin-like growth factor 1 (IGF1), [102]. Also, Klotho interferes with insulin/IGF-like signaling to improve longevity in *Caenorhabditis elegans* [103].
**(D) Chronic treatment with high doses of rapamycin** causes insulin resistance and glucose intolerance. This condition can be associated with normal/increased and decreased levels of insulin. Noteworthy, rapamycin induces Klotho [64].

## CONFLICT OF INTERESTS STATEMENT

The author of this manuscript has no conflict of interest to declare.

## REFERENCES


**1.** Lamming DW, Ye L, Katajisto P, Goncalves MD, Saitoh M, Stevens DM, Davis JG, Salmon AB, Richardson A, Ahima RS, Guertin DA, Sabatini DM, Baur JA. Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. Science. 2012; 335: 1638-1643.

**2.** Hughes KJ, Kennedy BK. Cell biology. Rapamycin paradox resolved. Science. 2012; 335: 1578-1579.

**3.** Fraenkel M, Ketzinel-Gilad M, Ariav Y, Pappo O, Karaca M, Castel J, Berthault MF, Magnan C, Cerasi E, Kaiser N, Leibowitz G. mTOR inhibition by rapamycin prevents beta-cell adaptation to hyperglycemia and exacerbates the metabolic state in type 2 diabetes. Diabetes. 2008; 57: 945-957.

**4.** Chang GR, Wu YY, Chiu YS, Chen WY, Liao JW, Hsu HM, Chao TH, Hung SW, Mao FC. Long-term administration of rapamycin reduces adiposity, but impairs glucose tolerance in high-fat diet-fed KK/HlJ mice. Basic Clin Pharmacol Toxicol. 2009; 105: 188-198.

**5.** Houde VP, Brule S, Festuccia WT, Blanchard PG, Bellmann K, Deshaies Y, Marette A. Chronic rapamycin treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and impairing lipid deposition in adipose tissue. Diabetes. 2010; 59: 1338-1348.

**6.** Yang SB, Lee HY, Young DM, Tien AC, Rowson-Baldwin A, Shu YY, Jan YN, Jan LY. Rapamycin induces glucose intolerance in mice by reducing islet mass, insulin content, and insulin sensitivity. J Mol Med (Berl). 2011.

**7.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandezr E, Miller RA. Rapamycin fed late in life extends lifespan in genetically heterogenous mice. Nature. 2009; 460: 392-396.

**8.** Miller RA, Harrison DE, Astle CM, Baur JA, Boyd AR, de Cabo R, Fernandez E, Flurkey K, Javors MA, Nelson JF, Orihuela CJ, Pletcher S, Sharp ZD, Sinclair D, Starnes JW, Wilkinson JE et al . Rapamycin, But Not Resveratrol or Simvastatin, Extends Life Span of Genetically Heterogeneous Mice. J Gerontol A Biol Sci Med Sci. 2011; 66: 191-201.

**9.** Lloberas N, Cruzado JM, Franquesa M, Herrero-Fresneda I, Torras J, Alperovich G, Rama I, Vidal A, Grinyo JM. Mammalian target of rapamycin pathway blockade slows progression of diabetic kidney disease in rats. J Am Soc Nephrol. 2006; 17: 1395-1404.

**10.** Sakaguchi M, Isono M, Isshiki K, Sugimoto T, Koya D, Kashiwagi A. Inhibition of mTOR signaling with rapamycin attenuates renal hypertrophy in the early diabetic mice. Biochem Biophys Res Commun. 2006; 340: 296-301.

**11.** Wittmann S, Daniel C, Stief A, Vogelbacher R, Amann K, Hugo C. Long-term treatment of sirolimus but not cyclosporine ameliorates diabetic nephropathy in the rat. Transplantation. 2009; 87: 1290-1299.

**12.** Flaquer M, Lloberas N, Franquesa M, Torras J, Vidal A, Rosa JL, Herrero-Fresneda I, Grinyo JM, Cruzado JM. The combination of sirolimus and rosiglitazone produces a renoprotective effect on diabetic kidney disease in rats. Life Sci. 2010; 87: 147-153.

**13.** Cruzado JM, Poveda R, Ibernon M, Diaz M, Fulladosa X, Carrera M, Torras J, Bestard O, Navarro I, Ballarin J, Romero R, Grinyo JM. Low-dose sirolimus combined with angiotensin-converting enzyme inhibitor and statin stabilizes renal function and reduces glomerular proliferation in poor prognosis IgA nephropathy. Nephrol Dial Transplant. 2011; 26: 3596-3602.

**14.** Lu MK, Gong XG, Guan KL. mTOR in podocyte function: is rapamycin good for diabetic nephropathy? Cell Cycle. 2011; 10: 3415-3416.

**15.** Demidenko ZN, Blagosklonny MV. The purpose of the HIF-1/PHD feedback loop: to limit mTOR-induced HIF-1alpha. Cell Cycle. 2011; 10: 1557-1562.

**16.** Kolosova NG, Muraleva NA, Zhdankina AA, Stefanova NA, Fursova AZ, Blagosklonny MV. Prevention of age-related macular degeneration (AMD)-like retinopathy by rapamycin in rats. Am J Pathol. 2012; in press

**17.** Pakala R, Stabile E, Jang GJ, Clavijo L, Waksman R. Rapamycin attenuates atherosclerotic plaque progression in apolipoprotein E knockout mice: inhibitory effect on monocyte chemotaxis. J Cardiovasc Pharmacol. 2005; 46: 481-486.

**18.** Mueller MA, Beutner F, Teupser D, Ceglarek U, Thiery J. Prevention of atherosclerosis by the mTOR inhibitor everolimus in LDLR-/- mice despite severe hypercholesterolemia. Atherosclerosis. 2008; 198: 39-48.

**19.** Chen WQ, Zhong L, Zhang L, Ji XP, Zhang M, Zhao YX, Zhang C, Zhang Y. Oral rapamycin attenuates inflammation and enhances stability of atherosclerotic plaques in rabbits independent of serum lipid levels. Br J Pharmacol. 2009; 156: 941-951.

**20.** Zhao L, Ding T, Cyrus T, Cheng Y, Tian H, Ma M, Falotico R, Pratico D. Low-dose oral sirolimus reduces atherogenesis, vascular inflammation and modulates plaque composition in mice lacking the LDL receptor. Br J Pharmacol. 2009; 156: 774-785.

**21.** Keogh A, Richardson M, Ruygrok P, Spratt P, Galbraith A, O'Driscoll G, Macdonald P, Esmore D, Muller D, Faddy S. Sirolimus in de novo heart transplant recipients reduces acute rejection and prevents coronary artery disease at 2 years: a randomized clinical trial. Circulation. 2004; 110: 2694-2700.

**22.** Rodriguez AE, Granada JF, Rodriguez-Alemparte M, Vigo CF, Delgado J, Fernandez-Pereira C, Pocovi A, Rodriguez-Granillo AM, Schulz D, Raizner AE, Palacios I, O'neill W, Kaluza GL, Stone G, Investigators OI. Oral Rapamycin After Coronary Bare-Metal Stent Implantation to Prevent Restenosis The Prospective, Randomized Oral Rapamycin in Argentina (ORAR II) Study. J Am Coll Cardiol. 2006; 47: 1522-1529.

**23.** Sarbassov dos D, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. Curr Opin Cell Biol. 2005; 17: 596-603.

**24.** Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell. 2006; 124: 471-484.

**25.** Blagosklonny MV, Hall MN. Growth and aging: a common molecular mechanism. Aging (Albany NY). 2009; 1: 357-362.

**26.** Loewith R, Hall MN. Target of rapamycin (TOR) in nutrient signaling and growth control. Genetics. 2011; 189: 1177-1201.

**27.** Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol. 2011; 12: 21-35.

**28.** Blagosklonny MV. Rapamycin-induced glucose intolerance: Hunger or starvation diabetes. Cell Cycle. 2011; 10: 4217 -4224.

**29.** Wang J, Obici S, Morgan K, Barzilai N, Feng Z, Rossetti L. Overfeeding rapidly induces leptin and insulin resistance. Diabetes. 2001; 50: 2786-2791.

**30.** Haruta T, Uno T, Kawahara J, Takano A, Egawa K, Sharma PM, Olefsky JM, Kobayashi M. A rapamycin-sensitive pathway down-regulates insulin signaling via phosphorylation and proteasomal degradation of insulin receptor substrate-1. Mol Endocrinol. 2000; 14: 783-794.

**31.** Ozes ON, Akca H, Mayo LD, Gustin JA, Maehama T, Dixon JE, Donner DB. A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. Proc Natl Acad Sci U S A. 2001; 98: 4640-4645.

**32.** Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, Fumagalli S, Allegrini PR, Kozma SC, Auwerx J, Thomas G. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature. 2004; 431: 200-205.

**33.** Shah OJ, Wang Z, Hunter T. Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. Curr Biol. 2004; 14: 1650-1656.

**34.** Tzatsos A, Kandror KV. Nutrients suppress phosphatidylinositol 3-kinase/Akt signaling via raptor-dependent mTOR-mediated insulin receptor substrate 1 phosphorylation. Mol Cell Biol. 2006; 26: 63-76.

**35.** Khamzina L, Veilleux A, Bergeron S, Marette A. Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: possible involvement in obesity-linked insulin resistance. Endocrinology. 2005; 146: 1473-1481.

**36.** Manning BD, Logsdon MN, Lipovsky A, Abbott D, Kwiatkowski DJ, Cantley LC. Feedback inhibition of Akt signaling limits the growth of tumors lacking Tsc2. Genes Dev. 2005; 19: 1773-1778.

**37.** Harrington LS, Findlay GM, Lamb RF. Restraining PI3K: mTOR signalling goes back to the membrane. Trends Biochem Sci. 2005; 30: 35-42.

**38.** Tremblay F, BržlŽ, S., Hee, Um. S., Li, Y., Masuda, K., Roden, M., Sun, X.J., Krebs, M., Polakiewicz, R.D., Thomas, G., Marette, A. Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance. Proc Natl Acad Sci U S A. 2007; 104: 14056-14061.

**39.** Tremblay F, Marette A. Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. J Biol Chem. 2001; 276: 38052-38060.

**40.** Tremblay F, Krebs M, Dombrowski L, Brehm A, Bernroider E, Roth E, Nowotny P, WaldhŠusl W, Marette A, Roden M. Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability. Diabetes. 2005; 54: 2674-2684.

**41.** Mordier S, Iynedjian PB. Activation of mammalian target of rapamycin complex 1 and insulin resistance induced by palmitate in hepatocytes. Biochem Biophys Res Commun. 2007; 362: 206-211.

**42.** Saha AK, Xu XJ, Balon TW, Brandon A, Kraegen EW, Ruderman NB. Insulin resistance due to nutrient excess: is it a consequence of AMPK downregulation? Cell Cycle. 2011; 10: 3447-3451.

**43.** Hsu PP, Kang SA, Rameseder J, Zhang Y, Ottina KA, Lim D, Peterson TR, Choi Y, Gray NS, Yaffe MB, Marto JA, Sabatini DM. The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. Science. 2011; 332: 1317-1322.

**44.** Yu Y, Yoon SO, Poulogiannis G, Yang Q, Ma XM, Villen J, Kubica N, Hoffman GR, Cantley LC, Gygi SP, Blenis J. Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. Science. 2011; 332: 1322-1326.

**45.** Korsheninnikova E, van der Zon GC, Voshol PJ, Janssen GM, Havekes LM, Grefhorst A, Kuipers F, Reijngoud DJ, Romijn JA, Ouwens DM, Maassen JA. Sustained activation of the mammalian target of rapamycin nutrient sensing pathway is associated with hepatic insulin resistance, but not with steatosis, in mice. Diabetologia. 2006; 49: 3049-3057.

**46.** Ueno M, Carvalheira JB, Tambascia RC, Bezerra RM, Amaral ME, Carneiro EM, Folli F, Franchini KG, Saad MJ. Regulation of insulin signalling by hyperinsulinaemia: role of IRS-1/2 serine phosphorylation and the mTOR/p70 S6K pathway. Diabetologia. 2005; 48: 506-518.

**47.** Barzilai N, She L, Liu BQ, Vuguin P, Cohen P, Wang J, Rossetti L. Surgical removal of visceral fat reverses hepatic insulin resistance. Diabetes. 1999; 48: 94-98.

**48.** Gabriely I, Ma XH, Yang XM, Atzmon G, Rajala MW, Berg AH, Scherer P, Rossetti L, Barzilai N. Removal of visceral fat prevents insulin resistance and glucose intolerance of aging: an adipokine-mediated process? Diabetes. 2002; 51: 2951-2958.

**49.** Muzumdar R, Allison DB, Huffman DM, Ma X, Atzmon G, Einstein FH, Fishman S, Poduval AD, McVei T, Keith SW, Barzilai N. Visceral adipose tissue modulates mammalian longevity. Aging Cell. 2008; 7: 438-440.

**50.** Krebs M, Brunmair B, Brehm A, Artwohl M, Szendroedi J, Nowotny P, Roth E, FŸrnsinn C, Promintzer M, Anderwald C, Bischof M, Roden M. The Mammalian target of rapamycin pathway regulates nutrient-sensitive glucose uptake in man. Diabetes. 2007; 56: 1600-1607.

**51.** Blagosklonny MV. Aging and immortality: quasi-programmed senescence and its pharmacologic inhibition. Cell Cycle. 2006; 5: 2087-2102.

**52.** Blagosklonny MV. An anti-aging drug today: from senescence-promoting genes to anti-aging pill. Drug Disc Today. 2007; 12: 218-224.

**53.** Blagosklonny MV. Validation of anti-aging drugs by treating age-related diseases. Aging (Albany NY). 2009; 1: 281-288.

**54.** Blagosklonny MV. mTOR-driven aging: speeding car without brakes. Cell Cycle. 2009; 8: 4055-4059.

**55.** Fontana L, Klein S, Holloszy JO. Effects of long-term calorie restriction and endurance exercise on glucose tolerance, insulin action, and adipokine production. Age (Dordr). 2010; 32: 97-108.

**56.** Rincon M, Muzumdar R, Atzmon G, Barzilai N. The paradox of the insulin/IGF-1 signaling pathway in longevity. Mech Ageing Dev. 2004; 125: 397-403.

**57.** Barzilai N, Huffman DM, Muzumdar RH, Bartke A. The critical role of metabolic pathways in aging. Diabetes. 2012; 61: 1315-1322.

**58.** Blagosklonny MV. Paradoxes of aging. Cell Cycle. 2007; 6: 2997-3003.

**59.** Weiss EP, Racette SB, Villareal DT, Fontana L, Steger-May K, Schechtman KB, Klein S, Holloszy JO, Group. WUSoMC. Improvements in glucose tolerance and insulin action induced by

increasing energy expenditure or decreasing energy intake: a randomized controlled trial. Am J Clin Nutr. 2006; 84: 1033-1042.

**60.** Fontana L. The scientific basis of caloric restriction leading to longer life. Curr Opin Gastroenterol. 2009; 25:144-150.

**61.** Fontana L, Partridge L, Longo VD. Extending healthy life span--from yeast to humans. Science. 2010; 328: 321-326.

**62.** Blagosklonny MV. Calorie restriction: Decelerating mTOR-driven aging from cells to organisms (including humans). Cell Cycle. 2010; 9: 683-688.

**63.** Di Paolo S, Teutonico A, Leogrande D, Capobianco C, Schena PF. Chronic inhibition of mammalian target of rapamycin signaling downregulates insulin receptor substrates 1 and 2 and AKT activation: A crossroad between cancer and diabetes? J Am Soc Nephrol. 2006; 17: 2236-2244.

**64.** Tataranni T, Biondi G, Cariello M, Mangino M, Colucci G, Rutigliano M, Ditonno P, Schena FP, Gesualdo L, Grandaliano G. Rapamycin-Induced Hypophosphatemia and Insulin Resistance Are Associated With mTORC2 Activation and Klotho Expression. Am J Transplant. 2011; 11: 1656-1664.

**65.** Velazquez-Garcia S, Valle S, Rosa TC, Takane KK, Demirci C, Alvarez-Perez JC, Mellado-Gil JM, Ernst S, Scott DK, Vasavada RC, Alonso LC, Garcia-Ocana A. Activation of protein kinase C-zeta in pancreatic beta-cells in vivo improves glucose tolerance and induces beta-cell expansion via mTOR activation. Diabetes. 2011; 60: 2546-2559.

**66.** Rachdi L, Balcazar N, Osorio-Duque F, Elghazi L, Weiss A, Gould A, Chang-Chen KJ, Gambello MJ, Bernal-Mizrachi E. Disruption of Tsc2 in pancreatic beta cells induces beta cell mass expansion and improved glucose tolerance in a TORC1-dependent manner. Proc Natl Acad Sci U S A. 2008; 105: 9250-9255.

**67.** Leibowitz G, Cerasi E, Ketzinel-Gilad M. The role of mTOR in the adaptation and failure of beta-cells in type 2 diabetes. Diabetes Obes Metab. 2008; 10 Suppl 4: 157-169.

**68.** Xie J, Herbert TP. The role of mammalian target of rapamycin (mTOR) in the regulation of pancreatic beta-cell mass: implications in the development of type-2 diabetes. Cell Mol Life Sci. 2011.

**69.** Blandino-Rosano M, Chen AY, Scheys JO, Alejandro EU, Gould AP, Taranukha T, Elghazi L, Cras-Meneur C, Bernal-Mizrachi E. mTORC1 signaling and regulation of pancreatic beta-cell mass. Cell Cycle. 2012; 11.

**70.** Hagiwara A, Cornu M, Cybulski N, Polak P, Betz C, Trapani F, Terracciano L, Heim MH, Ruegg MA, Hall MN. Hepatic mTORC2 Activates Glycolysis and Lipogenesis through Akt, Glucokinase, and SREBP1c. Cell Metab. 2012; 15: 725-738.

**71.** Teutonico A, Schena PF, Di Paolo S. Glucose metabolism in renal transplant recipients: effect of calcineurin inhibitor withdrawal and conversion to sirolimus. J Am Soc Nephrol. 2005; 16: 3128-3135.

**72.** Johnston O, Rose CL, Webster AC, Gill JS. Sirolimus is associated with new-onset diabetes in kidney transplant recipients. J Am Soc Nephrol. 2008; 19: 1411-1418.

**73.** Schwarz C, Oberbauer R. The future role of target of rapamycin inhibitors in renal transplantation. Curr Opin Urol. 2002; 12: 109-113.

**74.** Legendre C, Campistol JM, Squifflet JP, Burke JT. Cardiovascular risk factors of sirolimus compared with cyclosporine: early experience from two randomized trials in renal transplantation. Transplant Proc. 2003; 35: 151S-153S.

**75.** Gonwa T, Mendez R, Yang HC, Weinstein S, Jensik S, Steinberg S. Randomized trial of tacrolimus in combination with sirolimus or mycophenolate mofetil in kidney transplantation: results at 6 months. Transplantation. 2003; 75:1213-1220.

**76.** Romagnoli J, Citterio F, Violi P, Nanni G, Castagneto M. Posttransplant diabetes mellitus after kidney transplantation with different immunosuppressive agents. Transplant Proc. 2004; 36: 690-691.

**77.** Sulanc E, Lane JT, Puumala SE, Groggel GC, Wrenshall LE, Stevens RB. New-onset diabetes after kidney transplantation: an application of 2003 International Guidelines. Transplantation. 2005; 80: 945-952.

**78.** Araki M, Flechner SM, Ismail HR, Flechner LM, Zhou L, Derweesh IH, Goldfarb D, Modlin C, Novick AC, Faiman C. Posttransplant diabetes mellitus in kidney transplant recipients receiving calcineurin or mTOR inhibitor drugs. Transplantation. 2006; 81: 335-341.

**79.** Pavlakis M, Goldfarb-Rumyantzev AS. Diabetes after transplantation and sirolimus: what's the connection? J Am Soc Nephrol. 2008; 19: 1255-1256.

**80.** Blagosklonny MV. Aging, stem cells, and mammalian target of rapamycin: a prospect of pharmacologic rejuvenation of aging stem cells. Rejuvenation Res. 2008; 11: 801-808.

**81.** Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Antoch MP, Blagosklonny MV. Rapamycin extends maximal lifespan in cancer-prone mice. Am J Pathol. 2010; 176: 2092-2097.

**82.** Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Rosenfeld SV, Blagosklonny MV. Rapamycin increases lifespan and inhibits spontaneous tumorigenesis in inbred female mice. Cell Cycle. 2011; 10: 4230-4236.

**83.** Khanna A, Kapahi P. Rapamycin: killing two birds with one stone. Aging (Albany NY). 3: 1043-1044.

**84.** Spong A, Bartke A. Rapamycin slows aging in mice. Cell Cycle. 11.

**85.** Longo VD, Fontana L. Intermittent supplementation with rapamycin as a dietary restriction mimetic. Aging (Albany NY). 2011; 3: 1039-1040.

**86.** Selman C, Partridge L. A double whammy for aging? Rapamycin extends lifespan and inhibits cancer in inbred female mice. Cell Cycle. 11: 17-18.

**87.** Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol. 2004; 6: 1122-1128.

**88.** Markman B, Dienstmann R, Tabernero J. Targeting the PI3K/Akt/mTOR pathway--beyond rapalogs. Oncotarget. 2010; 1: 530-543.

**89.** Benjamin D, Colombi M, Moroni C, Hall MN. Rapamycin passes the torch: a new generation of mTOR inhibitors. Nat Rev Drug Discov. 2011; 10: 868-880.

**90.** Blagosklonny MV. Increasing healthy lifespan by suppressing aging in our lifetime: Preliminary proposal. Cell Cycle. 2010; 9: 4788-4794.

**91.** Kirkwood TB. Understanding the odd science of aging. Cell. 2005; 120: 437-447.

**92.** Bartke A. Long-lived Klotho mice: new insights into the roles of IGF-1 and insulin in aging. Trends Endocrinol Metab. 2006; 17: 33-35.

**93.** Bartke A. Single-gene mutations and healthy ageing in mammals. Philos Trans R Soc Lond B Biol Sci. 2011; 366: 28-34.

**94.** Selman C, Tullet JM, Wieser D, Irvine E, Lingard SJ, Choudhury AI, Claret M, Al-Qassab H, Carmignac D, Ramadani F, Woods A, Robinson IC, Schuster E, Batterham RL, Kozma SC, Thomas G et al. Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. Science. 2009; 326: 140-144.

**95.** Bartke A. Healthy Aging: Is Smaller Better? - A Mini-Review. Gerontology. 2012.

**96.** Guevara-Aguirre J, Balasubramanian P, Guevara-Aguirre M, Wei M, Madia F, Cheng CW, Hwang D, Martin-Montalvo A, Saavedra J, Ingles S, de Cabo R, Cohen P, Longo VD. Growth hormone receptor deficiency is associated with a major reduction in pro-aging signaling, cancer, and diabetes in humans. Sci Transl Med. 2011; 3: 70ra13.

**97.** Bartke A. Pleiotropic effects of growth hormone signaling in aging. Trends Endocrinol Metab. 2011; 22: 437-442.

**98.** Demidenko ZN, Blagosklonny MV. Growth stimulation leads to cellular senescence when the cell cycle is blocked. Cell Cycle. 2008; 7: 3355-3361.

**99.** Demidenko ZN, Zubova SG, Bukreeva EI, Pospelov VA, Pospelova TV, Blagosklonny MV. Rapamycin decelerates cellular senescence. Cell Cycle. 2009; 8: 1888-1895.

**100.** Wang Z, Al-Regaiey KA, Masternak MM, Bartke A. Adipocytokines and lipid levels in Ames dwarf and calorie-restricted mice. J Gerontol A Biol Sci Med Sci. 2006; 61: 323-331.

**101.** Selman C, Lingard S, Choudhury AI, Batterham RL, Claret M, Clements M, Ramadani F, Okkenhaug K, Schuster E, Blanc E, Piper MD, Al-Qassab H, Speakman JR, Carmignac D, Robinson IC, Thornton JM et al . Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice. Faseb J. 2008; 22: 807-818.

**102.** Kurosu H, Yamamoto M, Clark JD, Pastor JV, Nandi A, Gurnani P, McGuinness OP, Chikuda H, Yamaguchi M, Kawaguchi H, Shimomura I, Takayama Y, Herz J, Kahn CR, Rosenblatt KP, Kuro-o M. Suppression of aging in mice by the hormone Klotho. Science. 2005; 309: 1829-1833.

**103.** Chateau MT, Araiz C, Descamps S, Galas S. Klotho interferes with a novel FGF-signalling pathway and insulin/Igf-like signalling to improve longevity and stress resistance in Caenorhabditis elegans. Aging (Albany NY). 2010; 2: 567-581.

Research Paper

# Rapamycin increases oxidative stress response gene expression in adult stem cells

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**Abstract: Balancing quiescence with proliferation is of paramount importance for adult stem cells in order to avoid hyperproliferation and cell depletion. In some models, stem cell exhaustion may be reversed with the drug rapamycin, which was shown can suppress cellular senescence *in vitro* and extend lifespan in animals. We hypothesized that rapamycin increases the expression of oxidative stress response genes in adult stem cells, and that these gene activities diminish with age. To test our hypothesis, we exposed mice to rapamycin and then examined the transcriptome of their spermatogonial stem cells (SSCs). Gene expression microarray analysis revealed that numerous oxidative stress response genes were upregulated upon rapamycin treatment, including superoxide dismutase 1, glutathione reductase, and delta-aminolevulinate dehydratase. When we examined the expression of these genes in 55-week-old wild type SSCs, their levels were significantly reduced compared to 3-week-old SSCs, suggesting that their downregulation is coincident with the aging process in adult stem cells. We conclude that rapamycin-induced stimulation of oxidative stress response genes may promote cellular longevity in SSCs, while a decline in gene expression in aged stem cells could reflect the SSCs' diminished potential to alleviate oxidative stress, a hallmark of aging.**

## INTRODUCTION

Cell senescence may contribute to adult stem cell exhaustion, compromising the maintenance of cell lineages within the body [1]. Recent evidence suggests that the cumulative exposure to reactive oxygen species (ROS) and DNA damage can lead to the decline of adult stem cells both in population and in regenerative capacity. For example, hematopoietic stem cells (HSCs) from mice lacking forkhead box O (FOXO) family transcription factors exhibit higher levels of ROS, accompanied by short-term hyperproliferation that is then followed by increased apoptosis that depletes the HSC pool [2, 3]. Epithelial stem cells in the epidermis (ESCs) that are engineered to constitutively transduce wingless-related MMTV integration site (WNT) signals in mice rapidly divide in the short term, but then undergo cell senescence and disappear from the ESC compartment [4]. Neural stem cells (NSCs), meanwhile, decline in number and function within the subventricular zone of lateral ventricles in the aging mouse brain due to genomic instability and upregulated cyclin-dependent kinase inhibitor 2a (*Cdkn2a*; $p16^{Ink4a}$), which activates DNA-damage response pathways that induce apoptosis or senescence [5, 6]. Spermatogonial stem cells (SSCs) exhibit a loss of regenerative ability during aging *in vivo* and *in vitro*, with the downregulation of several genes important for self-renewal [7, 8, 9, 10].

Altered expression of the mammalian target of rapamycin complex 1 (mTORC1), a key regulator of cell metabolism and a kinase whose downstream activity is associated with phosphatidylinositol 3-kinase (PI3-K) signaling pathways, significantly alters the fate of adult stem cells. Studies in mice have revealed that hyperactive signaling through mTORC1 depletes HSCs, ESCs, and SSCs from their respective compartments [4, 11, 12]. This stem cell loss is rescued upon exposure to the drug rapamycin, with wild type SSCs undergoing active expansion *in vivo* [12]. Rapamycin specifically

inhibits mTORC1 and has been shown to increase the lifespan of organisms, including worms, flies, and aging mice [13, 14, 15, 16, 17]. Recent evidence demonstrated that rapamycin decreases mammalian cell senescence and delays spontaneous tumor development in mice at older ages [18, 19]. Insulin signaling and insulin-like growth factor 1 receptor activation, meanwhile, are known to modulate the levels of enzymes regulating numerous cellular processes. When wild type mice or cultured endothelial cells are exposed to high levels of glucose to establish diabetes-associated conditions, the transcriptional activity of superoxide dismutase 1 (*Sod1*) and the enzymatic activity of delta-aminolevulinate dehydratase (ALAD) are significantly lower than in controls [20, 21, 22]. Levels of glutathione and the enzyme glutathione reductase (GSR) are depleted in apoprotein E-deficient mutant mice [23]. As putative biomarkers for oxidative stress, *Sod1*, *Gsr*, and *Alad* transcript levels might also be expected to be altered in adult stem cells upon elevated mTORC1 activity or during the aging process.

Here, using mouse SSCs as an *in vivo* model system for studying adult stem cell maintenance and gene regulation downstream of mTORC1, we investigated the effect of rapamycin on the SSC transcriptome. We found that mTORC1 inhibition not only upregulates key genes important for SSC self-renewal, but also elevates transcript levels of oxidative stress response genes and downregulates genes associated with growth and metabolism. When aged SSCs were examined for *Sod1*, *Gsr*, and *Alad*, these transcript levels were significantly reduced when compared with those of younger SSCs. Our results implicate the aging process and mTORC1 in downregulating oxidative stress response genes in adult stem cells.

## RESULTS

### Magnetic-activated cell sorting enriches undifferentiated male germ cells from rapamycin-treated mice

To examine the effects of mTORC1 inhibition on SSC gene expression, we first implemented an established regimen in which juvenile male mice were administered intraperitoneal injections of rapamycin or control vehicle daily for two weeks (Figure 1A) [4, 12, 24]. Following these treatments, single cell suspensions of germ cells were prepared from isolated testes and subjected to magnetic-activated cell sorting (MACS). This procedure enriches the undifferentiated germ cell fraction, which represents the adult SSC population (Figure 1A) [25, 26, 27]. RNA from cells double-positive for the SSC surface markers thymus cell antigen 1, theta (THY1) and glial cell line-derived neurotrophic factor family receptor alpha 1 (GFRA1) was isolated for gene expression microarray analysis and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) validation. In order to verify that our MACS selection strategy was successfully enriching undifferentiated male germ cells, we performed qRT-PCR on MACS-enriched $THY1^+/GFRA1^+$ cells from non-injected mice. When compared to unsorted germ cells, the MACS-enriched cells exhibited a 16-fold increase in *Gfra1* transcripts, as well as 10-fold and 8-fold increases, respectively, in two additional SSC transcripts, zinc finger and BTB domain containing 16 (*Zbtb16*; referred to here as *Plzf*) and POU domain, class 5, transcription factor 1 (*Pou5f1*; referred to here as *Oct4*) (Figure 1B). MACS-enriched $THY1^+$ and $GFRA1^+$ germ cells have previously been shown to exhibit significantly increased stem cell activity over unsorted cells through transplantation assays [25, 28]. Thus, our experimental design favorably selected for SSCs and allowed for the examination of rapamycin-induced alterations of SSC gene activity.

### Rapamycin administration inhibits testis growth but expands the number of undifferentiated germ cells

Testicular weights and body weights of rapamycin-treated mice were both significantly reduced compared to those of control vehicle-treated mice (12.60 ± 0.492 mg vs 33.52 ± 0.968 mg average testis weight; 8.783 ± 0.155 g vs 13.35 ± 0.345 g average body weight; N=5), reflecting the effects of rapamycin on tissue growth (Figure 2 A-D). Administration of rapamycin or control vehicle began on postnatal day (P)12 and ended on P25, a period of mouse development in which the testis generates the first sets of meiotic and post-meiotic germ cells [29]. Equivalent numbers of terminally differentiated Sertoli cells, but fewer mitotic progenitor Leydig cells, were observed between rapamycin- and control vehicle-treated mice (data not shown). Rapamycin had previously been shown to inhibit the proliferation of differentiating germ cells through PI3-K signaling [30]. Conversely, mTORC1 inhibition by rapamycin expands the number of SSCs through glial cell line-derived neurotrophic factor (GDNF) signaling [12]. When we placed SSCs from individual mice into culture, stem cells from the rapamycin-treated mice formed more numerous and larger-sized colonies than the control SSCs during the first two weeks after plating (Figure 3). These results demonstrated that chronic rapamycin exposure selected for adult SSCs within the germ cell population of the juvenile testis. From this, we wondered whether the selection involved transcriptional networks independent of the GDNF signaling pathway.

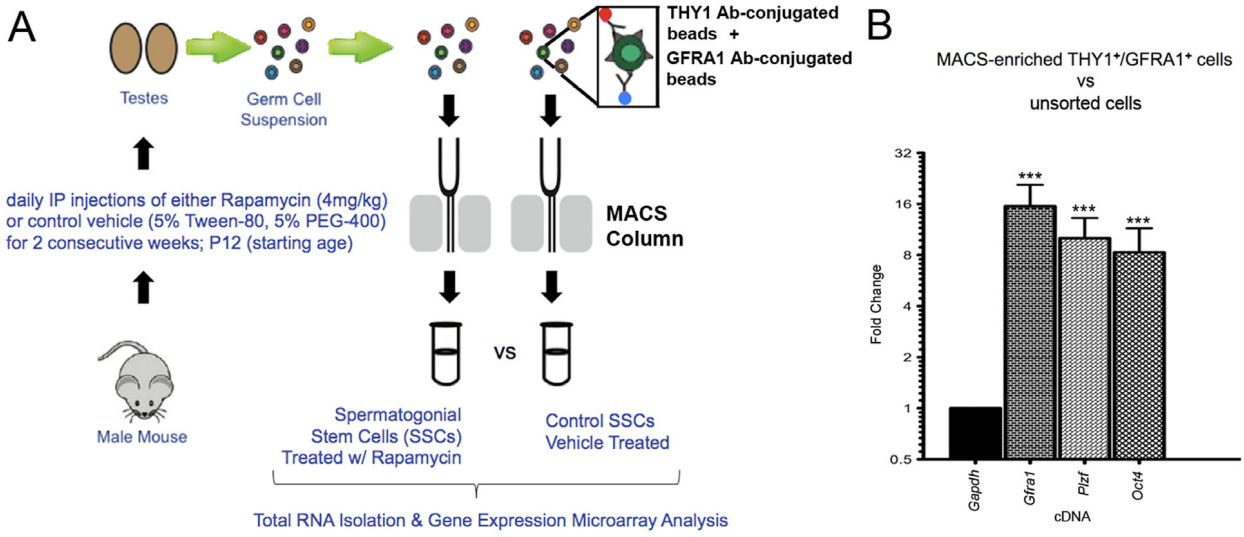


**Figure 1. Magnetic-activated cell sorting (MACS) significantly enriches for SSCs isolated from rapamycin- or control-treated mice.** Male animals were chronically administered rapamycin or control vehicle for 2 weeks, followed by MACS selection for undifferentiated germ cells. (**A**) Schematic diagram depicting the experimental design. Isolated testes were enzymatically digested to single cell suspensions containing germ cells (multi-colored circles). Incubation with magnetic beads conjugated to antibodies that recognize SSC surface proteins THY1 and GFRA1 was followed by the passage of the samples through separation columns attached to a magnet (gray bars). THY1+ and GFRA1+ cells were ultimately flushed out for cell culture or RNA isolation. (**B**) Quantitative RT-PCR was performed on THY1+/GFRA1+ cells enriched by MACS from age-matched wild type (non-injected) mice. When compared to the endogenous control *Gapdh* (assigned a relative value of "1"), the fold-changes in expression of SSC markers *Gfra1*, *Plzf*, and *Oct4* were all significantly elevated in the MACS-selected cells versus unsorted testicular cells (16-fold, 10-fold, and 8-fold, respectively). Data represent mean values +/- SEM from three biological replicates. Student's t-test was performed to assess significance between each SSC marker and the endogenous control; ***p<0.001.

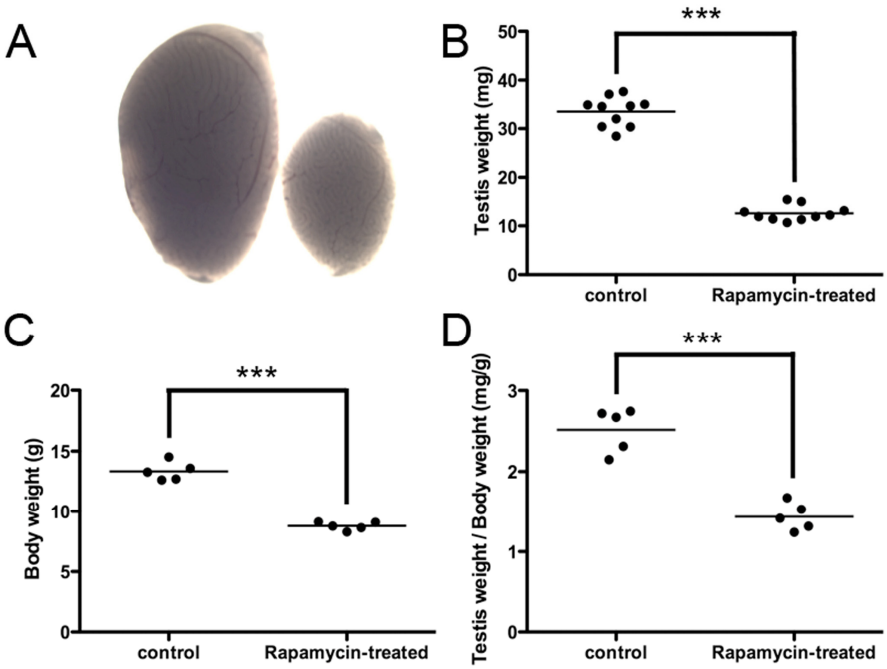


**Figure 2. Rapamycin inhibits testis growth and reduces body weight.** (**A**) Testes from mice chronically treated with rapamycin for 2 weeks (days 12 through 25) were significantly smaller in size. Representative images of a rapamycin-exposed testis (right) and a control vehicle-exposed testis (left). (**B**) Rapamycin-exposed mouse testes exhibited significantly diminished weights (12.60 ± 0.492 mg vs 33.52 ± 0.968 mg average testis weight; N=10, ***p<0.001). (**C**) Body weights of rapamycin-exposed mice were significantly reduced (8.783 ± 0.155 g vs 13.35 ± 0.345 g average body weight; N=5, ***p<0.001). (**D**) When controlled for body weight, rapamycin-exposed testes exhibited significantly lower values (1.44 mg/g body weight vs. 2.52 mg/g body weight; N=5, ***p<0.001).

### Identification and validation of a rapamycin-induced SSC transcriptome signature

To identify differentially expressed genes in rapamycin-exposed mouse SSCs, we used the Agilent Whole Mouse Genome oligonucleotide microarray 4x44K platform. Approximately 40% of the SSC transcriptome was significantly altered in expression levels, as measured by >2-fold change and p-value <0.01 (Figure 4A). A total of 7,741 oligo probes were upregulated, while 8,746 probes were downregulated, corresponding to an enhancement of 4,617 transcripts (unique Entrez gene IDs) and a reduction of 5,360 transcripts (Figure 4A, Supplemental Table 1). For data analysis, we used the non-hierarchical clustering method AutoSOME and generated a heat map of the cluster that specifically contained *Gfra1* (Figure 4B) [31]. The *Gfra1* transcript had previously been shown to be upregulated in SSCs following rapamycin exposure, and it exhibited a 3.13-fold enhancement in expression here (Table 1) [12]. Within the *Gfra1* cluster were additional SSC self-renewal-associated genes (*Ret*, *Lin28b*, *Nanos2*, *Foxo1*), all of which were significantly upregulated in our rapamycin-exposed SSCs (Figure 4B, Table 1). In addition to these genes important for SSC maintenance, the *Gfra1* cluster contained several oxidative stress response genes that were also significantly upregulated with rapamycin, including *Alad*, *Sod1*, and *Gsr* (Figure 4B, Table 1). In contrast, genes important for signal transduction in growth and metabolism (*Wnt3a*, *Wnt2*, *Stat4*, *Tgfbr1-3*) were significantly downregulated in our rapamycin-exposed SSCs (Table 1). To integrate these SSC transcriptome data into biological pathways, we used the knowledge-based database Ingenuity Pathway Analysis® (IPA). The top five canonical pathways, ranked by p-value and ratio of the number of genes (up/down) per category, are listed in Table 2. These include free radical scavenging, phosphatase and tensin homolog (PTEN) signaling, and nuclear factor (erythroid-derived 2)-related factor 2 (NRF2)-mediated oxidative stress response (Table 2). IPA identified several potential interactions among the oxidative stress response genes in our *Gfra1* cluster, with ALAD serving as a nodal point to connect tumor necrosis factor (TNF) and erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (ERBB2) with SOD1, GSR, and retinoblastoma protein (RB1) (Figure 4C). We validated the differential regulation of 15 selected transcripts (9 upregulated: *Gfra1*, *Ret*, *Lin28b*, *Nanos2*, *Foxo1*, *Alad*, *Sod1*, *Gsr*, *Erbb2*; 6 downregulated: *Wnt3a*, *Tgfbr1*, *Stat4*, *Tnf*, *Gsc*, *Meox2*) from the gene expression microarray using qRT-PCR (Figure 5A). All genes exhibited significant changes in expression as predicted from the microarray data.

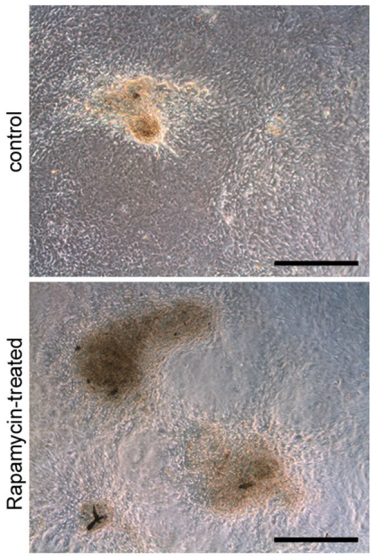


**Figure 3. Rapamycin expands SSC colonies in culture.** MACS-enriched THY1+/GFRA1+ cells cultured *in vitro* for two weeks from rapamycin-treated mice formed more numerous and larger-sized colonies (bottom) than the control SSCs (top). Scale bars = 200 m.

### Oxidative stress response genes upregulated by rapamycin are downregulated in aging SSCs

Given that rapamycin has been shown to increase lifespan in aging mice [13, 19], and that here it significantly enhanced the expression of *Alad*, *Sod1*, and *Gsr* in juvenile SSCs, we next asked whether the levels of these three oxidative stress response transcripts were diminished in the SSCs isolated from older versus younger wild type mice. When SSCs from 55-week-old males were compared to SSCs from 3-week-old males, the relative gene expression values for *Alad*, *Sod1*, and *Gsr* were all decreased (1.46-, 1.72-, and 1.62-fold, respectively; $p<0.05$) (Figure 5B). Morphologically, the SSCs from the two ages of mice were indistinguishable, although fewer SSCs were obtained from the older testes than from the younger testes (data not shown). These data suggest that as SSCs age *in vivo*, the aging process correlates with a downregulation in the expression of genes that respond to oxidative stress.

## DISCUSSION

The maintenance of adult stem cells, including SSCs, is critical to ensure the continuous production of differentiated cells within that lineage as organisms age. Mouse SSC self-renewal is promoted through GDNF signaling and mTORC1 antagonism by the PLZF-

mediated activation of *Redd1* [12]. Chronic exposure of mouse testes to rapamycin expands the SSC pool *in vivo* and increases *Gfra1* and *Ret* expression [12]. The present study demonstrated that along with *Gfra1* and *Ret*, additional SSC self-renewal genes (*Lin28b*, *Nanos 2*, *Foxo1*) and oxidative stress response genes (*Alad*, *Sod1*, *Gsr*) are upregulated in rapamycin-exposed SSCs. LIN28B suppresses microRNA biogenesis through interactions with the *let-7* precursor, and is enriched in undifferentiated germ cells within the testis [32]. The functional role of LIN28B in SSCs is not yet clear, but the protein exhibits a striking temporal co-expression in germ cells with PLZF, suggesting a possible regulatory association with this transcription factor (unpublished observations). NANOS2 is an RNA-binding protein that acts downstream of GFRA1 to promote SSC self-renewal, and is required for stem cell maintenance [33, 34]. FOXO1, a transcription factor, regulates the expression of *Ret* and other genes in SSCs and is required for their homeostasis [35]. Collectively, these findings identify a transcriptional network that is enhanced when mTORC1 is inhibited by rapamycin.

**Table 1. Selected differentially expressed genes from the oligo microarray (rapamycin-exposed SSCs vs controls)**

| Category | | Fold ⊗ | | p value |
|---|---|---|---|---|
| | | UP | DOWN | |
| SSC self-renewal | | | | |
| | *Gfra1* | 3.13411 | | 4.35E-10 |
| | *Ret* | 2.71904 | | 4.69E-06 |
| | *Lin28b* | 5.77946 | | 0.00615 |
| | *Nanos 2* | 3.00648 | | 0.00007 |
| | *Foxo1* | 2.3856 | | 8.50E-08 |
| Signal transduction | | | | |
| | *Wnt3a* | | 8.76346 | 1.98E-07 |
| | *Wnt2* | | 3.28504 | 4.55E-10 |
| | *Stat4* | | 7.53744 | 8.22E-18 |
| | *Tgfbr1* | | 2.52916 | 3.41E-08 |
| | *Tgfbr2* | | 2.41742 | 6.11E-08 |
| | *Tgfbr3* | | 2.98383 | 8.50E-06 |
| Oxidative stress response | | | | |
| | *Sod1* | 2.01295 | | 8.69E-06 |
| | *Gsr* | 2.74239 | | 2.40E-09 |
| | *Alad* | 5.23738 | | 1.91E-15 |
| | *Gstm6* | 6.59408 | | 5.00E-17 |
| | *Gstm7* | 7.34355 | | 1.15E-17 |
| | *Brca1* | 2.9183 | 0 | 0.00183 |
| | *Nfe2l1* | 2.21224 | | 5.94E-07 |
| | *Glrx2* | 2.75994 | | 0.00039 |
| Other | | | | |
| | *Erbb2* | 2.06434 | | 1.49E-10 |
| | *Rb1* | 2.35478 | | 3.73E-14 |
| | *Tnf* | | 2.67337 | 3.57E-18 |
| | *Gsc* | | 5.13706 | 1.74E-13 |
| | *Meox2* | | 7.68215 | 3.37E-06 |

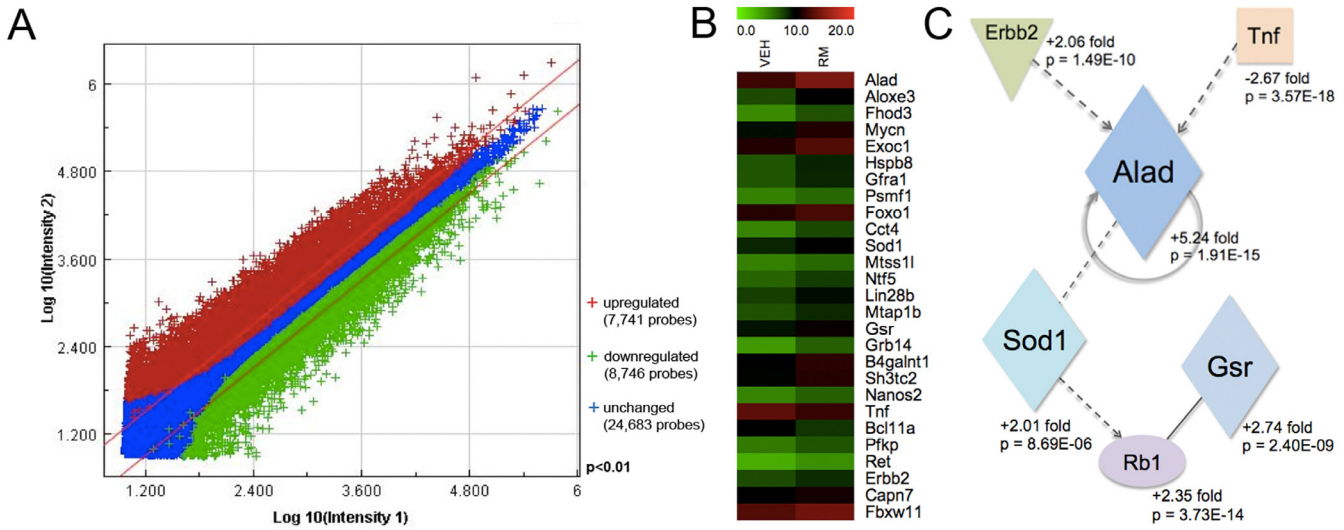


**Figure 4. Transcriptional profiling of rapamycin-exposed SSCs reveals an upregulation of oxidative stress response genes.** (**A**) Double-log scatter plot to visualize the signal intensities of all oligo probes on the Agilent Whole Mouse Genome oligonucleotide microarray 4x44K platform that were upregulated (red crosses), downregulated (green crosses), or unchanged (blue crosses). Filter conditions applied to the scatter plot: >2-fold change and p-value <0.01. (**B**) Heat map of genes within a cluster containing *Gfra1* that were significantly altered in THY1+/GFRA1+ cells in response to rapamycin exposure. Horizontal stripes represent genes and columns show the treatment conditions (VEH = control vehicle; RM = rapamycin). Log2-fold changes of gene ratios are color coded as shown in the top horizontal stripe, from a relative low value of 0.0 (green) to a high of 20.0 (red). (**C**) Potential relationships among the antioxidant gene products of *Sod1*, *Gsr*, and *Alad* in rapamycin-exposed SSCs, illustrated here as a schematic adapted from Ingenuity Pathways Analysis® (IPA). Parallelograms represent enzymes, ovals represent transcription factors, squares represent cytokines, and inverted triangles represent cell receptors. Solid lines indicate direct binding (only) between gene products, while dashed lines represent indirect associations. Arrows indicate that the first product acts upon the second product. A circular solid line with arrow represents direct auto regulation. Fold changes and p-values are listed next to the gene products.

Elevated levels of transcripts encoding antioxidant enzymes in our rapamycin-exposed SSCs suggest the possibility that the mitigation of ROS and DNA damage could facilitate adult stem cell expansion and that it might be counterbalanced by mTORC1 activity. An association between *Sod1* and the inhibition of mTORC1 by rapamycin was recently demonstrated in yeast, and enhanced GSR activity was observed in rapamycin-treated human corneal endothelial cells exposed to tert-butyl hydroperoxide [36, 37]. SOD1 binds to copper and zinc ions within the cytoplasm and mitochondrial intermembrane, and is one of three superoxide dismutase enzymes that destroy free superoxide radicals. GSR reduces glutathione disulfide, GSSG, into the sulfhydryl form, GSH. To our knowledge, no relationship between *Alad* and mTORC1 had previously been identified. ALAD activity is a biomarker for oxidative stress in human bone marrow transplant recipients, as well as for lead toxicity in human populations [38, 39]. ALAD catalyzes the second step in porphyrin and heme biosynthesis, condensing two molecules of delta-aminolevulinate to form porphobilinogen. We speculate that rapamycin might upregulate antioxidants in SSCs at the transcriptional level to promote cellular longevity, with the concomitant inhibition of metabolic events to reduce ROS and oxidative damage. Indeed, the top canonical pathways predicted by IPA to be differentially regulated upon rapamycin exposure involve biological processes known to associate with metabolism and its consequences: growth hormone signaling, NF-κB signaling, PTEN signaling, free radical scavenging, and the NRF2-mediated oxidative stress response. PTEN is a negative regulator of mTORC1, while NF-κB is activated downstream of mTORC1 to modulate specific sets of genes [11, 40]. Additional processes activated downstream of mTORC1 include ribosome biogenesis and protein synthesis, cellular events that, when

inhibited, can extend lifespan ( reviewed in [41] ). Interestingly, an association between NRF2 and mTORC1 was recently identified using *in vitro* and *in vivo* models of lung cancer [42].

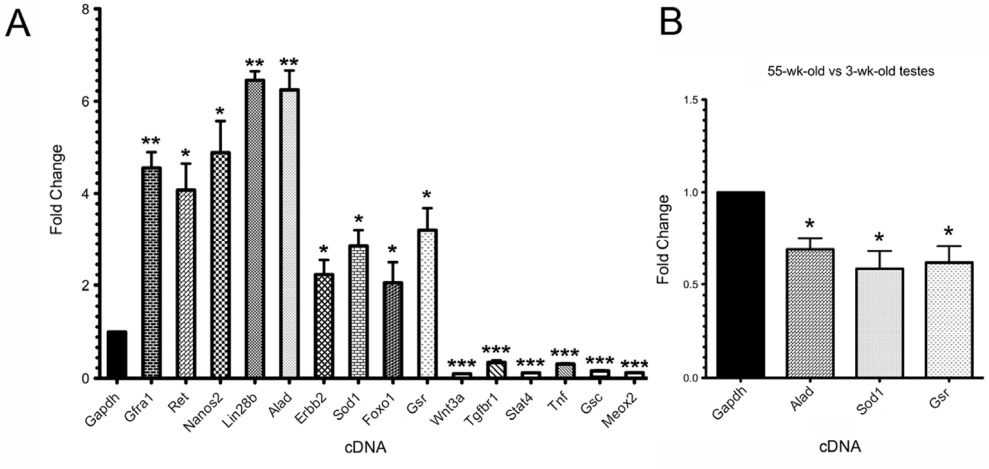


**Figure 5. Microarray validation and determination that genes encoding antioxidants are downregulated in aging SSCs.** (**A**) Quantitative RT-PCR validation of the differential gene expression of 15 selected transcripts observed in the microarray analysis of rapamycin-exposed SSCs (THY1+/GFRA1+ cells enriched by MACS). Nine upregulated genes, *Gfra1*, *Ret*, *Lin28b*, *Nanos2*, *Foxo1*, *Alad*, *Sod1*, *Gsr*, *Erbb2*, and six downregulated genes, *Wnt3a*, *Tgfbr1*, *Stat4*, *Tnf*, *Gsc*, *Meox2*, were compared to endogenous control *Gapdh* (assigned a relative value of "1"). (**B**) Quantitative RT-PCR compared THY1+/GFRA1+ cells enriched by MACS from 55-week-old wild type males to MACS-enriched cells from 3-week-old controls. When compared to endogenous control *Gapdh*, the fold-changes in expression of *Alad*, *Sod1*, and *Gsr* were all significantly diminished in the aged SSCs versus the young SSCs (1.46-, 1.72-, and 1.62-fold, respectively). Data represent mean values +/- SEM from three biological replicates. Student's t-test was performed to individually assess significance between each transcript and endogenous control *Gapdh*; *p<0.05, **p<0.01, ***p<0.001.

**Table 2. Canonical pathways identified by Ingenuity Pathway Analysis→**

| Category (up/down genes) | p value | ratio (# genes/category) |
|---|---|---|
| Free radical scavenging | 5.51E-04 | 26/57 (0.456) |
| PTEN signaling | 2.96E-04 | 48/114 (0.421) |
| Growth Hormone signaling | 5.50E-03 | 30/69 (0.435) |
| NF-κB signaling | 2.67E-03 | 64/165 (0.388) |
| NRF2-mediated oxidative stress response | 1.47E-02 | 64/180 (0.356) |

Many types of tissue-specific adult stem cells share common defects as they age within their respective niches. Aging mouse SSCs (>1 year) exhibit functional deficiencies, showing reduced proliferation *in vitro* and diminished colonization ability *in vivo* [7]. Kokkinaki et al. [8] compared 8-month-old SSCs to 1-week-old SSCs and identified a number of genes downregulated in the older cells, including membrane-spanning 4-domains, subfamily A, member 7 (*Ms4a7*), which mediates cell proliferation [8, 43]. *Drosophila* male germline stem cells (GSCs) experience a decline in self-renewal factors and a misorientation of their centrosomes as they age, contributing to fewer cell divisions [44, 45]. Aging mouse HSCs exhibit reduced numbers and diminished function, while aging melanocyte stem cells incur DNA damage and reflect inappropriate differentiation [46, 47]. Evidence of a decline in oxidative stress response gene activity during the aging process is contradictory. Aging rat bone marrow cells show no differences in ALAD levels in one study, while blood from aging rats contain progressively diminished ALAD in a different report [48, 49]. Mouse ovarian cells (2-, 6-, 9-, and 12-months) upregulate glutathione peroxidase 1 (*Gpx1*), but downregulate glutaredoxin 1 (*Glrx1*), glutathione S-transferase mu 2 (*Gstm2*), peroxiredoxin 3 (*Prdx3*), and thioredoxin 2 (*Txn2*) as they age [50]. *GPX1* expression and activity decrease, however, in aging human endothelial progenitor cells [51]. Overexpression of *SOD1* in aging *Drosophila* female GSCs and mouse NSCs increases their cellular proliferation and prolongs their maintenance, while endogenous SOD1 activity diminishes nearly 1.5-fold in aged rat gastrocnemius muscle (7-month-old versus 30-month-old) [52, 53, 54].

Here we have shown that aged mouse SSCs (>1-year-old) exhibit significantly reduced levels of *Sod1*, *Gsr*, and *Alad* transcripts when compared to young SSCs (<1-month-old). This reduction in gene expression likely affects the function of these stem cells within their niche, warranting further studies to examine the physiological consequences of this downregulation. While ROS accumulation is only one of many potential cellular mechanisms associated with aging that have yet to be fully investigated, the lower expression levels of oxidative stress response genes observed in aging SSCs could reinforce the diminished capacity for self-renewal in these stem cells. Such a process might, in turn, be reversed through rapamycin administration as a possible therapeutic endeavor.

## METHODS

Animals. Male FVB mice aged 12-days-old through 26-days-old were administered daily intraperitoneal (IP) injections of rapamycin (4mg/kg body weight) or control vehicle (5% Tween-80, 5% PEG-400), beginning at postnatal day (P)12. Mice were euthanized at P26 and their testes were isolated for germ cell enrichment. Untreated wild type male FVB mice aged 3-weeks-old and 55-weeks-old were also euthanized for experimental analysis. All procedures and care of animals were carried out according to the Children's Memorial Research Center Animal Care and Use Committee.

Isolation of mouse testicular germ cells. Testes were decapsulated and briefly minced in ice-cold 1:1 Dulbecco's Modified Eagle Medium–Ham's F-12 Medium. An initial enzymatic digestion using collagenase IV (1mg/ml) and DNase I (2mg/ml) at 37$^{o}$C for 30 min. was administered to remove interstitial Leydig cells and peritubular myoid cells from the seminiferous tubules. A second enzymatic digestion using collagenase IV (1 mg/ml), DNase I (2mg/ml), hyaluronidase (1.5mg/ml), and trypsin (1mg/ml) at 37$^{o}$C for 30 min. was administered to isolate germ cells and Sertoli cells from the remaining tissue. A final suspension of single cells was prepared in ice-cold PBS containing 0.5% BSA and 2mM EDTA (MACS Buffer) for subsequent spermatogonial stem cell enrichment.

Enrichment of SSCs using MACS separation. The single cell suspension containing germ cells in 80 µl MACS Buffer was first incubated with 20 µl rabbit anti-GFRA1 antibodies (Santa Cruz Biotechnology, CA) at 4$^{o}$C for 20 min. with rotation. After washes, a second incubation of cells in 80 µl MACS Buffer with 10 µl goat anti-rabbit antibody-conjugated MicroBeads and 10µl anti-THY1 antibody-conjugated MicroBeads (Miltenyi Biotech, Auburn, CA) was administered at 4$^{o}$C for 20 min. with rotation. The labeled cells were filtered through 30-µm pore size mesh to remove cell aggregates, and then sorted through a separation LS column attached to a MidiMACS separator (Miltenyi Biotec). THY1+ and GFRA1+ cells were retained inside the column within the magnetic field, while unlabeled cells passed through the column and were collected as the THY1-/GFRA1- cell fraction (flow through). After washes with MACS Buffer, the LS column was removed from the magnetic field and the THY1+ and GFRA1+ cells were flushed out.

Establishment of SSC cultures. Approximately 200,000 THY+/GFRA1+ cells were seeded into 35-mm round dishes containing irradiated Mouse Embryonic Fibroblast feeder layers (1.2 x $10^{6}$ MEFs per dish). Cells were maintained in optimized culture medium [55] (StemPro-34 supplemented with 1% FBS, 10 µg/mL GDNF, 10 ng/mL bFGF, 20 ng/mL EGF, 1,000 units/mL ESGRO/LIF), fed with new media every other

day. SSC colonies were visualized using a Leica DM-IRB Inverted Research Microscope.

RNA isolation and qRT-PCR analysis. Total RNA was extracted from MACS-separated cells using the RNeasy Micro Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. RNA samples were treated with RNase-free DNase I (Qiagen) on-column to remove genomic DNA. Yield and quality of RNA samples were determined using the NanoDrop 2000 Spectrophotometer (ThermoScientific, Wilmington, DE). Total RNA was reverse transcribed into cDNA using random hexamer primers (Life Technologies, Grand Island, NY). For qRT-PCR, cDNA was added to 2x Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) with specific oligonucleotide primer sets for the genes of interest (listed in Supplemental Table 2). Samples from three biological replicates were run in triplicate on an Applied Biosystems 7500 Real-Time PCR System using SYBR® Green dye for read-out and ROX™ dye as an internal reference. Each PCR reaction contained approximately 5–10 ng of cDNA, 1x Power SYBR® Green PCR Master Mix, and 500 nM of each forward and reverse primer for the desired gene. *Gapdh* was used as an endogenous control. The threshold cycle ($C_T$), indicating the relative abundance of a particular transcript, was calculated for each reaction by the system software. Quantification of the fold change in gene expression was determined by using the formula $2^{-\Delta\Delta CT}$, in which $\Delta\Delta C_T = [(C_T \text{ of gene of interest} - C_T \text{ of } Gapdh)_A - (C_T \text{ of gene of interest} - C_T \text{ of } Gapdh)_B]$. Fold change in transcript levels was plotted using Prism 5 software (GraphPad, La Jolla, CA). Values plotted are mean +/- SEM. Statistical analysis was performed using Prism 5, employing Student's t-test or ANOVA; *p<0.05; **p<0.01; ***p<0.001.

Gene expression microarrays. Total RNA samples were shipped on dry ice to the laboratories at Miltenyi Biotec (Auburn, CA), where they were quality-checked prior to processing with the Agilent 2100 Bioanalyzer platform (Agilent Technologies, Santa Clara, CA). Linear T7-based amplification was performed using 25 ng of each RNA sample. Amplification and Cy3-/Cy5-labeling was performed using the Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies) following the manufacturer's protocol. Yields of cRNA and the dye-incorporation rate were measured using the NanoDrop 2000 Spectrophotometer (ThermoScientific). Hybridization was performed according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression Hybridization Kit (Agilent Technologies). The Cy3- and Cy5-labeled cRNAs were combined and hybridized overnight (17 hours at 65°C) onto the Agilent Whole Mouse Genome oligo microarrays 4x44K using a hybridization chamber and oven. Fluorescence signals of the hybridized oligo microarrays were detected using Agilent's DNA microarray scanner (Agilent Technologies). Agilent Feature Extraction (AFE) software was used to process the microarray image files. AFE determines feature intensities and ratios (including background subtraction and normalization), rejects outliers and calculates statistical confidences (p-values). For determining differential gene expression, AFE-derived output data files were further analyzed using the Rosetta Resolver® gene expression data analysis system (Rosetta Biosoftware, Cambridge, MA). This software was used to generate a double-log scatter plot to visualize the signal intensities of all oligo probes (Figure 3A). AFE-derived output data files contained the gene lists with complete raw data sets.

SSC transcriptome data analysis. The unsupervised clustering method AutoSOME was used to assemble our gene expression data into distinct clusters for subsequent analysis [31]. We used Euclidean distance as a user-defined parameter, scaling our data sets in log2 (establishing a relative value range from 0.0 to 20.0) and employing unit variance normalization, median centering of genes (to eliminate amplitude shifts), and normalizing genes such that the sum of squares of each row/column from our data sets=1. All gene identifiers were collapsed into a non-redundant set by averaging expression values for genes represented by more than one probe. Probes without corresponding gene symbols were not analyzed. The AutoSOME output effectively generated clusters containing gene products assembled according to closely related and interconnected expression patterns. For this study, we focused on the cluster containing *Gfra1*.

To further analyze the SSC transcriptome, we used Ingenuity Pathways Analysis® (Ingenuity Systems, Redwood City, CA) to generate networks by uploading our data sets into the application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base (IPKB). A cutoff value of 2.00 was implemented to identify genes whose expression was significantly differentially regulated, overlaying them onto a global molecular network using information from the IPKB. Networks were algorithmically generated based on their connectivity, generating graphical representations of the molecular relationships among gene products (representing nodes) by depicting them as solid (direct) or dashed (indirect) lines. All lines are supported by at least one reference from the literature or from canonical information stored in the IPKB. Mouse, rat, and human orthologs of genes

are stored as separate objects in the IPKB, but are represented as single nodes in the network. Nodes are displayed using various shapes that represent the functional class of the gene product (i.e. parallelogram = enzyme). Canonical pathway analysis utilizes well characterized metabolic and cell signaling pathways that are generated prior to data input and on which identified gene products are overlaid.

The gene expression microarray data in this study are available in the public repository Gene Expression Omnibus; accession # GSE37062.

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## CONFLICT OF INTERESTS STATEMENT

The authors of this manuscript have no conflict of interest to declare.

## REFERENCES

**1.** Rossi DJ, Jamieson CH, Weissman IL. Stems cells and the pathways to aging and cancer. Cell. 2008;132:681-696.

**2.** Miyamoto K, Araki KY, Naka K, Arai F, Takubo K, Yamazaki S, Matsuoka S, Miyamoto T, Ito K, Ohmura M, Chen C, Hosokawa K, Nakauchi H, Nakayama K, Nakayama KI, Harada M, et al. Foxo3a is essential for maintenance of the hematopoietic stem cell pool. Cell Stem Cell. 2007;1:101-112.

**3.** Tothova Z, Kollipara R, Huntly BJ, Lee BH, Castrillon DH, Cullen DE, McDowell EP, Lazo-Kallanian S, Williams IR, Sears C, Armstrong SA, Passegue E, DePinho RA, Gilliland DG. Foxos are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell. 2007;128:325-339.

**4.** Castilho RM, Squarize CH, Chodosh LA, Williams BO, Gutkind JS. Mtor mediates wnt-induced epidermal stem cell exhaustion and aging. Cell Stem Cell. 2009;5:279-289.

**5.** Bailey KJ, Maslov AY, Pruitt SC. Accumulation of mutations and somatic selection in aging neural stem/progenitor cells. Aging Cell. 2004;3:391-397.

**6.** Molofsky AV, Slutsky SG, Joseph NM, He S, Pardal R, Krishnamurthy J, Sharpless NE, Morrison SJ. Increasing p16ink4a expression decreases forebrain progenitors and neurogenesis during ageing. Nature. 2006;443:448-452.

**7.** Schmidt JA, Abramowitz LK, Kubota H, Wu X, Niu Z, Avarbock MR, Tobias JW, Bartolomei MS, Brinster RL. In vivo and in vitro aging is detrimental to mouse spermatogonial stem cell function. Biol Reprod. 2011;84:698-706.

**8.** Kokkinaki M, Lee TL, He Z, Jiang J, Golestaneh N, Hofmann MC, Chan WY, Dym M. Age affects gene expression in mouse spermatogonial stem/progenitor cells. Reproduction. 2010;139:1011-1020.

**9.** Ryu BY, Orwig KE, Oatley JM, Avarbock MR, Brinster RL. Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. Stem Cells. 2006;24:1505-1511.

**10.** Zhang X, Ebata KT, Robaire B, Nagano MC. Aging of male germ line stem cells in mice. Biol Reprod. 2006;74:119-124.

**11.** Gan B, DePinho RA. Mtorc1 signaling governs hematopoietic stem cell quiescence. Cell Cycle. 2009;8:1003-1006.

**12.** Hobbs RM, Seandel M, Falciatori I, Rafii S, Pandolfi PP. Plzf regulates germline progenitor self-renewal by opposing mTORC1. Cell. 2010;142:468-479.

**13.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller RA. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009;460:392-395.

**14.** Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, Partridge L. Mechanisms of life span extension by rapamycin in the fruit fly drosophila melanogaster. Cell metabolism. 2010;11:35-46.

**15.** Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S. Regulation of lifespan in drosophila by modulation of genes in the tor signaling pathway. Current biology: CB. 2004;14:885-890.

**16.** Hansen M, Taubert S, Crawford D, Libina N, Lee SJ, Kenyon C. Lifespan extension by conditions that inhibit translation in caenorhabditis elegans. Aging Cell. 2007;6:95-110.

**17.** Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L, Muller F. Genetics: Influence of tor kinase on lifespan in c. Elegans. Nature. 2003;426:620.

**18.** Demidenko ZN, Zubova SG, Bukreeva EI, Pospelov VA, Pospelova TV, Blagosklonny MV. Rapamycin decelerates cellular senescence. Cell Cycle. 2009;8:1888-1895.

**19.** Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Rosenfeld SV, Blagosklonny MV. Rapamycin increases lifespan and inhibits spontaneous tumorigenesis in inbred female mice. Cell Cycle. 2011;10:4230-4236.

**20.** Ding H, Aljofan M, Triggle CR. Oxidative stress and increased enos and nadph oxidase expression in mouse microvessel endothelial cells. Journal of cellular physiology. 2007;212(3):682-689.

**21.** Brito VB, Folmer V, Soares JC, Silveira ID, Rocha JB. Long-term sucrose and glucose consumption decreases the delta-aminolevulinate dehydratase activity in mice. Nutrition. 2007;23:818-826.

**22.** Souza JB, Rocha JB, Nogueira CW, Borges VC, Kaizer RR, Morsch VM, Dressler VL, Martins AF, Flores EM, Schetinger MR. Delta-aminolevulinate dehydratase (delta-ala-d) activity in diabetes and hypothyroidism. Clinical biochemistry. 2007;40:321-325.

**23.** Biswas SK, Newby DE, Rahman I, Megson IL. Depressed glutathione synthesis precedes oxidative stress and atherogenesis in apo-e(-/-) mice. Biochemical and biophysical research communications. 2005;338:1368-1373.

**24.** Yilmaz OH, Valdez R, Theisen BK, Guo W, Ferguson DO, Wu H, Morrison SJ. Pten dependence distinguishes haematopoietic

stem cells from leukaemia-initiating cells. Nature. 2006;441:475-482.

**25.** Buageaw A, Sukhwani M, Ben-Yehudah A, Ehmcke J, Rawe VY, Pholpramool C, Orwig KE, Schlatt S. Gdnf family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes. Biol Reprod. 2005;73:1011-1016.

**26.** Gassei K, Ehmcke J, Schlatt S. Efficient enrichment of undifferentiated gfr alpha 1+ spermatogonia from immature rat testis by magnetic activated cell sorting. Cell Tissue Res. 2009;337:177-183.

**27.** Kubota H, Avarbock MR, Brinster RL. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proceedings of the National Academy of Sciences of the United States of America. 2003;100:6487-6492.

**28.** Oatley JM, Oatley MJ, Avarbock MR, Tobias JW, Brinster RL. Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. Development. 2009;136:1191-1199.

**29.** de Rooij DG, de Boer P. Specific arrests of spermatogenesis in genetically modified and mutant mice. Cytogenetic and genome research. 2003;103:267-276.

**30.** Feng LX, Ravindranath N, Dym M. Stem cell factor/c-kit up-regulates cyclin d3 and promotes cell cycle progression via the phosphoinositide 3-kinase/p70 s6 kinase pathway in spermatogonia. The Journal of biological chemistry. 2000;275:25572-25576.

**31.** Newman AM, Cooper JB. Autosome: A clustering method for identifying gene expression modules without prior knowledge of cluster number. BMC bioinformatics. 2010;11:117.

**32.** Gillis AJ, Stoop H, Biermann K, van Gurp RJ, Swartzman E, Cribbes S, Ferlinz A, Shannon M, Oosterhuis JW, Looijenga LH. Expression and interdependencies of pluripotency factors lin28, oct3/4, nanog and sox2 in human testicular germ cells and tumours of the testis. International journal of andrology. 2011;34:e160-174.

**33.** Sada A, Suzuki A, Suzuki H, Saga Y. The rna-binding protein nanos2 is required to maintain murine spermatogonial stem cells. Science. 2009;325:1394-1398.

**34.** Sada A, Hasegawa K, Pin PH, Saga Y. Nanos2 acts downstream of glial cell line-derived neurotrophic factor signaling to suppress differentiation of spermatogonial stem cells. Stem Cells. 2012;30:280-291.

**35.** Goertz MJ, Wu Z, Gallardo TD, Hamra FK, Castrillon DH. Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. The Journal of clinical investigation. 2011;121:3456-3466.

**36.** Neklesa TK, Davis RW. Superoxide anions regulate torc1 and its ability to bind fpr1:Rapamycin complex. Proceedings of the National Academy of Sciences of the United States of America. 2008;105:15166-15171.

**37.** Shin YJ, Cho DY, Chung TY, Han SB, Hyon JY, Wee WR. Rapamycin reduces reactive oxygen species in cultured human corneal endothelial cells. Current eye research. 2011;36:1116-1122.

**38.** Goncalves TL, Benvegnu DM, Bonfanti G, Frediani AV, Rocha JB. Delta-ala-d activity is a reliable marker for oxidative stress in bone marrow transplant patients. BMC cancer. 2009;9:138.

**39.** Kelada SN, Shelton E, Kaufmann RB, Khoury MJ. Delta-aminolevulinic acid dehydratase genotype and lead toxicity: A huge review. American journal of epidemiology. 2001;154:1-13.

**40.** Burow ME, Weldon CB, Melnik LI, Duong BN, Collins-Burow BM, Beckman BS, McLachlan JA. Pi3-k/akt regulation of nf-kappab signaling events in suppression of tnf-induced apoptosis. Biochemical and biophysical research communications. 2000;271:342-345.

**41.** Blagosklonny MV, Hall MN. Growth and aging: A common molecular mechanism. Aging. 2009;1:357-362.

**42.** Shibata T, Saito S, Kokubu A, Suzuki T, Yamamoto M, Hirohashi S. Global downstream pathway analysis reveals a dependence of oncogenic nf-e2-related factor 2 mutation on the mtor growth signaling pathway. Cancer research. 2010;70:9095-9105.

**43.** Brink TC, Sudheer S, Janke D, Jagodzinska J, Jung M, Adjaye J. The origins of human embryonic stem cells: A biological conundrum. Cells, tissues, organs. 2008;188:9-22.

**44.** Boyle M, Wong C, Rocha M, Jones DL. Decline in self-renewal factors contributes to aging of the stem cell niche in the drosophila testis. Cell Stem Cell. 2007;1:470-478.

**45.** Cheng J, Turkel N, Hemati N, Fuller MT, Hunt AJ, Yamashita YM. Centrosome misorientation reduces stem cell division during ageing. Nature. 2008;456:599-604.

**46.** Geiger H, Rudolph KL. Aging in the lympho-hematopoietic stem cell compartment. Trends in immunology. 2009;30:360-365.

**47.** Choi J, Artandi S. Stem cell aging and aberrant differentiation within the niche. Cell Stem Cell. 2009;5:6-8.

**48.** Ibraham NG, Lutton JD, Levere RD. Erythroid colony development as a function of age: The role of marrow cellular heme. Journal of gerontology. 1983;38:13-18.

**49.** Chiba M, Kikuchi M. Aging and activity of 5-aminolevulinate hydrolyase in the blood of humans and rats. Sangyo igaku Japanese journal of industrial health. 1976;18:391-394.

**50.** Lim J, Luderer U. Oxidative damage increases and antioxidant gene expression decreases with aging in the mouse ovary. Biol Reprod. 2011;84:775-782.

**51.** He T, Joyner MJ, Katusic ZS. Aging decreases expression and activity of glutathione peroxidase-1 in human endothelial progenitor cells. Microvascular research. 2009;78:447-452.

**52.** Pan L, Chen S, Weng C, Call G, Zhu D, Tang H, Zhang N, Xie T. Stem cell aging is controlled both intrinsically and extrinsically in the drosophila ovary. Cell Stem Cell. 2007;1:458-469.

**53.** Takemura S, Kayama T, Kuge A, Ali H, Kokubo Y, Sato S, Kamii H, Goto K, Yoshimoto T. Correlation between copper/zinc superoxide dismutase and the proliferation of neural stem cells in aging and following focal cerebral ischemia. Journal of neurosurgery. 2006;104:129-136.

**54.** Piec I, Listrat A, Alliot J, Chambon C, Taylor RG, Bechet D. Differential proteome analysis of aging in rat skeletal muscle. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2005;19:1143-1145.

**55.** Seandel M, James D, Shmelkov SV, Falciatori I, Kim J, Chavala S, Scherr DS, Zhang F, Torres R, Gale NW, Yancopoulos GD, Murphy A, Valenzuela DM, Hobbs RM, Pandolfi PP, Rafii S. Generation of functional multipotent adult stem cells from gpr125+ germline progenitors. Nature. 2007;449:346-350.

## SUPPLEMENTAL TABLES

For Supplemental Tables please see the full version of this manuscript on AGING web.

Research Perspective

# The implication of Sir2 in replicative aging and senescence in *Saccharomyces cerevisiae*

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**Abstract: The target of rapamycin (TOR) pathway regulates cell growth and aging in various organisms. In Saccharomyces cerevisiae, silent information regulator 2 (Sir2) modulates cellular senescence. Moreover, Sir2 plays a crucial role in promoting ribosomal DNA (rDNA) stability and longevity under TOR inhibition. Here we review the implication of rDNA stabilizers in longevity, discuss how Sir2 stabilizes rDNA under TOR inhibition and speculate on the link between sumoylation and Sir2-related pro-aging pathways.**

## INTRODUCTION

Aging and longevity have always been an important issue in biological sciences. Several reports have indicated that calorie restriction leads to lifespan extension from yeast to humans [1-3]. Longevity effect is also seen when nutrient-sensing signaling pathways such as the target of rapamycin (TOR) pathway and insulin/IGF pathway are inhibited [4-7], and the molecular mechanisms that regulate these nutrient-dependent pathways can substantially promote longevity in various organisms and mammalian cells [8-13]. The budding yeast *Saccharomyces cerevisiae* provides valuable information on understanding aging process and identifying the molecular mechanisms that regulate longevity in eukaryotic organisms. Two types of aging, replicative and chronological, have been described in *S. cerevisiae*. Replicative aging is an aging model of mitotically active cells in which the lifespan of a mother cell is measured by the number of daughter cells produced before death [14,15]. Chronological aging is an aging model of post-mitotic cells in which lifespan is defined by the survival time of cells in a non-dividing state [16]. In *S. cerevisiae*, a well-established mechanism for regulating longevity is the alteration of ribosomal DNA (rDNA) stability by the recombination between rDNA repeats and the accumulation of extra-chromosomal rDNA circles (ERCs) [17]. Recently, we confirmed that the inhibition of TOR pathway stabilizes rDNA locus by enhancing the association of silent information regulator 2 (Sir2) with rDNA, thereby leading to replicative lifespan extension in *S. cerevisiae* [18]. In this research perspective, we review the implication of rDNA stabilizers in longevity regulation, discuss how Sir2 stabilizes rDNA under TOR inhibition and speculate on the link between sumoylation and Sir2-related aging pathway.

### Sir2 and the epigenetic regulators of yeast rDNA locus

Sir2 proteins, or sirtuins, are a well-known, highly conserved, family of $NAD^+$-dependent deacetylases that are involved in the regulation of lifespan from yeast to humans [19-22]. Many reports have shown that sirtuins are linked to multiple physiological processes including metabolic regulation, DNA repair, stress response, apoptosis, cell survival and longevity [23-31]. Sirtuins also mediate the increased spontaneous physical activity in flies on calorie restriction and regulate p53 function via deacetylation in human cells [32,33]. In *S. cerevisiae*, Sir2 is a functional component of epigenetic complexes required for the establishment of silenced chromatin regions in rDNA locus, telomeres and silent

mating-type loci, *HML* and *HMR* [34-38]. *S. cerevisiae* rDNA consists of a 9.1-kb unit that contains RNA polymerase (Pol) I-transcribed 35S ribosomal RNA (rRNA) and Pol III-transcribed 5S rRNA gene, separated by a non-transcribed spacer, and is repeated 100-200 times on chromosome XII [39]. Because of highly repetitive nature, rDNA array is intrinsically unstable and is an easy target for homologous recombination. A primary cause of aging in *S. cerevisiae* is known to be the homologous recombination between rDNA repeats, which leads to the formation of ERCs that accumulate to toxic levels in mother cells [17]. Sir2 promotes replicative lifespan by repressing the homologous recombination between rDNA repeats and the subsequent formation of ERCs [40].

A well-known epigenetic regulator of yeast rDNA locus is the regulator of nucleolar silencing and telophase exit (RENT) complex that is composed of Sir2, Net1 and Cdc14 [41]. Net1, the core subunit of this complex, localizes to rDNA, recruits Sir2 to rDNA and is required for transcriptional silencing at rDNA [41-43]. Interestingly, several other epigenetic regulatory proteins also seem to be linked to rDNA stability and longevity. Tof2 interacts with Net1 and Sir2, binds to rDNA and induces rDNA silencing [43]. Lrs4 and Csm1, two of three subunits of monopolin complex that co-orients sister chromatids during meiosis I, interact with Tof2, associate with rDNA, establish rDNA silencing and suppress unequal recombination at rDNA [43-45]. Heh1 and Nur1, chromosome linkage inner nuclear membrane proteins, are physically linked to Lrs4 and Csm1, tether rDNA to nuclear periphery and promote rDNA stability [46]. Yeast linker histone Hho1, histone acetyltransferase Ada2 and Esa1 are required for rDNA silencing [47-49]. Histone methyltransferase Set1 is required for rDNA silencing in a Sir2-independent manner [50,51]. Chromatin remodeling proteins, such as Snf2, Fun30, Isw1 and Isw2, establish silencing at rDNA and maintain rDNA chromatin structure [52-56]. Collectively, these findings suggest that rDNA silencing factors, histone-modifying enzymes and chromatin remodeling factors promote rDNA stability and regulate ERC-mediated aging in yeast.

**Sir2 is part of a pathway mediating the longevity effect of TORC1 inhibition**

TOR kinase is a nutrient-responsive phosphatidyl-inositol kinase-related protein kinase structurally and functionally conserved from yeast to humans and plays critical roles in cell growth in response to nutrient availability by regulating transcription, translation, ribosome biogenesis and autophagy [13,57-59]. In yeast, TOR kinase exists in two functionally distinct multiprotein complexes, TOR complex1 (TORC1) and TOR complex2 (TORC2), each of which signals via a different set of effector pathways [60]. An immuno-suppressive and anticancer drug rapamycin specifically inhibits TORC1 and leads to a rapid decrease in ribosome biogenesis by regulating the transcription of all three kinds of RNA Pols [61,62]. In past decade, it has been reported that TORC1 signaling is deeply involved in eukaryotic cell aging and aging-related diseases. The inhibition of TORC1 signaling by rapamycin can delay aging and prolong lifespan in *S. cerevisiae, Caenorhabditis elegans* and *Drosophila melanogaster* [63-66]. Recent studies have shown that rapamycin can significantly increase lifespan also in genetically heterogeneous mice [67,68].

How the inhibition of TORC1 signaling extends lifespan is poorly understood. Meanwhile, whether Sir2 functions in rDNA stability and lifespan extension in yeast during TORC1 inhibition has been a matter of debate. Kaeberlein *et al.* reported that the inhibition of TORC1 signaling increases lifespan but has no effect on Sir2 activity [65], whereas Medvedik *et al.* indicated that TORC1 inhibition activates Sir2 by increasing the expression of *PNC1* encoding a nicotinamidase that depletes cellular nicotinamide, a physiological inhibitor of Sir2, thereby suppressing the formation of ERCs and leading to the extension of replicative lifespan [66]. Corroborating the latter study, we found that the inhibition of TORC1 signaling increases the association of Sir2 with rDNA in Pnc1- and Net1-dependent manners, enhances the transcriptional silencing of Pol II-transcribed genes at rDNA and induces the deacetylation of histones at rDNA, thereby promoting rDNA stability and replicative lifespan extension [18]. These findings suggest that Sir2 contributes to lifespan extension by enhancing rDNA silencing and rDNA stability under TORC1 inhibition.

Recent studies have reported that Sir2 inhibits replicative senescence by additional non-rDNA mechanisms. Sir2 is required for the asymmetric inheritance of oxidatively damaged proteins during cytokinesis, resulting in an enhanced capacity to respond to oxidative stress in daughter cells [69-72]. In addition, an age-associated decrease in Sir2 protein abundance is accompanied by the increase in histone acetylation and the loss of histones at specific subtelomeric regions in replicatively old yeast cells, indicating that Sir2 regulates replicative longevity through the maintenance of telomeric chromatin [73]. Whether TORC1 signaling modulates these non-rDNA functions of Sir2 is not known yet. It will be interesting to check whether the Sir2-dependent

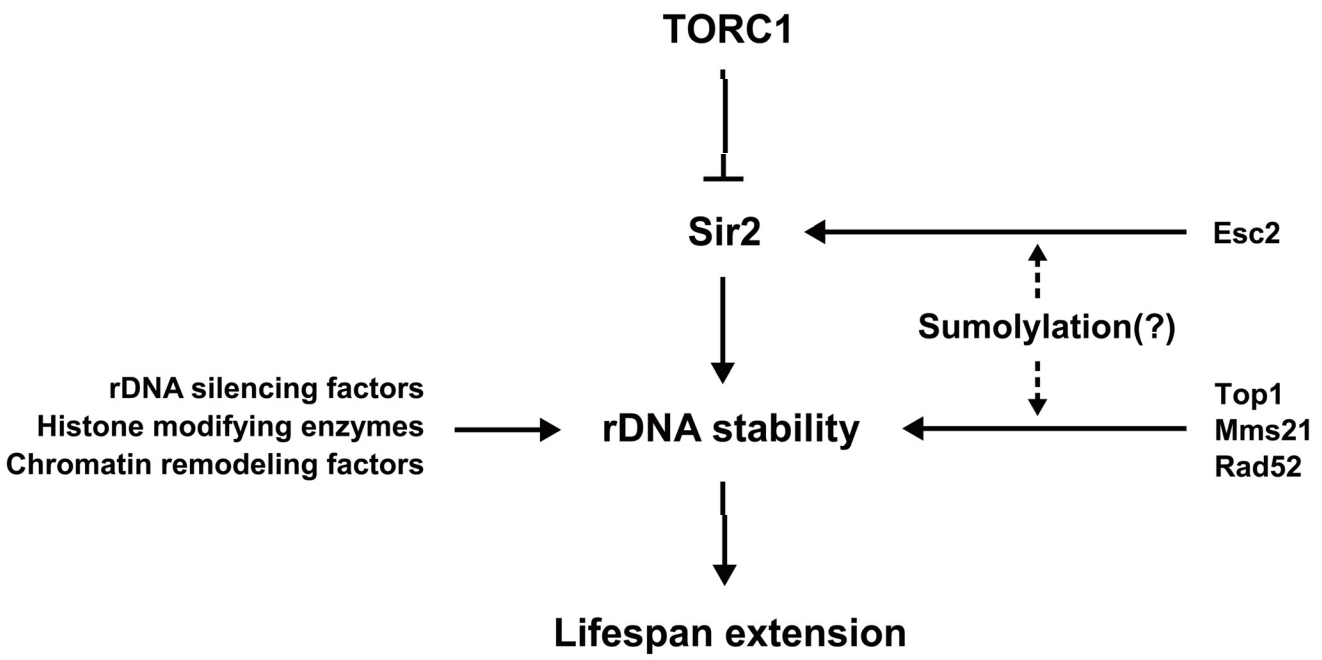


**Figure 1. A model for the link between sumoylation and Sir2-related aging pathway.** Various factors stabilize rDNA to affect lifespan extension in *S. cerevisiae*. TORC1 inhibition, Sir2 and several epigenetic regulators of rDNA promote rDNA stability and longevity. Sumoylation may regulate the activity of Sir2 and contribute to maintaining stable rDNA structure, leading to lifespan extension.

asymmetric inheritance of oxidatively damaged proteins and the Sir2-dependent maintenance of telomeric chromatin are influenced by TORC1 signaling.

**Sumoylation: a potential mechanism for the regulation of Sir2 function in rDNA maintenance**

Small ubiquitin-like modifier (SUMO) modification is an important posttranslational mechanism for regulating protein function, especially in the nucleus and nucleolus. Sumoylation is involved in various nuclear functions such as transcription, DNA repair and nuclear domain organization [74,75]. In yeast, SUMO is prominently enriched in the nucleolus and affects rDNA segregation and maintenance [76,77]. Topoisomerase Top1, which is sumoylated by Siz1 and Siz2, facilitates rDNA transcription and replication, is required for rDNA silencing and maintains rDNA integrity [77,78]. SUMO ligase Mms21, a subunit of the Smc5/Smc6 complex, binds to rDNA and maintains rDNA stability [79,80]. Moreover, cohesin and condensin subunits, which play important roles in rDNA stability and structures, are sumoylated by Mms21, and the association of these subunits with rDNA is regulated by Mms21 [77]. Additionally, sumoylation of Rad52 suppresses rDNA recombination and affects the efficiency of recombinational DNA repair [79,81]. These findings raise an interesting possibility that sumoylation may contribute to maintaining stable rDNA structure and thus promoting longevity.

Recent studies have shown that sumoylation is involved in the regulation of Sir2 function in the maintenance of chromatin structure at rDNA. Esc2, a member of a conserved family of proteins that contain SUMO-like domains, interacts with Sir2 through a SUMO-binding motif and is required for the maintenance of silent chromatin structure at rDNA [82]. A SUMO-binding motif of Esc2 is necessary and sufficient for interacting with both Sir2 and SUMO, and is required for the function of Esc2 in transcriptional silencing. These results raise a possibility that Sir2 is a sumoylated protein and Esc2 binds to sumoylated Sir2 via its SUMO-binding motif. A previous study has reported that the human sirtuin SIRT1 is sumoylated and this modification increases its deacetylase activity [83]. Although the sumoylation of yeast Sir2 has not been established yet, it is plausible that the activity of Sir2 may be regulated by its sumoylation. If Sir2 is not sumoylated, it is presumable that some sumoylated proteins may mediate its interaction with other proteins such as Esc2 and regulate its activity.

## CONCLUSION

Sirtuins are a highly conserved family of proteins that have been implicated in regulating diverse functions including longevity in a variety of species. Many studies suggest that Sir2 regulates rDNA recombination, DNA repair, chromosomal stability and longevity. In this research perspective, we briefly reviewed how Sir2 and other epigenetic regulators function as the stabilizer of rDNA and regulate longevity. Sir2 is part of a pathway mediating the longevity effect of TORC1 inhibition. Based on recent findings, we also propose that sumoylation may be involved in the regulation of Sir2 function in rDNA maintenance and thus longevity (Figure 1). Given that the human sirtuin SIRT1 is sumoylated and this modification increases its deacetylase activity [83], it will be interesting to investigate whether Sir2 and other sirtuins are sumoylated and, if so, whether their sumoylation is influenced by TORC1 signaling and how their functions are affected by sumoylation. These studies will provide deeper understanding of the mechanisms by which Sir2 and TORC1 signaling regulate longevity.

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## CONFLICT OF INTERESTS STATEMENT

The authors of this manuscript have no conflict of interests to declare.

## REFERENCES

**1.** Kennedy BK, Steffen KK, Kaeberlein M. Ruminations on dietary restriction and aging. Cell Mol Life Sci 2007;64:1323-1328.

**2.** Fontana L, Partridge L, Longo VD. Extending healthy life span--from yeast to humans. Science 2010;328:321-326.

**3.** Bishop NA, Guarente L. Genetic links between diet and lifespan: shared mechanisms from yeast to humans. Nat Rev Genet 2007; 8:835-844.

**4.** Kenyon C. The plasticity of aging: insights from long-lived mutants. Cell 2005; 120:449-460.

**5.** Clancy DJ, Gems D, Hafen E, Leevers SJ, Partridge L. Dietary restriction in long-lived dwarf flies. Science 2002; 296:319.

**6.** Flurkey K, Papaconstantinou J, Miller RA, Harrison DE. Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production. Proc Natl Acad Sci U S A 2001; 98:6736-6741.

**7.** Longo VD. Linking sirtuins, IGF-I signaling, and starvation. Exp Gerontol 2009; 44:70-74.

**8.** Pan Y, Shadel GS. Extension of chronological life span by reduced TOR signaling requires down-regulation of Sch9p and involves increased mitochondrial OXPHOS complex density. Aging (Albany NY) 2009; 1:131-145.

**9.** Heeren G, Rinnerthaler M, Laun P, von Seyerl P, Kossler S, Klinger H, Hager M, Bogengruber E, Jarolim S, Simon-Nobbe B and others. The mitochondrial ribosomal protein of the large subunit, Afo1p, determines cellular longevity through mitochondrial back-signaling via TOR1. Aging (Albany NY) 2009; 1:622-636.

**10.** Korotchkina LG, Leontieva OV, Bukreeva EI, Demidenko ZN, Gudkov AV, Blagosklonny MV. The choice between p53-induced senescence and quiescence is determined in part by the mTOR pathway. Aging (Albany NY) 2010; 2:344-352.

**11.** Lee JH, Bodmer R, Bier E, Karin M. Sestrins at the crossroad between stress and aging. Aging (Albany NY) 2010; 2:369-374.

**12.** Goldberg AA, Richard VR, Kyryakov P, Bourque SD, Beach A, Burstein MT, Glebov A, Koupaki O, Boukh-Viner T, Gregg C and others. Chemical genetic screen identifies lithocholic acid as an anti-aging compound that extends yeast chronological life span in a TOR-independent manner, by modulating housekeeping longevity assurance processes. Aging (Albany NY) 2010; 2:393-414.

**13.** Hands SL, Proud CG, Wyttenbach A. mTOR's role in ageing: protein synthesis or autophagy? Aging (Albany NY) 2009; 1:586-597.

**14.** Kaeberlein M. Lessons on longevity from budding yeast. Nature 2010; 464:513-519.

**15.** Steinkraus KA, Kaeberlein M, Kennedy BK. Replicative aging in yeast: the means to the end. Annu Rev Cell Dev Biol 2008; 24:29-54.

**16.** Kennedy BK, Smith ED, Kaeberlein M. The enigmatic role of Sir2 in aging. Cell 2005; 123:548-550.

**17.** Sinclair DA, Guarente L. Extrachromosomal rDNA circles--a cause of aging in yeast. Cell 1997; 91:1033-1042.

**18.** Ha CW, Huh WK. Rapamycin increases rDNA stability by enhancing association of Sir2 with rDNA in Saccharomyces cerevisiae. Nucleic Acids Res 2011; 39:1336-1350.

**19.** Finkel T, Deng CX, Mostoslavsky R. Recent progress in the biology and physiology of sirtuins. Nature 2009; 460:587-591.

**20.** Imai S, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 2000; 403:795-800.

**21.** Landry J, Sutton A, Tafrov ST, Heller RC, Stebbins J, Pillus L, Sternglanz R. The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. Proc Natl Acad Sci U S A 2000; 97:5807-5811.

**22.** Smith JS, Brachmann CB, Celic I, Kenna MA, Muhammad S, Starai VJ, Avalos JL, Escalante-Semerena JC, Grubmeyer C, Wolberger C and others. A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. Proc Natl Acad Sci U S A 2000; 97:6658-6663.

**23.** Blander G, Guarente L. The Sir2 family of protein deacetylases. Annu Rev Biochem 2004; 73:417-435.

**24.** Michan S, Sinclair D. Sirtuins in mammals: insights into their biological function. Biochem J 2007; 404:1-13.

**25.** Guarente L. Sirtuins as potential targets for metabolic syndrome. Nature 2006; 444:868-874.

**26.** Imai S, Guarente L. Ten years of NAD-dependent SIR2 family deacetylases: implications for metabolic diseases. Trends Pharmacol Sci 2010; 31:212-220.

**27.** Haigis MC, Sinclair DA. Mammalian sirtuins: biological insights and disease relevance. Annu Rev Pathol 2010; 5:253-295.

**28.** Bauer J, Antosh M, Chang C, Schorl C, Kolli S, Neretti N, Helfand SL. Comparative transcriptional profiling identifies takeout as a gene that regulates life span. Aging (Albany NY) 2010; 2:298-310.

**29.** Bauer JH, Morris SN, Chang C, Flatt T, Wood JG, Helfand SL. dSir2 and Dmp53 interact to mediate aspects of CR-dependent lifespan extension in D. melanogaster. Aging (Albany NY) 2009; 1:38-48.

**30.** Donehower LA. Longevity regulation in flies: a role for p53. Aging (Albany NY) 2009; 1:6-8.

**31.** Frankel S, Ziafazeli T, Rogina B. dSir2 and longevity in Drosophila. Exp Gerontol 2010.

**32.** Parashar V, Rogina B. dSir2 mediates the increased spontaneous physical activity in flies on calorie restriction. Aging (Albany NY) 2009; 1:529-541.

**33.** Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L, Weinberg RA. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell 2001; 107:149-159.

**34.** Aparicio OM, Billington BL, Gottschling DE. Modifiers of position effect are shared between telomeric and silent mating-type loci in S. cerevisiae. Cell 1991; 66:1279-1287.

**35.** Rusche LN, Kirchmaier AL, Rine J. The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annu Rev Biochem 2003; 72:481-516.

**36.** Guarente L. Diverse and dynamic functions of the Sir silencing complex. Nat Genet 1999; 23:281-285.

**37.** Bryk M, Banerjee M, Murphy M, Knudsen KE, Garfinkel DJ, Curcio MJ. Transcriptional silencing of Ty1 elements in the RDN1 locus of yeast. Genes Dev 1997; 11:255-269.

**38.** Smith JS, Boeke JD. An unusual form of transcriptional silencing in yeast ribosomal DNA. Genes Dev 1997; 11:241-254.

**39.** Johnston M, Hillier L, Riles L, Albermann K, Andre B, Ansorge W, Benes V, Bruckner M, Delius H, Dubois E and others. The nucleotide sequence of Saccharomyces cerevisiae chromosome XII. Nature 1997; 387:87-90.

**40.** Kaeberlein M, McVey M, Guarente L. The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. Genes Dev 1999; 13:2570-2580.

**41.** Huang J, Moazed D. Association of the RENT complex with nontranscribed and coding regions of rDNA and a regional requirement for the replication fork block protein Fob1 in rDNA silencing. Genes Dev 2003; 17:2162-2176.

**42.** Straight AF, Shou W, Dowd GJ, Turck CW, Deshaies RJ, Johnson AD, Moazed D. Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. Cell 1999; 97:245-256.

**43.** Huang J, Brito IL, Villen J, Gygi SP, Amon A, Moazed D. Inhibition of homologous recombination by a cohesin-associated clamp complex recruited to the rDNA recombination enhancer. Genes Dev 2006; 20:2887-2901.

**44.** Toth A, Rabitsch KP, Galova M, Schleiffer A, Buonomo SB, Nasmyth K. Functional genomics identifies monopolin: a kinetochore protein required for segregation of homologs during meiosis i. Cell 2000; 103:1155-1168.

**45.** Rabitsch KP, Petronczki M, Javerzat JP, Genier S, Chwalla B, Schleiffer A, Tanaka TU, Nasmyth K. Kinetochore recruitment of two nucleolar proteins is required for homolog segregation in meiosis I. Dev Cell 2003; 4:535-548.

**46.** Mekhail K, Seebacher J, Gygi SP, Moazed D. Role for perinuclear chromosome tethering in maintenance of genome stability. Nature 2008; 456:667-670.

**47.** Levy A, Eyal M, Hershkovits G, Salmon-Divon M, Klutstein M, Katcoff DJ. Yeast linker histone Hho1p is required for efficient RNA polymerase I processivity and transcriptional silencing at the ribosomal DNA. Proc Natl Acad Sci U S A 2008; 105:11703-11708.

**48.** Jacobson S, Pillus L. The SAGA subunit Ada2 functions in transcriptional silencing. Mol Cell Biol 2009; 29:6033-6045.

**49.** Clarke AS, Samal E, Pillus L. Distinct roles for the essential MYST family HAT Esa1p in transcriptional silencing. Mol Biol Cell 2006; 17:1744-1757.

**50.** Bryk M, Briggs SD, Strahl BD, Curcio MJ, Allis CD, Winston F. Evidence that Set1, a factor required for methylation of histone H3, regulates rDNA silencing in S. cerevisiae by a Sir2-independent mechanism. Curr Biol 2002; 12:165-170.

**51.** Briggs SD, Bryk M, Strahl BD, Cheung WL, Davie JK, Dent SY, Winston F, Allis CD. Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in Saccharomyces cerevisiae. Genes Dev 2001; 15:3286-3295.

**52.** Dror V, Winston F. The Swi/Snf chromatin remodeling complex is required for ribosomal DNA and telomeric silencing in Saccharomyces cerevisiae. Mol Cell Biol 2004; 24:8227-8235.

**53.** Mueller JE, Bryk M. Isw1 acts independently of the Isw1a and Isw1b complexes in regulating transcriptional silencing at the ribosomal DNA locus in Saccharomyces cerevisiae. J Mol Biol 2007; 371:1-10.

**54.** Mueller JE, Li C, Bryk M. Isw2 regulates gene silencing at the ribosomal DNA locus in Saccharomyces cerevisiae. Biochem Biophys Res Commun 2007; 361:1017-1021.

**55.** Saha A, Wittmeyer J, Cairns BR. Chromatin remodelling: the industrial revolution of DNA around histones. Nat Rev Mol Cell Biol 2006; 7:437-447.

**56.** Neves-Costa A, Will WR, Vetter AT, Miller JR, Varga-Weisz P. The SNF2-family member Fun30 promotes gene silencing in heterochromatic loci. PLoS One 2009; 4:e8111.

**57.** Schneper L, Duvel K, Broach JR. Sense and sensibility: nutritional response and signal integration in yeast. Curr Opin Microbiol 2004; 7:624-630.

**58.** Dennis PB, Fumagalli S, Thomas G. Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. Curr Opin Genet Dev 1999; 9:49-54.

**59.** Schmelzle T, Hall MN. TOR, a central controller of cell growth. Cell 2000; 103:253-262.

**60.** Loewith R, Jacinto E, Wullschleger S, Lorberg A, Crespo JL, Bonenfant D, Oppliger W, Jenoe P, Hall MN. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol Cell 2002; 10:457-468.

**61.** Mayer C, Grummt I. Ribosome biogenesis and cell growth: mTOR coordinates transcription by all three classes of nuclear RNA polymerases. Oncogene 2006; 25:6384-6391.

**62.** Powers T, Walter P. Regulation of ribosome biogenesis by the rapamycin-sensitive TOR-signaling pathway in Saccharomyces cerevisiae. Mol Biol Cell 1999; 10:987-1000.

**63.** Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L,

Muller F. Genetics: influence of TOR kinase on lifespan in C. elegans. Nature 2003; 426:620.

**64.** Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S. Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. Curr Biol 2004; 14:885-890.

**65.** Kaeberlein M, Powers RW, 3rd, Steffen KK, Westman EA, Hu D, Dang N, Kerr EO, Kirkland KT, Fields S, Kennedy BK. Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. Science 2005; 310:1193-1196.

**66.** Medvedik O, Lamming DW, Kim KD, Sinclair DA. MSN2 and MSN4 link calorie restriction and TOR to sirtuin-mediated lifespan extension in Saccharomyces cerevisiae. PLoS Biol 2007; 5:e261.

**67.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS and others. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature 2009; 460:392-395.

**68.** Miller RA, Harrison DE, Astle CM, Baur JA, Boyd AR, de Cabo R, Fernandez E, Flurkey K, Javors MA, Nelson JF and others. Rapamycin, But Not Resveratrol or Simvastatin, Extends Life Span of Genetically Heterogeneous Mice. J Gerontol A Biol Sci Med Sci 2010.

**69.** Aguilaniu H, Gustafsson L, Rigoulet M, Nystrom T. Asymmetric inheritance of oxidatively damaged proteins during cytokinesis. Science 2003; 299:1751-1753.

**70.** Erjavec N, Nystrom T. Sir2p-dependent protein segregation gives rise to a superior reactive oxygen species management in the progeny of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 2007; 104:10877-10881.

**71.** Erjavec N, Cvijovic M, Klipp E, Nystrom T. Selective benefits of damage partitioning in unicellular systems and its effects on aging. Proc Natl Acad Sci U S A 2008; 105:18764-18769.

**72.** Hekimi S, Guarente L. Genetics and the specificity of the aging process. Science 2003; 299:1351-1354.

**73.** Dang W, Steffen KK, Perry R, Dorsey JA, Johnson FB, Shilatifard A, Kaeberlein M, Kennedy BK, Berger SL. Histone H4 lysine 16 acetylation regulates cellular lifespan. Nature 2009; 459:802-807.

**74.** Johnson ES. Protein modification by SUMO. Annu Rev Biochem 2004; 73:355-382.

**75.** Geiss-Friedlander R, Melchior F. Concepts in sumoylation: a decade on. Nat Rev Mol Cell Biol 2007; 8:947-956.

**76.** Takahashi Y, Strunnikov A. In vivo modeling of polysumoylation uncovers targeting of Topoisomerase II to the nucleolus via optimal level of SUMO modification. Chromosoma 2008; 117:189-198.

**77.** Takahashi Y, Dulev S, Liu X, Hiller NJ, Zhao X, Strunnikov A. Cooperation of sumoylated chromosomal proteins in rDNA maintenance. PLoS Genet 2008; 4:e1000215.

**78.** Smith JS, Caputo E, Boeke JD. A genetic screen for ribosomal DNA silencing defects identifies multiple DNA replication and chromatin-modulating factors. Mol Cell Biol 1999; 19:3184-3197.

**79.** Torres-Rosell J, Machin F, Farmer S, Jarmuz A, Eydmann T, Dalgaard JZ, Aragon L. SMC5 and SMC6 genes are required for the segregation of repetitive chromosome regions. Nat Cell Biol 2005; 7:412-419.

**80.** Zhao X, Blobel G. A SUMO ligase is part of a nuclear multiprotein complex that affects DNA repair and chromosomal organization. Proc Natl Acad Sci U S A 2005; 102:4777-4782.

**81.** Altmannova V, Eckert-Boulet N, Arneric M, Kolesar P, Chaloupkova R, Damborsky J, Sung P, Zhao X, Lisby M, Krejci L. Rad52 SUMOylation affects the efficiency of the DNA repair. Nucleic Acids Res 2010; 38:4708-4721.

**82.** Yu Q, Kuzmiak H, Olsen L, Kulkarni A, Fink E, Zou Y, Bi X. Saccharomyces cerevisiae Esc2p interacts with Sir2p through a small ubiquitin-like modifier (SUMO)-binding motif and regulates transcriptionally silent chromatin in a locus-dependent manner. J Biol Chem 2010; 285:7525-7536.

**83.** Yang Y, Fu W, Chen J, Olashaw N, Zhang X, Nicosia SV, Bhalla K, Bai W. SIRT1 sumoylation regulates its deacetylase activity and cellular response to genotoxic stress. Nat Cell Biol 2007; 9:1253-1262.

# Potential of mTOR inhibitors for the treatment of subependymal giant cell astrocytomas in tuberous sclerosis complex

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**Abstract: Rapamycin inhibits the mTOR (target of rapamycin) pathway and extends lifespan in multiple species. The tuberous sclerosis complex (TSC) protein is a negative regulator of mTOR. In humans, loss of the TSC protein results in a disorder characterized clinically by the growth of benign tumors in multiple organs, due to overactivation of mTOR inhibition. Subependymal giant cell astrocytomas (SEGAs) are benign brain tumors associated with TSC that have traditionally been treated by surgery, but for which mTOR inhibitors have recently been suggested as potential alternative treatments. The duration of mTOR treatment for SEGAs might have to be prolonged, probably lifelong, because SEGAs usually grow back after treatment is stopped. This cohort of patients who will experience prolonged exposure to mTOR inhibitors should be carefully followed longitudinally to better document long term side effects, but also to compare their longevity with the one of similar patients with TSC. These patients represent a unique opportunity to study the potential anti-aging properties of mTOR inhibitors in humans.**

Rapamycin (also called sirolimus) is an immunosuppressive drug that has recently been shown to extend lifespan in multiple species including mammals [1]. This anti-aging property is presumably related to the mTOR (mammalian target of rapamycin) inhibition properties of rapamycin. The mTOR pathway is crucial for the coordination of growth in response to energy status, stress, and nutrient availability [2,3].

The potential anti-aging properties of rapamycin and of other mTOR inhibitors, such as RAD001 (everolimus), and CCI-779 (temsirolimus) are of great interest. Unfortunately, the side effects related to these drugs preclude the undertaking of research trials about their impacts on aging in healthy individuals. Considering this obstacle, experts in the field of aging have suggested that the potential anti-aging drugs should be introduced to the clinical trials for therapy of particular diseases and then be approved for prevention of all age-related diseases in healthy individuals [4]. In this context, tuberous sclerosis complex (TSC) seems to be an ideal disease model where the potential of mTOR inhibitors can be assessed because these drugs are increasingly being tested and used clinically to treat certain aspects of this condition [5].

TSC is an autosomal dominant disorder caused by the inactivation in one of two tumor suppressor genes, hamartin (TSC1) or tuberin (TSC2). In the normal state, the hamartin–tuberin complex activates the protein Rheb, which inhibits mTOR. If a TSC mutation is present, mTOR is constitutively activated, leading to abnormal cellular proliferation, ribosome biogenesis, and mRNA translation (see [2] for complete review of the mTOR molecular pathway). In consequence, TSC is characterized clinically by the growth of benign tumors in multiple organs, including the brain, the heart, the kidneys, the lungs, and the skin [6]. Its incidence is estimated at 1 in 6000 live births [7]. The severity of the disease is highly variable, ranging from mild skin manifestations to intractable epilepsy, mental retardation, and autism [8].

The only report studying specifically the causes of death in TSC was performed at the Mayo clinic [9]. Overall, the survival curves showed a decreased survival for

patients with TSC compared with the general population. Of the 355 patients with TSC followed, 40 died of causes related to TSC, with renal disease being the most common cause of death (11/40). Ten patients died as a consequence of brain tumors and four patients died of lymphangioleiomyomatosis (LAM). Thirteen patients with severe mental impairment passed away due to status epilepticus or bronchopneumonia. One baby died of cardiac failure and one child died of rupture of an aneurysm of the thoracic aorta.

The main current clinical complication related to TSC for which treatment with mTOR inhibitors is indicated are subependymal giant cell astrocytomas (SEGA). This complication affects approximately 15% of patients with TSC and it occurs in the pediatric age group [10]. SEGAs tend to lose their propensity to grow in the early twenties. They are slow-growing benign tumors of mixed glioneuronal lineage that arise from the growth of pre-existing subependymal nodules, which are asymptomatic lesions that protrude from the walls of the ventricles [10]. SEGAs most commonly grow near the foramen of Monro. This can lead to obstruction of the normal cerebrospinal fluid circulation and subsequent intracranial hypertension that can potential be fatal if left untreated. The distinction between a SEGA and a subependymal nodule is still debated. Generally, a clinical diagnosis of SEGA is made when there are symptoms of intracranial hypertension, papilledema, or radiological evidence of hydrocephalus or interval growth.

The traditional management approach is to monitor SEGAs with periodic neuroimaging and to resect those that exhibit growth and/or cause clinical signs of intracranial hypertension. This approach is being challenged by recent observations that suggest that mTOR inhibitors, such as rapamycin (sirolimus) and RAD001 (everolimus), can induce partial regression of SEGAs [11,12,13]. The first report showing clear regression of SEGAs in five patients with the use of rapamycin was published in 2006 [11]. Recently, a phase II trial [13] using everolimus to treat SEGAs in 28 patients with TSC showed SEGA reduction of at least 30% in 21 patients (75%) and at least 50% in 9 patients (32%). Everolimus was well tolerated as only single cases of grade 3 treatment-related sinusitis, pneumonia, viral bronchitis, tooth infection, stomatitis, and leukopenia were reported.

These observations suggest that mTOR inhibitors could serve as an acceptable alternative treatment to SEGA surgery. Renal angiomyolipomas and lymphangioleimyomatosis (LAM) are other TSC manifestations for which mTOR inhibitors have proven potential efficacy [14]. In addition, animal models of TSC have suggested that mTOR inhibitors could have beneficial effects on cognitive deficits [15] and on epileptogenesis [16]. Whether similar benefits would be observed in humans with TSC is still unknown. Research trials are ongoing and should soon provide answers to these questions.

Other important questions remain regarding the use of mTOR inhibitors for the treatment of SEGA in TSC. Side effects, especially long term side effects, and optimal duration of treatment are still under investigation. The short-term side effects related to rapamycin are generally considered acceptable. The most common side effects are oral ulcers, acneiform rash, thrombocytopenia, hyperlipidemia, impaired wound healing, and immunosuppression [14]. Long term side effects are less known. For example, reports from the literature related to the use of rapamycin for kidney transplant prevention suggested that rapamycin might be associated with impaired spermatogenesis and, as a corollary, may reduce male fertility [17]. This observation might not be applicable to other patients populations, but requires further investigation.

The duration of treatment might be prolonged, probably lifelong. There is clear evidence that SEGAs grow back after the mTOR inhibitor is stopped [11]. Most experts currently recommend continuation of mTOR inhibitors at the lowest efficacious dose. This cohort of patients who will experience prolonged exposure to mTOR inhibitors should be carefully followed longitudinally to better document long term side effects, but also to compare their longevity with the one of similar patients with TSC. These patients represent a unique opportunity to study the potential anti-aging properties of mTOR inhibitors in humans.

In conclusion, a new treatment era has begun in the field of TSC since the discovery of the potential beneficial effects of mTOR inhibitors. Although the use of mTOR inhibitors is becoming increasingly accepted, especially for the treatment of SEGAs in TSC, questions now remain about the duration of treatment and long term side effects. Whether mTOR inhibitors will have a significant impact on longevity in TSC is unknown, but warrants attention as mTOR inhibitors are increasingly recognized as anti-aging drugs in animal models. Long-term prospective studies in patients with TSC might provide evidence about the potential anti-aging properties of mTOR inhibitors in humans.

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## CONFLICT OF INTERESTS STATEMENT

The author of this manuscript has no conflict of interests to declare.

## REFERENCES


1. Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller RA. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009; 460(7253): 392-395.

2. Orlova KA, Crino PB. The tuberous sclerosis complex. Ann N Y Acad Sci. 2010; 1184: 87-105.

3. Reiling JH, Sabatini DM. Stress and mTORture signalling. Oncogene. 2006; 25: 6373-6383.

4. Blagosklonny MV. Validation of anti-aging drugs by treating age-related diseases. Aging. 2009; 1(3): 281-288.

5. Franz DN, Bissler JJ, McCormack FX. Tuberous sclerosis complex: neurological, renal and pulmonary manifestations. Neuropediatrics. 2010; 41: 199-208.

6. Crino PB, Nathanson KL, Henske EP. The Tuberous Sclerosis Complex. N Engl J Med. 2006; 355: 1345-1356.

7. Osborne JP, Fryer A, Webb D. Epidemiology of tuberous sclerosis. Ann NY Acad Sci. 1991; 615: 125–127.

8. Lyczkowski DA, Conant KD, Pulsifer MB, Jarrett DY, Grant PE, Kwiatkowski DJ, Thiele EA: Intrafamilial phenotypic variability in tuberous sclerosis complex. J Child Neurol. 2007; 22(12):1348-1355.

9. Shepherd CW, Gomez MR, Lie JT, Crowson BA. Causes of death in patients with tuberous sclerosis. Mayo Clin Proc. 1991; 66: 792-796.

10. Goh S, Butler W, Thiele EA. Subependymal giant cell tumors in tuberous sclerosis complex. Neurology. 2004; 63: 1457-1461.

11. Franz DN, Leonard J, Tudor C, Chuck G, Care M, Sethuraman G, Dinopoulos A, Thomas G, Crone KR. Rapamycin causes regression of astrocytomas in tuberous sclerosis complex. Ann Neurol. 2006; 59: 490-498.

12. Birca A, Mercier C, Major P. Rapamycin as an alternative to surgical treatment of subependymal giant cell astrocytomas in a patient with tuberous sclerosis complex. J Neurosurg Pediatrics. 2010; 6: 381-384.

13. Krueger DA, Care MM, Holland KM, Agricola K, Tudor C, Mangeshkar P, Wilson KA, Byars A, Sahmoud T, Franz DN. Everolimus for Subependymal Giant-Cell Astrocytomas in Tuberous Sclerosis. N Engl J Med. 2010; 363:1801-1811.

14. Bissler JJ, McCormack FX, Young LR, Elwing JM, Chuck G, Leonard JM, Schmithorst VJ, Laor T, Brody AS, Bean J, Salisbury S, Franz DN. Sirolimus for angiomyolipoma in tuberous sclerosis complex or lymphangioleiomyomatosis. N Engl J Med. 2008; 358: 140-151.

15. Ehninger D, Han S, Shilyansky C, Zhou Y, Li W, Kwiatkowski DJ, Ramesh V, Silva AJ. Reversal of learning deficits in a Tsc2+/- mouse model of tuberous sclerosis. Nat Med. 2008; 14: 843-848.

16. Wong M. Mammalian target of rapamycin (mTOR) inhibition as a potential antiepileptogenic therapy: From tuberous sclerosis to common acquired epilepsies. Epilepsia. 2010; 51: 27-36.

17. Boobes Y, Bernieh B, Saadi H, Raafat Al Hakim M, Abouchacra S. Gonadal dysfunction and infertility in kidney transplant patients receiving sirolimus. Int Urol Nephrol. 2010; 42(2): 493-498.

Hypothesis

# Xenohormetic, hormetic and cytostatic selective forces driving longevity at the ecosystemic level

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**Abstract: We recently found that lithocholic acid (LCA), a bile acid, extends yeast longevity. Unlike mammals, yeast do not synthesize bile acids. We therefore propose that bile acids released into the environment by mammals may act as interspecies chemical signals providing longevity benefits to yeast and, perhaps, other species within an ecosystem.**

## Bile acids delay aging in yeast via two different mechanisms

We recently found that LCA greatly (and some other bile acids to a lesser degree) increases the chronological life span of yeast under caloric restriction (CR) conditions [1]. Our findings provided evidence that LCA extends longevity of chronologically aging yeast through two different mechanisms (Figure 1).

In one mechanism, this bile acid targets longevity pathways that control chronological aging irrespective of the number of calories available to yeast. Because these pathways modulate longevity regardless of calorie availability, we called them "constitutive" or "housekeeping" [1]. LCA modulates these house-keeping longevity assurance pathways by suppressing lipid-induced necrosis, attenuating mitochondrial fragmentation, altering oxidation-reduction processes in mitochondria, enhancing resistance to oxidative and thermal stresses, suppressing mitochondria-controlled apoptosis, and enhancing stability of nuclear and mitochondrial DNA ([1]; Figure 1C). The housekeeping longevity pathways do not overlap with the TOR (target of rapamycin) and cAMP/PKA (cAMP/protein kinase A) signaling pathways ([1]; Figure 1A), both of which are "adaptable" by nature because they are under the stringent control of calorie and/or nutrient availability ([2-6]; Figure 1B).

In the other mechanism, LCA targets the adaptable cAMP/PKA pathway by unmasking an anti-aging potential of PKA under non-CR conditions, perhaps by activating PKA-dependent phosphorylation of the cytosolic pool of the key nutrient-sensory protein kinase Rim15p [1]. The phosphorylation of Rim15p by PKA inactivates its protein kinase activity [7]. Hence, the LCA-driven inactivation of Rim15p may reduce the phosphorylation status of its known [8] target proteins in the cytosol, thereby lowering their pro-aging efficacy ([1]; Figure 1A).

## Bile acids are beneficial to health and longevity in animals

Although bile acids in mammals have been traditionally considered only as trophic factors for the enteric epithelium and detergents for the emulsification and absorption of dietary lipids [9-11], they are now also recognized for their essential role as signaling molecules regulating lipid, glucose and energy homeostasis and activating detoxification of xenobiotics ([9-14]; Figure 2A). Many of the numerous health-improving metabolic effects caused by bile acids and

their demonstrated ability to protect mammals from xenobiotic toxins ([9-14]; Figure 2A) suggest that, by improving overall health, these amphipathic molecules may delay the onset of age-related diseases and have beneficial effect on longevity. Furthermore, because of the elevated levels of several bile acids in the long-lived Ghrhr$^{lit/lit}$ mice and due to the ability of cholic acid administered to food of wild-type mice to activate transcription of numerous xenobiotic detoxification genes, it has been proposed that, by promoting chemical hormesis in mammals, these mildly toxic molecules with detergent-like properties may extend their longevity by acting as endobiotic regulators of aging [15-18]. Moreover, bile acid-like dafachronic acids (including 3-keto-LCA) in worms function as endocrine regulators of aging by activating an anti-aging transcriptional program governed by the DAF-12/DAF-16 signaling cascade ([19-21]; Figure 2B). Altogether, these findings support the notion that bile acids are beneficial to health and longevity in animals because of their ability to operate as potent signaling molecules that modulate a compendium health- and longevity-related processes. Noteworthy, by modulating many of these processes also in yeast, LCA extends their longevity [1]. It is likely therefore that the life-extending capacity of LCA and other bile acids as well as, perhaps, the mechanisms underlying their anti-aging action are conserved across animal species and other phyla.

## Bile acids may function as interspecies chemical signals extending yeast longevity within ecosystems

Importantly, yeast do not synthesize LCA or any other bile acid found in mammals [1,11,22]. We therefore hypothesize that bile acids released into the environment by mammals may act as interspecies chemical signals providing longevity benefits to yeast. In our hypothesis, these mildly toxic compounds released into the environment by mammals may create selective pressure for the evolution of yeast species that can respond to the resulting mild cellular damage by developing the most efficient stress protective mechanisms. Such mechanisms may provide effective protection of yeast not only against cellular damage caused by bile acids (and, perhaps, by other environmental xenobiotics) but also against molecular and cellular damage accumulated with age. In our hypothesis, yeast species that have been selected for the most effective mechanisms providing protection against bile acids (and other environmental xenobiotics) are expected to evolve the most effective anti-aging mechanisms that are sensitive to regulation by bile acids (and, perhaps, by other environmental xenobiotics). Thus, the ability of yeast to sense bile acids produced by mammals and then to respond by undergoing certain life-extending changes to their physiology (Figure 1) is expected to increase their chances of survival, thereby creating selective force aimed at maintaining such ability.

## Natural variations of bile acid levels within ecosystems may modulate both housekeeping and adaptable longevity pathways in yeast

Noteworthy, the bulk quantity of bile acids in mammals exists as an organismal pool which cycles between intestine and liver in the enterohepatic circulation due to the efficient reabsorption of bile acids in the terminal ileum [10,11]. However, about 5% (up to 600 mg/day) of this pool escapes each reabsorption cycle, being continuously released into the large intestine and ultimately into the environment [10,11]. Thus, yeast are permanently exposed to bile acids due to their fecal loss by mammals. It is conceivable therefore that, in yeast exposed to bile acids released by mammals, these interspecies chemical signals modulate housekeeping longevity assurance pathways that 1) regulate yeast longevity irrespective of the state of the environment or food supply (*i.e.*, the number of available calories and nutrients); and 2) do not overlap (or only partially overlap) with the adaptable TOR and cAMP/PKA longevity pathways that are under the stringent control of calorie and nutrient availability.

It should be stressed, however, that the quantity of bile acids released into the environment by mammals could vary due to changes in the density of mammalian population and, perhaps, due to other environmental factors (including the abundance of food available to mammals, its nutrient and caloric content, and its fat mass and quality). In fact, the organismal pool of bile acids in mammals is under the stringent control of regulatory mechanisms operating in the liver during the fasting-refeeding transition [9-11]. Hence, it is likely that, in addition to the ability of yeast to respond to the permanently available exogenous pool of bile acids by modulating some housekeeping longevity assurance pathways, they have also evolved the ability to sense the environmental status-dependent variations of bile acids abundance by modulating the adaptable TOR and cAMP/PKA longevity pathways. Importantly, our recent study provided evidence for two mechanisms underlying the life-extending effect of LCA in yeast; one mechanism involves the calorie supply-independent modulation of a compendium of housekeeping longevity assurance processes that are not regulated by the TOR and cAMP/PKA pathways, whereas the other mechanism operates only in yeast on a calorie-rich diet by unmasking the previously unknown anti-aging potential of the calorie supply-dependent PKA [1].

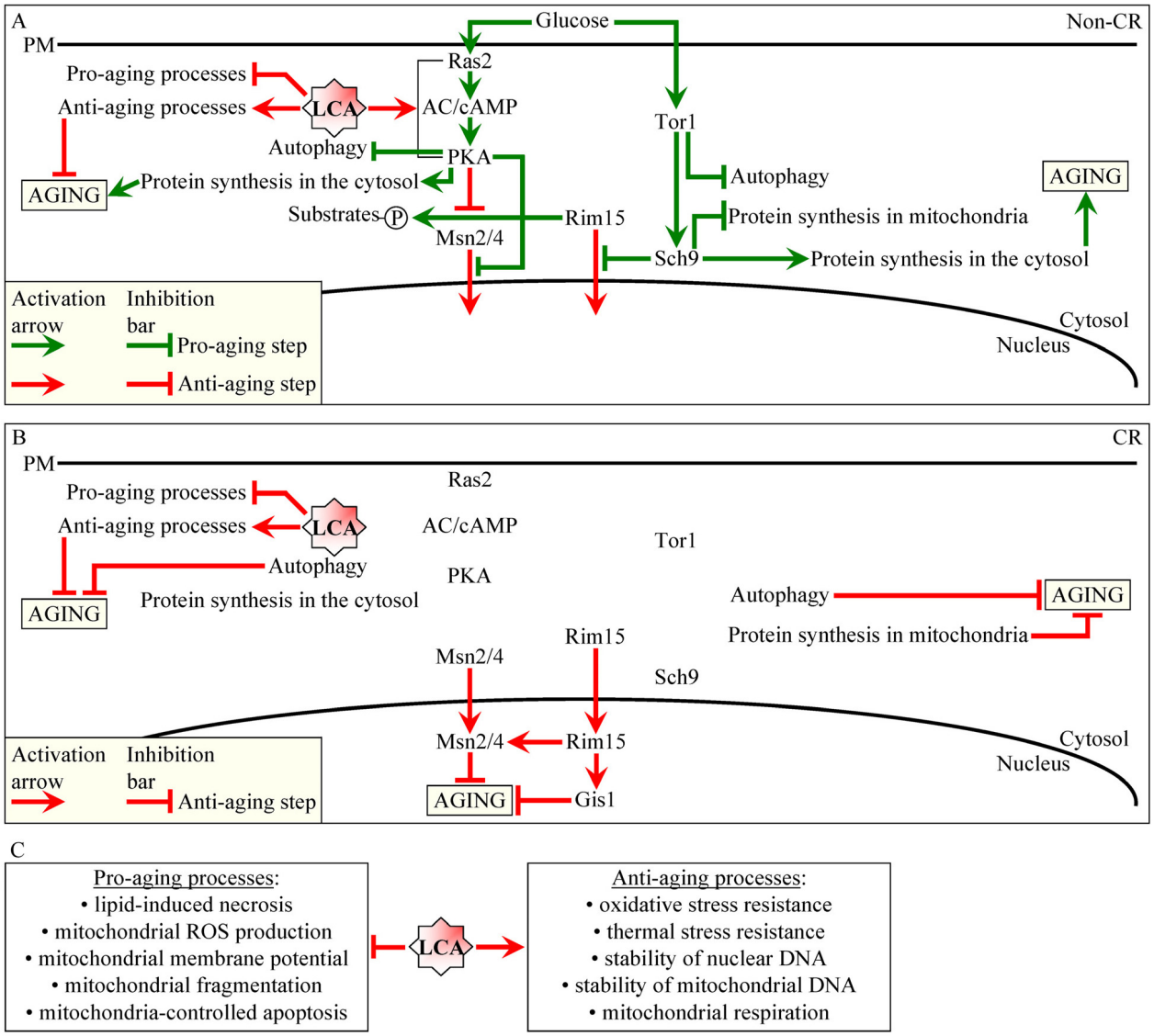


**Figure 1. Lithocholic acid (LCA) extends longevity of chronologically aging yeast through two different mechanisms.** (**A** and **B**) Outline of pro- and anti-aging processes that are controlled by the TOR and/or cAMP/PKA signaling pathways and are modulated by LCA in yeast cells grown under non-CR (**A**) or CR (**B**) conditions. Activation arrows and inhibition bars denote pro-aging (displayed in green color) or anti-aging (displayed in red color) processes. Under both non-CR and CR conditions, LCA targets housekeeping longevity assurance processes listed in (**C**). Under non-CR conditions only, LCA also targets the adaptable cAMP/PKA pathway. By activating PKA-dependent phosphorylation of the cytosolic pool of the key nutrient-sensory protein kinase Rim15p, LCA causes the inactivation of Rim15p. The resulting reduction of the phosphorylation status of several Rim15p target proteins in the cytosol lowers their pro-aging efficacy. Abbreviations: CR, caloric restriction; PM, plasma membrane.

It remains to be seen if our hypothesis on the essential role of bile acids as interspecies chemical signals regulating longevity in yeast is applicable to other species routinely exposed to bile acids within an ecosystem, such as plants and bacteria.

## Rapamycin may also act as an interspecies chemical signal modulating longevity at the ecosystemic level

Our hypothesis on longevity regulation by bile acids within ecosystems may explain the evolutionary origin of the life-extending effect of another anti-aging compound, called rapamycin. Synthesized by soil bacteria to inhibit growth of fungal competitors, this macrocyclic lactone provides longevity benefit to yeast, fruit flies and mice by specifically inhibiting TOR (Tor1p in yeast), a nutrient-sensory protein kinase that operates as a master negative regulator of the key adaptable longevity pathway [3,4,23-25]. Because rapamycin delays proliferative growth of organisms across phyla by causing G1 cell cycle arrest [3,4,26], it could be considered as a mildly cytotoxic compound, akin to bile acids (Our recent unpublished data revealed that rapamycin is a more toxic hormetic molecule than LCA and other bile acids). We propose therefore that, following its release into the environment by soil bacteria, rapamycin may create selective pressure for the evolution of yeast, fly and mammalian species that can respond to rapamycin-induced growth retardation by developing certain mechanisms aimed at such remodeling of their anabolic and catabolic processes that would increase their chances of survival under conditions of slow growth. It is plausible that some of these mechanisms delay aging by optimizing essential longevity-related processes and remain sensitive to modulation by rapamycin. Hence, the ability of yeast, fruit flies and mice to sense rapamycin produced by soil bacteria and then to respond by undergoing certain life-extending changes to their physiology is expected to increase their chances of survival, thereby creating selective force for maintaining such ability.

Interestingly, rapamycin has been shown to increase life span in fruit flies under dietary restriction conditions [25], when the TOR-governed adaptable pro-aging pathways are fully suppressed and the TOR-governed adaptable anti-aging pathways are fully activated [3,4]. It is plausible therefore that - similar to the proposed above anti-aging mechanism of LCA in yeast - rapamycin in fruit flies can modulate both the housekeeping (TOR-independent) and adaptable (TOR-dependent) longevity pathways. Hence, it is tempting to speculate that, in addition to the ability of fruit flies to respond to the permanently available exogenous pool of rapamycin by modulating some housekeeping longevity assurance pathways, they have also evolved the ability to sense the environmental status-dependent variations of rapamycin abundance (due to, *e.g.*, changes in the density of soil bacteria population) by modulating the TOR-governed adaptable longevity pathways. Of note, recent findings in yeast imply that - in addition to its role as a master negative regulator of the key adaptable longevity pathway - Tor1p may also operate as a positive longevity regulator, in particular by stimulating nuclear import of the transcriptional factors Sfp1p, Rtg1 and Rtg3 in response to partial mitochondrial dysfunction or changes in the exogenous and endogenous levels of glutamate and glutamine [27-29]. The ability of these transcriptional factors to regulate metabolism, ribosome biogenesis and growth is crucial for longevity [28,30,31].

## The "xenohormesis" hypothesis: a case of xenohormetic phytochemicals

Our hypothesis on longevity regulation by bile acids and rapamycin within ecosystems complements the "xenohormesis" hypothesis, in which plants and other autotrophic organisms respond to various environmental stresses (*i.e.*, UV light, dehydration, infection, predation, cellular damage and nutrient deprivation) by synthesizing a compendium of secondary metabolites [32-34]. Within plants and other autotrophs producing these phytochemicals in response to environmental stresses, they activate defense systems protecting the host organisms against such stresses. In addition, these phytochemicals constitute a chemical signature of the environmental status of an ecosystem. As such, they provide to heterotrophic organisms (*i.e.*, animals and fungi) within the ecosystem an advance warning about deteriorating environmental conditions [33]. By operating as interspecies chemical signals, they could create selective pressure for the evolution of heterotrophic organisms that can sense these signals and then to respond by altering their metabolism in defensive preparation for the imminent adversity while conditions are still favorable. The resulting metabolic remodeling causes such specific changes in physiology of heterotrophs that are beneficial to their health and longevity [33]. Although xenohormetic phyto-chemicals are produced by autotrophic organisms only in response to hormetic environmental stresses, it is unlikely that they function as mildly toxic hormetic molecules within heterotrophic organisms; rather, the xenohormesis hypothesis proposes that the beneficial to health and longevity effects of xenohormetic phytochemicals are due to their well known ability to modulate the key enzymes of stress-response pathways governing numerous longevity-related processes in heterotrophic organisms [33-42]. The xenohormetic

mode of positive selection for the most efficient longevity regulation mechanisms has been proposed to be driven by such phytochemicals as resveratrol, butein, fisetin and other polyphenols, as well as by curcumin [32-34]. The ability of caffeine to increase yeast chronological life span by decreasing the catalytic activity of Tor1p [43] suggests that this xanthine alkaloid could also operate as a xenohormetic phytochemical signal providing an advance warning about deteriorating environmental conditions to yeast, thereby driving the evolution of their longevity regulation mechanisms.

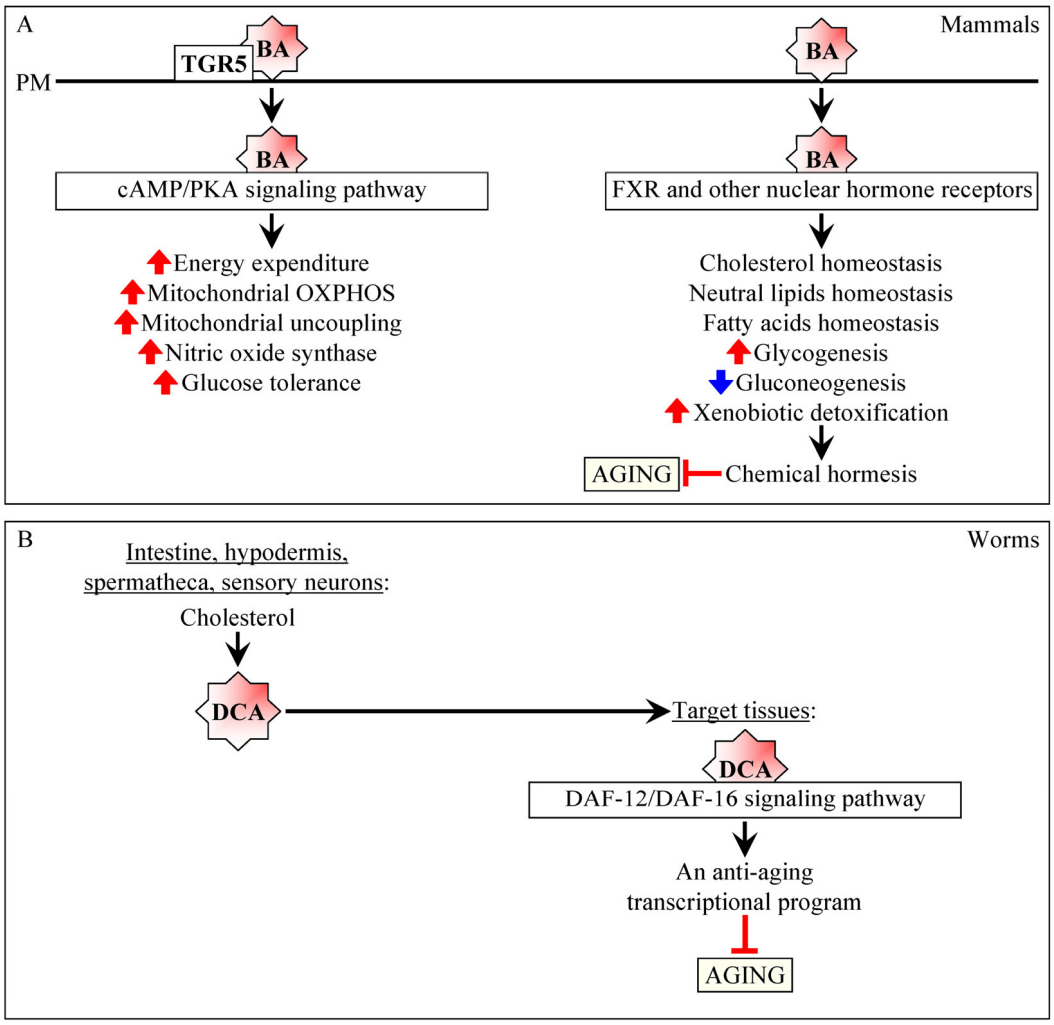


**Figure 2. Bile acids are beneficial to health and longevity in animals.** (**A**) In mammals, bile acids (BA) function not only as trophic factors for the enteric epithelium and detergents for the emulsification and absorption of dietary lipids, but also as signaling molecules that regulate lipid, glucose and energy homeostasis and activate detoxification of xenobiotics. By improving overall health, BA may delay the onset of age-related diseases and have beneficial effect on longevity. By activating transcription of numerous xenobiotic detoxification genes and thus promoting chemical hormesis, BA may extend their longevity by acting as endobiotic regulators of aging. (**B**) In worms, following their synthesis from cholesterol in the intestine, hypodermis, spermatheca and sensory neurons, bile acid-like dafachronic acids (DCA) are delivered to other tissues where they activate the DAF-12/DAF-16 signaling cascade, thereby orchestrating an anti-aging transcriptional program and increasing the life span of the entire organism.

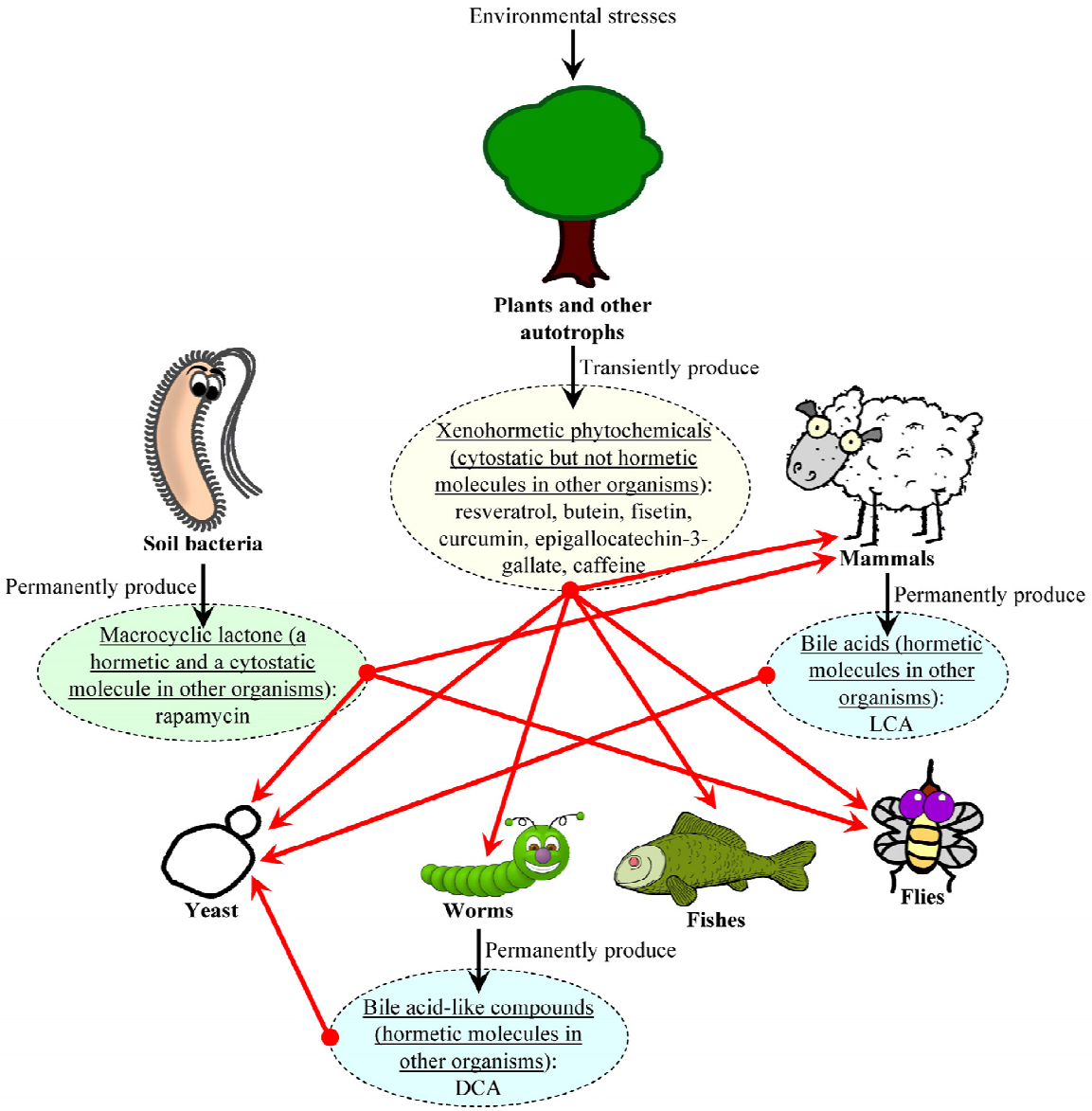


**Figure 3. The xenohormetic, hormetic and cytostatic selective forces may drive the evolution of longevity regulation mechanisms within an ecosystem.** We propose that organisms from all domains of life within an ecosystem synthesize chemical compounds that 1) are produced and then released into the environment permanently or only in response to deteriorating environmental conditions, increased population density of competitors and/or predators, or changes in food availability and its nutrient and/or caloric content; 2) are mildly toxic compounds that trigger a hormetic response in an organism that senses them or, alternatively, are not toxic for any organism within the ecosystem and do not cause a hormetic response; 3) are cytostatic compounds that attenuate the TOR-governed signaling network or, alternatively, do not modulate this growth-promoting network; and 4) extend longevity of organisms that can sense these compounds (red arrows), thereby increasing their chances of survival and creating selective force aimed at maintaining the ability of organisms composing the ecosystem to respond to these compounds by undergoing specific life-extending changes to their physiology. In our hypothesis, the evolution of longevity regulation mechanisms in each group of the organisms composing an ecosystem is driven by the ability of this group of organisms to undergo specific life-extending changes to their physiology in response to a compendium of "critical" chemical compounds that are permanently or transiently released to the ecosystem by other groups of organisms. Abbreviations: LCA, lithocholic acid; DCA, bile acid-like dafachronic acids.

## The "anti-aging side effect" hypothesis: delaying aging by attenuating the growth-promoting TOR signaling pathway

A common feature of many anti-aging compounds - some of which are mildly toxic hormetic molecules, whereas the others are non-toxic xenohormetic phytochemicals - is that they exhibit a cytostatic effect by inhibiting TOR, a nutrient-sensing signaling pathway that promotes proliferative growth in all heterotrophic organisms. A recently proposed "anti-aging side effect" hypothesis envisions that the primary objective for the synthesis of these cytostatic compounds by a group of the organisms composing an ecosystem is to suppress growth of other group(s) of organisms within this ecosystem, thereby killing competitors and/or protecting themselves from predators [39]. Due to its central role in promoting proliferative growth of all heterotrophic organisms, the TOR signaling pathway is a preferable target of such cytostatic compounds [3,26,39,44,45]. Because the TOR pathway provides a molecular link between growth and aging by driving a so-called quasi-programmed aging [3,44,45], these compounds exhibit a side effect of suppressing aging [39]. In fact, soil bacteria synthesize rapamycin to suppress growth of fungal competitors by inhibiting the TOR protein kinase, a master positive regulator of the TOR signaling pathway that drives developmental growth of young organisms [3,23-25]. However, since - according to the anti-aging side effect hypothesis - in heterotrophic organisms across phyla this pathway also drives aging after their developmental growth is completed [44,45], rapamycin has a side effect of suppressing aging of all groups of heterotrophic organisms within an ecosystem [39]. Moreover, the anti-aging side effect hypothesis predicts that plants synthesize resveratrol in part to protect their grapes by inhibiting fungal growth [39]. Yet, because this small polyphenol attenuates the TOR signaling pathway by modulating key upstream regulators and downstream targets of the TOR protein kinase [35-42], resveratrol also displays a side effect of slowing down quasi-programmed TOR-driven aging of various species of heterotrophic organisms within an ecosystem [39].

In the anti-aging side effect hypothesis, cytostatic compounds attenuating the TOR pathway operate as interspecies chemical signals that provide longevity benefits to a range of heterotrophic organisms composing an ecosystem [39]. We propose that, following their release into the environment by soil bacteria or plants, these growth suppressing chemical compounds may create selective pressure for the evolution of yeast, worm, fly and mammalian species that can respond to the resulting retardation of their growth by developing certain mechanisms aimed at specific remodeling of the TOR-governed signaling network. By targeting the TOR protein kinase itself and/or its numerous upstream regulators and downstream targets, such mechanisms may attenuate the hyper-activation of TOR-governed cellular signaling pathways and cellular functions that - according to the concept of quasi-programmed TOR-driven aging [44,45] - are initiated after developmental growth of a heterotrophic organism is completed. In our hypothesis, the species of heterotrophic organisms that have been selected for the most efficient mechanisms preventing the hyper-activation of TOR-governed cellular signaling pathways and cellular functions following the completion of developmental growth are expected to evolve the most effective anti-aging mechanisms. Such mechanisms may be sensitive to various environmental factors, including the density of organism population and abundance of nutrients within an ecosystem.

## The xenohormetic, hormetic and cytostatic selective forces may drive the evolution of longevity regulation mechanisms within ecosystems

Unlike xenohormetic phytochemicals that are non-toxic compounds transiently synthesized and released by autotrophs only in response to environmental stresses [33,34], bile acids are mildly toxic hormetic molecules that are permanently synthesized and released by mammals [9-11,14-18]. Furthermore, rapamycin is a more toxic hormetic molecule than bile acids (our unpublished data) that is permanently synthesized and released by soil bacteria [46]. Moreover, many xenohormetic phytochemicals and mildly toxic hormetic molecules exhibit a cytostatic effect by attenuating TOR-governed cellular signaling pathways and cellular functions [39]. Therefore, by fusing the xenohormesis hypothesis [32-34], the anti-aging side effect hypothesis [39] and the proposed here hypothesis on longevity regulation by bile acids and rapamycin within ecosystems, we put forward a unified hypothesis of the xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms at the ecosystemic level.

In our unified hypothesis (Figure 3), organisms from all domains of life (*i.e.*, bacteria, fungi, plants and animals) within an ecosystem are able to synthesize chemical compounds that 1) are produced and then released into the environment permanently or only in response to deteriorating environmental conditions, increased population density of competitors and/or predators, or changes in food availability and its nutrient and/or caloric content; 2) are mildly toxic compounds that trigger a hormetic response in an organism that senses

them or, alternatively, are not toxic for any organism within the ecosystem and do not cause a hormetic response; 3) are cytostatic compounds that attenuate the TOR-governed signaling network (*e.g.*, rapamycin and resveratrol) or, alternatively, do not modulate this growth-promoting network (*e.g.*, LCA and other bile acid) and 4) extend longevity of organisms that can sense these compounds, thereby increasing their chances of survival and creating selective force aimed at maintaining the ability of organisms composing the ecosystem to respond to these compounds by undergoing specific life-extending changes to their physiology. Our hypothesis implies that the evolution of longevity regulation mechanisms in each group of the organisms composing an ecosystem is driven by the ability of this group of organisms to undergo specific life-extending physiological changes in response to a compendium of "critical" chemical compounds that are permanently or transiently released to the ecosystem by other groups of organisms.

### Verification of our hypothesis

As the first step towards testing the validity of our hypothesis of the xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms within ecosystems, we are currently carrying out the LCA-driven experimental evolution of longevity regulation mechanisms in chronologically aging yeast cultured under laboratory conditions. If we could select long-lived yeast species following a long-term exposure of wild-type yeast to LCA, we would be able to begin addressing the following intriguing questions: 1) what genes are affected by mutations responsible for the extended longevity of selected long-lived yeast species? 2) how these mutations influence a compendium of the housekeeping longevity-related processes modulated by LCA in chronologically aging yeast ([1]; Figure 1); 3) will these mutations affect the growth rate of yeast in media with or without LCA? 4) will selected long-lived yeast species be able to maintain their ability to live longer than wild-type yeast if they undergo several successive passages in medium without LCA? - and, thus, is there selective pressure aimed at maintaining of an "optimal" rather than a "maximal" chronological life span of yeast (due to, *e.g.*, a proposed selective advantage of the envisioned "altruistic" program [47-52] of chronological aging in yeast)? and 5) if mixed with an equal number of wild-type yeast cells, will selected long-lived yeast species out-grow and/or out-live them in medium without LCA or the opposite will happen (due to selective pressure on yeast aimed at maintaining of the so-called "altruistic" program [47-52] of their chronological aging)?


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## CONFLICT OF INTERESTS STATEMENT

The authors of this manuscript have no conflict of interests to declare.

## REFERENCES


1. Goldberg AA et al. Chemical genetic screen identifies lithocholic acid as an anti-aging compound that extends yeast chronological life span in a TOR-independent manner, by modulating housekeeping longevity assurance processes. Aging 2010; 2: 393-414.
2. Greer EL, Brunet A. Signaling networks in aging. J Cell Sci. 2008; 121: 407-412.
3. Blagosklonny MV, Hall MN. Growth and aging: a common molecular mechanism. Aging 2009; 1: 357-362.
4. Hands SL, Proud CG, Wyttenbach A. mTOR's role in ageing: protein synthesis or autophagy? Aging 2009; 1: 586-597.
5. Narasimhan SD, Yen K, Tissenbaum HA. Converging pathways in lifespan regulation. Curr Biol. 2009; 19: R657-R666.
6. Fontana L, Partridge L, Longo VD. Extending healthy life span - from yeast to humans. Science 2010; 328: 321-326.
7. Smets B, Ghillebert R, De Snijder P, Binda M, Swinnen E, De Virgilio C, Winderickx J. Life in the midst of scarcity: adaptations to nutrient availability in *Saccharomyces cerevisiae*. Curr Genet. 2010; 56: 1-32.
8. Ptacek J, Devgan G, Michaud G, Zhu H, Zhu X, Fasolo J, Guo H, Jona G, Breitkreutz A, Sopko R, McCartney RR, Schmidt MC, Rachidi N et al. Global analysis of protein phosphorylation in yeast. Nature 2005; 438: 679-684.
9. Thomas C, Pellicciari R, Pruzanski M, Auwerx J, Schoonjans K. Targeting bile-acid signalling for metabolic diseases. Nat Rev Drug Discov. 2008; 7: 678-693.
10. Hylemon PB, Zhou H, Pandak WM, Ren S, Gil G, Dent P. Bile acids as regulatory molecules. J Lipid Res. 2009; 50: 1509-1520.
11. Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile acid receptors in metabolic regulation. Physiol Rev. 2009; 89: 147-191.
12. Ramalho RM, Viana RJ, Low WC, Steer CJ, Rodrigues CM. Bile acids and apoptosis modulation: an emerging role in experimental Alzheimer's disease. Trends Mol. Med. 2008; 14: 54-62.

**13.** Amaral JD, Viana RJ, Ramalho RM, Steer CJ, Rodrigues CM. Bile acids: regulation of apoptosis by ursodeoxycholic acid. J Lipid Res. 2009; 50: 1721-1734.

**14.** Vallim TQ, Edwards PA. Bile acids have the gall to function as hormones. Cell Metab. 2009; 10: 162-164.

**15.** Amador-Noguez D, Yagi K, Venable S, Darlington G. Gene expression profile of long-lived Ames dwarf mice and Little mice. Aging Cell 2004; 3: 423-441.

**16.** Amador-Noguez D, Dean A, Huang W, Setchell K, Moore D, Darlington G. Alterations in xenobiotic metabolism in the long-lived Little mice. Aging Cell 2007; 6: 453-470.

**17.** Gems D. Long-lived dwarf mice: are bile acids a longevity signal? Aging Cell 2007; 6: 421-423.

**18.** Gems D, Partridge L. Stress-response hormesis and aging: "that which does not kill us makes us stronger". Cell Metab. 2008; 7: 200-203.

**19.** Motola DL, Cummins CL, Rottiers V, Sharma KK, Li T, Li Y, Suino-Powell K, Xu HE, Auchus RJ, Antebi A, Mangelsdorf DJ. Identification of ligands for DAF-12 that govern dauer formation and reproduction in *C. elegans*. Cell 2006; 124: 1209-1223.

**20.** Gerisch B, Rottiers V, Li D, Motola DL, Cummins CL, Lehrach H, Mangelsdorf DJ, Antebi A. A bile acid-like steroid modulates *Caenorhabditis elegans* lifespan through nuclear receptor signaling. Proc Natl Acad Sci USA 2007; 104: 5014-5019.

**21.** Russell SJ, Kahn CR. Endocrine regulation of ageing. Nat Rev Mol Cell Biol. 2007; 8: 681-691.

**22.** Monte MJ, Marin JJ, Antelo A, Vazquez-Tato J. Bile acids: chemistry, physiology, and pathophysiology. World J Gastroenterol. 2009; 15: 804-816.

**23.** Powers RW 3rd, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S. Extension of chronological life span in yeast by decreased TOR pathway signaling. Genes Dev. 2006; 20: 174-184.

**24.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller RA. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature 2009; 460: 392-395.

**25.** Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, Partridge L. Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila melanogaster*. Cell Metab. 2010; 11: 35-46.

**26.** Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell 2006; 124: 471-484.

**27.** Dann SG, Thomas G. The amino acid sensitive TOR pathway from yeast to mammals. FEBS Lett. 2006; 580: 2821-2829.

**28.** Heeren G, Rinnerthaler M, Laun P, von Seyerl P, Kössler S, Klinger H, Jarolim S, Simon-Nobbe B, Hager M, Schüller C, Carmona-Gutierrez D, Breitenbach-Koller L, Mück C, Jansen-Dürr P, Criollo A, Kroemer G, Madeo F, Breitenbach M. The mitochondrial ribosomal protein of the large subunit, Afo1p, determines cellular longevity through mitochondrial back-signaling via TOR1. Aging 2009; 1: 622-636.

**29.** Ralser M, Lehrach H. Building a new bridge between metabolism, free radicals and longevity. Aging 2009; 1: 836-838.

**30.** Butow RA, Avadhani NG. Mitochondrial signaling: the retrograde response. Mol Cell 2004; 14: 1-15.

**31.** Jazwinski SM. Rtg2 protein: at the nexus of yeast longevity and aging. FEMS Yeast Res. 2005; 5: 1253-1259.

**32.** Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. Nature 2003; 425: 191-196.

**33.** Howitz KT, Sinclair DA. Xenohormesis: sensing the chemical cues of other species. Cell 2008; 133: 387-391.

**34.** Lamming DW, Wood JG, Sinclair DA. Small molecules that regulate lifespan: evidence for xenohormesis. Mol Microbiol. 2004; 53: 1003-1009.

**35.** Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le Couteur D, Shaw RJ, Navas P, Puigserver P, Ingram DK, de Cabo R, Sinclair DA. Resveratrol improves health and survival of mice on a high-calorie diet. Nature 2006; 444: 337-342.

**36.** Dasgupta B, Milbrandt J. Resveratrol stimulates AMP kinase activity in neurons. Proc Natl Acad Sci USA 2007; 104: 7217-7222.

**37.** Armour SM, Baur JA, Hsieh SN, Land-Bracha A, Thomas SM, Sinclair DA. Inhibition of mammalian S6 kinase by resveratrol suppresses autophagy. Aging 2009; 1: 515-528.

**38.** Demidenko ZN, Blagosklonny MV. At concentrations that inhibit mTOR, resveratrol suppresses cellular senescence. Cell Cycle 2009; 8: 1901-1904.

**39.** Blagosklonny MV. Inhibition of S6K by resveratrol: in search of the purpose. Aging 2009; 1: 511-514.

**40.** Morselli E, Galluzzi L, Kepp O, Criollo A, Maiuri MC, Tavernarakis N, Madeo F, Kroemer G. Autophagy mediates pharmacological lifespan extension by spermidine and resveratrol. Aging 2009; 1: 961-970.

**41.** Shakibaei M, Harikumar KB, Aggarwal BB. Resveratrol addiction: to die or not to die. Mol Nutr Food Res. 2009; 53: 115-128.

**42.** Morselli E, Maiuri MC, Markaki M, Megalou E, Pasparaki A, Palikaris K, Galluzzi L, Criollo A, Malik SA, Madeo F, Tavernarakis N, Kroemer G. Caloric restriction and resveratrol prolong longevity via the sirtuin-1 dependent induction of autophagy. Cell Death Disease 2010; 1: e10.

**43.** Wanke V, Cameroni E, Uotila A, Piccolis M, Urban J, Loewith R, De Virgilio C. Caffeine extends yeast lifespan by targeting TORC1. Mol Microbiol. 2008; 69: 277-285.

**44.** Blagosklonny MV. Aging and immortality: quasi-programmed senescence and its pharmacologic inhibition. Cell Cycle 2006; 5: 2087-2102.

**45.** Blagosklonny MV. Rapamycin and quasi-programmed aging: Four years later. Cell Cycle 2010; 9: 1859-1862.

**46.** Vezina C, Kudelski A, Sehgal SN. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. J Antibiot. 1975; 28: 721-726.

**47.** Fabrizio P, Battistella L, Vardavas R, Gattazzo C, Liou LL, Diaspro A, Dossen JW, Gralla EB, Longo VD. Superoxide is a mediator of an altruistic aging program in *Saccharomyces cerevisiae*. J Cell Biol. 2004; 166: 1055-1067.

**48.** Herker E, Jungwirth H, Lehmann KA, Maldener C, Frohlich KU, Wissing S, Buttner S, Fehr M, Sigrist S, Madeo F. Chronological aging leads to apoptosis in yeast. J Cell Biol. 2004; 164: 501-507.

**49.** Longo VD, Mitteldorf J, Skulachev VP. Programmed and altruistic ageing. Nat Rev Genet. 2005; 6: 866-872.

**50.** Vachova L, Palkova Z. Physiological regulation of yeast cell death in multicellular colonies is triggered by ammonia. J Cell Biol. 2005; 169: 711-717.

**51.** Büttner S, Eisenberg T, Herker E, Carmona-Gutierrez D, Kroemer G, Madeo F. Why yeast cells can undergo apoptosis: death in times of peace, love, and war. J Cell Biol. 2006; 175: 521-525.

**52.** Severin FF, Meer MV, Smirnova EA, Knorre DA, Skulachev VP. Natural causes of programmed death of yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 2008; 1783: 1350-1353.

Research Paper

# Chemical genetic screen identifies lithocholic acid as an anti-aging compound that extends yeast chronological life span in a TOR-independent manner, by modulating housekeeping longevity assurance processes

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**Abstract: In chronologically aging yeast, longevity can be extended by administering a caloric restriction (CR) diet or some small molecules. These life-extending interventions target the adaptable target of rapamycin (TOR) and cAMP/protein kinase A (cAMP/PKA) signaling pathways that are under the stringent control of calorie availability. We designed a chemical genetic screen for small molecules that increase the chronological life span of yeast under CR by targeting lipid metabolism and modulating housekeeping longevity pathways that regulate longevity irrespective of the number of available calories. Our screen identifies lithocholic acid (LCA) as one of such molecules. We reveal two mechanisms underlying the life-extending effect of LCA in chronologically aging yeast. One mechanism operates in a calorie availability-independent fashion and involves the LCA-governed modulation of housekeeping longevity assurance pathways that do not overlap with the adaptable TOR and cAMP/PKA pathways. The other mechanism extends yeast longevity under non-CR conditions and consists in LCA-driven unmasking of the previously unknown anti-aging potential of PKA. We provide evidence that LCA modulates housekeeping longevity assurance pathways by suppressing lipid-induced necrosis, attenuating mitochondrial fragmentation, altering oxidation-reduction processes in mitochondria, enhancing resistance to oxidative and thermal stresses, suppressing mitochondria-controlled apoptosis, and enhancing stability of nuclear and mitochondrial DNA.**

## INTRODUCTION

Aging of multicellular and unicellular eukaryotic organisms is a multifactorial biological phenomenon that has various causes and affects a plethora of cellular activities [1]. These numerous activities are modulated by only a few nutrient- and energy-sensing signaling pathways that are conserved across phyla and include the insulin/insulin-like growth factor 1 (IGF-1), AMP-activated protein kinase/target of rapamycin (AMPK/TOR) and cAMP/protein kinase A (cAMP/PKA) pathways [2-5]. By sharing a compendium of protein kinases and adaptor proteins, the insulin/IGF-1, AMPK/TOR and cAMP/PKA pathways in yeast,

worms, fruit flies and mammals converge into a network regulating longevity [2-4,6,7]. This network may also include several proteins that currently are not viewed as being in any of these three pathways [2,3,8,9]. Moreover, this network responds to the age-related partial mitochondrial dysfunction and is modulated by mitochondrially produced reactive oxygen species (ROS) [3,8,10,11]. By sensing the nutritional status of the whole organism as well as the intracellular nutrient and energy status, functional state of mitochondria, and concentration of ROS produced in mitochondria, the longevity network regulates life span across species by coordinating information flow along its convergent, divergent and multiply branched signaling pathways.

By defining the organismal and intracellular nutrient and energy status, nutrient intake plays an important role in modulating life span and influences age-related pathologies [12,13]. Two dietary regimens are known to have the most profound life-extending effects across species and to improve overall health by delaying the onset of age-related diseases. They include: 1) caloric restriction (CR), a diet in which only calorie intake is reduced but the supply of amino acids, vitamins and other nutrients is not compromised [13-15]; and 2) dietary restriction (DR), in which the intake of nutrients (but not necessarily of calories) is reduced by limiting food supply without causing malnutrition [16-18]. In a "TOR-centric" view of longevity regulation, TOR alone governs the life-extending and health-improving effects of CR/DR by: 1) integrating the flow of information on the organismal and intracellular nutrient and energy status from the protein kinases AMPK, PKA, PKB/AKT (the insulin/IGF-1 pathway) and ERK1/2 (the PKA-inhibited Raf/MEK/ERK protein kinase cascade) as well as from the mitochondrial redox protein $P66^{Shc}$; 2) sensing the intracellular levels of amino acids in an AMPK-independent manner; and 3) operating as a control center which, based on the information it has gathered and processed, modulates many longevity-related processes in a sirtuin-independent fashion [19-21]. The inability of CR to increase the replicative life span (RLS) of yeast mutants lacking components of the TOR pathway [22] and the lack of the beneficial effect of DR on life span in worms with reduced TOR signaling [23,24] support the proposed central role for TOR in orchestrating the life-extending effect of CR/DR in these two longevity paradigms. Moreover, while the postulated by the TOR-centric model dispensability of sirtuins for the longevity benefit associated with DR has been confirmed in worms [24], the importance of the sirtuin Sir2p in mediating the life-extending effect of CR in replicatively aging yeast is debated [22,25-27]. Noteworthy, while TOR is a central regulator of the life-extending effect of CR in replicatively aging yeast, the longevity benefit associated with CR in chronologically aging yeast is mediated by a signaling network that includes: 1) the TOR and cAMP/PKA pathways converged on Rim15p, which therefore acts as a nutritional integrator; and 2) some other, currently unknown pathways that are not centered on Rim15p [6]. Considering the likely convergence of the insulin/IGF-1, AMPK/TOR and cAMP/PKA signaling pathways into a network regulating longevity in worms, fruit flies and mammals (see above), it is conceivable that - akin to TOR - the insulin/IGF-1 and cAMP/PKA pathways may contribute to the beneficial effect of CR/DR on their longevity. Although some findings in worms, fruit flies and mammals support the involvement of the insulin/IGF-1 pathway in the longevity benefit associated with CR/DR, other data imply that such benefit is independent of insulin/IGF-1 (reviewed by Narasimhan *et al*. [3]). The role of cAMP/PKA signaling in the life-extending effect of CR/DR in these multicellular eukaryotes remains to be tested. Importantly, the recently reported in worms involvement of both independent and overlapping pathways in life span extension by different DR regimens [28] supports the notion that the longevity benefit associated with nutrient limitation is mediated by a signaling network that integrates several pathways.

Akin to CR and DR regimens, certain pharmacological interventions can extend longevity across phyla and improve health by beneficially influencing age-related pathologies. Noteworthy, all of the currently known anti-aging compounds increase life span under non-CR or non-DR conditions (Table S1). Under such conditions, these compounds have been shown to: 1) provide the longevity and health benefits associated with CR and DR, but without restricting caloric and nutrient intake; and 2) mimic numerous life-extending effects of CR and DR on gene expression pattern, metabolic and physiological processes, and stress response pathways. Therefore, the names "CR mimetics" and "DR mimetics" have been coined for them [29,30]. Importantly, most CR mimetics and DR mimetics target signaling pathways that modulate longevity in response to the organismal and intracellular nutrient and energy status, including the insulin/IGF-1 and AMPK/TOR pathways as well as the sirtuin-governed protein deacetylation module of the longevity signaling network integrating these pathways (Table S1). Furthermore, such compounds as resveratrol, metformin and mianserin increase life span only under non-CR or non-DR conditions, but are unable to do so if the supply of calories or nutrients is limited [31-35]. Hence, one could envision that most, if not all,

longevity pathways are "adaptable" by nature, *i.e.*, that they modulate longevity only in response to certain changes in the extracellular and intracellular nutrient and energy status of an organism. However, $Li^+$ in worms and rapamycin in fruits flies extend life span even under DR conditions [36,37]. It is likely therefore that some longevity pathways could be "constitutive" or "housekeeping" by nature, *i.e.*, that they: 1) modulate longevity irrespective of the organismal and intracellular nutrient and energy status; and 2) do not overlap (or only partially overlap) with the adaptable longevity pathways that are under the stringent control of calorie and/or nutrient availability. The challenge is to identify such housekeeping longevity pathways, perhaps by using a chemical screen for compounds that can extend longevity even under CR/DR conditions. Because under such conditions the adaptable pro-aging pathways are fully suppressed and the adaptable anti-aging pathways are fully activated, a compound that can increase life span is expected to target the housekeeping longevity pathways.

Noteworthy, two anti-aging compounds alter lipid levels in mammals and fruit flies under non-DR conditions. In fact, resveratrol treatment reduces the levels of the neutral lipids triacylglycerols (TAG) and increases free fatty acid (FFA) levels in mouse adipocytes [38]. Furthermore, feeding rapamycin to fruit flies results in elevated TAG levels [37]. Although it remains to be seen if such effects of resveratrol and rapamycin on lipid levels play a casual role in their anti-aging action under non-DR conditions, it should be stressed that lipid metabolism has been shown to be involved in longevity regulation in yeast [39,40], worms [9,41-43], fruit flies [41,44] and mice [38,41,45-48]. We recently proposed a mechanism linking yeast longevity and lipid dynamics in the endoplasmic reticulum (ER), lipid bodies and peroxisomes. In this mechanism, a CR diet extends yeast chronological life span (CLS) by activating FFA oxidation in peroxisomes [39,40]. It is conceivable that the identification of small molecules targeting this mechanism could yield novel anti-aging compounds. Such compounds can be used as research tools for defining the roles for different longevity pathways in modulating lipid metabolism and in integrating lipid dynamics with other longevity-related processes. Furthermore, the availability of such compounds would enable a quest for housekeeping longevity assurance pathways that do not overlap (or only partially overlap) with the adaptable TOR and cAMP/PKA pathways. Moreover, such compounds would have a potential to be used as pharmaceutical agents for increasing life span and promoting healthy aging by delaying the onset of age-related diseases, regardless of an organism's dietary regimen.

We sought to identify small molecules that increase the CLS of yeast under CR conditions by targeting lipid metabolism and modulating housekeeping longevity assurance pathways. Our chemical genetic screen identified lithocholic acid (LCA) as one of such small molecules. We provide evidence that LCA extends longevity of chronologically aging yeast through two different mechanisms. In one mechanism, this bile acid targets - regardless of the number of available calories - housekeeping longevity assurance pathways that do not overlap with the adaptable TOR and cAMP/PKA pathways and modulate a compendium of pro- and anti-aging processes. In the other mechanism, LCA targets the adaptable cAMP/PKA pathway under non-CR conditions by unmasking the previously unknown anti-aging potential of PKA.

## RESULTS

### Our rationale for choosing a mutant strain and growth conditions to screen compound libraries for anti-aging small molecules

To perform a chemical genetic screen for small molecules that increase the CLS of yeast by targeting lipid metabolism, we chose the single-gene-deletion mutant strain *pex5Δ*. Because *pex5Δ* lacks a cytosolic shuttling receptor for peroxisomal import of Fox1p and Fox2p, these two enzymes of the β-oxidation of FFA reside in the cytosol of *pex5Δ* cells [49] (Figure 1A). In contrast, the Pex5p-independent peroxisomal import of Fox3p, the third enzyme of the FFA β-oxidation pathway, sorts it to the peroxisome in *pex5Δ* cells [49]. By spatially separating Fox1p and Fox2p from Fox3p within a cell, the *pex5Δ* mutation impairs FFA oxidation (Figure 1A). In chronologically aging yeast grown under CR conditions on 0.2% or 0.5% glucose, peroxisomal FFA oxidation regulates longevity by 1) efficiently generating acetyl-CoA to synthesize the bulk of ATP in mitochondria; and 2) acting as a rheostat that modulates the age-related dynamics of FFA and diacylglycerol (DAG), two regulatory lipids known to promote longevity-defining cell death [39,40,50]. Unlike CR yeast, chronologically aging non-CR yeast grown on 1% or 2% glucose are unable to generate significant quantities of ATP by oxidizing peroxisome-derived acetyl-CoA in mitochondria and, instead, produce the bulk of ATP via glycolytic oxidation of glycogen- and trehalose-derived glucose [39,40]. Consistent with the essential role of peroxisomal FFA oxidation as a longevity assurance process only under CR, the *pex5Δ* mutation substantially shortened the CLS of CR yeast but caused a significantly lower reduction of longevity in non-CR yeast, especially in yeast grown on 2% glucose (Figures 1B to F).

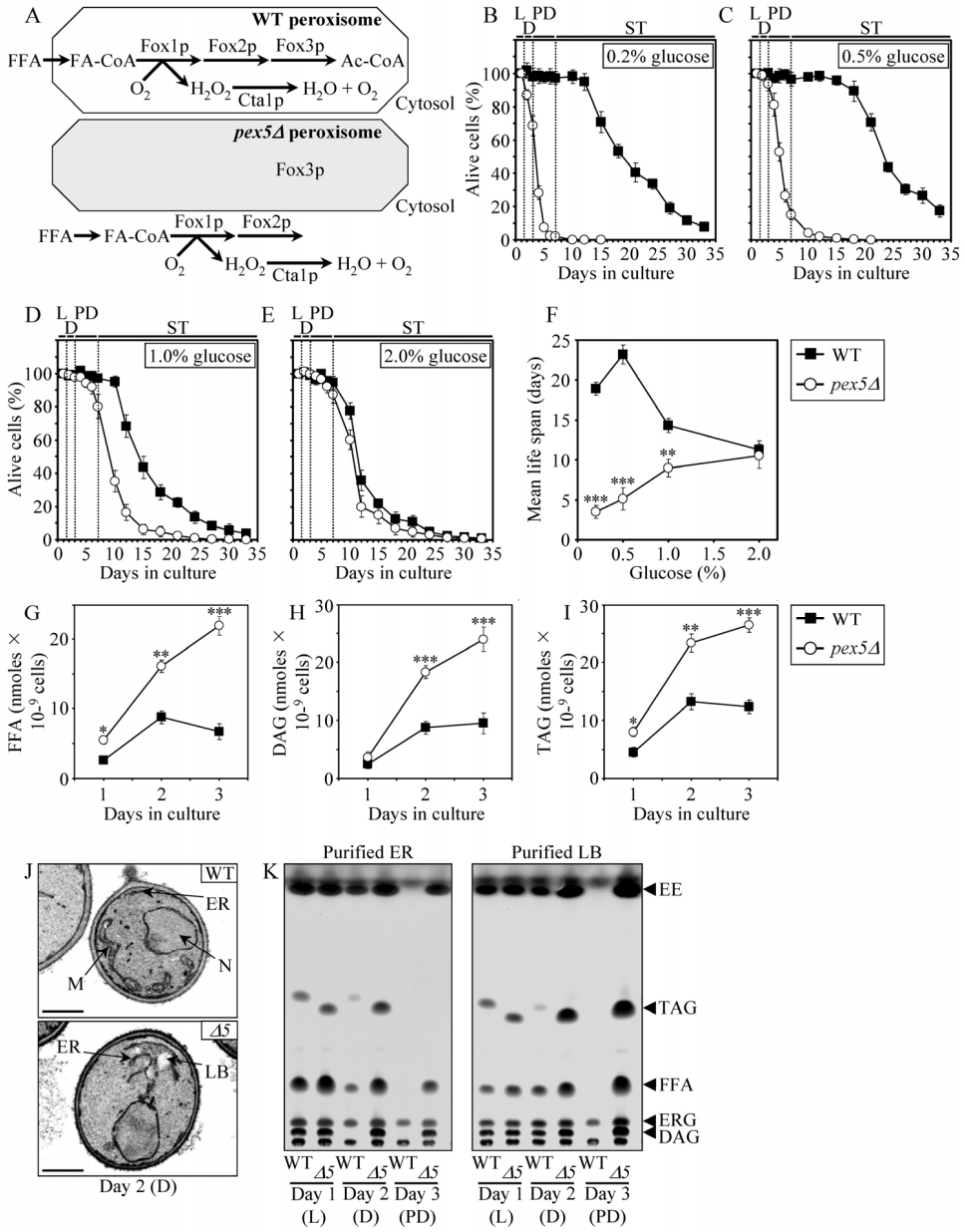



**Figure 1. The *pex5Δ* mutation shortens chronological life span (CLS), alters cell morphology and remodels lipid metabolism in CR yeast.** (**A**) Outline of subcellular localization of the Fox1p, Fox2p and Fox3p enzymes of fatty acid ß-oxidation in WT and *pex5Δ* cells. (**B** - **F**) Survival and the mean life spans of chronologically aging WT and *pex5Δ* yeast cultured in medium initially containing 0.2%, 0.5%, 1% or 2% glucose. Data are presented as means ± SEM (n = 16-38; ***p < 0.001; **p < 0.01). (**G** - **I**) Levels of free fatty acids (FFA), diacylglycerols (DAG) and triacylglycerols (TAG) in WT and *pex5Δ* cells grown on 0.2% glucose and taken for analyses at the indicated time-points. FFA and TAG were measured by quantitative mass spectrometry. The levels of DAG were quantitated by densitometric analysis of TLC plates. Data are presented as means ± SEM (n = 3-8; ***p < 0.001; **p < 0.01; *p < 0.05). (**J** and **K**) Transmission electron micrographs (**J**) and spectra of lipids extracted from purified endoplasmic reticulum (ER) and lipid bodies (LB) and analyzed by TLC (**K**) for WT and *pex5Δ* (*Δ5*) yeast grown on 0.2% glucose and taken for analyses at the indicated time-points. Abbreviations: Cta1p, peroxisomal catalase; D, diauxic growth phase; EE, ethyl esters; ERG, ergosterol; FA-CoA, CoA esters of fatty acids; L, logarithmic growth phase; M, mitochondrion; N, nucleus; PD, post-diauxic growth phase; ST, stationary growth phase.

In chronologically aging CR yeast, peroxisomal FFA oxidation modulates, perhaps via several negative feedback loops, the following three processes: 1) the ER-confined biosynthesis of TAG from FFA and DAG; 2) the subsequent deposition of TAG, the major neutral lipid reserves, in lipid bodies; and 3) the consequent lipolysis of deposited TAG and the resulting formation of FFA and DAG [39,40]. By impairing the ability of peroxisomal FFA oxidation to act as a rheostat that regulates cellular aging by modulating the age-related dynamics of FFA, DAG and TAG in the ER and lipid bodies, the *pex5Δ* mutation caused the accumulation of the closely apposed ER membranes and ER-originated lipid bodies in CR yeast (Figure 1J). Of note, these morphological features of *pex5Δ* yeast under CR were similar to those observed in a mouse model for the peroxisome biogenesis disorder Zellweger syndrome with hepatocyte-specific elimination of the *PEX5* gene [51]. Furthermore, the *pex5Δ* mutation increased the concentrations of FFA, DAG and TAG in CR yeast (Figures 1G to I), promoting their buildup in the ER and lipid bodies (Figure 1K). CR yeast carrying the *pex5Δ* mutation also accumulated the ER-derived and lipid bodies-deposited ergosteryl esters (EE) neutral lipid species (Figure 1K).

Following a short-term exposure to exogenous FFA (palmitoleic acid or oleic acid) or DAG, wild-type (WT) cells grown under CR conditions died (Figure S1A). The vast majority of these WT cells displayed propidium iodide (PI) positive staining characteristic of the loss of plasma membrane integrity, a hallmark event of necrotic cell death (Figures S1B and S1C). In contrast, only a minor portion of these WT cells displayed Annexin V positive staining used to visualize the externalization of phosphatidylserine, a hallmark event of apoptotic cell death (Figures S1B and S1C). Thus, a brief exposure of WT cells grown under CR conditions to exogenous FFA or DAG caused their necrotic, not apoptotic, death. Importantly, we found that the *pex5Δ* mutation enhances the susceptibility of CR yeast to necrotic death caused by a short-term exposure to exogenous FFA or DAG (Figures S1A and S1C), perhaps due to the increased concentrations of endogenous FFA and DAG seen in *pex5Δ* cells under CR (Figures 1G and H).

In addition to its effect on lipid metabolism and lipid-induced necrotic cell death, the *pex5Δ* mutation also altered mitochondrial morphology and oxidation-reduction processes in mitochondria of CR yeast. In fact, this mutation caused the fragmentation of a tubular mitochondrial network into individual mito-chondria under CR conditions (Figures S2A and S2B).

Furthermore, in CR yeast the *pex5Δ* mutation 1) greatly reduced the rate of oxygen consumption by mitochondria (Figure S2C); 2) substantially decreased the mito-chondrial membrane potential (Figure S2D); and 3) diminished the level of intracellular ROS (Figure S2E), known to be generated mostly as by-products of mitochondrial respiration [10,52]. Interestingly, all these mitochondrial abnormalities in *pex5Δ* yeast under CR were reminiscent of changes in mitochondrial morpholo-gy and functions seen in mice with hepatocyte-specific elimination of the *PEX5* gene, a model for the peroxi-some biogenesis disorder Zellweger syndrome [51].

Besides its profound effect on lipid metabolism, lipid-induced necrosis, mitochondrial morphology and functions, the *pex5Δ* mutation also 1) reduced the resistance of chronologically aging CR yeast to chronic oxidative, thermal and osmotic stresses (Figure S3A); 2) sensitized CR yeast to death that was initiated in response to a short-term exposure to exogenous hydrogen peroxide or acetic acid (Figure S3B) and that is known to be caused by mitochondria-controlled apoptosis [53,54]; and 3) elevated the frequencies of deletion and point mutations in mitochondrial and nuclear DNA of CR yeast (Figures S3C to S3E).

The profound changes in cell morphology andphysiology, stress resistance, susceptibility to lipid-induced necrosis and mitochondria-controlled apoptosis, and stability of nuclear and mitochondrial DNA seen in *pex5Δ* yeast under CR conditions coincided with considerable changes in their proteome. Indeed, our mass spectrometry-based quantitative proteomic analysis of proteins recovered in total cell lysates as well as in purified ER and mitochondria revealed that the *pex5Δ* mutation altered the abundance of many proteins (Figure 2A). Protein species that were depleted or enriched in the total cell lysate, ER and mitochondria of *pex5Δ* yeast grown under CR conditions included proteins involved in a number of cellular processes (Figure 2B). Importantly, lack of 91 of these proteins increased the CLS of yeast under CR (Figure 2B), suggesting their essential pro-aging role in longevity regulation when calorie supply is limited. Noteworthy, 58 of the genes encoding these proteins and termed gerontogenes (*i.e.*, the genes whose mutant alleles extend life span; [55]) have not been previously known as being critical for defining the CLS of yeast. The identities of protein species that were depleted or enriched in *pex5Δ* yeast grown under CR conditions, the extent to which their levels were altered and the names of gerontogenes identified in our functional analysis will be reported elsewhere (Goldberg et al., manuscript in preparation). Importantly, for most of these proteins

(with some exceptions, see Figures S4C and S4D) the fold increase or decrease in the level of a protein enriched or depleted in *pex5Δ* was found to be in good correlation with the fold increase or decrease (respectively) in the mean CLS of a mutant strain lacking it (Figures S4A and S4B).

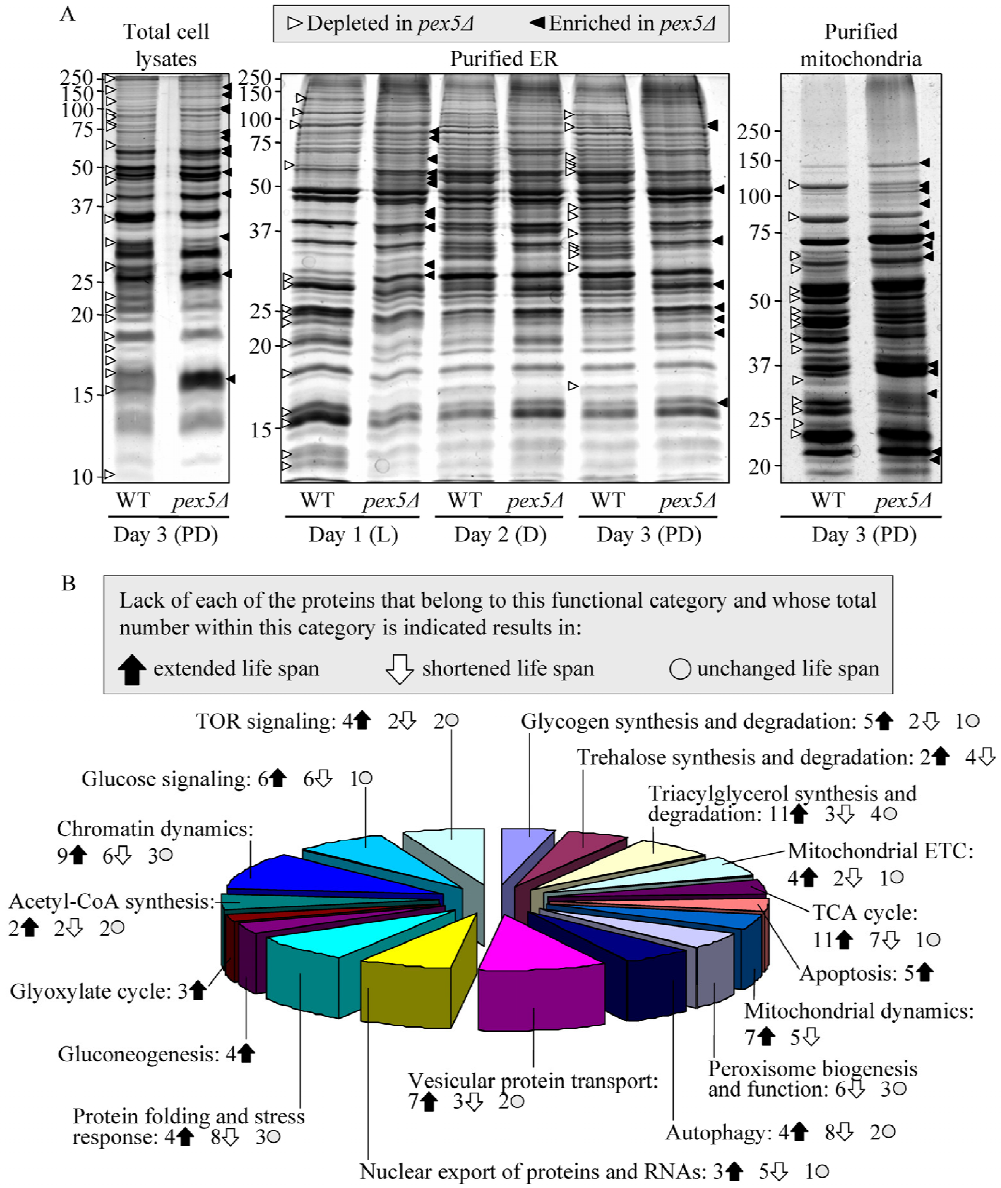



**Figure 2. The *pex5Δ* mutation alters the abundance of many proteins recovered in total cell lysates, purified ER and mitochondria of CR yeast.** (**A**) The spectra of proteins recovered in total cell lysates, purified ER and mitochondria of WT and *pex5Δ* cells that were grown under CR on 0.2% glucose and taken for analyses at the indicated time-points. (**B**) Functional categories of proteins that were enriched or depleted in the total cell lysate, ER and mitochondria of *pex5Δ* cells (as compared to WT cells) under CR conditions. Lack of 91 of these proteins increased the CLS of yeast under CR, suggesting their essential pro-aging role in longevity regulation when calorie supply is limited.

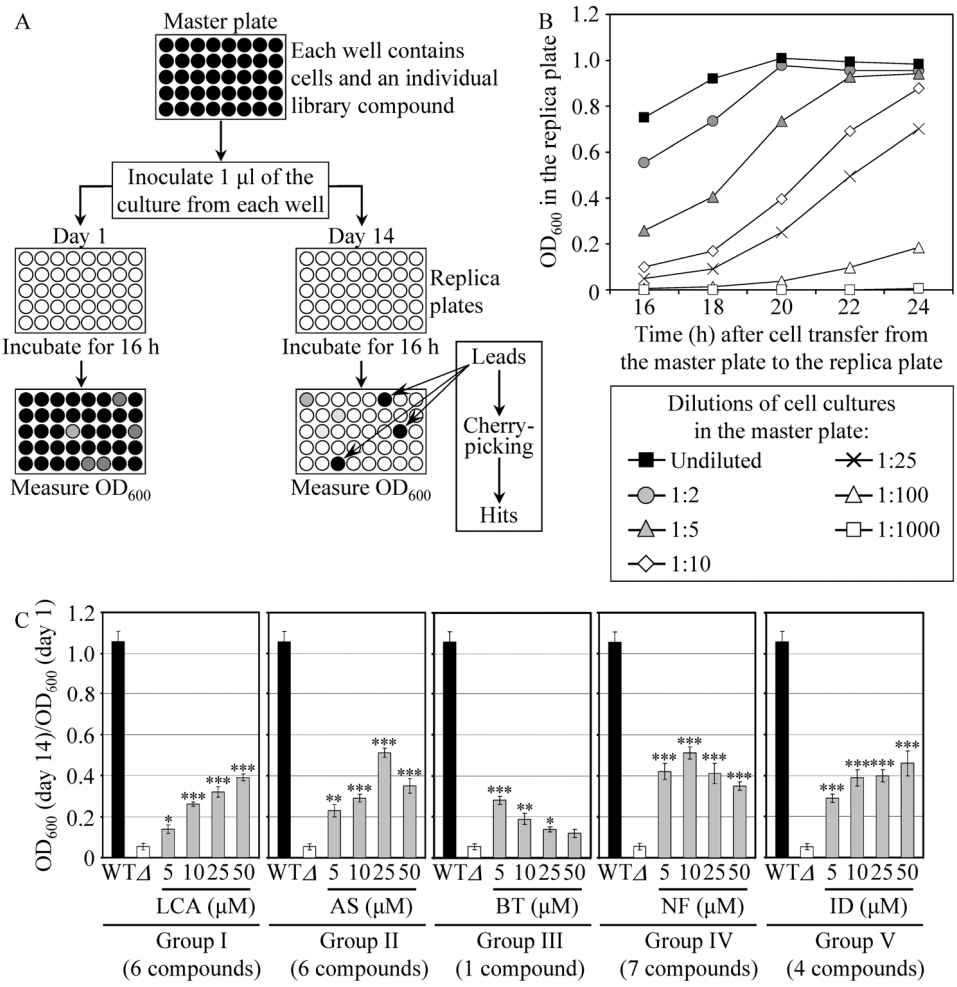



**Figure 3. A high-throughput screen of compound libraries for small molecules that extend the CLS of yeast under CR conditions.** (**A**) A microplate assay for measuring yeast CLS by monitoring optical density at 600 nm ($OD_{600}$) was used for screening representative compounds from several commercial libraries for small molecules that extend the CLS of *pex5Δ* cells grown under CR on 0.5% glucose. (**B**) The $OD_{600}$ of a cell culture in the replica microplate following incubation for 16 to 24 hours correlates with the number of viable cells present in this culture before it was taken from the master microplate for replica plating. (**C**) The effect of various concentrations of the identified anti-aging small molecules on the CLS of the *pex5*Δ (Δ) strain under CR conditions. The "$OD_{600}$ at day 14/$OD_{600}$ at day 1" ratio was used as a measure of CLS. Data are presented as means ± SEM (n = 3-5; ***p < 0.001; **p < 0.01; *p < 0.05). The anti-aging small molecules LCA, AS, BT, NF and ID belong to five chemical groups.

Altogether, these findings imply that, by impairing peroxisomal FFA oxidation and affecting lipid metabolism in the ER and lipid bodies, the *pex5Δ* mutation alters the levels of numerous pro- and anti-aging proteins and impacts many longevity-related processes, thereby shortening the CLS of yeast when calorie supply is limited. We therefore chose the short-lived *pex5Δ* strain to carry out a chemical genetic screen for anti-aging compounds that target lipid metabolism to extend CLS in yeast placed on a CR diet.

## A chemical genetic screen for small molecules that extend the CLS of yeast under CR conditions

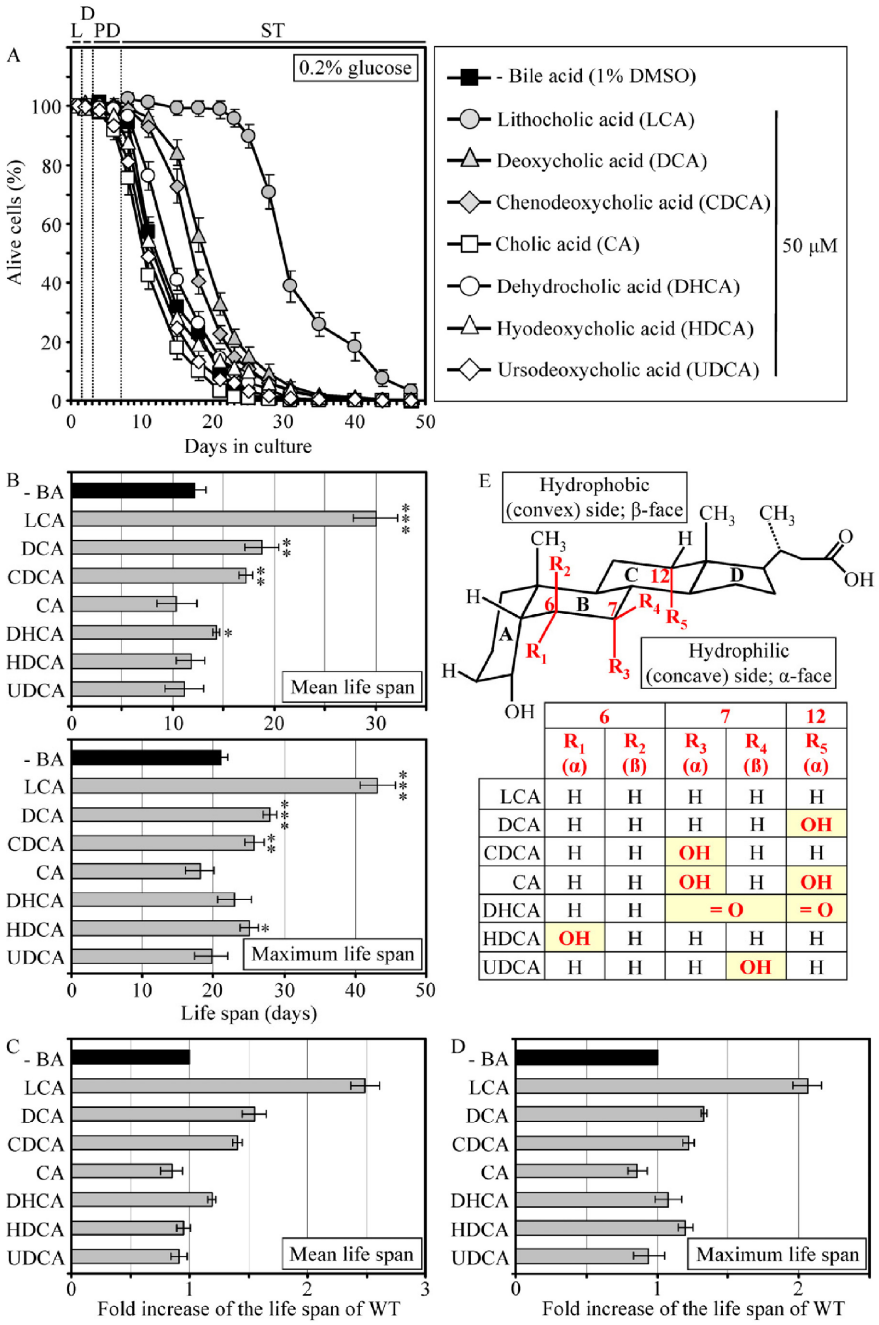
To facilitate a high-throughput screen of compound libraries for anti-aging small molecules, we adopted a previously described microplate assay [56] for measuring CLS by monitoring optical density at 600 nm ($OD_{600}$) (Figure 3A). In our assay, a small aliquot of the *pex5Δ* culture grown in a nutrient-rich medium containing 0.5% glucose and recovered from mid-

logarithmic phase was transferred into each well of a 96-well master microplate containing the same growth medium and a compound from a commercially available library. At days 1, 7, 10 and 14 of the incubation of master microplates, a small aliquot of each culture was transferred into individual wells of a new (replica) microplate containing growth medium only. Following incubation of replica microplates for 16 hours, the $OD_{600}$ of the culture in each well of the replica microplate was measured. Importantly, we found that under such conditions the $OD_{600}$ of a cell culture in a well of the replica microplate correlates with the number of viable cells in the corresponding well of the master microplate (Figure 3B). To calculate survival at each time point, the $OD_{600}$ at a particular time point was divided by the $OD_{600}$ at day 1. By translating our microplate assay into high-throughput format and screening representative compounds from the NIH Clinical Collection, Prestwick Chemical Inc. and Sigma-LOPAC commercial libraries, we identified "lead" compounds. The subsequent "cherry-picking" analysis of these small molecules revealed "hit" compounds that in our microplate assay reproducibly extended the CLS of *pex5Δ*. Using the web-based eMolecules searching engine, we identified commercially available structural analogs of the hit compounds and then tested their life-extending efficacy in our microplate assay for measuring the CLS of *pex5Δ*. By screening the total of approximately 19,000 representative compounds from seven commercial libraries, we identified 24 small molecules that greatly extend the CLS of *pex5Δ* under CR and belong to 5 chemical groups (Figure 3C). Group I consisted of 6 bile acids, including lithocholic acid (LCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), cholic acid (CA), dehydrocholic acid (DHCA) and hyodeoxycholic acid (HDCA) (Figures 3C and S5). Noteworthy, the anti-aging efficacy of these bile acids correlated with their hydrophobicity. In fact, LCA - the most hydrophobic bile acid species [57] - displayed the highest ability to delay chronological aging of *pex5Δ* under CR conditions in the microplate assay (Figure S5). The identities of small molecules that belong to groups II to V (Figure 3C) of the anti-aging compounds identified in our screen and the structure-activity analysis of their life-extending potential will be reported elsewhere (Goldberg et al., manuscript in preparation).

Noteworthy, none of the small molecules that has been shown to extend CLS (*i.e.*, caffeine, methionine sulfoximine, rapamycin and spermidine; Table S1; [56,58,59]) and/or RLS (*i.e.*, rapamycin and resveratrol; Table S1; [27,31]) in yeast has been identified in our screen for compounds capable of increasing the CLS of *pex5Δ* under CR. Furthermore, none of these currently known life-extending molecules is structurally related to the anti-aging compounds that we revealed. Thus, it is likely that LCA and all other novel anti-aging compounds identified in our screen target longevity- related cellular processes that are not modulated by the presently known anti-aging small molecules. Because our screen was aimed at identifying compounds that extend yeast longevity by targeting lipid metabolism, it is conceivable that the age-related dynamics of TAG, FFA and DAG is one of such cellular processes.

**Pharmacophore modeling of the anti-aging potential of bile acids**

Similar to their effect on *pex5Δ*, some of the group I anti-aging compounds extended the CLS of WT strain under CR conditions. Specifically, LCA and two other bile acids - DCA and CDCA - increased both the mean and maximum CLS of WT yeast grown under CR on 0.2% glucose (Figures 4A to 4D). Moreover, DHCA increased only the mean CLS of WT yeast under CR at 0.2% glucose, whereas HDCA increased only their maximum CLS (Figures 4A to 4D). Akin to its highest life-extending efficacy in *pex5Δ* under CR, the most hydrophobic bile acid - LCA [57] - provided WT cells with the greatest longevity benefit when calorie supply was limited. In fact, LCA increased the mean CLS of WT strain under CR at 0.2% glucose by almost 250% and its maximum CLS by more than 200% (Figures 4A to D). Our comparative analysis of the structural differences between various bile acids and their relative life-extending efficacies revealed that the positions 6, 7 and 12 in the six-member rings B and C of the steroid nucleus are important for the anti-aging potential of a bile acid. Indeed, the ability of LCA to extend both the mean and maximum CLS of WT yeast under CR can be: 1) eliminated (with respect to the mean CLS) or greatly reduced (with respect to the maximum CLS) by attaching an α-oriented hydroxyl group at the position 6 (as in HDCA); 2) greatly reduced (with respect to both the mean and maximum CLS) by attaching an α- oriented hydroxyl group at the position 7 (as in CDCA); and 3) greatly reduced (with respect to both the mean and maximum CLS) by attaching an α-oriented hydroxyl group at the position 12 (as in DCA) (Figures 4B to E). All these modifications to the structure of LCA increase polarity of the hydrophilic (concave) side [α-face] of the steroid nucleus by positioning a hydroxyl group below the nucleus and axially to its plane (Figure 4E). Furthermore, the anti-aging potential of LCA can be abolished by attaching a β-oriented hydroxyl group at the position 7 (as in UDCA), thereby conferring polarity to the hydrophobic (convex) side [β-face] of the steroid nucleus by positioning a hydroxyl group above the nucleus and equatorially to its plane (Figures 4B to E).




| | 6 | | 7 | | 12 |
|---|---|---|---|---|---|
| | $R_1$ (α) | $R_2$ (ß) | $R_3$ (α) | $R_4$ (ß) | $R_5$ (α) |
| LCA | H | H | H | H | H |
| DCA | H | H | H | H | OH |
| CDCA | H | H | OH | H | H |
| CA | H | H | OH | H | OH |
| DHCA | H | H | = O | | = O |
| HDCA | OH | H | H | H | H |
| UDCA | H | H | H | OH | H |

**Figure 4. LCA and some other bile acids extend the CLS of WT strain under CR conditions.** (**A** - **D**) Effect of various bile acids on survival (**A**) and on the mean and maximum life spans (**B** - **D**) of chronologically aging WT strain grown under CR conditions on 0.2% glucose. Data are presented as means ± SEM (n = 3-28; ***p < 0.001; **p < 0.01; *p < 0.05). (**E**) Structure and hydrophilic/hydrophobic properties of bile acids. The R1 (α), R3 (α) and R5 (α) hydroxyl groups at the positions 6, 7 and 12 in the six-member rings **B** and **C** of the steroid nucleus increase polarity of the hydrophilic (concave) side [α-face] of the nucleus by being located below the nucleus and axially to its plane. The R4 (β) hydroxyl group at the position 7 in the six-member ring B of the steroid nucleus confers polarity of the hydrophobic (convex) side [β-face] of the nucleus by being located above the nucleus and equatorially to its plane.

Moreover, the simultaneous attachments of two α-oriented hydroxyl groups (as in CA) or two keto groups (as in DHCA) at the positions 7 and 12 eliminated the ability of LCA to extend both the mean and maximum CLS of WT yeast under CR (Figures 4B to E). Altogether, the results of our pharmacophore modeling of the anti-aging potential of bile acids imply that the maintenance of the minimal polarity of both the hydrophilic (concave) and hydrophobic (convex) sides of the steroid nucleus - by avoiding the presence of polar substituents at the positions 6, 7 and 12 - is mandatory for the extreme life-extending efficacy of LCA under CR conditions. Such stringent structural requirements are consistent with a target specificity of LCA action as an anti-aging small molecule.

**LCA extends the CLS of WT yeast under both CR and non-CR conditions, although to a different extent**

If added to growth medium at the time of cell inoculation, LCA increased both the mean and maximum CLS of WT strain not only under CR at 0.2% or 0.5% glucose (Figures 5A, 5B and 5G - 5I) but also under non-CR conditions administered by culturing yeast in medium initially containing 1% or 2% glucose (Figures 5C, 5D and 5G - 5I). At any tested concentration of glucose in growth medium, LCA displayed the greatest beneficial effect on both the mean and maximum CLS of WT strain if used at a final concentration of 50 μM (Figures 5E and 5F). It should be stressed that the life-extending efficacy of 50 μM LCA under CR exceeded that under non-CR conditions, being inversely proportional to the concentration of glucose in growth medium and thus in correlation with the extent of calorie supply limitation (Figures 5G to 5I). Importantly, although 50 μM LCA displayed a profound effect on CLS, it did not cause significant changes in growth of WT strain at any tested concentration of glucose in medium. In fact, both growth rate in logarithmic phase and time prior to entry into stationary (ST) phase were similar for WT cells cultured in medium with or without LCA (Figure S6).

**LCA extends the CLS of WT yeast under CR by modulating a compendium of longevity-related processes**

Our chemical genetic screen identified LCA as a compound that under CR conditions extends the CLS of *pex5Δ*, a prematurely aging mutant strain displaying profound changes in lipid metabolism, lipid-induced necrotic cell death, mitochondrial morphology and functions, stress resistance, mitochondria-controlled apoptosis, and stability of nuclear and mitochondrial DNA. We found that LCA also greatly increases the mean and maximum CLS of WT yeast limited in calorie supply. This finding prompted us to investigate how the exposure of WT cells to LCA under CR conditions influences a compendium of longevity-related processes impaired in *pex5Δ*.

Consistent with its sought-after effect on lipid metabolism in the ER, lipid bodies and peroxisomes, LCA elevated the concentration of TAG in WT cells that entered the non-proliferative ST phase under CR at 0.2% glucose (Figure 6A). Furthermore, under these conditions LCA also substantially reduced the intracellular levels of FFA and DAG in WT yeast that reached reproductive maturation by entering into ST phase (Figures 6B and 6C). Moreover, LCA greatly reduced the susceptibility of reproductively mature WT cells under CR to necrotic cell death that was caused by a short-term exposure to exogenous FFA or DAG and defined by Annexin $V^-/PI^+$ staining (Figures 6D to 6I).

The exposure of reproductively mature WT cells to LCA under CR conditions also influenced other longevity-related processes impaired in *pex5Δ*, including those confined to mitochondria. Indeed, in WT cells that entered the non-proliferative ST phase under CR at 0.2% glucose, LCA 1) attenuated the fragmentation of a tubular mitochondrial network into individual mitochondria (Figure 7A); 2) elevated the rate of oxygen consumption by mitochondria (Figure 7B); 3) reduced the mitochondrial membrane potential (Figure 7C); and 4) decreased the level of intracellular ROS (Figure 7D) known to be generated mainly in mitochondria [10,52].

Moreover, in WT yeast that under CR conditions reached reproductive maturation by entering into ST phase, LCA 1) enhanced cell resistance to oxidative and thermal (but not to osmotic) stresses (Figure 7E); 2) reduced cell susceptibility to death triggered by a short-term exposure to exogenous hydrogen peroxide or acetic acid (Figure 7F) known to be caused by mitochondria-controlled apoptosis [53,54]; and 3) decreased the frequencies of deletions and point mutations in mitochondrial and nuclear DNA (Figures 7G to I).

**LCA extends yeast CLS independent of TOR, by modulating housekeeping longevity assurance pathways**

Our chemical genetic screen was aimed at identifying small molecules that can increase the CLS of yeast under CR by modulating housekeeping longevity pathways. Such pathways may regulate yeast longevity irrespective of the number of available calories and may not necessarily overlap (or may only partially overlap)

with the adaptable longevity pathways that are under the stringent control of calorie availability. In chronologically aging yeast, the TOR and cAMP/PKA signaling pathways are the two adaptable longevity pathways that govern the life-extending effect of CR (Figure 10A) [5,6,60-62].

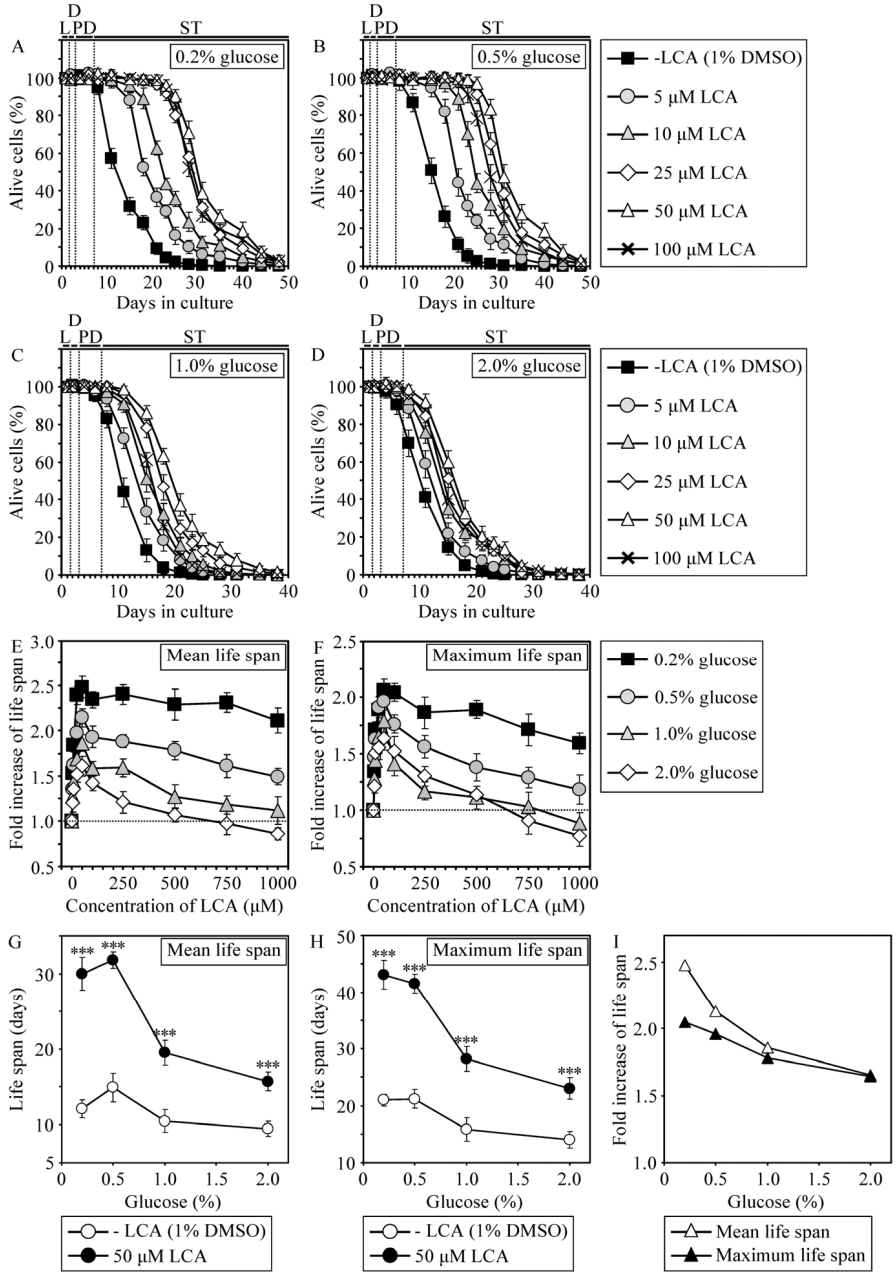



**Figure 5. In chronologically aging WT yeast, the life-extending efficacy of LCA under CR exceeds that under non-CR conditions.** (**A** - **F**) Effect of various concentrations of LCA on survival (**A** - **D**) and on the fold increase in the mean (**E**) or maximum (**F**) life span of chronologically aging WT strain cultured in medium initially containing 0.2%, 0.5%, 1% or 2% glucose. Data are presented as means ± SEM (n = 3-28). (**G** - **I**) Effect of 50 μM LCA on the mean or maximum CLS of WT yeast cultured in medium initially containing 0.2%, 0.5%, 1% or 2% glucose. Data are presented as means ± SEM (n = 12-28; ***p < 0.001).

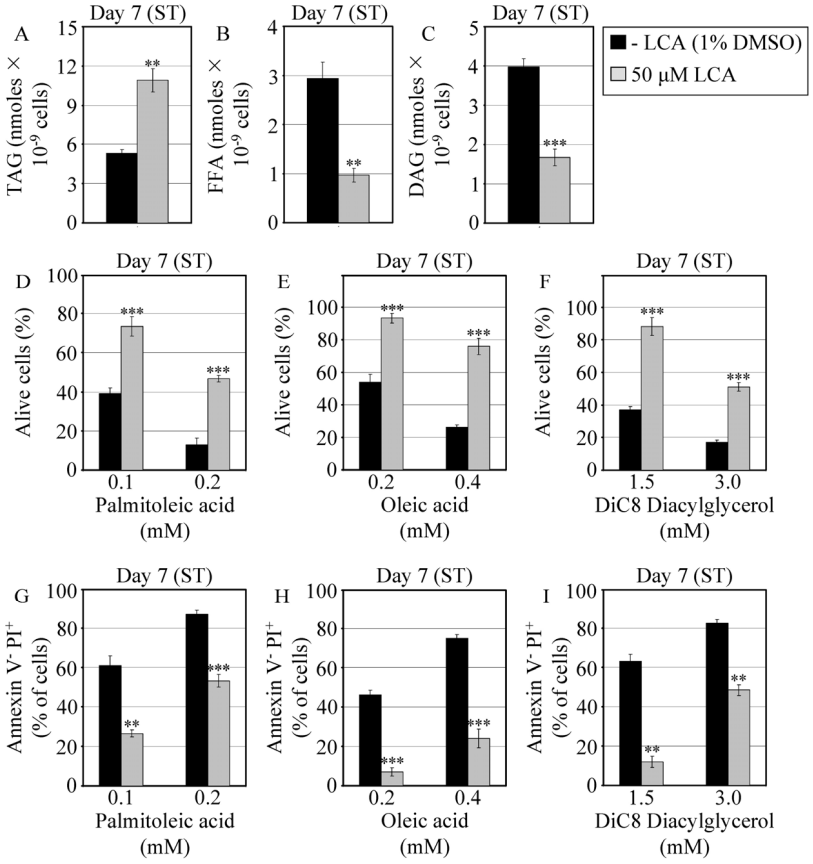



**Figure 6. In chronologically aging WT yeast that entered the non-proliferative stationary (ST) phase under CR, LCA alters the levels of lipids and protects cells from lipid-induced necrotic death.** (**A** - **C**) Levels of triacylglycerols (TAG) and free fatty acids (FFA) measured by quantitative mass spectrometry (**A** and **B**, respectively) and of diacylglycerols (DAG) monitored by TLC (**C**) in WT cells grown in medium with or without LCA. (**D** - **F**) Viability of WT cells pre-grown in medium with or without LCA and then treated for 2 h with palmitoleic acid (**D**), oleic acid (**E**) or DiC8 diacylglycerol (**F**). (**G** - **I**) Percent of WT cells (pre-grown in medium with or without LCA) that following their treatment with palmitoleic acid (G), oleic acid (H) or DiC8 diacylglycerol (**I**) displayed Annexin V negative and PI positive (Annexin $V^-$ and $PI^+$) staining characteristic of necrotic cell death. Data are presented as means ± SEM (n = 3-9; ***p < 0.001; **p < 0.01). WT cells grown on 0.2% glucose in the presence or absence of LCA were taken for analyses at day 7, when they reached reproductive maturation by entering into ST phase.

Reduction of the Tor1p protein kinase activity in yeast placed on a CR diet or exposed to rapamycin prevents inhibitory phosphorylation of Atg13p, a key positive regulator of autophagy, thereby activating this essential anti-aging process (Figure 10A) [63,64]. Under CR conditions or in response to rapamycin, Tor1p is also unable to phosphorylate and activate the nutrient-sensory protein kinase Sch9p [60,65]. The resulting inhibition of the Sch9p kinase activity suppresses its ability to attenuate protein synthesis in mitochondria, thus turning on this essential anti-aging process [61].

Furthermore, by inhibiting the Sch9p kinase activity, CR restrains Sch9p from activating protein synthesis in the cytosol, thereby slowing down this essential pro-aging process [60,62,65]. Moreover, the attenuation of the Sch9p kinase activity in CR yeast prevents the retention of Rim15p in the cytosol, hence allowing this nutrient-sensory protein kinase to enter the nucleus where it orchestrates an anti-aging transcriptional program by activating the stress response transcriptional activators Msn2p, Msn4p and Gis1p [58,62]. The longevity benefit associated with CR in chronologically

aging yeast is also due to the attenuation of signaling through the cAMP/PKA pathway, which is driven by glucose deprivation [5,6,62]. By preventing inhibitory phosphorylation of Atg13p, the reduction of the PKA kinase activity in CR yeast results in activation of autophagy (Figure 10A) [63,66]. In addition, by inhibiting the PKA kinase activity, CR suppresses the ability of PKA to activate protein synthesis in the cytosol [62]. Moreover, reduced PKA kinase activity in CR yeast enables nuclear import of Msn2p and Msn4p, thus turning on an anti-aging transcriptional program driven - in a Rim15p-dependent fashion - by these two transcriptional activators [27,62,67]. Noteworthy, the kinase activity of the cytosolic pool of Rim15p is inactivated through PKA-dependent phosphorylation (Figure 10A) [62]. Although some of the Rim15p phosphorylation targets are involved in longevity regulation and reside outside the nucleus [68], a role of such phosphorylation in the life-extending effect of CR in yeast remains to be established.

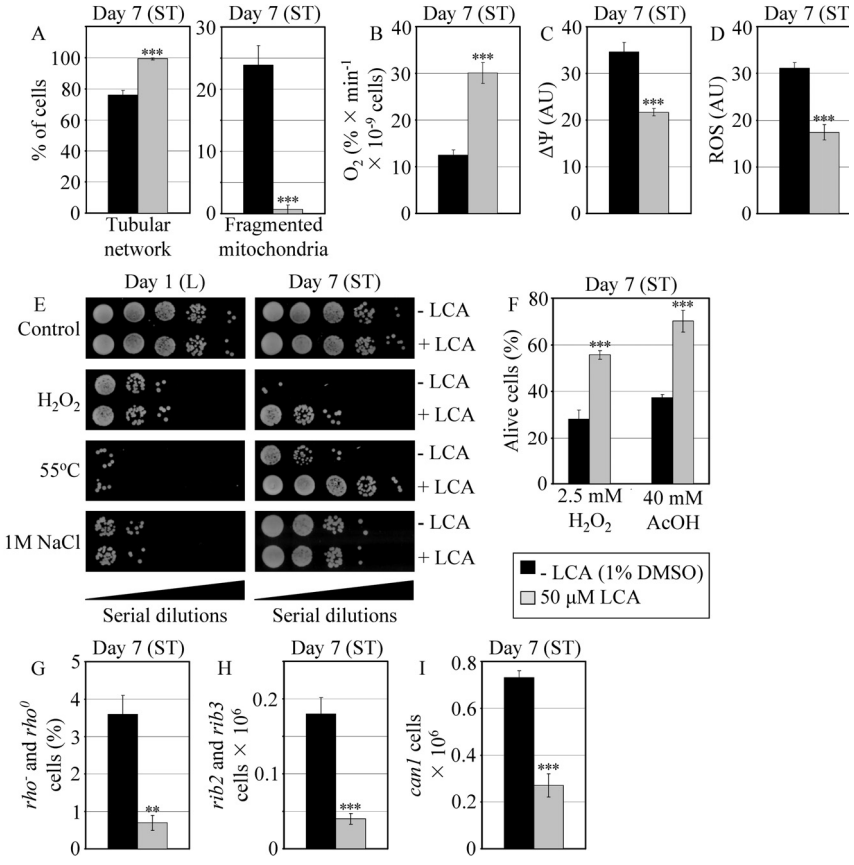



**Figure 7. In reproductively mature WT yeast that entered the non-proliferative stationary (ST) phase under CR, LCA modulates mitochondrial morphology and functions, enhances stress resistance, attenuates mitochondria-controlled apoptosis, and increases stability of nuclear and mitochondrial DNA.** (**A**) Percent of WT cells grown in medium with or without LCA and exhibiting a tubular mitochondrial network or fragmented mitochondria. Mitochondria were visualized by indirect immunofluorescence microscopy using monoclonal anti-porin primary antibodies and Alexa Fluor 568-conjugated goat anti-mouse IgG secondary antibodies. (**B** - **D**) Oxygen consumption by WT cells grown in medium with or without LCA (**B**), their mitochondrial membrane potential ΔΨ (**C**) and their ROS levels (D). ΔΨ and ROS were visualized in living cells by fluorescence microscopy using fluorescent dyes Rhodamine 123 or Dihydrorhodamine 123, respectively. (**E**) The resistance of WT cells pre-grown in medium with or without LCA to chronic oxidative, thermal and osmotic stresses. (**F**) Viability of WT cells pre-grown in medium with or without LCA and then treated for 1 h with hydrogen peroxide or acetic acid (AcOH) to induce mitochondria-controlled apoptosis. (**G - I**) The frequencies of *rho*$^-$ and *rho*$^0$ mutations in mitochondrial DNA (**G**), *rib2* and *rib3* mutations in mitochondrial DNA (**H**), and of *can1* (Can$^r$) mutations in nuclear DNA (I) of WT cells grown in medium with or without LCA. Data in **A** - **D** and **F** - **I** are presented as means ± SEM (n = 4-17; ***p < 0.001; **p < 0.01). WT cells grown on 0.2% glucose in the presence or absence of LCA were taken for analyses at day 7, when they reached reproductive maturation by entering into ST phase.

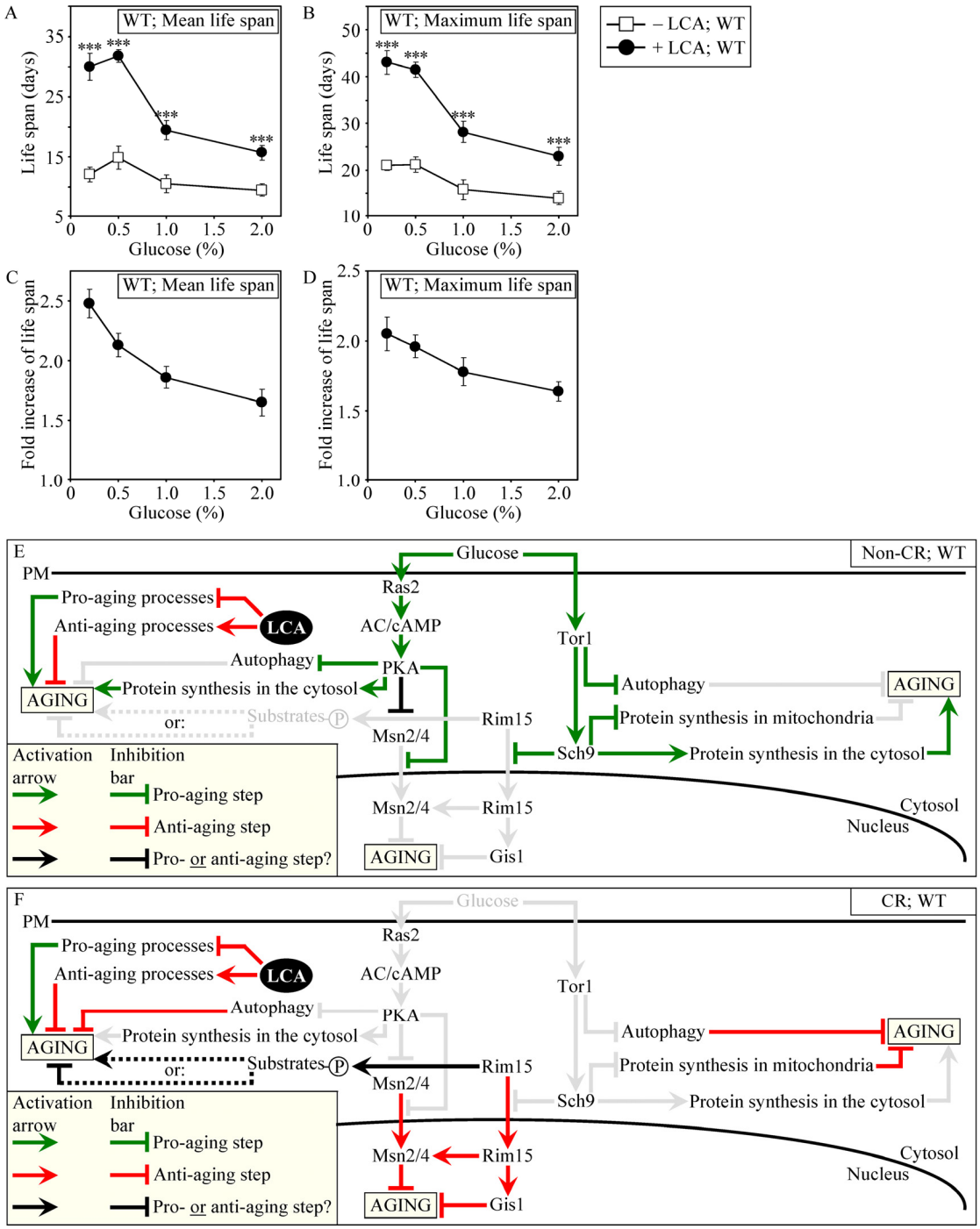



**Figure 8. LCA increases the CLS of WT strain to the highest extent under CR conditions.** (**A** and **B**) Effect of LCA on the mean (**A**) and maximum (**B**) life spans of chronologically aging WT strain. Data are presented as means ± SEM (n = 12-28; ***p < 0.001). (**C** - **E**) Effect of LCA on the fold increase in the mean (**C**) or maximum (**D**) life span of chronologically aging WT strain. Data are presented as means ± SEM (n = 12-28). Cells in **A** to **D** were cultured in medium initially containing 0.2%, 0.5%, 1% or 2% glucose in the presence of LCA (50 μM) or in its absence. Survival data are provided in Figure S9. (**E** and **F**) Outline of pro- and anti-aging processes that are controlled by the TOR and/or cAMP/PKA signaling pathways and are modulated by LCA in WT cells grown under non-CR (**E**) or CR (**F**) conditions. Activation arrows and inhibition bars denote pro-aging processes (displayed in green color), anti-aging processes (displayed in red color) or processes whose role in longevity regulation is presently unknown (displayed in black color). Doted lines denote hypothetical processes. Abbreviations: PM, plasma membrane.

Our evaluation of the life-extending efficacy of LCA in WT strain on a high- or low-calorie diet revealed that this compound increased CLS irrespective of the number of available calories (Figures 8A and 8B). Intriguingly, the extent to which LCA extended longevity was highest under CR conditions (Figures 8C and 8D), when the pro-aging processes modulated by the adaptable TOR and cAMP/PKA pathways are suppressed and the anti-aging processes are activated (Figure 8F). The life-extending efficacy of LCA in CR yeast significantly exceeded that in yeast on a high-calorie diet (Figures 8C and 8D), in which the adaptable TOR and cAMP/PKA pathways greatly activate the pro-aging processes and suppress the anti-aging processes (Figure 8E). Altogether, these findings suggest that, consistent with its sought-after effect on a longevity signaling network, LCA mostly targets certain housekeeping longevity assurance pathways that do not overlap (or only partially overlap) with the adaptable TOR and cAMP/PKA pathways modulated by calorie availability (Figures 8E and 8F).

Consistent with our assumption that LCA extends longevity not by modulating the adaptable TOR pathway (Figures 9E and 9F), lack of Tor1p did not impair the life-extending efficacy of LCA under CR (Figures 9A to 9D). Importantly, by eliminating a master regulator of this key adaptable pathway that shortens the CLS of yeast on a high-calorie diet, the *tor1Δ* mutation abolished the dependence of the anti-aging efficacy of LCA on the number of available calories. In fact, LCA extended longevity of the *tor1Δ* mutant strain to a very similar degree under CR and non-CR conditions (Figures 9C and 9D).

We next assessed how the adaptable cAMP/PKA pathway influences the life-extending efficacy of LCA in yeast on a high- or low-calorie diet. Although the *ras2Δ* mutation greatly decreases the PKA protein kinase activity by eliminating a GTP-binding protein that activates adenylate cyclase responsible for the synthesis of the PKA activator cAMP (Figures S7E and S7F) [62], it did not abolish the ability of LCA to extend CLS under CR and non-CR conditions (Figures S7A and S7B). However, the life-extending efficacy of LCA was decreased by the *ras2Δ* mutation, as compared to that seen in WT cells exposed to this compound (Figures S7C and S7D). In spite of such partial reduction of the anti-aging potential of LCA in *ras2Δ*, LCA still significantly increased its CLS under CR and non-CR conditions (Figures S7C and S7D).

Thus, it seems that LCA extends longevity of chronologically aging yeast through two different mechanisms. Firstly, irrespective of the number of available calories, this bile acid targets certain housekeeping longevity assurance pathways that 1) inhibit some pro-aging processes and/or activate some anti-aging processes; and 2) do not overlap with the adaptable cAMP/PKA pathway modulated by calorie availability (Figure 10B). Secondly, we propose that LCA unmasks the anti-aging potential of PKA by activating PKA-dependent phosphorylation of the cytosolic pool of Rim15p (Figure 10B). Because such phosphorylation of Rim15p is known to inactivate its protein kinase activity (Figure 10A) [62], we hypothesize that, while the nuclear pool of Rim15p has a well established anti-aging function [5,6,27,62], the cytosolic pool of this nutrient-sensory protein kinase plays an essential pro-aging role by phosphorylating a compendium of proteins that promote aging only if phosphorylated (Figure 10B). Noteworthy, some of the Rim15p phosphorylation targets are involved in longevity regulation and reside outside the nucleus [68]. In our hypothesis, LCA can unmask the anti-aging potential of PKA only when PKA is activated by cAMP, *i.e.*, under non-CR conditions (Figures 8E and F). Consistent with our hypothesis on the two mechanisms underlying the anti-aging effect of LCA, lack of Ras2p only partially and to the same extent reduced the life- extending potential of LCA under both CR and non-CR conditions (Figures S7C and S7D), likely by impairing the mechanism in which LCA unmasks the anti-aging potential of PKA. The resulting inability of PKA to inhibit the proposed pro-aging role of the cytosolic pool of Rim15p in *ras2Δ* cells would make the Rim15p- dependent pro-aging mechanism constitutively active in these cells, regardless of the number of available calories or presence of LCA (Figures S7E and S7F).

The TOR and cAMP/PKA pathways converge on Rim15p whose nuclear pool plays a pivotal role in governing the life-extending effect of CR by enabling the establishment of an anti-aging transcriptional program driven by Msn2p, Msn4p and Gis1p (Figure 10A) [5,6,27,62]. Our evaluation of the life-extending efficacy of LCA in yeast lacking Rim15p further supported the notion that one of the two mechanisms underlying the anti-aging effect of this bile acid involves its ability to modulate certain housekeeping longevity assurance pathways that are not centered on Rim15p and do not overlap with the adaptable TOR and cAMP/PKA pathways. In fact, although the life-extending potential of LCA in rim15Δ was partially reduced (Figures S8C and S8D) due to the impairment of the Rim15p-centered mechanism of its anti-aging action (Figures S8E and S8F), LCA still significantly increased the CLS of rim15Δ under CR and non-CR conditions (Figures S8A to S8D). Importantly, by eliminating a key nutrient-sensory protein kinase on which the adaptable TOR and cAMP/PKA pathways converge to regulate longevity in a

calorie availability-dependent fashion, the *rim15Δ* mutation abolishedthe dependence of the anti-aging efficacy of LCA on the number of available calories (Figures S8C and S8D).

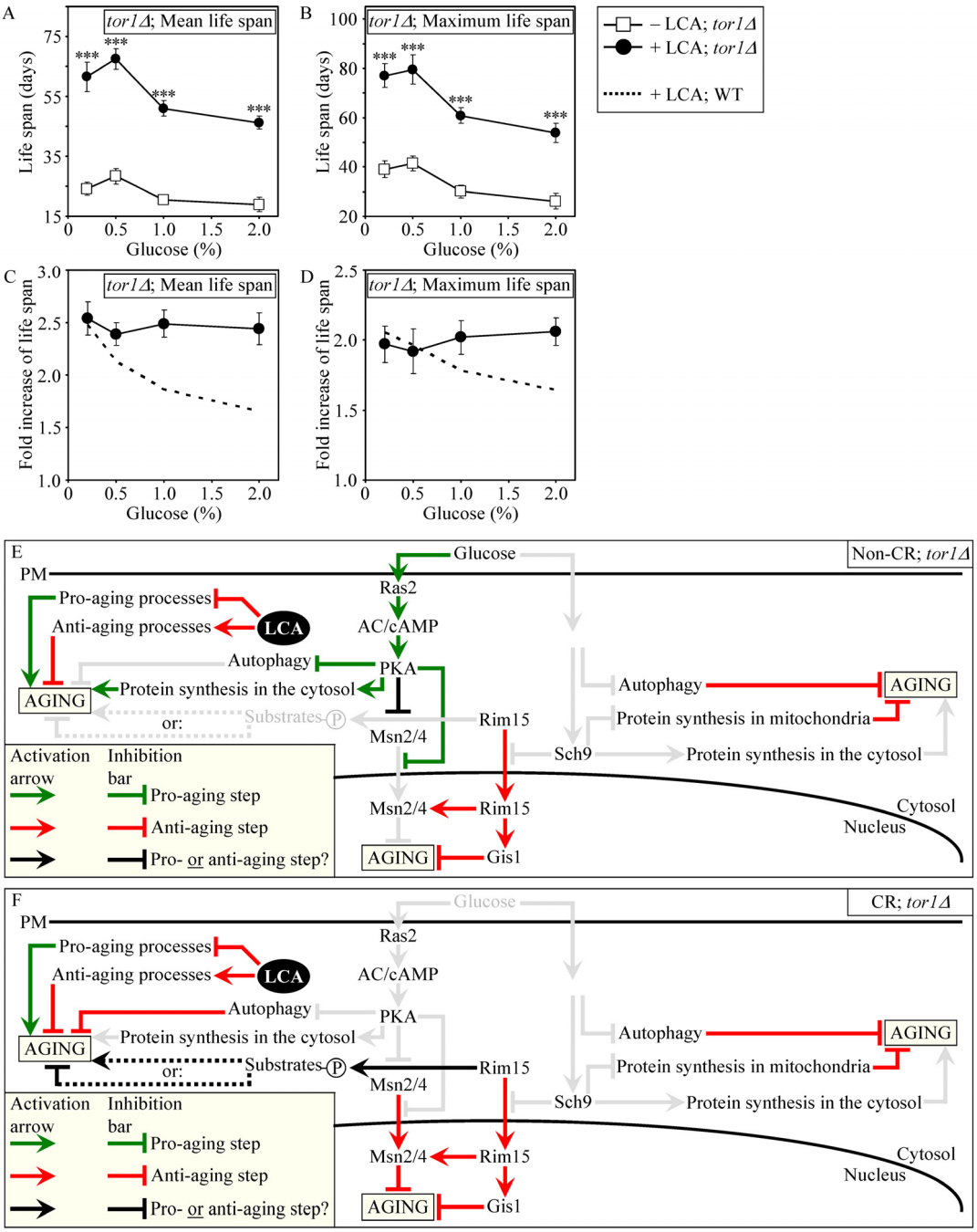



**Figure 9. Lack of Tor1p does not impair the life-extending effect of LCA and abolishes the dependence of the anti-aging efficacy of LCA on the number of available calories.** (**A** and **B**) Effect of LCA on the mean (**A**) and maximum (**B**) life spans of chronologically aging *tor1⊗* strain. Data are presented as means ± SEM (n = 4-7; ***p < 0.001). (**C** and **D**) Effect of LCA on the fold increase in the mean (**C**) or maximum (**D**) life spans of chronologically aging *tor1⊗* and WT strains. Data are presented as means ± SEM (n = 4-7). Cells in **A** to **D** were cultured in medium initially containing 0.2%, 0.5%, 1% or 2% glucose in the presence of LCA (50 μM) or in its absence. Survival data are provided in Figure S10. (**E** and **F**) Outline of pro- and anti-aging processes that are controlled by the TOR and/or cAMP/PKA signaling pathways and are modulated by LCA in *tor1⊗* cells grown under non-CR (**E**) or CR (**F**) conditions.

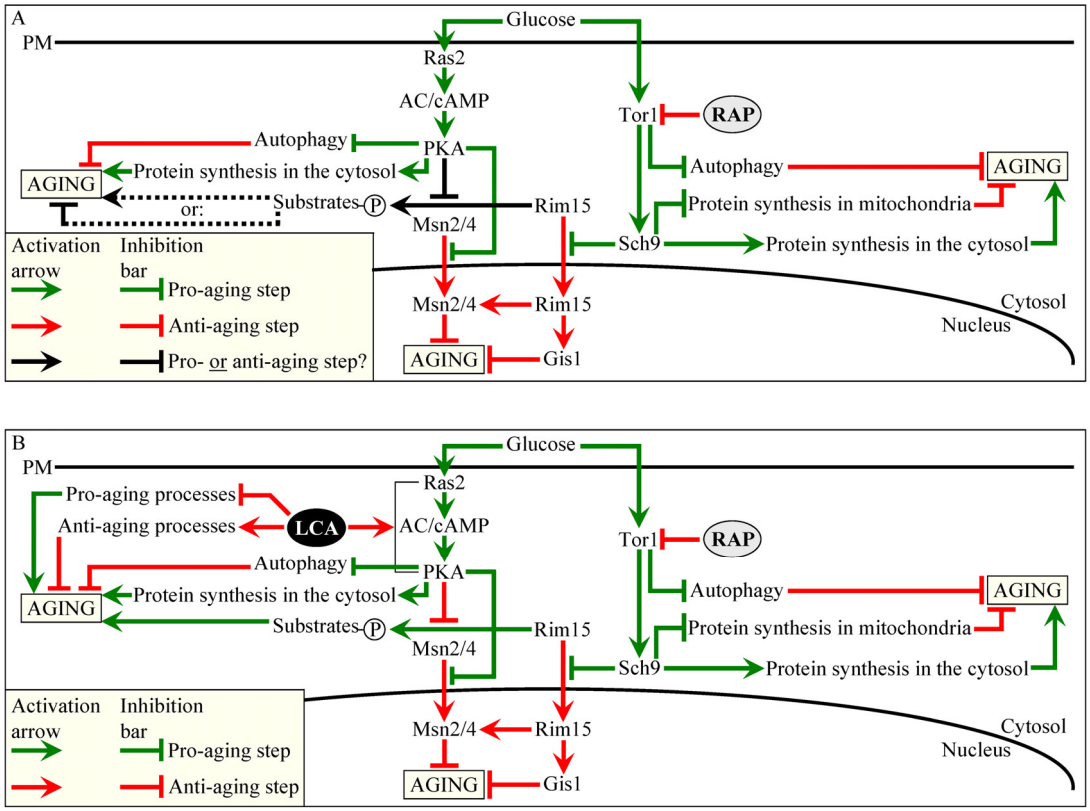



**Figure 10. Outline of pro- and anti-aging processes that are controlled by the TOR and/or cAMP/PKA signaling pathways and are modulated by LCA or rapamycin (RAP) in chronologically aging yeast.** The currently accepted (**A**) and updated, based on this study (**B**), outlines of pro- and anti-aging processes are shown. Activation arrows and inhibition bars denote pro-aging processes (displayed in green color), anti-aging processes (displayed in red color) or processes whose role in longevity regulation was unknown (displayed in black color). Doted lines denote hypothetical, until this study, processes. See text for details.

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## DISCUSSION

In this study, we designed a chemical genetic screen for small molecules that increase the CLS of yeast under CR conditions by targeting lipid metabolism and modulating housekeeping longevity pathways that regulate longevity irrespective of the number of available calories. Our screen identifies LCA as one of such molecules. Our analysis of how LCA influences various longevity-related processes and how it affects the CLS of yeast mutants impaired in the adaptable TOR and cAMP/PKA longevity pathways provided important new insights into mechanisms of longevity regulation, as outlined below.

### LCA extends yeast CLS by modulating housekeeping longevity assurance processes that are not regulated by the adaptable TOR and cAMP/PKA signaling pathways

Our findings imply that LCA extends longevity of chronologically aging yeast by targeting two different mechanisms. One mechanism extends longevity regardless of the number of available calories. This mechanism involves the LCA-governed modulation of certain housekeeping longevity assurance pathways that do not overlap with the adaptable TOR and cAMP/PKA pathways (Figure 10B). We identify a compendium of processes that compose LCA-targeted housekeeping longevity assurance pathways. Our data provide

evidence that LCA modulates these pathways by 1) suppressing the pro-aging process [39,40,50] of lipid-induced necrotic cell death, perhaps due to its observed ability to reduce the intracellular levels of FFA and DAG that trigger such death; 2) attenuating the pro-aging process [69,70] of mitochondrial fragmentation, a hallmark event of age-related cell death; 3) altering oxidation-reduction processes in mitochondria - such as oxygen consumption, the maintenance of membrane potential and ROS production - known to be essential for longevity regulation [8,10,11,71]; 4) enhancing cell resistance to oxidative and thermal stresses, thereby activating the anti-aging process [11,39,40,72,73] of stress response; 5) suppressing the pro-aging process [69,70] of mitochondria-controlled apoptosis; and 6) enhancing stability of nuclear and mitochondrial DNA, thus activating the anti-aging process [74,75] of genome maintenance. The observed pleiotropic effect of LCA on a compendium of housekeeping longevity assurance processes implies that this bile acid is a multi-target life-extending compound that increases CLS in yeast by modulating a network of the highly integrated processes that are not controlled by the adaptable TOR and cAMP/PKA pathways. The major challenge now is to define the molecular mechanisms by which LCA modulates each of these pro- and anti-aging housekeeping processes and integrates them in chronologically aging yeast.

The other mechanism underlying the life-extending effect of LCA in chronologically aging yeast increases life span only under non-CR conditions. This mechanism consists in LCA-driven unmasking of the previously unknown anti-aging potential of PKA, a key player in the adaptable cAMP/PKA pathway. We propose that LCA unveils the anti-aging potential of PKA by activating PKA-dependent phosphorylation of the cytosolic pool of Rim15p, a key nutrient-sensory protein kinase on which the adaptable TOR and cAMP/PKA pathways converge to regulate longevity in a calorie availability-dependent fashion (Figure 10B). Of note, the nuclear pool of Rim15p is well known for its anti-aging role in governing the life-extending effect of CR by enabling a pro-longevity transcriptional program driven by Msn2p, Msn4p and Gis1p (Figure 10B) [6,62]. In our hypothesis 1) unlike its nuclear pool, the cytosolic pool of Rim15p has an essential pro-aging function in phosphorylating a compendium of its cytosolic target proteins [68] some of which promote aging only if phosphorylated (Figure 10B); 2) under non-CR conditions LCA activates the PKA-dependent phosphorylation of Rim15p (Figure 10B); and 3) because the phosphorylation of Rim15p inactivates its protein kinase activity [62], the dephosphorylation of pro-aging target proteins of Rim15p in the cytosol by phosphatases inhibits the ability of these target proteins to promote aging (Figure 10B). To test the validity of our hypothesis, we are currently evaluating how genetic manipulations that alter the abundance of various extra-nuclear target proteins of Rim15p or affect their phosphorylation status influence the life-extending efficacy of LCA.

**Bile acids are beneficial to health and longevity across phyla**

It should be stressed that, although we found that LCA greatly extends yeast longevity, yeast do not synthesize this or any other bile acid found in mammals [57,76]; our mass spectrometry-based analysis of the total yeast lipidome has confirmed lack of endogenous bile acids. One could envision that during evolution yeast have lost the ability to synthesize bile acids but have maintained the life-extending response to these biologically active molecules by retaining certain longevity-related processes that are sensitive to regulation by bile acids. Alternatively, one could think that during evolution yeast have developed the ability to sense bile acids produced by mammals (and/or bile acid-like lipids synthesized by worms), recognize these mildly toxic molecules as environmental stressors providing hormetic benefits and/or as indicators of the state of the environment or food supply, and then to respond by undergoing certain life-extending changes to their physiology that ultimately increase their chances of survival. It is conceivable therefore that the life-extending potential of LCA and other bile acids as well as, probably, the mechanisms underlying their anti-aging action are evolutionarily conserved.

In fact, following their synthesis from cholesterol in the intestine, hypodermis, spermatheca and sensory neurons of worms, bile acid-like dafachronic acids (including 3-keto-LCA) are delivered to other tissues where they activate the DAF-12/DAF-16 signaling cascade that in turn orchestrates an anti-aging transcriptional program, thereby increasing the life span of the entire organism [41]. Bile acids also provide health benefits to mammals. Synthesized from cholesterol in hepatocytes of the liver, these amphipathic molecules have been for a long time considered to function only as trophic factors for the enteric epithelium and as detergents for the emulsification and absorption of dietary lipids and fat-soluble vitamins [57,76,77]. Recent years have been marked by a significant progress in our understanding of the essential role that bile acids play as signaling molecules regulating lipid, glucose and energy homeostasis and activating detoxification of xenobiotics [57,77,78]. By stimulating the G-protein-coupled receptor TGR5, bile acids activate the cAMP/PKA

signaling pathway that 1) enhances energy expenditure in brown adipose tissue and muscle by stimulating mitochondrial oxidative phosphorylation and uncoupling; 2) improves liver and pancreatic function by activating the endothelial nitric oxide synthase; and 3) enhances glucose tolerance in obese mice by inducing intestinal glucagon-like peptide-1 release [57,76,78]. Furthermore, by activating the farnesoid X receptor (FXR) and several other nuclear hormone receptors inside mammalian cells, bile acids 1) modulate the intracellular homeostasis of cholesterol, neutral lipids and fatty acids; 2) regulate glucose metabolism by enhancing glycogenesis and attenuating gluconeogenesis; and 3) stimulate clearance of xenobiotic and endobiotic toxins by activating transcription of numerous xenobiotic detoxification genes [57,76-78]. All these health-improving, beneficial metabolic effects of bile acids prevent the development of obesity following administration of high-fat diet [57,76,77]. Thus, bile acids have a great potential as pharmaceutical agents for the treatment of diabetes, obesity and various associated metabolic disorders, all of which are age-related [57,76]. Moreover, bile acids have been shown to inhibit neuronal apoptosis in experimental rodent models of neurodegenerative disorders by promoting mitochondrial membrane stability, preventing the release of cytochrome c from mitochondria, reducing activities of various caspases, and activating the NF-κB, PI3K and MAPK survival pathways [79,80].

It should be stressed that many of the metabolic, stress response and apoptotic processes modulated by bile acids in mammals are essential for healthy aging and longevity regulation. Importantly, we found that, by modulating several of these health- and longevity-related processes in chronologically aging yeast, LCA increases their life span. Moreover, the long-lived $Ghrhr^{lit/lit}$ mice displayed elevated levels of several bile acids and exhibited increased FXR-dependent transcription of numerous xenobiotic detoxification genes; if administered to food consumed by wild-type mice, cholic acid - one of these bile acids - mimicked the FXR-governed gene expression pattern observed in $Ghrhr^{lit/lit}$ mice [81,82]. It has been therefore proposed that, by promoting chemical hormesis in mammals, these mildly toxic molecules with detergent-like properties may extend their longevity by acting as endobiotic regulators of aging [73,82,83].

Altogether, these findings support the notion that bile acids act as endobiotic and xenobiotic regulators of aging that are beneficial to health and longevity across phyla. A comparative analysis of the mechanisms underlying such health-improving and life-extending action of bile acids implies that these mechanisms are likely to be evolutionarily conserved.

## METHODS

Yeast strains and growth conditions. The WT strain BY4742 (*MATα his3Δ 1 leu2Δ 0 lys2Δ 0 ura3Δ 0*) and single-gene-deletion mutant strains in the BY4742 genetic background (all from Open Biosystems) were grown in YP medium (1% yeast extract, 2% peptone) containing 0.2% 0.5%, 1% or 2% glucose as carbon source. Cells were cultured at 30°C with rotational shaking at 200 rpm in Erlenmeyer flasks at a "flask volume/medium volume" ratio of 5:1.

Chemical genetic screen for compounds that increase chronological life span (CLS). The screen was conducted at the High Throughput/Content Screening Facility at McGill University. The single-gene-deletion mutant strain *pex5Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ 0 pex5Δ::kanMX4*) from Open Biosystems was grown in YPA0.5D medium (1% yeast extract, 2% peptone, 50 µg/ml ampicillin, 0.5% glucose). 3-µl aliquots of the *pex5Δ* culture recovered from mid-logarithmic phase at a cell titre of $2 \times 10^7$ cells/ml were aliquoted into 96-well master microplates using a Beckman Coulter high density Biomek FXII replica pinning robot. Each well of a master microplate contained 96 µl of YPA0.5D medium. 1 µl of a compound stock solution from a commercially available library (each compound at 5 mM in dimethylsulfoxide (DMSO)) was added to each well using a Beckman Coulter high density Biomek FXII replica pinning robot. Wells of a master microplate supplemented with 1% DMSO (1 µl of DMSO per a well containing 3 µl of the *pex5Δ* culture and 96 µl of YPA0.5D medium) were used as negative controls. Each master plate was created in duplicate. The master microplates were sealed and incubated without shaking at 30°C in a moist chamber. At days 1, 7, 10 and 14 of the incubation of master microplates, a 3-µl aliquot of each culture was transferred into individual wells of a new (replica) microplate containing 97 µl of YPA0.5D medium. Following incubation of sealed replica microplates in a moist chamber for 16 hours at 30oC (to allow for growth of cells that were still viable), the optical density at 600 nm (OD600) of the culture in each well of the replica microplate was measured using a Molecular Devices Analyst HT plate reader. To calculate survival at each time point, the OD600 at a particular time point was divided by the OD600 at day 1. "Cherry-picking" of the identified "lead" compounds for possible "hits" was carried out as described above, with each lead compound being used at a final

concentration of 5, 10, 25 or 50 μM and assessed in triplicate for validation. Commercially available structural analogs of hit compounds were identified using the web-based eMolecules searching engine. In total, approximately 19,000 representative compounds from the BIOMOL, Chembridge, Maybridge, MicroSource Discovery, NIH Clinical Collection, Prestwick Chemical Inc. and Sigma-LOPAC commercial libraries were tested using the screen for chemical modulators of longevity.

Pharmacological manipulation of CLS. CLS analysis was performed as previously described [39]. The chenodeoxycholic (C9377), cholic (C1129), dehydrocholic (D3750), deoxycholic (D2510), hyodeoxycholic (H3878), lithocholic (L6250) and ursodeoxycholic (U5127) bile acids were from Sigma. Their stock solutions in DMSO were made on the day of adding each of these compounds to cell cultures. Compounds were added to growth medium at the indicated concentration immediately following cell inoculation. The final concentration of DMSO in yeast cultures supplemented with a bile acid (and in the corresponding control cultures supplemented with drug vehicle) was 1% (v/v).

Miscellaneous procedures. Fluorescence [39], immuno-fluorescence [39] and electron [84] microscopies followed by morphometric analyses of the resulting images have been described elsewhere. Extraction of lipids and their separation, identification and quantitation with the help of TLC were performed according to established procedures [84]. Mass spectrometric identification and quantitation of various lipid species were carried as previously described [85]. Subcellular fractionation and organelle purification, cell viability and stress resistance assays, oxygen consumption assay, the measurement of the frequencies of spontaneous point and deletion mutations in mitochondrial and nuclear DNA, total cell lysates preparation, and mass spectrometric identification and quantitation of proteins were performed according to established procedures [39].

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## CONFLICT OF INTERESTS STATEMENT

The authors of this manuscript have no conflict of interests to declare.

## REFERENCES

**1.** Guarente LP, Partridge L and Wallace DC. Molecular Biology of Aging. 2008; (Cold Spring Harbor: Cold Spring Harbor Laboratory Press).

**2.** Greer EL, Brunet A. Signaling networks in aging. J Cell Sci. 2008; 121: 407-412.

**3.** Narasimhan SD, Yen K, Tissenbaum HA. Converging pathways in lifespan regulation. Curr Biol. 2009; 19: R657-R666.

**4.** Shaw RJ. LKB1 and AMP-activated protein kinase control of mTOR signalling and growth. Acta Physiol. 2009; 196: 65-80.

**5.** Fontana L, Partridge L, Longo VD. Extending healthy life span - from yeast to humans. Science 2010; 328: 321-326.

**6.** Wei M, Fabrizio P, Hu J, Ge H, Cheng C, Li L, Longo VD. Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9. PLoS Genet. 2008; 4: e13.

**7.** Laplante M, Sabatini DM. mTOR signaling at a glance. J Cell Sci. 2009; 122: 3589-3594.

**8.** Finley LW, Haigis MC. The coordination of nuclear and mitochondrial communication during aging and calorie restriction. Ageing Res Rev. 2009; 8: 173-188.

**9.** Soukas AA, Kane EA, Carr CE, Melo JA, Ruvkun G. Rictor/TORC2 regulates fat metabolism, feeding, growth, and life span in *Caenorhabditis elegans*. Genes Dev. 2009; 23: 496-511.

**10.** Giorgio M, Trinei M, Migliaccio E, Pelicci PG. Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? Nat Rev Mol Cell Biol. 2007; 8: 722-728.

**11.** Lapointe J, Hekimi S. When a theory of aging ages badly. Cell Mol Life Sci. 2010; 67: 1-8.

**12.** Mair W, Dillin A. Aging and survival: the genetics of life span extension by dietary restriction. Annu Rev Biochem. 2008; 77: 727-754.

**13.** Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, Allison DB, Cruzen C, Simmons HA, Kemnitz JW, Weindruch R. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science 2009; 325: 201-204.

**14.** Masoro EJ. (2002). Caloric Restriction: A Key to Understanding and Modulating Aging (Amsterdam: Elsevier).

**15.** Min KJ, Flatt T, Kulaots I, Tatar M. Counting calories in *Drosophila* diet restriction. Exp Gerontol. 2007; 42: 247-251.

**16.** Weindruch R and Walford RL. (1988). The Retardation of Aging and Disease by Dietary Restriction (Springfield: Thomas).

**17.** Zimmerman JA, Malloy V, Krajcik R, Orentreich N. Nutritional control of aging. Exp Gerontol. 2003; 38: 47-52.

**18.** Piper MD, Mair W, Partridge L. Counting the calories: the role of specific nutrients in extension of life span by food restriction. J Gerontol A Biol Sci Med Sci. 2005; 60: 549-555.

**19.** Blagosklonny MV. Aging and immortality: quasi-programmed senescence and its pharmacologic inhibition. Cell Cycle 2006; 5: 2087-2102.

**20.** Blagosklonny MV. Aging: ROS or TOR. Cell Cycle 2008; 7: 3344-3354.

**21.** Blagosklonny MV. TOR-driven aging: speeding car without brakes. Cell Cycle 2009; 8: 4055-4059.
**22.** Kaeberlein M, Powers RW 3rd, Steffen KK, Westman EA, Hu D, Dang N, Kerr EO, Kirkland KT, Fields S, Kennedy BK. Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. Science 2005; 310: 1193-1196.
**23.** Meissner B, Boll M, Daniel H, Baumeister R. Deletion of the intestinal peptide transporter affects insulin and TOR signaling in *Caenorhabditis elegans*. J Biol Chem. 2004; 279: 36739-36745.
**24.** Hansen M, Taubert S, Crawford D, Libina N, Lee SJ, Kenyon C. Lifespan extension by conditions that inhibit translation in *Caenorhabditis elegans*. Aging Cell 2007; 6: 95-110.
**25.** Chen D, Guarente L. SIR2: a potential target for calorie restriction mimetics. Trends Mol Med. 2007; 13: 64-71.
**26.** Kaeberlein M, Powers RW 3rd. Sir2 and calorie restriction in yeast: a skeptical perspective. Ageing Res Rev. 2007; 6: 128-140.
**27.** Medvedik O, Lamming DW, Kim KD, Sinclair DA. MSN2 and MSN4 link calorie restriction and TOR to sirtuin-mediated lifespan extension in *Saccharomyces cerevisiae*. PLoS Biol. 2007; 5: e261.
**28.** Greer EL, Brunet A. Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in *C. elegans*. Aging Cell 2009; 8: 113-127.
**29.** Ingram DK, Zhu M, Mamczarz J, Zou S, Lane MA, Roth GS, deCabo R. Calorie restriction mimetics: an emerging research field. Aging Cell 2006; 5: 97-108.
**30.** Lane MA, Roth GS, Ingram DK. Caloric restriction mimetics: a novel approach for biogerontology. Methods Mol Biol. 2007; 371: 143-149.
**31.** Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. Nature 2003; 425: 191-196.
**32.** Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, Sinclair D. Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature 2004; 430: 686-689.
**33.** Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG et al. Resveratrol improves health and survival of mice on a high-calorie diet. Nature 2006; 444: 337-342.
**34.** Petrascheck M, Ye X, Buck LB. An antidepressant that extends lifespan in adult *Caenorhabditis elegans*. Nature 2007; 450: 553-556.
**35.** Onken B, Driscoll M. Metformin induces a dietary restriction-like state and the oxidative stress response to extend *C. elegans* healthspan via AMPK, LKB1, and SKN-1. PLoS ONE 2010; 5: e8758.
**36.** McColl G, Killilea DW, Hubbard AE, Vantipalli MC, Melov S, Lithgow GJ. Pharmacogenetic analysis of lithium-induced delayed aging in *Caenorhabditis elegans*. J Biol Chem. 2008; 283: 350-357.
**37.** Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, Partridge L. Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila melanogaster*. Cell Metab. 2010; 11: 35-46.
**38.** Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, Machado De Oliveira R, Leid M, McBurney MW, Guarente L. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-γ. Nature 2004; 429: 771-776.
**39.** Goldberg AA, Bourque SD, Kyryakov P, Gregg C, Boukh-Viner T, Beach A, Burstein MT, Machkalyan G, Richard V, Rampersad S, Cyr D, Milijevic S, Titorenko VI. Effect of calorie restriction on the metabolic history of chronologically aging yeast. Exp Gerontol. 2009; 44: 555-571.
**40.** Goldberg AA, Bourque SD, Kyryakov P, Boukh-Viner T, Gregg C, Beach A, Burstein MT, Machkalyan G, Richard V, Rampersad S, Titorenko VI. A novel function of lipid droplets in regulating longevity. Biochem Soc Trans. 2009; 37: 1050-1055.
**41.** Russell SJ, Kahn CR. Endocrine regulation of ageing. Nat Rev Mol Cell Biol. 2007; 8: 681-691.
**42.** Wang MC, O'Rourke EJ, Ruvkun G. Fat metabolism links germline stem cells and longevity in *C. elegans*. Science 2008; 322: 957-960.
**43.** Narbonne P, Roy R. *Caenorhabditis elegans* dauers need LKB1/AMPK to ration lipid reserves and ensure long-term survival. Nature 2009; 457: 210-214.
**44.** Grönke S, Mildner A, Fellert S, Tennagels N, Petry S, Müller G, Jäckle H, Kühnlein RP. Brummer lipase is an evolutionary conserved fat storage regulator in *Drosophila*. Cell Metab. 2005; 1: 323-330.
**45.** Blüher M, Kahn BB, Kahn CR. Extended longevity in mice lacking the insulin receptor in adipose tissue. Science 2003; 299: 572-574.
**46.** Chiu CH, Lin WD, Huang SY, Lee YH. Effect of a C/EBP gene replacement on mitochondrial biogenesis in fat cells. Genes Dev. 2004; 18: 1970-1975.
**47.** Haemmerle G, Lass A, Zimmermann R, Gorkiewicz G, Meyer C, Rozman J, Heldmaier G, Maier R, Theussl C, Eder S, Kratky D, Wagner EF, Klingenspor M et al. Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. Science 2006; 312: 734-737.
**48.** Gerhart-Hines Z, Rodgers JT, Bare O, Lerin C, Kim SH, Mostoslavsky R, Alt FW, Wu Z, Puigserver P. Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1α. EMBO J. 2007; 26: 1913-1923.
**49.** Léon S, Goodman JM, Subramani S. Uniqueness of the mechanism of protein import into the peroxisome matrix: transport of folded, co-factor-bound and oligomeric proteins by shuttling receptors. Biochim Biophys Acta 2006; 1763: 1552-1264.
**50.** Low CP, Liew LP, Pervaiz S, Yang H. Apoptosis and lipoapoptosis in the fission yeast *Schizosaccharomyces pombe*. FEMS Yeast Res. 2005; 5: 1199-1206.
**51.** Dirkx R, Vanhorebeek I, Martens K, Schad A, Grabenbauer M, Fahimi D, Declercq P, Van Veldhoven PP, Baes M. Absence of peroxisomes in mouse hepatocytes causes mitochondrial and ER abnormalities. Hepatology 2005; 41: 868-878.
**52.** D'Autréaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nat Rev Mol Cell Biol. 2007; 8: 813-824.
**53.** Eisenberg T, Büttner S, Kroemer G, Madeo F. The mitochondrial pathway in yeast apoptosis. Apoptosis 2007; 12: 1011-1023.
**54.** Pereira C, Silva RD, Saraiva L, Johansson B, Sousa MJ, Côrte-Real M. Mitochondria-dependent apoptosis in yeast. Biochim Biophys Acta 2008; 1783: 1286-1302.
**55.** Sinclair DA. Toward a unified theory of caloric restriction and longevity regulation. Mech. Ageing Dev. 2005; 126: 987-1002.

**56.** Powers RW 3rd, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S. Extension of chronological life span in yeast by decreased TOR pathway signaling. Genes Dev. 2006; 20: 174-184.
**57.** Thomas C, Pellicciari R, Pruzanski M, Auwerx J, Schoonjans K. Targeting bile-acid signalling for metabolic diseases. Nat Rev Drug Discov. 2008; 7: 678-693.
**58.** Wanke V, Cameroni E, Uotila A, Piccolis M, Urban J, Loewith R, De Virgilio C. Caffeine extends yeast lifespan by targeting TORC1. Mol Microbiol. 2008; 69: 277-285.
**59.** Eisenberg T, Knauer H, Schauer A, Büttner S, Ruckenstuhl C, Carmona-Gutierrez D, Ring J, Schroeder S, Magnes C, Antonacci L, Fussi H, Deszcz L, Hartl R et al. Induction of autophagy by spermidine promotes longevity. Nat Cell Biol. 2009; 11: 1305-1314.

**60.** Huber A, Bodenmiller B, Uotila A, Stahl M, Wanka S, Gerrits B, Aebersold R, Loewith R. Characterization of the rapamycin-sensitive phosphoproteome reveals that Sch9 is a central coordinator of protein synthesis. Genes Dev. 2009; 23: 1929-1943.
**61.** Pan Y, Shadel GS. Extension of chronological life span by reduced TOR signaling requires down-regulation of Sch9p and involves increased mitochondrial OXPHOS complex density. Aging 2009; 1: 131-145.
**62.** Smets B, Ghillebert R, De Snijder P, Binda M, Swinnen E, De Virgilio C, Winderickx J. Life in the midst of scarcity: adaptations to nutrient availability in *Saccharomyces cerevisiae*. Curr Genet. 2010; 56: 1-32.
**63.** Stephan JS, Yeh YY, Ramachandran V, Deminoff SJ, Herman PK. The Tor and PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to control autophagy. Proc Natl Acad Sci USA 2009; 106: 17049-17054.
**64.** Kamada Y, Yoshino K, Kondo C, Kawamata T, Oshiro N, Yonezawa K, Ohsumi Y. Tor directly controls the Atg1 kinase complex to regulate autophagy. Mol Cell Biol. 2010; 30: 1049-1058.
**65.** Urban J, Soulard A, Huber A, Lippman S, Mukhopadhyay D, Deloche O, Wanke V, Anrather D, Ammerer G, Riezman H, Broach JR, De Virgilio C, Hall MN et al. Sch9 is a major target of TORC1 in *Saccharomyces cerevisiae*. Mol Cell (2007) 26: 663-674.
**66.** Yorimitsu T, Zaman S, Broach JR, Klionsky DJ. Protein kinase A and Sch9 cooperatively regulate induction of autophagy in *Saccharomyces cerevisiae*. Mol Biol Cell 2007; 18: 4180-4189.
**67.** Lee P, Cho BR, Joo HS, Hahn JS. Yeast Yak1 kinase, a bridge between PKA and stress-responsive transcription factors, Hsf1 and Msn2/Msn4. Mol Microbiol. 2008; 70: 882-895.
**68.** Ptacek J, Devgan G, Michaud G, Zhu H, Zhu X, Fasolo J, Guo H, Jona G, Breitkreutz A, Sopko R, McCartney RR, Schmidt MC, Rachidi N et al. Global analysis of protein phosphorylation in yeast. Nature 2005; 438: 679-684.
**69.** Fabrizio P, Longo VD. Chronological aging-induced apoptosis in yeast. Biochim. Biophys. Acta 2008; 1783: 1280-1285.
**70.** Hamann A, Brust D, Osiewacz HD. Apoptosis pathways in fungal growth, development and ageing. Trends Microbiol. 2008; 16: 276-283.
**71.** Skulachev VP, Anisimov VN, Antonenko YN, Bakeeva LE, Chernyak BV, Erichev VP, Filenko OF, Kalinina NI, Kapelko VI, Kolosova N*G*, Kopnin BP, Korshunova GA, Lichinitser MR et al. An attempt to prevent senescence: a mitochondrial approach. Biochim Biophys Acta 2009; 1787: 437-461.
**72.** Schulz TJ, Zarse K, Voigt A, Urban N, Birringer M, Ristow M. Glucose restriction extends *Caenorhabditis elegans* life span by inducing mitochondrial respiration and increasing oxidative stress. Cell Metab. 2007; 6: 280-293.
**73.** Gems D, Partridge L. Stress-response hormesis and aging: "that which does not kill us makes us stronger". Cell Metab. 2008; 7: 200-203.
**74.** Pang CY, Ma YS, Wei YU. MtDNA mutations, functional decline and turnover of mitochondria in aging. Front Biosci. 2008; 13: 3661-3675.
**75.** Sinclair DA, Oberdoerffer P. The ageing epigenome: damaged beyond repair? Ageing Res Rev. 2009; 8: 189-198.
**76.** Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile acid receptors in metabolic regulation. Physiol Rev. 2009; 89: 147-191.
**77.** Hylemon PB, Zhou H, Pandak WM, Ren S, Gil G, Dent P. Bile acids as regulatory molecules. J Lipid Res. 2009; 50: 1509-1520.
**78.** Vallim TQ, Edwards PA. Bile acids have the gall to function as hormones. Cell Metab. 2009; 10: 162-164.
**79.** Ramalho RM, Viana RJ, Low WC, Steer CJ, Rodrigues CM. Bile acids and apoptosis modulation: an emerging role in experimental Alzheimer's disease. Trends Mol. Med. 2008; 14: 54-62.
**80.** Amaral JD, Viana RJ, Ramalho RM, Steer CJ, Rodrigues CM. Bile acids: regulation of apoptosis by ursodeoxycholic acid. J Lipid Res. 2009; 50: 1721-1734.
**81.** Amador-Noguez D, Yagi K, Venable S, Darlington G. Gene expression profile of long-lived Ames dwarf mice and Little mice. Aging Cell 2004; 3: 423-441.
**82.** Amador-Noguez D, Dean A, Huang W, Setchell K, Moore D, Darlington G. Alterations in xenobiotic metabolism in the long-lived Little mice. Aging Cell 2007; 6: 453-470.
**83.** Gems D. Long-lived dwarf mice: are bile acids a longevity signal? Aging Cell 2007; 6: 421-423.
**84.** Guo T, Gregg C, Boukh-Viner T, Kyryakov P, Goldberg A, Bourque S, Banu F, Haile S, Milijevic S, San KH, Solomon J, Wong V, Titorenko VI. A signal from inside the peroxisome initiates its division by promoting the remodeling of the peroxisomal membrane. J Cell Biol. 2007; 177: 289-303.
**85.** Bourque SD, Titorenko VI. A quantitative assessment of the yeast lipidome using electrospray ionization mass spectrometry. J Vis Exp. 2009; 30: doi: 10.3791/1513.

## SUPPLEMENTAL DATA

Please check the link "Supplemental Data" in full text version of this manuscript to find the related Supplemental Figures and Tables.

Hypothesis

# Why men age faster but reproduce longer than women: mTOR and evolutionary perspectives

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***Key words:*** *aging, reproduction, longevity, lifespan, rapamycin, menopause*
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**Abstract: Women live longer than men. Yet, it is believed that men do not age faster than women but simply are weaker at every age. In contrast, I discuss that men age faster. From evolutionary perspective, high accidental death rate in young males is compatible with fast aging. Mechanistically, hyper-activated mTOR (Target of Rapamycin) may render young males robust at the cost of accelerated aging. But if women age slower, why then is it women who have menopause? Some believe that menopause is programmed and purposeful (grandmother theory). In contrast, I discuss how menopause is not programmed but rather is an aimless continuation of the same program that initially starts reproduction at puberty. This quasi-program causes over-activation of female reproductive system, which is very vulnerable to over-activation. Mechanisms of aging and menopause are discussed.**

## Longevity: men and women

Women have lived longer than men in different countries and in every era [1]. In 1980 in the USA, the estimated life expectancy at birth was 70 years for men and 77.5 years for women [2]. In the world, 75% and 90% of people older than 100 years and 110 years (respectively) are women. And the longest living person (122 years old) was a woman. But do women age slower than men? The conventional opinion is that women and men age at the same rate but men are 'less robust' than women [1]. Seemingly in agreement, the mortality rate is lower in young women compared with young men. In women, the mortality rate is lower at every age, even in childhood. In other words, "women do not live longer than men because they age slowly, but because they are more robust at every age" [1]. This reasoning would be correct if causes of death were the same at every age. However, young and old men die from different causes. Young men die from accidents, while old men die from aging (technically speaking, from age-related diseases).

## High accidental death rate and fast aging (evolutionary perspective)

There is a very noticeable jump of mortality in the late teens in men [1]. Young men are often engaged in competitive, reckless, and dangerous activities. Therefore, even in modern society, the accidental death rate is high in young men. Historically, the accidental death rate in men was much higher than it is now. (Due to a fierce competition for status and mates, due to fights and wars, young men were killed at a very high rate). So, historically, men had lower chances to survive into old age than women had. And, according to evolutionary theory, a high accidental death rate determines fast aging [3-5]. If most men died young from accidental death, then they could not live long enough to experience aging. Then there was no natural selection to postpone aging. So accelerated aging in men is predictable from evolutionary perspective. But accelerated aging is also predictable mechanistically.

## Mechanistic explanation: antagonistic pleiotropy and mTOR

In males, muscle hypertrophy and heavy body helps to compete with other males. (In fact, men are larger than women.) Cellular growth and hypertrophy are stimulated by the mTOR (mammalian Target of Rapamycin) intracellular signaling pathway. Insulin, growth factors, amino acids, glucose lipoproteins, and testosterone all activate the mTOR pathway [6-9]. In turn, the mTOR pathway stimulates protein synthesis and cell size growth [10]. For example, skeletal muscle hypertrophy depends on the mTOR pathway [11, 12]. In addition, inhibition of the mTOR pathway decreases testosterone levels and spermatogenesis [13]. Thus, activation of mTOR may provide a selective advantage to young males.

On the other hand, the mTOR pathway is required forcellular senescence of mammalian cells [14-18]. Cellular aging is driven by the remaining activation of mitogenic signaling pathways in post-mitotic cells [19, 20]. In fact, mechanistically, aging is a continuation of growth, driven in part by mTOR [21]. In agreement, mTOR is involved in age-related diseases such as atherosclerosis, neurodegeneration, cancer, which are deadly manifestations of aging. (see for review [22-24]. And rapamycin prolongs lifespan in mammals [25].

Thus, over-activation of mTOR may provide an advantage (muscle hypertrophy, high levels of testosterone and high spermatogenesis) in early life at the cost of accelerated aging later in life. As an illuminating example, mice over-expressing growth hormone exhibit increased levels of IGF-I and adult body size, reduced life span and reproductive life span [26]. (Note: IGF-I stimulates mTOR, Figure 1).

## Accelerated age-related diseases in men

Humans do not die from "healthy" aging. Humans die from age-related diseases. The mTOR pathway is involved in age-related diseases such as cancer, atherosclerosis, hypertension, heart failure, osteoporosis, type II diabetes [22, 24, 27]. These diseases are deadly manifestations of aging. When aging is accelerated, age-related diseases occur earlier in life too. Healthy aging (a late onset of diseases) is associated with longevity (see for discussion [28]). For example, centenarians (100 years old or older) show a delay in the onset of age-related diseases, including cardiovascular disease, type 2 diabetes, cancer and Alzheimer's disease. In other words, those who age slower are healthier [29, 30].

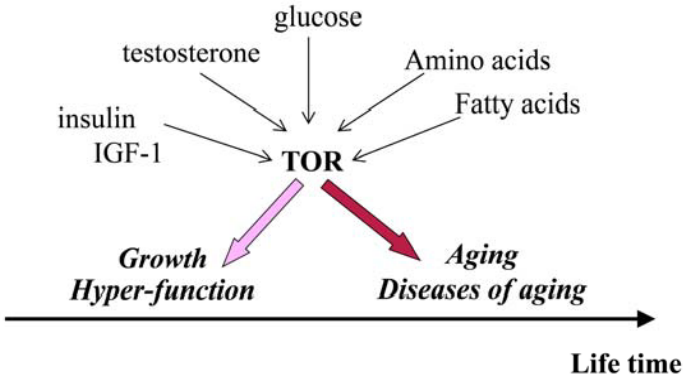


**Figure 1. Program of growth and quasi-program of aging.** The TOR pathway is activated by growth factors, hormones and nutrients. This activation is beneficial early in life by stimulating growth and muscle hypertrophy. Evolutionary perspective: This was especially important for prehistoric men, living in dangerous environment that required physical strength. mTOR is involved in aging later in life, but most men died young from accidental death. Thus, robustness early in life is associated with accelerated aging.

If women age slower than men, then age-related diseases must be delayed in women. In fact, most age-related diseases are delayed in women compared with men. For example, coronary atherosclerosis is postponed in women. Not only atherosclerosis, but also cancer and most other diseases of aging occur earlier in men than in women [31]. Women also live more years than men free of each of these diseases with the exception of arthritis [32]. Women rarely die from age-related diseases before menopause. The later onset of diseases in women compared with men suggests that women age slower than men.

Intriguingly, slower erosion of human telomeres favor females [33] and, even further, the rate of leukocyte telomere shortening predicts mortality from cardio-vascular disease in elderly men [34]. I speculate that high rate of telomere shortening reflects cellular hyper-activation and may be suppressed by rapamycin.

## Aging *versus* reproductive aging

Yet common wisdom holds that women age faster than men. One should not confuse aging and subjective perception of youthfulness and sexual attractiveness, which reflects fertility. Aging is an increase of the probability of death. And a 50-year-old man has higher chances to die than a 50- year-old woman. Furthermore, men acquire grey hair and wrinkles faster than women and thus men even 'look' older [35]. Although men age faster, they can reproduce longer. And here is another puzzle: why women undergo menopause.

Like aging itself, menopause is tolerated by natural selection, because women (until recently) did not live long enough to experience it. (In modern society, there must be a very strong natural selection for delayed menopause). So an evolutionary explanation is simple: ancestral women did not live long enough to have menopause. But male lifespan was even shorter: why then do men not have menopause? What is so special about female reproduction?

## Can menopause be programmed?

There is common opinion among traditional gerontologists that menopause is beneficial for women, has an evolutionary advantage and is adaptive [36-38]. It was suggested, for instance, that menopause prevents death of women in labor. The most popular is a "grandmother hypothesis" that menopause frees older women to help their daughters to raise grandchildren. This is a sort of group-selection hypothesis. Why do not daughters delay reproduction just in order to help their mothers raise siblings? Or what is the biological sense to stop reproduction, if a woman has no grandchildren living with her? The crucial assumption of 'grandmother' hypothesis is that menopause occurs only in humans [37]. Yet, menopause was documented in non-human primates, rodents, whales, dogs, rabbits, elephants and domestic livestock [39]. It was shown, for instance, that mice eventually undergo ovarian changes analogous to menopause in humans [40, 41].

It was shown that grandmothers may promote survival of their maternal grandchildren in Gambia [37]. Grandmothers are useful but menopause is not. There is no experimental evidence that menopause is beneficial even when women live with grandchildren in Gambia. Menopause accelerates age-related diseases such as atherosclerosis, osteoporosis and cancer [42, 43]. Reproductive death provides no selective benefit (unless group-selection theories of aging are correct) and 'grandmother hypothesis' contradicts the evolutionary theory. If aging is not programmed, then reproductive aging is not programmed too.

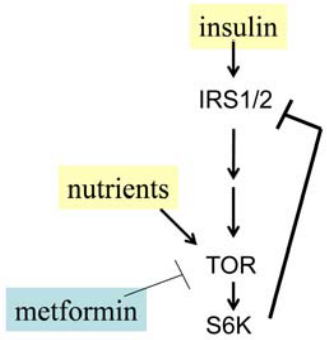


**Figure 2. Negative feedback and insulin resistance.** TOR is activated by nutrients and insulin and in turn causes depletion of IRS1/2 and insulin resistance. Whereas nutrients activate TOR, low nutrients and metformin deactivate TOR.

## TOR-driven quasi-programmed aging

Aging is not programmed but quasi-programmed [22, 44-46]. ("Quasi-" means "as if, resembling"). Quasi-program is an aimless continuation of a useful program that was not switched off upon its completion. Unlike a program, a quasi-program has no purpose. Develop-mental programs become aimless quasi-programs later in life. Quasi-programs are driven by antagonistic pleiotropic genes, which are beneficial early in life on the cost of aging later in life. Most genes that control aging and longevity constitute the mTOR pathway [22,

23]. mTOR is absolutely essential during embryonic development [47, 48]. In post-development, mTOR is involved in aging and age-related diseases [22].

Nutrients activate mTOR and cause insulin-resistance in cell culture [49, 50] as well as systemically in rodents and humans [51-54]. There is a negative feedback loop between insulin signaling and TOR (Figure 2). When mTOR is activated, it blocks insulin signaling (insulin resistance) [49, 55]. Noteworthy, insulin resistance is associated with premature menopause in some patients [56].

## The menstrual cycle is fragile

Since aging is not programmed, it does not hurt on purpose. It does not cause ovarian failure (menopause) on purpose. The logic of aging is simple: the most fragile systems fail first. A female reproductive system is fragile because it depends on exact interactions between The hypothalamus and ovaries, communicating via dozens of hormones. The menstrual cycle is regulated by interplay of negative and positive feedback loops. The hypothalamus stimulates the pituitary gland to secrete Follicle-Stimulating Hormone (FSH), which in turn stimulates follicles in the ovaries (Figure 3). Follicles maturate and secrete estrogens. Estrogens inhibit the hypothalamus, decreasing secretion of FSH (a negative feedback loop). In turn, FSH stimulates ovarian follicles, which produce estrogens, which in turn inhibit FSH production. Also, estrogens stimulate secretion of Lutenizing Hormone (LH). LH in turn causes ovulation. So for the normal menstrual cycle, the hypothalamus should have a narrow range of sensitivity to estrogens. Both too high and too low sensitivities are not compatible with menstrual cycles. In comparison, regulation of reproduction in men is simpler. There is a gradual decrease in fertility in men too (analogous to pre-menopause), although this usually does not result in testicular failure during a man's lifetime [57, 58].

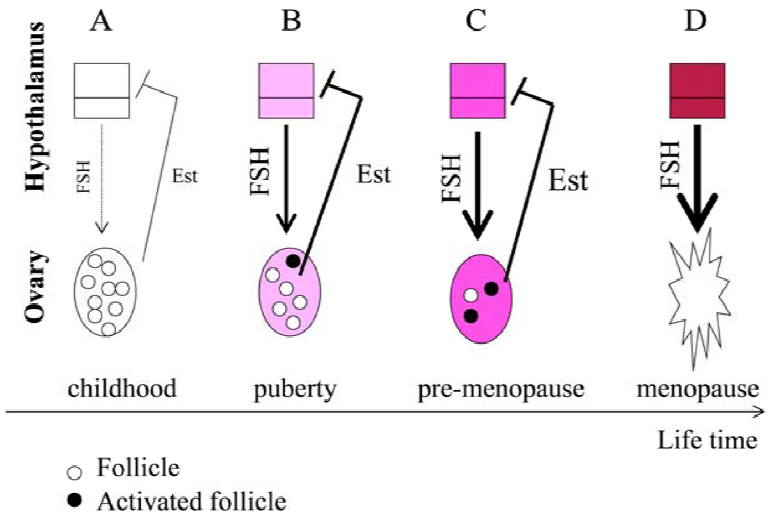


**Figure 3. From programmed puberty to quasi-programmed menopause.** For simplicity, only the FSH-estrogen feedback loop is shown. FSH stimulates follicles and production of estrogens (Est). Estrogens inhibit FSH production (negative feedback). (**A**) In girls, the hypothalamus is extremely sensitive to estrogens and even low levels of estrogens inhibit FSH. (**B**) The onset of menstrual cycle. While the hypothalamus is becoming resistant to estrogens, FSH stimulates the ovaries and estrogen production. Progressive activation of follicles from the dormant pool serves as the source of fertilizable ova. (**C**) Pre-menopause. While the hypothalamus is becoming progressively resistant to estrogens, FSH progressively over-stimulates the ovaries. (**D**) The ovaries fail. Menopause occurs when the primordial follicle pool is exhausted. Estrogen levels drop. The feedback between hypothalamus and the ovaries is disrupted.

## Quasi-programmed menopause

A half century ago, Vladimir Dilman proposed a "biological clock" that initially launches reproduction in puberty and then causes menopause [59, 60]. This idea is absolutely compatible with quasi-programmed nature of menopause, as discussed herein.

Before puberty, the hypothalamus is extremely sensitive to estrogens (Figure 3 A). Even low levels of estrogens suppress FSH production and, therefore, levels of FSH are low. At puberty, the hypothalamus becomes more resistant to estrogens. Then low levels of estrogens cannot suppress FSH. FSH in turn stimulates the ovarian follicles. Follicles produce estrogens, which in turn inhibit FSH production (Figure 3 B). During lifetime, resistance to estrogens continues to increase (Figure 3C). This ever-increasing resistance is an aimless continuation of the same program that initiated menstrual cycle at puberty. FSH is elevated in pre-menopause and rising serum FSH levels is one of the earliest signs of human female reproductive aging [61], [62]. Rising FSH levels over-stimulate the ovaries (Figure 3C), thus depleting follicles (Figure 3D).

FSH hyper-stimulates the ovaries, causing more follicles to be recruited simultaneously (Figure 3 C). This may explain the increased tendency of older mothers to have dizygotic twins [63]. Due to hypothalamic resistance to estrogens, estrogens cannot induce LH surges, which are necessary for ovulation. Therefore, follicles are recruited without progression to ovulation. Therefore, fertility gradually decreases long before menopause.

Hypothalamic resistance to estrogens causes higher FSH levels and lower LH pulses, disturbed feedback relationships and decrease in fertility [64]. Levels of estrogens tend to be increased in pre-menopause [64], but even increased estrogens cannot suppress FSH [61]. FSH over-stimulates follicle recruitment, leading eventually to follicular depletion (Figure 3D). This process eventually results in ovarian failure (Figure 3 D). Post-menopause is characterized by a drop in estrogen levels because of the depletion of follicular oocytes that normally produce estrogen (Figure 3 D).

Noteworthy, aged mouse ovaries possess rare premeiotic germ cells that can generate oocytes following transplantation into a young host environment [65], and even further young adult donor bone marrow infusions into female mice postpone age-related reproductive failure [66]. In other words, some follicles may become unresponsive due to age-associated over-stimulation but can be rejuvenated.

Thus, reproductive aging is set in motion at puberty by an ever-increasing hypothalamic resistance to estrogens. By increasing resistance of the hypothalamus to estrogens, the developmental program establishes the menstrual cycle at puberty. There is no program to cause menopause. It simply happens because resistance to estrogens (and some other hormones) is ever-increasing. This is an example of a quasi-program, a continuation of a program that was not switched off upon its completion (at puberty). The quasi-program interrupts the same reproductive function that the program establishes. The same mechanism (resistance of the hypothalamus to estrogen) first starts and then ends reproduction in women. An increased resistance to estrogens can explain both initiation and termination of the menstrual cycle.

How may we explain an increased resistance to estrogens? Resistance may be secondary to hyper-stimulation by estrogens themselves. In fact, in old acyclic mice, ovariectomy for 2 months partially reversed the hypothalamic resistance [41]. Hyper-stimulation of the hypothalamus by estrogens may cause resistance, in turn increasing stimulation of the ovary, until failure occurs. Alternatively, overstimulation of the hypothalamus with hormones and nutrients can cause estrogen-resistance. Is there a feedback resistance to overstimulation as shown in Figure 2? Then over-stimulation, with secondary resistance, is the driving cause of reproductive program and quasi-program. And most importantly over-stimulation occurs simultaneously both in the ovary and the brain.

### mTOR and menopause

I propose that the increasing activation of mTOR (both in the hypothalamus and the ovary) drives hormone resistance, causing the onset of reproduction and then hyper-stimulation of the ovary and the hypothalamus and finally menopause (Figure 4). Let us bring together several pieces of data.

First, mTOR is a regulator of puberty onset via modulation of the hypothalamus [67]. Also, both FSH and estrogens activate the mTOR pathway [68], [69]. So if TOR is activated constantly, it may not respond further to stimulation (hormone resistance).

Second, in mice lacking PTEN in oocytes, the entire primordial follicle pool is activated. Subsequently, all primordial follicles become depleted in early adulthood, causing premature ovarian failure [70]. PTEN loss results in suppression of Foxo, so the Foxo was a primer suspect [70]. Yet, in theory loss of PTEN must also result in mTOR overactivation (Figure 1). I suggest that

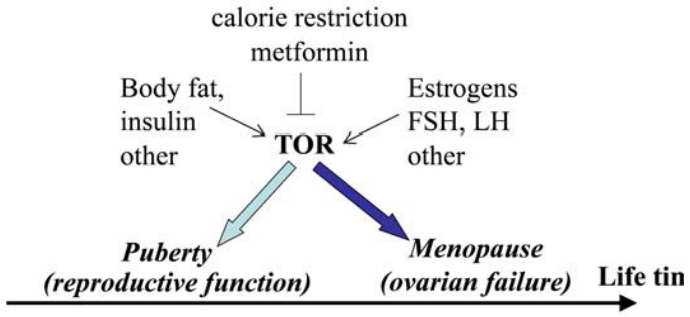


**Figure 4. Program of puberty and quasi-program of menopause.** The TOR pathway in the hypothalamus and the ovary is activated by growth factors, hormones (leptin, estrogens and FSH, LH, respectively) and nutrients. This activation starts menarche and then leads to menopause.

premature ovarian failure is caused by over-activation of TOR. (Note: this paper was initially written in 2008 and was ahead of its time and was not well received by conventional journals. Now it can be updated). It was shown tuberous sclerosis complex (Tsc), which negatively regulates mTOR, functions in oocytes to maintain the quiescence of primordial follicles. In mutant mice lacking the Tsc1 gene in oocytes, the entire pool of primordial follicles is activated prematurely due to elevated mTORC1 activity in the oocyte, ending up with follicular depletion in early adulthood and causing premature ovarian failure [71, 72].

Third, calorie restriction (CR) prevents age-related increase in estrogen resistance in the hypothalamus of old female mice [73]. As already discussed, CR de-activates TOR [74]. I speculate that CR de-activates mTOR and delays estrogen resistance in the hypothalamus. Simultaneously, by deactivating mTOR in the oocytes, it may delay their depletion.

It was shown almost a century ago [75] and then reproduced numerous times that CR extends lifespan and prevents age-related infertility in rodents. In most of these studies, CR was initiated at weaning, causing a delayed onset of sexual maturation. So, the same condition (CR) delays both puberty and menopause. This is consistent with the notion that a quasi-program (menopause) is a mere continuation of the program (puberty). But quasi-programs can be manipulated, exactly like programs. Recently it has been shown that a moderate caloric restriction initiated in rodents during adulthood sustains reproductive function of the female reproductive axis into advanced chronological age [76].

Fifth, metformin, an antidiabetic drug, activates AMPK and thus inhibits mTOR [77]. Furthermore, metformin inhibits mTOR in AMPK-independent manner too. Metformin restores ovulations in patients with premature menopause associated with polycystic ovary syndrome [56]. On the other hand, metformin delays a premature onset of the menstrual cycle [78]. So the same agent that inhibits the onset of reproductive function also inhibits its termination. This antagonistic pleiotropic effect is consistent with the notion of the same mechanism switching reproduction on and off. Metformin slowed down aging and the age-related switch-off of estrous function in mice [79]. Thus menopause can be delayed pharmacologically.

## CONCLUSION

This article presents two hypotheses. The first hypothesis explains (from both an evolutionary and mechanistic perspective) why aging is accelerated in men. From the evolutionary perspective, the high accidental death rate in young men determines an accelerated aging. A model of TOR-driven aging provides a mechanistic explanation. When the accidental death rate is high, it is important to be bigger and stronger. And the mTOR pathway is involved in growth and cellular hypertrophy. So, overactivated mTOR may be adaptive for young men.

But this can accelerate aging. At the cost of accelerated aging, over-stimulated mTOR pathway may provide an advantage earlier in life. And vice versa as discussed, “weak mTOR” provides disadvantage earlier in life and, vice versa, robustness and fast aging are associated

[28]. Noteworthy, "competitive, aggressive personality" among men is associated with atherosclerosis and earlier death from age-related coronary disease [80].

The second hypothesis explains why menopause in women occurs despite slow-aging. Simply, the regulation of the menstrual cycle is fragile. There is a fine balance between ovarian stimulation by FSH and feedback hypothalamic responsiveness to estrogens. The menstrual cycle is vulnerable. Menopause is an example of a quasi-program (a program that was not switched off after its completion). In puberty, an increasing resistance to estrogen starts reproduction (a program). A further increase in the resistance (a quasi-program) causes overactivation of the ovary, decreasing fertility. This process can be treated pharmacologically (as any other age-related disease) to postpone menopause Potential therapeutic interventions to postpone menopause (as well as abolishment of the harmful consequences of menopause) will be discussed in forthcoming book The *Origin of Aging*.

## CONFLICT OF INTERESTS STATEMENT

The author of this manuscript has no conflict of interests to declare.

## REFERENCES


**1.** Austad SN. Why women live longer than men: sex differences in longevity. Gend Med. 2006, 3: 79-92.

**2.** Verbrugge LM,Wingard DL. Sex differentials in health and mortality. Women Health 1987, 12: 103-145.

**3.** Medawar PB An unsolved problem in biology. London: HK Lewis, 1952.

**4.** Keller L,Genoud M. Extraordinary lifespans in ants: a test of evolutionary theories of ageing. Nature 1997, 389: 958-960.

**5.** Gardner MP, Gems D, Viney ME. Extraordinary plasticity in aging in Strongyloides ratti implies a gene-regulatory mechanism of lifespan evolution. Aging Cell 2006, 5: 315-323.

**6.** Kim E,Guan KL. RAG GTPases in nutrient-mediated TOR signaling pathway. Cell Cycle 2009, 8: 1014-1018.

**7.** Xu Y, Chen SY, Ross KN, Balk SP. Androgens induce prostate cancer cell proliferation through mammalian target of rapamycin activation and post-transcriptional increases in cyclin D proteins. Cancer Res. 2006, 66: 7783-7792.

**8.** Glazer HP, Osipov RM, Clements RT, Sellke FW, Bianchi C. Hypercholesterolemia is associated with hyperactive cardiac mTORC1 and mTORC2 signaling. Cell Cycle 2009, 8: 1738-1746.

**9.** Hands SL, Proud CG, Wyttenbach A. mTOR's role in ageing: protein synthesis or autophagy? Aging 2009, 586-597.

**10.** Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell 2006, 124: 471-484.

**11.** Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol. 2001, 3: 1014-1019.

**12.** Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD, Glass DJ. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat Cell Biol. 2001, 3: 1009-1013.

**13.** Skrzypek J,Krause W. Azoospermia in a renal transplant recipient during sirolimus (rapamycin) treatment. Andrologia 2007, 39: 198-199.

**14.** Demidenko ZN,Blagosklonny MV. Growth stimulation leads to cellular senescence when the cell cycle is blocked. Cell Cycle 2008, 7: 3355-3361.

**15.** Demidenko ZN, Zubova SG, Bukreeva EI, Pospelov VA, Pospelova TV, Blagosklonny MV. Rapamycin decelerates cellular senescence. Cell Cycle 2009, 8: 1888-1895.

**16.** Demidenko ZN, Shtutman M, Blagosklonny MV. Pharmacologic inhibition of MEK and PI-3K converges on the mTOR/S6 pathway to decelerate cellular senescence. Cell Cycle 2009, 8: 1896-1900.

**17.** Demidenko ZN,Blagosklonny MV. Quantifying pharmacologic suppression of cellular senescence: prevention of cellular hypertrophy versus preservation of proliferative potential. Aging 2009, 1: 1008-1016.

**18.** Pospelova TV, Demidenk ZN, Bukreeva EI, Pospelov VA, Gudkov AV, Blagosklonny MV. Pseudo-DNA damage response in senescent cells. Cell Cycle 2009, 8: 4112-4118.

**19.** Blagosklonny MV. Cell senescence and hypermitogenic arrest. EMBO Rep. 2003, 4: 358-362.

**20.** Blagosklonny MV. Cell senescence: hypertrophic arrest beyond restriction point. J Cell Physiol. 2006, 209:592-7.

**21.** Blagosklonny MV,Hall MN. Growth and aging: a common molecular mechanism. Aging 2009, 1: 357-362.

**22.** Blagosklonny MV. Aging and immortality: quasi-programmed senescence and its pharmacologic inhibition. Cell Cycle 2006, 5: 2087-2102.

**23.** Blagosklonny MV. An anti-aging drug today: from senescence-promoting genes to anti-aging pill. Drug Disc Today 2007, 12: 218-224.

**24.** Blagosklonny MV. Validation of anti-aging drugs by treating age-related diseases. Aging 2009, 1: 281-288.

**25.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandezr E, Miller RA. Rapamycin fed late in life extends lifespan in genetically heterogenous mice. Nature 2009, 460: 392-396.

**26.** Bartke A. Insulin and aging. Cell Cycle 2008, 7: 3338-3343.

**27.** Tsang CK, Qi H, Liu LF, Zheng XFS. Targeting mammalian target of rapamycin (mTOR) for health and diseases. Drug Disc Today 2007, 12: 112-124.

**28.** Blagosklonny MV. Why human lifespan is rapidly increasing: solving "longevity riddle" with "revealed-slow-aging" hypothesis. Aging 2: 177-182.

**29.** Perls T, Kunkel L, Puca A. The genetics of aging. Curr. Opin. Genet. Dev. 2002, 12: 362-369.

**30.** Curtis R, Geesaman BJ, DiStefano PS. Ageing and metabolism: drug discovery opportunities. Nat Rev Drug Discov. 2005, 4:569-80.

**31.** Giampaoli S. Epidemiology of major age-related diseases in women compared to men. Aging 2000, 12: 93-105.

**32.** Crimmins EM, Kim JK, Hagedorn A. Life with and without disease: women experience more of both. J Women Aging 2002,

14: 47-59.

**33.** Moller P, Mayer S, Mattfeldt T, Muller K, Wiegand P, Bruderlein S. Sex-related differences in length and erosion dynamics of human telomeres favor females. Aging 2009, 1: 733-739.

**34.** Epel ES, Merkin SS, Cawthon R, Blackburn EH, Adler NE, Pletcher MJ, Seeman TE. The rate of leukocyte telomere shortening predicts mortality from cardiovascular disease in elderly men. Aging 2009, 1: 81-88.

**35.** Bulpitt CJ, Markowe HL, Shipley MJ. Why do some people look older than they should? Postgrad Med J 2001, 77: 578-581.

**36.** Shanley DP,Kirkwood TB. Evolution of the human menopause. Bioessays 2001, 23: 282-287.

**37.** Shanley DP, Sear R, Mace R, Kirkwood TB. Testing evolutionary theories of menopause. Proc Biol Sci. 2007, 274: 2943-2949.

**38.** Kirkwood TB. Understanding ageing from an evolutionary perspective. J Intern Med. 2008, 263: 117-127.

**39.** Packer C, Tatar M, Collins A. Reproductive cessation in female mammals. Nature 1998, 392: 807-811.

**40.** Gee DM, Flurkey K, Finch CE. Aging and the regulation of luteinizing hormone in C57BL/6J mice: impaired elevations after ovariectomy and spontaneous elevations at advanced ages. Biol Reprod. 1983, 28: 598-607.

**41.** Mobbs CV, Gee DM, Finch CE. Reproductive senescence in female C57BL/6J mice: ovarian impairments and neuroendocrine impairments that are partially reversible and delayable by ovariectomy. Endocrinology 1984, 115: 1653-1662.

**42.** Horiuchi S. Postmenopausal acceleration of age-related mortality increase. J Gerontol A Biol Sci Med Sci. 1997, 52: B78-92.

**43.** Krum SA,Brown M. Unraveling estrogen action in osteoporosis. Cell Cycle 2008, 7: 1348-1352.

**44.** Blagosklonny MV. Program-like aging and mitochondria: instead of random damage by free radicals. J Cell Biochem 2007, 102: 1389-1399.

**45.** Blagosklonny MV. TOR-driven aging: speeding car without brakes. Cell Cycle 2009, 8: 4055-4059.

**46.** Blagosklonny MV. Rapamycin and quasi-programmed aging: four years later. Cell Cycle 2010, 9:

**47.** Gangloff YG, Mueller M, Dann SG, Svoboda P, Sticker M, Spetz JF, Um SH, Brown EJ, Cereghini S, Thomas G, Kozma SC. Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. Mol Cell Biol. 2004, 24: 9508-2516.

**48.** Murakami M, Ichisaka T, Maeda M, Oshiro N, Hara K, Edenhofer F, Kiyama H, Yonezawa K, Yamanaka S. mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells. Mol Cell Biol. 2004, 24: 6710-6718.

**49.** Shah OJ, Wang Z, Hunter T. Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. Curr Biol. 2004, 14: 1650-1656.

**50.** Tremblay F,Marette A. Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. J Biol Chem. 2001, 276: 38052-38060.

**51.** Liu Z, Wu Y, Nicklas EW, Jahn LA, Price WJ, Barrett EJ. Unlike insulin, amino acids stimulate p70S6K but not GSK-3 or glycogen synthase in human skeletal muscle. Am J Physiol Endocrinol Metab. 2004, 286: E523-528.

**52.** Tremblay F, Krebs M, Dombrowski L, Brehm A, Bernroider E, Roth E, Nowotny P, Waldhäusl W, Marette A, Roden M. Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability. Diabetes 2005, 54: 2674-2684.

**53.** Krebs M, Brunmair B, Brehm A, Artwohl M, Szendroedi J, Nowotny P, Roth E, Fürnsinn C, Promintzer M, Anderwald C, Bischof M, Roden M. The Mammalian target of rapamycin pathway regulates nutrient-sensitive glucose uptake in man. Diabetes 2007, 56: 1600-1607.

**54.** Khamzina L, Veilleux A, Bergeron S, Marette A. Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: possible involvement in obesity-linked insulin resistance. Endocrinology 2005, 146: 1473-1481.

**55.** Briaud I, Dickson LM, Lingohr MK, McCuaig JF, Lawrence JC, Rhodes CJ. Insulin receptor substrate-2 proteasomal degradation mediated by a mammalian target of rapamycin (mTOR)-induced negative feedback down-regulates protein kinase B-mediated signaling pathway in beta-cells. J Biol Chem. 2005, 280: 2282-2293.

**56.** Cheang KI, Sharma ST, Nestler JE. Is metformin a primary ovulatory agent in patients with polycystic ovary syndrome? Gynecol Endocrinol. 2006, 22: 595-604.

**57.** Wu FC, Tajar A, Pye SR, Silman AJ, Finn JD, O'Neill TW, Bartfai G, Casanueva F, Forti G, Giwercman A, Huhtaniemi IT, Kula K, Punab M, Boonen S, Vanderschueren DEMASG. Hypothalamic-pituitary-testicular axis disruptions in older men are differentially linked to age and modifiable risk factors: the European Male Aging Study. J Clin Endocrinol Metab. 2008 Jul;93(7):. Epub 2008 2008, 93: 2737-2745.

**58.** Veldhuis JD, Keenan DM, Liu PY, Iranmanesh A, Takahashi PY, Nehra AX. The aging male hypothalamic-pituitary-gonadal axis: Pulsatility and feedback. Mol Cell Endocrinol. 2009;299:14-22.

**59.** Dilman VM. Age-associated elevation of hypothalamic, threshold to feedback control, and its role in development, ageine, and disease. Lancet 1971, 1: 1211-1219.

**60.** Dilman VM,Anisimov VN. Hypothalmic mechanisms of ageing and of specific age pathology--I. Sensitivity threshold of hypothalamo-pituitary complex to homeostatic stimuli in the reproductive system. Exp Gerontol. 1979, 14: 161-174.

**61.** Weiss G, Skurnick JH, Goldsmith LT, Santoro NF, Park SJ. Menopause and hypothalamic-pituitary sensitivity to estrogen. JAMA 2004, 292: 2991-2996.

**62.** Sherman BM, West JH, Korenman SG. The menopausal transition: analysis of LH, FSH, estradiol, and progesterone concentrations during menstrual cycles of older women. J Clin Endocrinol Metab. 1976, 42: 629-636.

**63.** Beemsterboer SN, Homburg R, Gorter NA, Schats R, Hompes PG, Lambalk CB. The paradox of declining fertility but increasing twinning rates with advancing maternal age. Hum Reprod. 2006, 21: 1531-1532.

**64.** Prior JC. Ovarian aging and the perimenopausal transition: the paradox of endogenous ovarian hyperstimulation. Endocrine 2005, 26: 297-300.

**65.** Niikura Y, Niikura T, Tilly JL. Aged mouse ovaries possess rare premeiotic germ cells that can generate oocytes following transplantation into a young host environment. Aging 2009, 1: 971-978.

**66.** Selesniemi K, Lee HJ, Niikura T, Tilly JL. Young adult donor bone marrow infusions into female mice postpone age-related reproductive failure and improve offspring survival. Aging 2009, 1: 49-57.

**67.** Roa J, Garcia-Galiano D, Varela L, Sanchez-Garrido MA, Pineda R, Castellano JM, Ruiz-Pino F, Romero M, Aguilar E, Lopez M, Gaytan F, Dieguez C, Pinilla L, Tena-Sempere M. The mammalian target of rapamycin as novel central regulator of puberty onset via modulation of hypothalamic Kiss1 system. Endocrinology 2009, 150: 5016-5026.

**68.** Kayampilly PP,Menon KM. Follicle-stimulating hormone increases tuberin phosphorylation and mammalian target of rapamycin signaling through an extracellular signal-regulated kinase-dependent pathway in rat granulosa cells. Endocrinology 2007, 148: 3950-3957.

**69.** Yu J,Henske EP. Estrogen-induced activation of mammalian target of rapamycin is mediated via tuberin and the small GTPase Ras homologue enriched in brain. Cancer Res. 2006, 66: 9461-9466.

**70.** Reddy P, Liu L, Adhikari D, Jagarlamudi K, Rajareddy S, Shen Y, Du C, Tang W, Hämäläinen T, Peng SL, Lan ZJ, Cooney AJ, Huhtaniemi I, Liu K. Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool. Science 2008, 319: 611-613.

**71.** Adhikari D, Flohr G, Gorre N, Shen Y, Yang H, Lundin E, Lan Z, Gambello MJ, Liu K. Disruption of Tsc2 in oocytes leads to overactivation of the entire pool of primordial follicles. Mol Hum Reprod 2009, 15: 765-770.

**72.** Adhikari D, Zheng W, Shen Y, Gorre N, Hamalainen T, Cooney AJ, Huhtaniemi I, Lan ZJ, Liu K. Tsc/mTORC1 signaling in oocytes governs the quiescence and activation of primordial follicles. Hum Mol Genet 19: 397-410.

**73.** Yaghmaie F, Saeed O, Garan SA, Freitag W, Timiras PS, Sternberg H. Caloric restriction reduces cell loss and maintains estrogen receptor-alpha immunoreactivity in the pre-optic hypothalamus of female B6D2F1 mice. Neuro Endocrinol Lett. 2005, 26: 197-203.

**74.** Blagosklonny MV. Calorie restriction: decelerating mTOR-driven aging from cells to organisms (including humans). Cell Cycle 9: 683-688.

**75.** Osborne TB, Mendel LB, Ferry EL. The effect of retardation of growth upon the breeding period and duration of life of rats. Science 1917, 45: 294-295.

**76.** Selesniemi K, Lee H-J, Tilly J. Moderate caloric restriction initiated in rodents during adulthood sustains function of the female reproductive axis into advanced chronological age. Aging Cell 2008, 7:622-9.

**77.** Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, Montminy M, Cantley LC. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science 2005, 310: 1642-1646.

**78.** Palomba S, Orio FJ, Falbo A, Russo T, Tolino A, Zullo F. Clomiphene citrate versus metformin as first-line approach for the treatment of anovulation in infertile patients with polycystic ovary syndrome. J Clin Endocrinol Metab. 2007, 92: 3498-3503.

**79.** Anisimov VN, Berstein LM, Egormin PA, Piskunova TS, Popovich IG, Zabezhinski MA, Tyndyk ML, Yurova MV, Kovalenko IG, Poroshina TE, Semenchenko AV. Metformin slows down aging and extends life span of female SHR mice. Cell Cycle 2008, 7: 2769-2773.

**80.** Waldron I,Johnston S. Why do women live longer than men? J Human Stress 1976, 2: 19-30.

Hypothesis

# Why human lifespan is rapidly increasing: solving "longevity riddle" with "revealed-slow-aging" hypothesis

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***Key words:*** *aging, senescence, longevity, diseases, health span, lifespan, rapamycin, mTOR*
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**Abstract: Healthy life span is rapidly increasing and human aging seems to be postponed. As recently exclaimed in *Nature*, these findings are so perplexing that they can be dubbed the 'longevity riddle'. To explain current increase in longevity, I discuss that certain genetic variants such as hyper-active mTOR (mTarget of Rapamycin) may increase survival early in life at the expense of accelerated aging. In other words, robustness and fast aging may be associated and slow-aging individuals died prematurely in the past. Therefore, until recently, mostly fast-aging individuals managed to survive into old age. The progress of civilization (especially 60 years ago) allowed slow-aging individuals to survive until old age, emerging as healthy centenarians now. I discuss why slow aging is manifested as postponed (healthy) aging, why the rate of deterioration is independent from aging and also entertain hypothetical use of rapamycin in different eras as well as the future of human longevity.**

## Unexpected increase in longevity

Death from aging is technically death from age-related diseases, which are manifestations of advanced aging [1]. But, historically, most people died young and, of course, not from age-related diseases but, rather, from starvation and epidemics (cholera, smallpox, tuberculosis and many currently rare infections) as well as from physical violence. Just three centuries ago, life expectancy was less than 16 years and 75% of people born in London in 1662 died before they reached the age of 26 (Graunt's life table). The progress of civilization eliminated many causes of death that killed young people in the past. This dramatically increased the average lifespan. In addition, modern medicine extended lifespan of old people by treating age-related diseases. But maximal lifespan seemed to be not affected. It was assumed that human life span is close to its upper limits. However, surprising demographists and gerontologists, it was shown that life expectancy continues to increase at an astonishing pace [2, 3]. In the countries with the highest life expectancies, the long term increase in life expectancy proceeds at a pace of 2.5 years per 10 years, or six hours per day [4]. A century ago, the chance to become centenarian (a person older than 100 years) was a hundred times lower. Furthermore, as calculated, most babies born since 2000 in countries with long life expectancies will celebrate their 100th birthdays [5]. Most astonishingly, people are reaching very old age in better health. But then they deteriorate fast, seemingly indicating that the rate of aging was not changed but just aging was postponed [3]. "Taken together, these findings are so perplexing that they can be dubbed the 'longevity riddle': why do the evolutionary forces that shaped human aging provide a license to alter the level of health but not the rate of debilitation?" [3]. So why can aging be delayed but not slowed? Or can aging be slowed? In order to solve the longevity riddle, we should turn gerontology on its head. It has been always assumed that aging is

caused by damage. As recently argued, aging is not driven by damage, but, in contrast, leads to damage (organ damage) [6-8]. And aging is driven in part by mTOR (mammalian target of rapamycin).

## TOR-driven quasi-programmed aging and age-related diseases

The mTOR intracellular signaling pathway is activated by numerous signals including glucose, amino acids, fat acids and other nutrients, insulin and some other hormones, growth factors and cytokines [9-11]. In response, it increases cellular functions and cellular mass growth [12]. When the cell cycle is blocked, mTOR drives cellular senescence [13]. Cellular aging can be defined as over-activation of signaling pathways (such as mTOR) with secondary signal resistance [14]. In turn this slowly leads to diseases of aging (hypertension, atherosclerosis, macular degeneration, insulin resistance, obesity, neurodegeneration, cancer, osteoporosis, organ hypertrophy). For example, TOR-dependent activation of osteoclasts causes bone resorption (osteoporosis) [15]. But these aging processes are relatively silent (subclinical, no obvious deterioration) until aging culminates in "catastrophes" – organ damage. For example, osteoporosis can lead to broken hip and atherosclerosis can lead to infarction. Then deterioration can be quick, leading to death in a mater of hours or years or decades, depending on the level of medical care.

## Morbid phase

When diseases become clinical then deterioration may be fast. For example, high blood pressure, thrombosis and atherosclerosis can culminate in stroke. This will initiate a chain of deteriorations (immobility – pneumonia, etc.) that are TOR-independent. The duration of this morbid (deterioration) phase is almost solely determined by the level of medical care. Furthermore, age-related blindness and Alzheimer's disease are rarely lethal anymore. Medicine may dramatically prolong the morbidity phase, delaying death. Thus, the speed of deterioration is almost independent from the aging process and cannot serve as a marker of aging or the rate of aging. The rate of aging is actually determined by the age of the onset of age-related diseases. Slowing down the aging process (by calorie restriction, rapamycin or genetic manipulation) delays diseases.

## "Thought experiment": how would rapamycin affect longevity in 1667 versus 1967

Rapamycin is an anti-aging drug, which is currently used to prevent donor organ rejections [16]. Rapamycin delays cancer in animals and humans (see for review [17]). It also delays other age-related diseases in animal models of accelerated diseases. For example, rapamycin and its analogs delay atherosclerosis [18-23]. mTOR is involved in age-related diseases exactly because it is involved in aging. In fact, rapamycin prolongs life span in mice and flies [24-27]. It is expected that, in adult humans, rapamycin (at correct doses and schedules) will prolong healthy and maximal lifespan [16]. But consider rapamycin administered for life, starting from childhood. Then its effect on longevity will depend on the level of civilization and will be opposite in the 17$^{th}$ and 20$^{th}$ centuries.

**Scenario 1**. Assume that in 1667, 3 out of 4 newborns were randomly prescribed rapamycin for life. Rapamycin would slow down developmental growth (a disadvantage for survival, especially for orphans). Malnutrition and stresses would be less tolerated, because the nutrient sensing pathway is deactivated by rapamycin. Reduced muscle mass and fat stores would increase chances of death from violence and famine. In infants with natural immunotolerance, rapamycin would further decrease immunity against infections, which were numerous, incurable and non-preventable in 17$^{th}$ century. So, if 3 out of 4 people must die before the age of 26 (1667 in London), they would be those who were treated with rapamycin. The control group would survive and develop diseases of aging at normal (early) age.

**Scenario 2**. In 20$^{th}$ century London, sanitation, vaccination and other measures have greatly reduced epidemics. The discovery of antibiotics has further prevented death from infections. Famine and violent death are not common either. Those who were treated with rapamycin for life will survive into adulthood and then will age slowly. In the rapamycin-treated group, diseases will be delayed. Furthermore, even its ability to cause immunologic tolerance ('rejuvenate' immunity) will be beneficial in the elderly by decreasing hyper-immunity and autoimmunity. (Note: rapamycin improves immunity in old animals [28]). So, now, the rapamycin treated group becomes centenarians in good heath. But because deterioration is mTOR-independent, this group will deteriorate at the same rate (but later in life) as the control group, assuming that the medical treatment is equal in both groups (in reality, younger patients are treated more intensively.)

## The revealed-slow-aging hypothesis

Thus, while slow aging was a disadvantage in 1667, it became an advantage in 1967. In the past, mostly fast-aging individuals could survive into chronologically old

age (Figure 2A). Now, slow-aging individuals can survive into chronologically old age (Figure 2B). Therefore, demographists observe an increasing number of individuals who are healthy at advanced chronological ages with delayed onset of diseases, who then deteriorate at the same rate as younger patients (Figure 1A vs 1B).

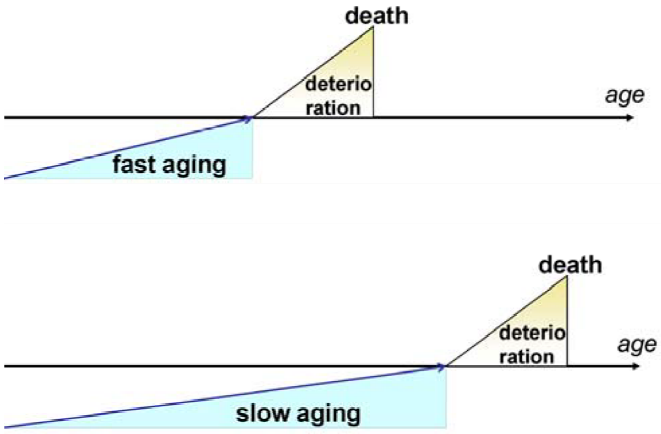


**Figure 1. Fast and slow aging.** In slow aging, the onset of deterioration is postponed but the rate of deterioration is not changed.

Importantly, current increase in healthy lifespan (increased longevity with late onset of age-related diseases) is not caused by natural selection. It happens in the same generation. Slow aging was not selected but was simply revealed (Figure 2 B). Until recently, most slow-aging individuals died prematurely. They (we) did not necessarily die young but nevertheless died not from aging. For example, at the same chronological age when fast-aging individuals died from heart attack, healthy slow-aging individuals died from malnutrition and infections, for instance. Elimination of premature death greatly enriched chronologically old population with slow-aging (biologically young) individuals (Figure 2).

To be possibly correct, the hypothesis requires a high proportion of slow-aging individuals at birth (Figure 2). Otherwise, there would be too few slow-aging individuals to make a difference later (Figure 2 A vs B). Why was not slow aging selected out? Slow aging must be beneficial for women, by increasing their reproductive period. In fact, female's fertility is decreasing early in life (starting from late twenties, long before menopause). This reproductive aging is one of the earliest manifestations of aging in females. So slow aging benefits females. Also, as I will discuss elsewhere, women do not need to be as robust as men, so can afford to age slower (see forthcoming article "Why men age faster but reproduce longer: mTOR perspective"). In turn, males inherit genes for longevity too, explaining a high proportion of slow-aging individuals at birth.

The revealed-slow-aging hypothesis predicts that certain very harsh conditions may result in a decrease in healthy lifespan decades later. For example, perhaps it is robust (and therefore fast-aging later) young men who predominantly survived wars, camps and orphanages. (If so, the death of weak slow-aging young men during $1940^{th}$-$1950^{th}$ might explain a drop in healthy lifespan of Russian men 50 years later.) Also, the hypothesis explains data on early-age mortality and subsequent mortality in the same cohorts. Thus Finch and Crimmins showed that increasing longevity and declining mortality in the elderly occurred among the same birth cohorts that experienced a reduction in mortality at younger ages [29, 30]. The revealed-slow-aging hypothesis suggests that high levels of infection early in life eliminate young individuals with a 'weak' mTOR (slow-aging individuals, who otherwise would live longer).

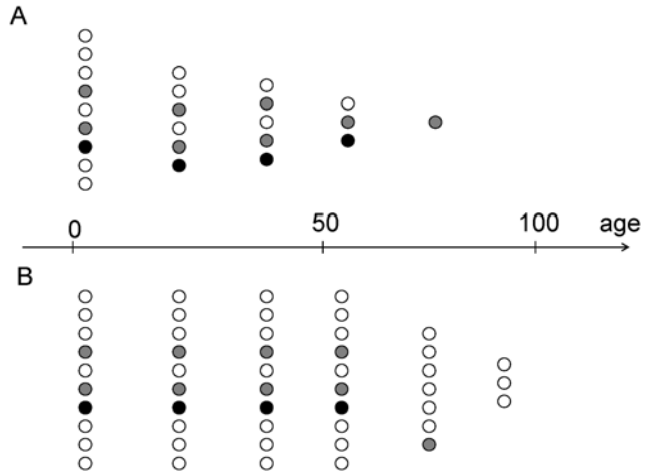


**Figure 2. Preferential survival fast- versus slow-aging individuals.** (**A**) In the past, slow-aging individuals (open circles) died prematurely and fast-aging individuals (closed circles) survived into old age. (**B**) Now, slow-aging individuals (open circles) survived into old age as healthy (biologically young) and outlive faster aging individuals (closed circles).

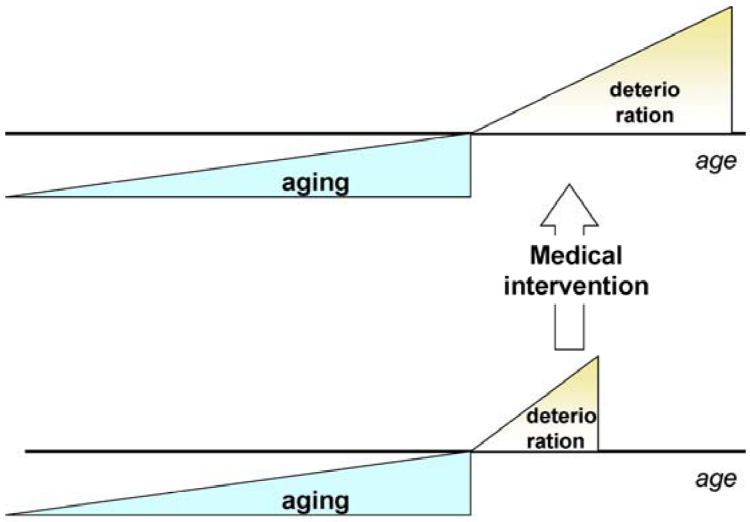


**Figure 3. Traditional medicine increases survival (extends deterioration phase) without affecting the onset of deterioration.**

## The prospect of longevity

Today, most slow-aging individuals, with less active mTOR, do not die early in life from malnutrition and infections and can reach chronologically old age. Exactly because they are slow-aging (young biologically), they are able to reach old age in good health. This may explain the current increase in longevity. But this trend is probably close to saturation and will be saturated by 2050 (a century after invention of antibiotics) in the countries with the highest longevity. The reason is that the rate of aging was not affected by elimination of death from famine and infections.

Yet, aging could be slowed by rapamycin, a drug currently approved to prevent organ rejection. (Note: rapamycin, as an anti-aging drug, perhaps should not be administrated until after growth is completed). Based on data with calorie restriction and rapamycin in mice, lifespan might be increased on 30 percent. Then we will observe 140-150 years old individuals and average lifespan will exceed 100.

## Solution of heath care crisis and further prospect on longevity

Currently, by treating each disease individually and focusing on advanced diseases, traditional medical interventions lengthen the morbidity phase (Figure 3).

So, traditional medicine increases number of old people in bad health. However, extension of lifespan by lengthening only the morbidity phase will make the cost of medical care unsustainable for society. Anti-aging medicine can solve this crisis by delaying the morbidity (deterioration) phase (Figure 4).

There is incorrect perception that anti-aging drugs would increase a number of people suffering with age-related diseases. In contrast, such *old* people will be healthy because they will be only chronologically old but biologically young. They will be healthier for longer (until they reach biological age of deterioration). Biological age is by itself determined by the sum of all diseases of aging [1]. In other words, diseases of aging are manifestations of biological aging. It is impossible to dissociate biological aging and diseases of aging. Healthy aging is healthy *non-aging* (or slow aging).

Deceleration of aging, manifested as "healthy aging", increases the ratio of healthy to unhealthy people (Figure 4). Furthermore, the ability to work is determined by biological age. Slow aging may delay retirement until later in life (as also suggested by Vaupel [3]) and in turn may provide the means for society to support further development of increasingly powerful (and expensive) conventional medicine. Then lifespan can be extended by both anti-aging medical intervention (to delay morbidity) and specialized medical intervention (to prolong morbidity stage).

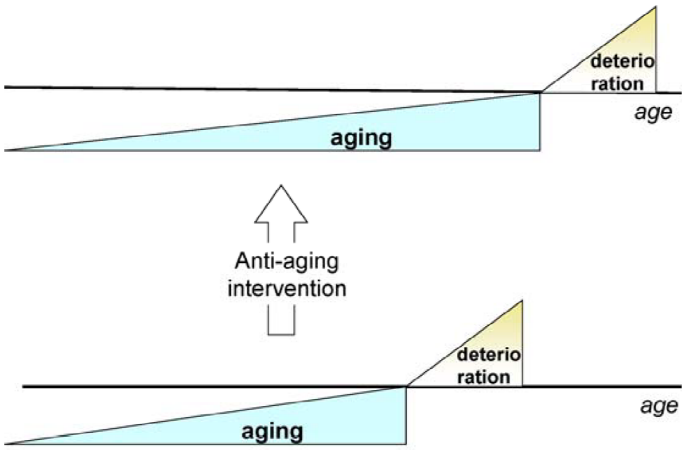


**Figure 4. Anti-aging drugs will delay the onset of deterioration without affecting deterioration.**

In conclusion, the progress of medicine 60-100 years ago (in prevention and treatment of non-age-related diseases) allowed slow-aging individuals to survive long enough to die from late onset age-related diseases (in other words to die from postponed aging). Civilization increased a proportion of slow-aging persons among the elderly, without actually slowing the aging process. Rapamycin will be used to slow down aging itself, further extending healthy lifespan. The extent of lifespan extension will depend on the future discoveries. And future discoveries are predictably unpredictable [31].

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## CONFLICT OF INTERESTS STATEMENT

The author of this manuscript has no conflict of interests to declare.

## REFERENCES

**1.** Blagosklonny MV. Validation of anti-aging drugs by treating age-related diseases. Aging 2009; 1: 281-288.

**2.** Wilmoth JR, Deegan LJ, Lundstrom H, Horiuchi S. Increase of maximum life-span in Sweden, 1861-1999. Science. 2000; 289: 2366-2368.

**3.** Vaupel JW. Biodemography of human ageing. Nature. 2010; 464: 536-542.

**4.** Oeppen J, Vaupel JW. Demography. Broken limits to life expectancy. Science. 2002; 296: 1029-1031.

**5.** Christensen K, Doblhammer G, Rau R, Vaupel JW. Ageing populations: the challenges ahead. Lancet. 2009; 374: 1196-1208.

**6.** Blagosklonny MV. Aging and immortality: quasi-programmed senescence and its pharmacologic inhibition. Cell Cycle 2006, 5: 2087-2102.

**7.** Blagosklonny MV. Aging: ROS or TOR. Cell Cycle. 2008; 7: 3344-3354.

**8.** Blagosklonny MV. TOR-driven aging: speeding car without brakes. Cell Cycle. 2009; 8: 4055-4059.

**9.** Hay N,Sonenberg N. Upstream and downstream of mTOR. Genes Dev. 2004; 18: 1926-1945.

**10.** Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell. 2006; 124: 471-484.

**11.** Hands SL, Proud CG, Wyttenbach A. mTOR's role in ageing: protein synthesis or autophagy? Aging. 2009; 586-597.

**12.** Blagosklonny MV and Hall MN. Growth and aging: a common molecular mechanism. Aging. 2009; 1: 357-362.

**13.** Demidenko ZN and Blagosklonny MV. Growth stimulation leads to cellular senescence when the cell cycle is blocked. Cell Cycle. 2008; 7: 3355-3361.

**14.** Blagosklonny MV. Aging-suppressants: cellular senescence (hyperactivation) and its pharmacological deceleration. Cell Cycle. 2009; 8: 1883-1887

**15.** Kneissel M, Luong-Nguyen NH, Baptist M, Cortesi R, Zumstein-Mecker S, Kossida S, O'Reilly T, Lane H, Susa M. Everolimus suppresses cancellous bone loss, bone resorption, and cathepsin K expression by osteoclasts. Bone. 2004; 35: 1144-1156.

**16.** Blagosklonny MV. An anti-aging drug today: from

senescence-promoting genes to anti-aging pill. Drug Discov Today. 2007; 12: 218-224.

**17.** Blagosklonny MV. Prevention of cancer by inhibiting aging. Cancer Biol Ther. 2008; 7: 1520-1524.

**18.** Elloso MM, Azrolan N, Sehgal SN, Hsu PL, Phiel KL, Kopec CA, Basso MD, Adelman SJ. Protective effect of the immunosuppressant sirolimus against aortic atherosclerosis in apo E-deficient mice. Am J Transplant. 2003; 3: 562-569.

**19.** Castro C, Campistol JM, Sancho D, Sanchez-Madrid F, Casals E, Andres V. Rapamycin attenuates atherosclerosis induced by dietary cholesterol in apolipoprotein-deficient mice through a p27 Kip1 -independent pathway. Atherosclerosis. 2004; 172: 31-38.

**20.** Pakala R, Stabile E, Jang GJ, Clavijo L, Waksman R. Rapamycin attenuates atherosclerotic plaque progression in apolipoprotein E knockout mice: inhibitory effect on monocyte chemotaxis. J Cardiovasc Pharmacol. 2005; 46: 481-486.

**21.** Gadioli AL, Nogueira BV, Arruda RM, Pereira RB, Meyrelles SS, Arruda JA, Vasquez EC. Oral rapamycin attenuates atherosclerosis without affecting the arterial responsiveness of resistance vessels in apolipoprotein E-deficient mice. Braz J Med Biol Res. 2009; 42: 1191-1195.

**22.** Zhao L, Ding T, Cyrus T, Cheng Y, Tian H, Ma M, Falotico R, Pratico D. Low-dose oral sirolimus reduces atherogenesis, vascular inflammation and modulates plaque composition in mice lacking the LDL receptor. Br J Pharmacol. 2009; 156: 774-785.

**23.** Mueller MA, Beutner F, Teupser D, Ceglarek U, Thiery J. Prevention of atherosclerosis by the mTOR inhibitor everolimus in LDLR-/- mice despite severe hypercholesterolemia. Atherosclerosis. 2008; 198: 39-48.

**24.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandezr E, Miller RA. Rapamycin fed late in life extends lifespan in genetically heterogenous mice. Nature. 2009; 460: 392-396.

**25.** Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Antoch MP, Blagosklonny MV. Rapamycin Extends Maximal Lifespan in Cancer-Prone Mice. Am J Pathol. 2010; in press

**26.** Moskalev AA, Shaposhnikov MV. Pharmacological Inhibition of Phosphoinositide 3 and TOR Kinases Improves Survival of Drosophila melanogaster. Rejuvenation Res. 2009; in press

**27.** Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, Partridge L. Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. Cell Metab. 2010; 11: 35-46.

**28.** Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, Larsen CP, Ahmed R. mTOR regulates memory CD8 T-cell differentiation. Nature. 2009; 460: 108-112.

**29.** Finch CE, Crimmins EM. Inflammatory exposure and historical changes in human life-spans. Science. 2004; 305: 1736-1739.

**30.** Crimmins EM, Finch CE. Infection, inflammation, height, and longevity. Proc Natl Acad Sci U S A. 2006; 103: 498-503.

**31.** Taleb NN. The black swan: the impact of the highly improbable. 2007. Random House, New York.

Research paper

# Extension of chronological life span by reduced TOR signaling requires down-regulation of Sch9p and involves increased mitochondrial OXPHOS complex density

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**Abstract: The nutrient-sensing target of rapamycin (TOR) pathway appears to have a conserved role in regulating life span. This signaling network is complex, with many downstream physiological outputs, and thus the mechanisms underlying its age-related effects have not been elucidated fully. We demonstrated previously that reduced TOR signaling (in *tor1Δ* strains) extends yeast chronological life span (CLS) by increasing mitochondrial oxygen consumption, in part, by up-regulating translation of mtDNA-encoded oxidative phosphorylation (OXPHOS) subunits. Here, we have examined in greater detail how TOR signaling influences mitochondrial function and CLS and the role of the Sch9p kinase in the TOR-mitochondria pathway. As is the case for oxygen consumption, mitochondrial translation is elevated in *tor1Δ* strains only during active growth and early stationary phase growth points. This is accompanied by a corresponding increase in the abundance of both mtDNA-encoded and nucleus-encoded OXPHOS subunits per mitochondrial mass. However, this increased OXPHOS complex density is not associated with more mitochondria/cell or cellular ATP and leads to an overall decrease in membrane potential, suggesting that TOR signaling may influence respiration uncoupling. Finally, we document that the Sch9p kinase is a key downstream effector of OXPHOS, ROS and CLS in the TOR-mitochondria pathway. Altogether, our results demonstrate that TOR signaling has a global role in regulating mitochondrial proteome dynamics and function that is important for its role in aging and provide compelling evidence for involvement of a "mitochondrial pre-conditioning" effect in CLS determination.**

## INTRODUCTION

How and why we age has long been a fascination of humans. In addition to being of intrinsic philosophical, evolutionary and biological interest, determining the molecular and cellular mechanisms underlying the aging process is relevant to understanding age-related pathology that ultimately limits human life and health span. Model organism studies have been instrumental in understanding aging, with many conserved pathways and factors having been identified in files, worms and yeast (and other organisms) that have physiological and pathological relevance in humans [1]. One general area that has been implicated strongly in aging and life span determination is nutrient availability/sensing. For example, dietary (i.e. caloric) restriction extends life span and ameliorates many of the age-associated declines in cellular function in virtually all organisms examined to date [2].

One major consequence of changing nutrient availability/sensing is alternation of cellular metabolism and mitochondrial respiration. Life span extension by caloric restriction, for instance, usually involves enhanced mitochondrial activity [2, 3]. While best known for providing ATP via oxidative phosphorylation (OXPHOS), mitochondria are a major crossroads for anabolic and catabolic metabolism, as well as many other critical cellular functions such as apoptosis, signal transduction, and ion homeostasis [4]. Mitochondria also contain a DNA genome (mitochondrial DNA; mtDNA) that harbors a set of genes involved in OXPHOS and requires dedicated machinery for organellar DNA replication and gene expression that is encoded primarily by genes in the nucleus (e.g. mitochondrial DNA and RNA polymerase, ribosomes, transcription and translation factors, etc) [5, 6]. Mitochondria also generate reactive oxygen species (ROS) as byproducts of the electron transport process, which is a major way they are thought to contribute to the aging process. For example, the "mitochondrial theory of aging", which builds on Harman's "free-radical" theory, posits that ROS from mitochondrial respiration damage cellular components, including mtDNA, and lead to declines in cell, tissue and organismal function over time [7, 8]. As ROS are also signaling molecules, altered signal transduction is another potential contributor to aging phenotypes due to mitochondrial dysfunction [9]. While the mechanisms through which altered respiration affects life span are complex and have not been defined fully, differential ROS production is likely involved. For example, aberrant respiration due to defective RAS signaling [10], pharmacological inhibition [11], or imbalanced translation of mtDNA-encoded OXPHOS subunits [9] elevates cellular ROS and severely curtails yeast chronological life span (CLS). Conversely, mild uncoupling of mitochondrial respiration extends yeast CLS and decreases ROS [11].

Several kinase pathways serve as physiological switches in response to nutrient availability. For example, the conserved target of rapamycin (TOR) signaling pathway controls growth by positively regulating the processes of ribosome biogenesis and cytoplasmic translation when preferred nutrient supplies are available. In yeast, the TOR pathway also negatively regulates stress response genes, autophagy, and usage of alternate carbon and nitrogen sources [12]. Thus, when nutrients are limiting, TOR activity is reduced, energy is conserved (by shutting down expensive growth-promoting pathways) and diverted to provide stress resistance and access to alternate energy stores. The TOR kinase forms two multi-protein complexes, TORC1 and TORC2, with TORC1 functioning as the nutrient sensor [12]. In yeast, there are two TOR kinase genes *TOR1* and *TOR2*. Both Tor1p and Tor2p can function in the TORC1 complex, but only Tor2p can function in the TORC2 complex. Thus, deletion of *TOR1* results in reduced TORC1 signaling, but is not lethal. This is because Tor2p can partially cover the loss of Tor1p in TORC1, while still also functioning in TORC2. In contrast, deletion of *TOR2* is lethal [13]. Reduced TORC1 signaling extends life span in a number of model organisms including yeast (*S. cerevisiae*), worms (*C. elegans*) and flies (*D. melanogaster*) [14-17]. We recently reported that a major mechanism underlying this phenotype in yeast is enhanced mitochondrial respiration driven, at least in part, by increased translation of mtDNA-encoded OXPHOS subunits [18]. In that study, we speculated that the extension of CLS by reduced TOR signaling involves an increase in the number of OXPHOS complexes per organelle that increases oxygen consumption, decreases ROS production in stationary phase, and thereby limits damage to cellular components. However, since mtDNA encodes only minority of the OXPHOS complex subunits (i.e. of the ~80 OXPHOS subunits only seven in yeast and thirteen in mammals are encoded by mtDNA) and mitochondria contain >1,000 proteins (encoded by nuclear genes and imported into the organelle), the possibility that TOR signaling regulates mitochondria in a more global fashion is likely. In fact, TOR-dependent changes in the mitochondrial proteome have been documented in human Jurkat T cells [19].

Sch9p belongs to the AGC family of kinases and is a key downstream target of TORC1 signaling in yeast. For example, Sch9p is a functional ortholog of ribosomal protein S6 kinase, a key mediator of mTOR signaling in mammalian cells [20]. TORC1 directly phosphorylates Sch9p at multiple sites, which is important for modulating cytoplasmic translation and cell cycle progression. Sch9p is also a negative regulator of both chronological and replicative aging [14, 21] and has recently been shown to similarly regulate mitochondrial respiration [22]. In fact, like deletion of *TOR1*, deletion of *SCH9* extends yeast CLS in a respiration-dependent fashion, suggesting that Sch9p could be a downstream mediator of TOR-dependent mitochondrial OXPHOS regulation in this regard. In the current study, we have examined in greater mechanistic detail how the yeast TOR pathway influences mitochondrial gene expression, OXPHOS activity, and proteome composition, and the role of the Sch9p kinase as a downstream mediator of its effects on mitochondria.

## RESULTS

### Reduced TOR signaling globally increases mitochondrial translation and results in a greater number of OXPHOS complexes per organelle

We demonstrated previously that reduced TOR signaling (in *tor1* null yeast strains; *tor1*) results in increased mitochondrial translation rates, oxygen consumption, and life span [18]. This is accompanied by a corresponding increase in the steady-state levels of mtDNA-encoded OXPHOS subunits. However, whether there is global up-regulation of mitochondrial translation was not addressed in that study and only a single, late culture growth point was analyzed. To better understand the mitochondrial translation response to reduced TOR signaling, we labeled all mtDNA-encoded subunits at three growth points and visualized the individual products by autoradiography of SDS-PAGE gels. Compared to wild-type strains, we observed global up-regulation of mitochondrial translation products in log-phase and early stationary phase (day 1) cultures in *tor1*⊗ strains (Figure 1). One day later in stationary phase (day 2) the wild-type and *tor1*⊗ strains showed similar rates of mitochondrial translation, due to an increase in the rate in the wild-type strains (Figure 1). These results mirrored closely our previously published results on oxygen consumption as a function of growth state and demonstrate that the major differences in mitochondrial function in these strains are manifest during growth and early stationary phase, which is when TOR signaling is at its highest in wild-type strains.

The observed increase in mitochondrial translation in *tor1*⊗ strains prompted us to examine additional mitochondrial parameters. Here, we focused on mid-log growth points, where the largest differences in mitochondrial translation and oxygen consumption are observed. First, consistent with the increase in mitochondrial translation, there was an increase in the steady-state levels of mtDNA-encoded OXPHOS subunits (3-12 fold) per mitochondrial mass as judged by western blotting of Cox1p, Cox2p and Cox3p in mitochondrial extracts (Figure 2A). This was accompanied by an increase in the Cox4p OXPHOS subunit (2.2 fold), but not of porin, both of which are encoded by nuclear genes (Figure 2A). This result suggested to us that the OXPHOS machinery was up-regulated more or less specifically and that an overall increase in mitochondrial biogenesis was not occurring. To test this hypothesis, we transformed the strains with a plasmid encoding a mitochondria-targeted GFP protein and measured mitochondrial content by FACS, as well as determined mtDNA copy number, amounts of which usually correlate with mitochondrial abundance. No significant differences in mitochondrial mass (Figure 2B) or mtDNA (Figure 2C) were observed between the wild-type and *tor1Δ* strains. There also were no obvious differences in mitochondrial distribution or morphology observed by fluorescence microscopy of the GPF-containing strains (data not shown). Altogether, these data indicate that there is an increase in the number of OXPHOS complexes per organelle mass in *tor1Δ* strains, as opposed to a global up-regulation of the amount of mitochondria per cell. However, despite the fact there is increased mitochondrial OXPHOS components and oxygen consumption, there was a reduction in mitochondrial membrane potential (Figure 2D) and no significant difference in total cellular ATP in *tor1Δ* strains (data not shown).

To better understand how reduced TOR signaling dynamically effects respiration, we used the TOR kinase inhibitor rapamycin under a variety of conditions. Addition of rapamycin to a wild-type culture from the beginning of growth resulted in a significant and sustained increase in mitochondrial oxygen consumption (Figure S1A), similar to that observed in *tor1Δ* strains. However, rapamycin greatly inhibited the growth rate of these strains (data not shown). In contrast, adding rapamycin at a later point during growth (after the strains reached OD ~1.0) only increased oxygen consumption by ~30% (Figure S1B). This increase required the presence of the drug for 2-4 hours, was sustained for at least 30 hours (Figure S1B), and depended on both cytoplasmic and mitochondrial translation (i.e. was inhibited by addition of either cycloheximide or chloramphenicol; data not shown).

### Reduced TOR signaling increases the steady-state levels of mitochondrial transcripts

Given that the overall rates of mitochondrial translation were higher in *tor1Δ* strains, but mtDNA copy number was not, led us to investigate the whether there were changes in steady-state levels of mitochondrial transcripts that might indicate a mtDNA transcriptional response. Northern blots of three mitochondrial mRNA transcripts revealed that there is a 1.5- to 2.1-fold increase in *tor1Δ* strains (using 25S rRNA as a loading control; Figure 3). Similar changes were observed in the 14S rRNA (data not shown). These data indicate that there is a moderate increase in mitochondrial transcripts in *tor1Δ* strains, but that this is unlikely to be the primary driving force behind the significantly greater rates of mitochondrial translation and OXPHOS complex abundance observed.

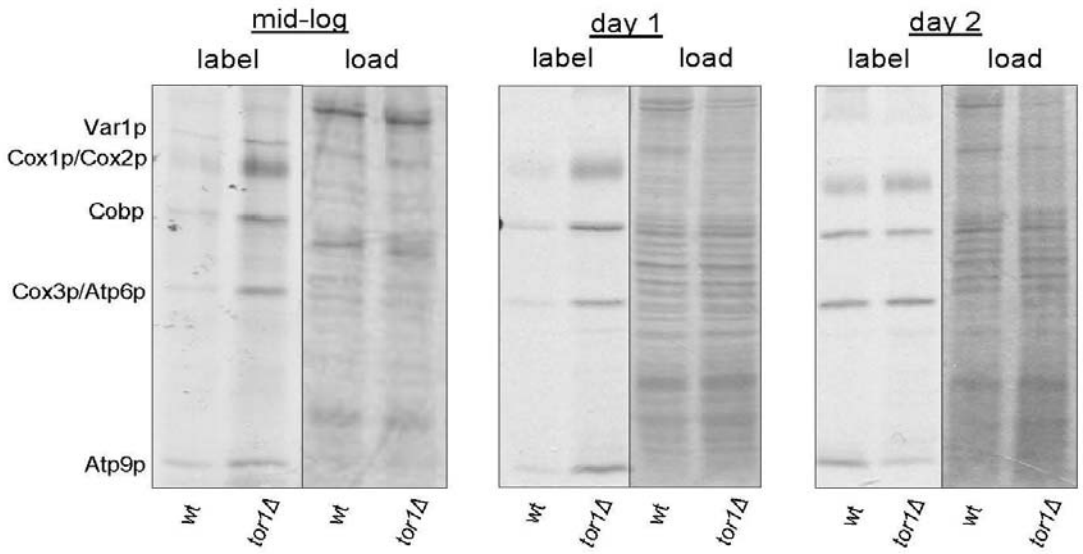


**Figure 1. Elevated mitochondrial translation rates in *tor1Δ* strains during the exponential and early stationary growth phases.** Results of an *in vivo*-labeling experiment in which the mtDNA-encoded gene products are labeled specifically and visualized by autoradiography after separation by SDS-PAGE (see Materials and Methods). Wild-type (wt) and *tor1* null (*tor1Δ*) strains labeled at mid-log, early stationary (day 1) and later stationary (day 2) are shown. The left-half panel under each time point is the autoradiogram showing the labeled mitochondrial gene products (with each product indicated on the left) and the right-hand panel is the respective Coomassie blue-stained gel as a control for total protein loading.

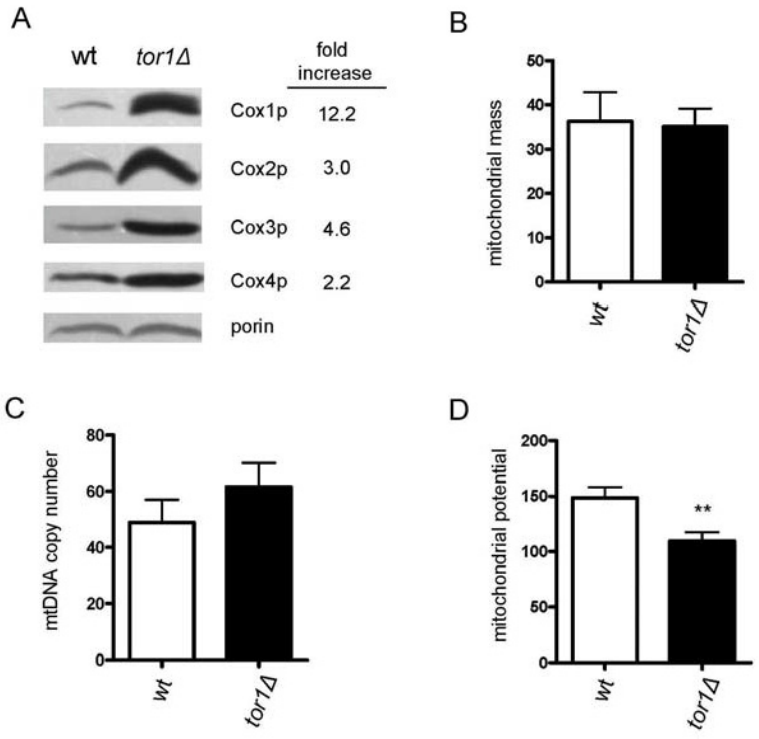


**Figure 2. Reduced TOR signaling increases the number of mitochondrial OXPHOS complexes per organelle, as opposed to the number of mitochondria/cell.** Comparative analysis of four mitochondria-related parameters in wild-type (wt) and *tor1Δ* strains is shown. (**A**) Western blot analysis of four OXPHOS subunits (Cox1p-4p) and porin (as a mitochondrial normalization control). Fifty μg of mitochondrial extract was loaded in each lane. The fold difference between wt and *tor1Δ* normalized to the porin signal is shown on the right. (**B**) Mitochondrial mass as estimated by the amount of mitochondrial-GFP signal determined by FACS (see Materials and Methods). (**C**) mtDNA copy number determined by real-time PCR (measured as the ratio of the mitochondrial gene target *COX1* relative to the nuclear gene target *ACT1*). (**D**) Mitochondrial membrane potential determined by $DiOC_6$ staining and FACS analysis. In B-D means of at least three biological replicates +/- one standard deviation are graphed (** represents a p-value from a student t-test that is <0.01).

**Global up-regulation of OXPHOS-related proteins in *tor1Δ* mitochondria revealed by 2D-DIGE**

To gain a better understanding of how reduced TOR signaling affects mitochondria, we have begun to characterize changes in the mitochondrial proteome in *tor1Δ* strains by two-dimensional, differential gel electrophoresis (2D-DIGE), coupled to mass spectrometry-based identification of differentially regulated proteins. Given that we observed an increase in OXPHOS subunits/mitochondrial mass by western blot (Figure 2A), we have focused initially on those proteins that were up-regulated by 2-fold or greater in mitochondria from *tor1Δ* strains (see Materials and Methods). Of the 26 up-regulated spots picked and analyzed based on this 2-fold cutoff, we have unambiguously identified eleven proteins that are at higher steady state-levels in mitochondria purified from *tor1Δ* strains in the mid-log growth phase (Table 1). In addition to Cox4p, which we had already documented as increased by western blot (Figure 2A), we identified five other OXPHOS components: Cox13p (another subunit of Complex IV), Qcr7 (subunit of Complex III), and Atp2p, Atp5p and Atp7p (subunits of Complex V/ATP synthase). In addition to OXPHOS components, we have thus far identified five other proteins that are up-regulated in mitochondria from *tor1Δ* strains (Table 1). Three of these (Dld2p, Gcv3p, and Ilv6p) are involved in various aspects of metabolism, one (Om45p) is an abundant outer mitochondrial membrane protein of unknown function, and the final one (Yhb1p) is involved in nitric oxide detoxification. Altogether, these data solidify our contention that that there is global up-regulation of OXPHOS machinery/organelle in response to reduced TOR signaling, but also indicate that TOR activity impacts mitochondrial proteome composition in other interesting ways. Furthermore, in the case of Gcv1p and Ilv6p, the spots identified of wild-type differ in molecular weight and/or PI from those of *tor1Δ* (data not shown), suggesting that TOR regulates expression and/or processing of these proteins in a unique manner.

**Balanced expression of mitochondrial OXPHOS components is required for extension of chronological lifespan mediated by reduced TOR signaling**

We previously documented that strains with imbalanced expression of mtDNA-encoded OXPHOS subunits have reduced chronological life span (CLS) [9]. One strain (GS129), in particular, has a severely curtailed CLS due to a point mutation in the amino-terminal domain of mtRNA polymerase (Rpo41p) that results in increased ROS [9]. Given that reduced TOR signaling (due to *TOR1* deletion) increases CLS, in part by increasing the rate of mitochondrial translation [18], we used the GS129 strain background to address the requirement for

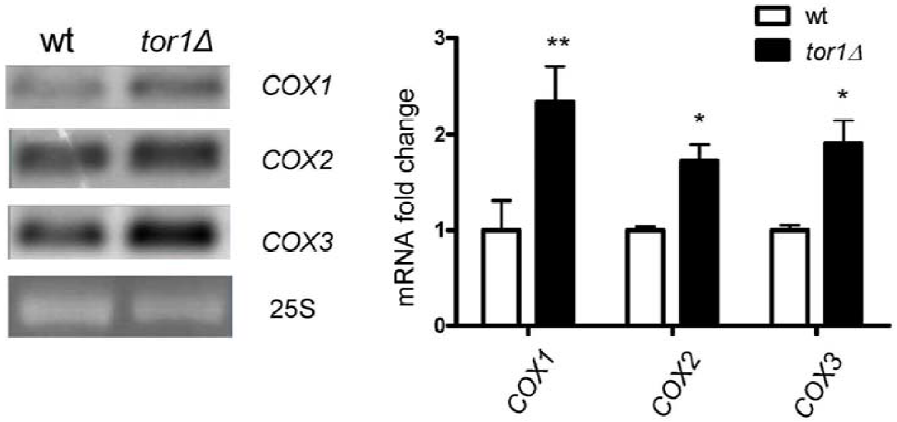


**Figure 3. Increase of mitochondrial transcript abundance in *tor1Δ* strains.** Northern analysis of the mtDNA-encoded mRNA transcripts *COX1-COX3* from wild-type (wt) and *tor1Δ* strains is shown, along with ethidium bromide-stained nuclear 25S rRNA as a loading control. Graphed on the right is the mean fold difference in COX1, COX2, and COX3 abundance normalized to 25S rRNA +/- one standard deviation (* designates a p-value <0.05) and ** designates a p-value <0.01 based on a student's t-test).

balanced mtDNA expression in this regard. Deletion of *TOR1* in the GS129 background resulted in an increase in translation of most mtDNA-encoded products to a degree that exceeded that in the isogenic wild-type strain GS122, but less than that observed in the isogenic wild-type *tor1Δ* strain (GS122 *tor1Δ*) (Figure 4A). However, unlike in the wild-type strain, there was no significant increase in Cox1p translation when *TOR1* was deleted in the GS129 background (Figure 4A). In other words, translation was increased in the GS129 strain in response to reduced TOR signaling, but not in a balanced manner. Analysis of CLS in these strains revealed that deletion of *TOR1* extended life span in the wild-type (GS122) background as expected, but did not significantly increase CLS in the "imbalanced" GS129 strain (Figure 4B). These data indicate that extension of life span by reduced TOR signaling requires balanced up-regulation of OXPHOS components encoded by mtDNA.

### *SCH9* is a downstream target of TOR signaling in the regulation of mitochondrial function

Recently, deletion of *SCH9* was also shown to increase expression of mitochondrial OXPHOS genes and mitochondrial respiration [22]. Given that these mitochondrial phenotypes are similar to those we have documented here and previously in *tor1Δ* strains, we tested the hypothesis that *SCH9* is downstream of *TOR1* with regard to mitochondrial regulation by simultaneously analyzing isogenic single (*tor1Δ* or *sch9Δ*) and double (*tor1Δ sch9Δ*) knock-out strains. As reported previously [18], we observed an increase in mitochondrial oxygen consumption in the *sch9Δ* strain that was similar in magnitude (2-fold) to the increase observed in the isogenic *tor1Δ*strain (Figure 5A). However, this increase was not enhanced further in the *tor1Δ sch9Δ* double-mutant strain (Figure 5A), consistent with these genes

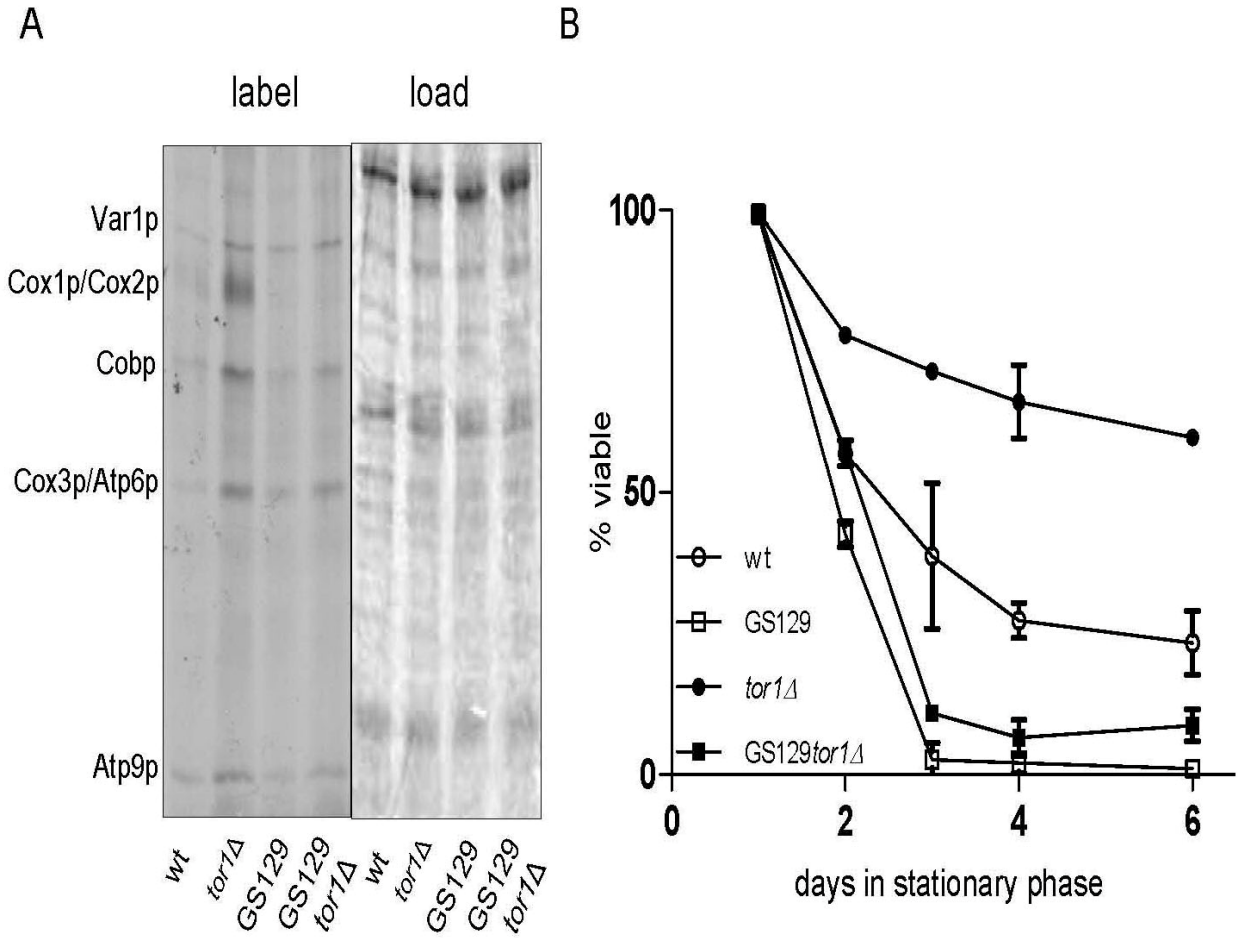


**Figure 4. Reduced TOR signaling does not rescue chronological life span in the short-lived GS129 strain with imbalanced mitochondrial translation.** (**A**) Results of mitochondrial translation assays are shown as described in Figure 1. The strains analyzed are GS122 (wt with regard to *RPO41*) and GS129 (containing the *rpo41-R129D* point mutation) in which the *TOR1* gene was (*tor1Δ*) or was not (wt) disrupted (see Materials and Methods). (**B**) Chronological life span curves of the same strains in A. are shown. Three independent colonies of each strain were analyzed and the mean % viability +/- one standard deviation is plotted according to the key in the lower left corner.

being in the same pathway with regard to mitochondrial respiration. Similar results were obtained for mitochondrial translation rates (Figure 5B) and steady-state levels of nucleus and mtDNA-encoded OXPHOS proteins (Figure 5C). However, the *sch9Δ* single mutant had a greater effect than the *tor1Δ* single mutant on these latter three parameters, and there was no synergistic effect observed in the double-mutant strains (Figures 5A and 5B). The fact that the double-mutant strain more closely resembled the *sch9Δ* strain is most consistent with *SCH9* being downstream of *TOR1* in this pathway controlling mitochondrial translation and respiration. This was evidenced further by the fact that addition of rapamycin to wild-type strains caused an increase in mitochondrial translation that was greater in magnitude to that observed in the *tor1Δ* strain (Figure. 5B). That is, rapamycin or *SCH9* deletion appears to represent a more complete down-regulation of TOR signaling than deletion of *TOR1*. Finally, comparison of the actin and porin ratio (an indicator of mitochondrial abundance) in the single and double mutant strains (Figure 5C) confirmed that, as was the case for *tor1Δ* there was no significant increase in overall mitochondrial biogenesis in the *sch9Δ* and *tor1Δ sch9Δ* strains, but rather an increase in the number of OXPHOS complexes per organelle mass, again placing these two genes in the same pathway with regard to mitochondrial function.

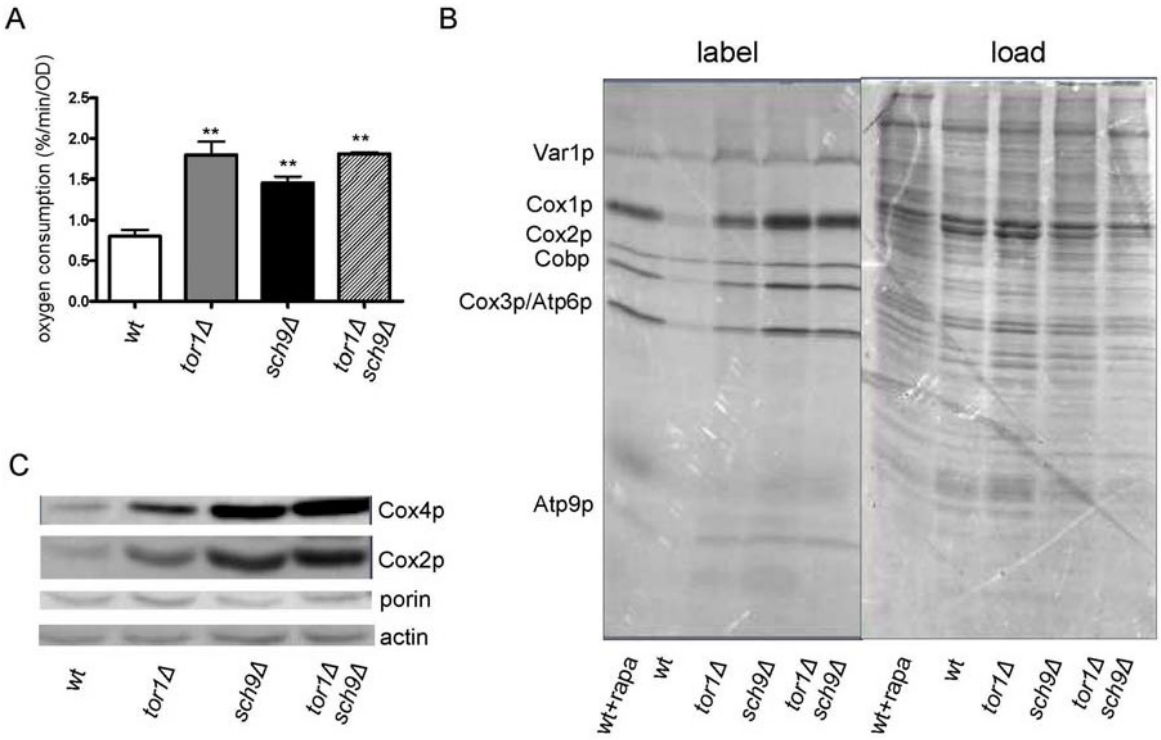


**Figure 5. Sch9p mediates TOR-dependent increases in mitochondrial function.** Comparative analysis of mitochondria-related parameters in wild-type (wt), *tor1Δ*, *sch9Δ* and *sch9Δtor1Δ* strains in the DBY2006 genetic background. (**A**) Mitochondrial oxygen consumption. (**B**) Mitochondrial translation as described in Figure 1. (**C**) Western blot of the Cox1p, Cox4p, porin and actin OXPHOS components in the four strains using 100 μg of whole cell extract in each lane. We use the ratio of porin to actin as one measure of mitochondrial abundance per cell (which is virtually the same between the strains) and the ratio of Cox subunits to porin to demonstrate their specific increase per mitochondrial mass.

| Protein | ID | Protein Function | Expression Ratio *tor1*/wt |
|---|---|---|---|
| **OXPHOS Components** | | | |
| Atp2p | gi\|151945186 | F1F0 ATP synthase beta subunit | 2.09 |
| Atp5p | gi\|6320504 | Subunit 5 of the stator stalk of mitochondrial F1F0 ATP synthase | 2.51 |
| Atp7p | gi\|151941529 | F1F0 ATP synthase subunit d | 2.48 |
| Cox13p | gi\|6321247 | Subunit VIa of cytochrome c oxidase | 3.33 |
| Cox4p | gi\|6321251 | Subunit IV of cytochrome c oxidase | 2.19 |
| Qcr7p | gi\|6320738 | Subunit 7 of the ubiquinol cytochrome-c reductase complex | 2.20 |
| **Outer Membrane Protein** | | | |
| Om45p | gi\|6322055 | Protein of unknown function, major constituent of the mitochondrial outer membrane | 2.33 |
| **Metabolic Enzymes** | | | |
| Dld2p | gi\|51830216 | D-lactate dehydrogenase, located in the mitochondrial matrix | 2.51 |
| Gcv3p* | gi\|595540 | H-protein subunit of the glycine cleavage system | 2.60 |
| Ilv6p* | gi\|6319837 | Regulatory subunit of acetolactate synthase, which catalyzes the first step of branched-chain amino acid biosynthesis | 2.53 |
| **Detoxification Enzyme** | | | |
| Yhb1p | gi\|6321673 | Nitric oxide oxidoreductase, flavohemoglobin involved in nitric oxide detoxification | 2.45 |

*proteins were found in different spots in wildtype than in *tor1Δ*.

**Table 1. Mitochondrial Proteins Identified as Up-regulated in *tor1Δ* Yeast Strains by 2D-DIGE**

### *SCH9* is downstream of *TOR1* in the regulation of chronological life span

We previously implicated reduced ROS in stationary phase as a significant factor that increases the CLS of *tor1Δ* strains [18]. A similar reduction in ROS was also observed in *sch9Δ* and *tor1Δ sch9Δ* strains (Figure 6A), again consistent with *SCH9* working in the same genetic pathway as *TOR1* with regard to mitochondria-derived ROS. Finally, as was the case for mitochondrial translation and OXPHOS complex abundance, we found that deletion of *SCH9* increased CLS to a greater degree than deletion of *TOR1*, but that there was no further increase in CLS in the *tor1Δ sch9Δ* double mutant strain (Figure 6B). Altogether, these data solidify the connections between mitochondrial OXPHOS, ROS and CLS and demonstrate that Sch9p is a key downstream factor that mediates the effects of TOR signaling on mitochondrial function and yeast aging.

## DISCUSSION

This study provides significant new insight into the mechanism through which TOR signaling controls mitochondrial function to influence yeast CLS and elucidates which arm of the TORC1 pathway is involved. The primary conclusions we draw from the results obtained are that 1) reduced TORC1 signaling (via deletion of the TOR1 gene) increases respiration primarily through up-regulation of the number of OXPHOS complexes/organelle, not by increasing overall mitochondrial biogenesis, 2) the up-regulation of OXPHOS complexes involves both mtDNA-encoded and nucleus-encoded subunits and, in terms of mtDNA expression, occurs primarily via translational regulation, 3) in addition to its effects on OXPHOS complex abundance, TOR signaling controls other aspects of mitochondrial proteome dynamics, 4) TOR-dependent changes in mitochondrial function and CLS are

mediated by the downstream Sch9p kinase, and 5) it is TOR-dependent alterations of mitochondrial function in the exponential and/or post-diauxic-early stationary growth phases that subsequently impact late stationary- phase survival and extend CLS, which suggests a role of "mitochondrial pre-conditioning" on yeast aging. The basis of these conclusions and additional interpretations are discussed below.

The increase in cellular mitochondrial oxygen consumption (i.e. respiration) in response to reduced TOR signaling reported herein (Figure 5A) and previously [18] could occur by one of several mechanisms that are not mutually exclusive. For example, it could be mediated by direct effects on the activity of existing OXPHOS complexes, by increasing overall mitochondrial biogenesis (resulting in more mitochondria/cell), or by increasing the number of OXPHOS complexes per organelle. Our results demons-trate that increasing organelle OXPHOS complex density is definitely one mechanism at play.

The basis for this conclusion is that, in mitochondrial extracts, we observe increased abundance of both nuclear and mitochondrial OXPHOS subunits, but not other mitochondrial markers (e.g. porin; Figure 2A). This result was substantiated by our initial 2D-DIGE proteomic analysis of highly purified mitochondria from wild-type and *tor1Δ* strains, in which we identified OXPHOS complex subunits (from three separate complexes) as proteins that are in significantly higher abundance in mitochondria from *tor1Δ* cells (Table 1). Finally, there was no increase in overall mitochondrial biogenesis as judged by mtDNA content (Figure 2C), labeling of mitochondria with a GFP marker and analyzing them by FACS (Figure 2B), and western blot comparisons of mitochondrial and cytoplasmic markers (Figure 5C). While increased OXPHOS complex density is clearly occurring, we have not eliminated the possibility that there are also TOR-dependent effects on the enzymatic activity of the complexes that contribute to the increase in oxygen consumption.

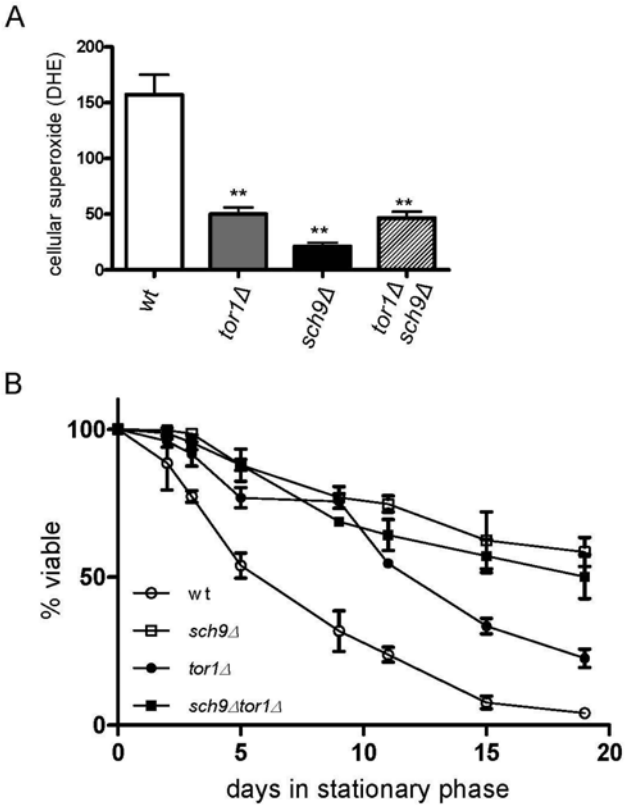


**Figure 6. Sch9p is the downstream mediator of TOR-dependent decreases in ROS level and chronological life span extension.** Analysis of cellular ROS and chronological life span in the same strains shown in Figure 5. (**A**) FACS analysis of day 2 stationary phase cells stained for cellular superoxide using dihyroethidium (DHE) is shown. The mean fluorescence intensity is plotted +/- one standard deviation (** represents a p-value <0.01 according to a student's t-test). (**B**) Chronological life span plotted as described in Figure 4B.

Our 2D-DIGE results are not entirely consistent with a recent proteomic study of rapamycin-treated yeast cell [23], where fewer OXPHOS proteins were identified as up-regulated. We found that addition of rapamycin during the growth phase impacts mitochondrial oxygen consumption on a longer time scale and to a lesser degree compared to adding rapamycin from the beginning of the growth experiment (i.e. at inoculation; Supplemental Figure S1). The latter condition is in some ways more similar to the *tor1Δ* strains analyzed in this study in that, in this case, TOR signaling is reduced throughout all stages of growth. Thus, differences in the timing and/or degree of TOR inhibition may explain the different results obtained in the two studies.

To increase OXPHOS complex density as a means to increase mitochondrial oxygen consumption is to our knowledge a unique mechanism of mitochondrial regulation levied by the TOR pathway. We originally hypothesized that this would lead to greater mitochondrial membrane potential due to the increase in electron transport activity and perhaps also a higher cellular ATP. However, this was not the case; there was instead a decrease in membrane potential in *tor1Δ* strains (Figure 2D) and no change in cellular ATP (data not shown). Thus, in *tor1Δ* strains, there in an increase in electron transport activity (i.e. oxygen consumption) and a decrease in mitochondrial membrane potential, which equates to a mitochondrial network with overall lower energy capacity on average. One potential explanation for this result is that reduced TOR signaling is leading to an increase in uncoupled respiration. This would lead to increased oxygen consumption in an attempt to maintain the membrane potential in the face of the proton leak and an inability to simultaneously increase ATP production. Since mild uncoupling also increases CLS [11], this indeed may prove to be the mechanism through which TOR signaling influences aging in yeast. Testing this hypothesis is a logical area of future investigation, but certainly other explanations can be envisioned.

In order to affect an increase in OXPHOS complexes per mitochondrion, the cell needs to increase the production and/or stability of both mtDNA-encoded and nucleus-encoded OXPHOS subunits, while not inducing a full mitochondrial biogenesis response. How reduced TOR signaling accomplishes this remains to be determined, yet several insights are gleaned from our results. First, we observe an increase in both mtDNA-encoded and nucleus-encoded OXPHOS subunits (Figure 2A, Table 1), thus TOR signaling is affecting both mitochondrial and nuclear gene expression simultaneously. According to our results, this is occurring both at the mRNA level (Figure 3) and at the translational level (Figure 1) in mitochondria, but not at the level of protein stability to any obvious degree (Figure 5B, Supplemental Figure S2). Since transcription and translation are coupled in mitochondria [24-26], these changes probably work together to mediate the increase in OXPHOS complex abundance in *tor1Δ* strains. Although, the translational control appears to contribute to a greater extent, given only modest changes in mitochondrial transcript levels are observed. However, the change in mitochondrial transcripts of *tor1Δ* strains might represent a reduction of glucose repression, which is known to induce mitochondrial transcription [27, 28] and mimic the effects of *tor1Δ* on respiration and CLS based on our previous study [18]. Interesting in this regard is the key role of the Snf1p kinase in the glucose repression phenomenon [29]. Snf1p is the yeast ortholog of mammalian AMP kinase, which negatively regulates mTOR signaling in response to energy charge by activating Tsc2, an inhibitor of mTORC1 [30]. Though a Tsc2 ortholog appears to be absent in yeast, these correlations might suggest an evolutionarily conserved regulatory framework that links glucose metabolism, TOR signaling, mitochondrial gene expression and life span.

Whether the increase in nuclear OXPHOS gene expression is mediated at the transcriptional or post-transcriptional level remains to be determined, as does the identity of the putative TOR-regulated mitochondrial factors that meditate the increase in mitochondrial mRNA transcription/stability and translation of mtDNA-encoded OXPHOS subunits. Certainly, nuclear transcription factors that are known to be downstream of TORC1 [31], involved in nuclear-mitochondrial signaling [32], or in glucose repression of mitochondrial function [33] are obvious candidates to test with regard to the nuclear gene expression response. And, with regard to TOR-dependent factors that regulate mitochondrial gene expression directly, the mitochondrial transcription machinery, mitochondrial ribosomes, or the various general and specific translational activators [5] are likely candidates to consider in future studies. Furthermore, since our results clearly implicate Sch9p as the key mediator of the TORC1-mitochondria-CLS pathway (Figure 6B), searching for mitochondrial substrates of Sch9p as potential downstream targets that execute changes in mitochondrial gene expression and OXPHOS activity would likely be fruitful.

The fact that up-regulation of mitochondrial oxygen consumption [18] and mitochondrial translation (Figure 1) in *tor1Δ* strains occurs only in log-phase and early stationary phase cultures (and not later in stationary phase) strongly suggests that TOR-dependent

mitochondrial changes that occur early are responsible for the life span extension later in stationary phase. The concept of early mitochondrial-related events effecting life span has been promoted by others in aging studies in *C. elegans* [34, 35] and is also consistent with the observation of Piper and colleagues that previous conditioning of yeast to respiratory conditions extends CLS in subsequent cultures [36]. While, at this point, the molecular explanation for this "mitochondrial pre-conditioning" effect is not clear, we consider ROS signaling as one potential model. This idea is attractive because the rate of production of ROS from the mitochondrial electron transport chain is likely an accurate reflection of mitochondrial OXPHOS activity and/or redox status that could be used by cells as a retrograde signal to modulate nutrient-sensing pathways. Although we have not observed a significant change in the steady-state level of superoxide in log-phase *tor1Δ* cells (data not shown), it is possible that other ROS species may be relevant or that the steady- state measurements are not accurately predicting the rate of mitochondrial ROS production. Alternatively, we observed up-regulation of Yhb1, a nitric oxide detoxifying enzyme in *tor1Δ* mitochondria, but not Sod2p (data not shown; [18]). These results might suggest a role for NO and/or other reactive nitrogen species as relevant. Interesting in this regard, as is the case in *tor1Δ* cells (Table 1), Yhb1p localizes to mitochondria under anaerobic condition [37]. This, coupled to our observation that hypoxic conditions bypass the extension of CLS by *TOR1* deletion [18] might suggest that reduced TOR signaling and an-aerobic conditions share a common route to impact life span that may involve NO metabolism. Future studies along these and related lines, as well as further characterization of TOR-dependent changes in the mitochondrial proteome should be most revealing in terms of understanding how the TOR-mitochondria axis controls aging and deciphering the complex relationships between OXPHOS activity, ROS (and/or other reactive species), nutrient sensing, and life span. This, in turn, may provide new inroads into understanding and perhaps counteracting age-related pathology in humans.

## MATERIALS AND METHODS

Yeast strains. Unless otherwise stated, strains of the DBY2006 (*MATa his3-Δ200 leu2-3,-112 ura3-52 trp1-Δ1 ade2-1*) background were used exclusively. The GS122 and GS129 strains are derivatives of DBY2006 that have plasmid-borne RPO41 and rpo41-R129D alleles covering a chromosomal disruption of the endogenous *RPO41* gene and have been described previously [24]. These strains were used for the experiments presented in Figures 1 and 4. The *TOR1* gene was disrupted in these strains as described previously [18]. The *SCH9* gene was disrupted using a standard *HIS3* knockout cassette [38]. Briefly, the *HIS3* in pRS313 was PCR amplified with primers ACCACCGCTATTAGTCAGGACTTATATGCAATG GGCACAACAGGAATAACAAGATTGTACTGAGA GTGCAC (*SCH9*_LeftDel) and CATCATTGATGTCC TCGTCCCCGTCATCATCGATGACATCTTCGTCTG GACTGTGCGGTATTTCACACCG (SCH9_RightDel). Gel-purified amplicons were used to transform wild-type and *tor1Δ* DBY2006. His+ transformants were selected on his- plates and single colonies were picked and verified by PCR. The mitochondrial GFP expres-sing yeast strains were generated by transforming wild-type DBY2006 and *tor1Δ* with pYX142-SU9-GFP [39].

Mitochondria purification. Mitochondria were isolated from yeast (from cultures grown to $OD_{600}$=1.0 in selective media) by differential centrifugation followed by sucrose-gradient fractionation as described [40]. For 2D-DIGE, the purity of mitochondrial preparations was checked by western blot analysis with anti-actin (Chemicon, 1:1000), anti-alkaline phosphatase (Molecular Probe 1:1000), anti-Dol-P-Man synthase (Molecular Probes, 1:1000) antibodies to control for contamination of cytoplasm, vacuolar membrane, and ER membrane respectively. Only residual ER contamination was present in the purified mitochondrial preparations (data not shown).

Mitochondrial translation assay. Unless otherwise stated, mitochondrial translation assays were performed as described [25], except the following: all reactions were carried out at $30^0$C, gradient gels (6-20%, Figures 1 and 4; 15-22.5%, Figure 5) were used to resolve translation products and electrophoresis was conducted at a constant current of 30 mA.

Chronological life Span assay. Chronological life span was assayed as described previously [9, 18] Unless otherwise stated, viability was determined by staining with 0.4% trypan blue.

Measurement of mtDNA copy number. The mtDNA copy number was determined using a quantitative real-time PCR procedure as described previously [41, 42].

Northern analysis. Northern blots were performed as described previously [24, 42]. Briefly, 5 µg of total RNA extracted from yeast (cultured to an $OD_{600}$=1) was separated on a 1.5% agarose-formaldehyde gel and then transferred to a nylon membrane by capillary action. Radiolabeled probes were synthesized by PCR with $^{32}$P-

dCTP an added to the membranes in rapid-hyb buffer (GE Healthcare) and incubated overnight at 42 $^0$C. The membrane was washed at room temperature with increasing stringency before visualization by auto-radiography as described in the references cited above.

Western blot analysis. Western blots of mitochondria and total cell extracts (from cultures at ($OD_{600}$=1) was performed as described previously [18, 25]. Proteins were separated on a 10% SDS-PAGE, transferred to a PVDF membrane, and incubated with the indicated primary and HRP-conjugated anti-mouse secondary (Molecular Probes) antibodies as described previously [18, 25]. Anti-Cox4p (MitoSciences) antibody (not used previously) was diluted 1:1000 for incubation with the blocked membrane.

2D-DIGE. Mitochondrial extraction followed steps mentioned in the "mitochondrial extraction" section. 2D-DIGE was conducted by the W.M. Keck Facility of Yale University (http://keck.med.yale.edu/dige/). Briefly, protein samples were prepared by TCA-precipitation of mitochondrial extracts from DBY2006 and *tor1Δ*. The samples were further cleaned with 2-D Clean-Up Kit (GE Healthcare) and labeled with CyDye DIGE fluors (Amersham). 50 μg of the labeled samples were resolved on a 2D gel (Ettan DIGE system from Amersham). A representative 2D gel and the distribution of up-regulated and down-regulated proteins is shown in Supplemental Figure S3. In this study, 26 proteins that were up-regulated by 2-fold or more were selected for MALDI-MS/MS analysis. We were able to unambiguously identify 11 of these based on multiple peptide matches.

Flow cytometry. All analysis was performed on a Beckton-Dickenson FACSCalibur. Analysis of yeast ROS using DHE was performed as described previously [9]. For measurement of mitochondrial potential, cells from a growing culture were pelleted by centrifugation and washed with phosphate-buffered saline (PBS). $DiOC_6$ (Molecular Probes) was diluted to a final concentration of 200 nM in PBS and used to resuspend the cells. The cell suspension was then incubated for 30 minutes at 30 $^0$C, washed twice with PBS, and analyzed by flow cytometry using the FL3 channel without compensation. For measurement of mitochondrial mass, cultured GFP expressing yeast cells were pelleted, washed once and then re-suspended in PBS, and subject to flow cytometry analysis using the FL1 channel without compensation.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTERESTS STATEMENT

The authors have no conflict of interests to declare.

## REFERENCES

**1.** Tissenbaum H, Guarente L. Model organisms as a guide to mammalian aging. Dev Cell. 2002;2:9-19.

**2.** Mair W, Dillin A. Aging and survival: the genetics of life span extension by dietary restriction. Annu Rev Biochem. 2008;77:727-754.

**3.** Guarente L. Mitochondria--a nexus for aging, calorie restriction, and sirtuins? Cell. 2008;132:171-176.

**4.** Wallace D. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu Rev Genet. 2005;39:359-407.

**5.** Costanzo MC, Fox TD. Control of mitochondrial gene expression in Saccharomyces cerevisiae. Annu Rev Genet. 1990;24:91-113.

**6.** Bonawitz ND, Clayton DA, Shadel GS. Initiation and beyond: multiple functions of the human mitochondrial transcription machinery. Mol Cell. 2006;24:813-825.

**7.** Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. Cell. 2005;120:483-495.

**8.** Bonawitz ND, Shadel GS. Rethinking the mitochondrial theory of aging: the role of mitochondrial gene expression in lifespan determination. Cell Cycle. 2007;6:1574-1578.

**9.** Bonawitz ND, Rodeheffer MS, Shadel GS. Defective mitochondrial gene expression results in reactive oxygen species-mediated inhibition of respiration and reduction of yeast life span. Mol Cell Biol. 2006;26:4818-4829.

**10.** Hlavatá L, Nachin L, Jezek P, Nyström T. Elevated Ras/protein kinase A activity in Saccharomyces cerevisiae reduces proliferation rate and lifespan by two different reactive oxygen species-dependent routes. Aging Cell. 2008;7:148-157.

**11.** Barros M, Bandy B, Tahara E, Kowaltowski A. Higher respiratory activity decreases mitochondrial reactive oxygen release and increases life span in Saccharomyces cerevisiae. J Biol Chem. 2004;279:49883-49888.

**12.** Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell. 2006;124:471-484.

**13.** Kunz J, Henriquez R, Schneider U, Deuter-Reinhard M, Movva NR, Hall MN. Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. Cell. 1993;73:585-596.

**14.** Kaeberlein M, Powers R 3rd, Steffen K, Westman EA, Hu D, Dang N, Kerr EO, Kirkland KT, Fields S, Kennedy BK. Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. Science. 2005;310:1193-1196.

**15.** Powers R 3rd, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S. Extension of chronological life span in yeast by decreased TOR pathway signaling. Genes Dev. 2006;20:174-184.

**16.** Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L, Müller F. Genetics: influence of TOR kinase on lifespan in C. elegans. Nature. 2003; 426:620.

**17.** Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S. Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. Curr Biol. 2004 May 25;14:885-890.

**18.** Bonawitz ND, Chatenay-Lapointe M, Pan Y, Shadel GS. Reduced TOR signaling extends chronological life span via increased respiration and upregulation of mitochondrial gene expression. Cell Metab. 2007;5:265-277.

**19.** Schieke SM, Phillips D, McCoy JP Jr, Aponte AM, Shen RF, Balaban RS, Finkel T. The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. J Biol Chem. 2006;281:27643-27652.

**20.** Urban J, Soulard A, Huber A, Lippman S, Mukhopadhyay D, Deloche O, Wanke V, Anrather D, Ammerer G, Riezman H, Broach JR, De Virgilio C, Hall M, Loewith R. Sch9 is a major target of TORC1 in Saccharomyces cerevisiae. Mol Cell. 2007;26:663-674.

**21.** Fabrizio P, Pozza F, Pletcher SD, Gendron CM, Longo VD. Regulation of longevity and stress resistance by Sch9 in yeast. Science. 2001;292:288-290.

**22.** Lavoie H, Whiteway M. Increased respiration in the sch9Δ mutant is required for increasing chronological life span but not replicative life span. Eukaryot Cell. 2008;7:1127-1135.

**23.** Bandhakavi S, Xie H, O'Callaghan B, Sakurai H, Kim D, Griffin T. Hsf1 activation inhibits rapamycin resistance and TOR signaling in yeast revealed by combined proteomic and genetic analysis. PLoS ONE. 2008;3:e1598.

**24.** Rodeheffer MS, Boone BE, Bryan AC, Shadel GS. Nam1p, a protein involved in RNA processing and translation, is coupled to transcription through an interaction with yeast mitochondrial RNA polymerase. J Biol Chem. 2001 Mar 16;276(11):8616-8622.

**25.** Rodeheffer MS, Shadel GS. Multiple interactions involving the amino-terminal domain of yeast mtRNA polymerase determine the efficiency of mitochondrial protein synthesis. J Biol Chem. 2003;278:18695-18701.

**26.** Bryan AC, Rodeheffer MS, Wearn CM, Shadel GS. Sls1p is a membrane-bound regulator of transcription-coupled processes involved in Saccharomyces cerevisiae mitochondrial gene expression. Genetics. 2002 Jan;160(1):75-82.

**27.** Ulery TL, Jang SH, Jaehning JA. Glucose repression of yeast mitochondrial transcription: kinetics of derepression and role of nuclear genes. Mol Cell Biol. 1994;14:1160-1170.

**28.** Amiott EA, Jaehning JA. Mitochondrial transcription is regulated via an ATP "sensing" mechanism that couples RNA abundance to respiration. Mol Cell. 2006; 22: 329-338.

**29.** Hardie DG, Carling D, Carlson M. The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu Rev Biochem. 1998;67:821-855.

**30.** Inoki, K., Zhu, T., and Guan, K.L. TSC2 mediates cellular energy response to control cell growth and survival. Cell 2003; 115, 577–590.

**31.** Beck T and Hall M. The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature. 1999;402:689-692.

**32.** Liu Z, Butow R. Mitochondrial retrograde signaling. Annu Rev Genet. 2006;40:159-185.

**33.** Carlson M. Glucose Repression in yeast. Curr Opin Microbiol. 1999 Apr;2(2):202-207.

**34.** Dillin A, Hsu AL, Arantes-Oliveira N, Lehrer-Graiwer J, Hsin H, Fraser AG, Kamath RS, Ahringer J, Kenyon C. Rates of behavior and aging specified by mitochondrial function during development. Science. 2002;298:2398-2401.

**35.** Schulz T, Zarse K, Voigt A, Urban N, Birringer M, Ristow M. Glucose restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress. Cell Metab. 2007;6:280-293.

**36.** Piper PW, Harris NL, MacLean M. Preadaptation to efficient respiratory maintenance is essential both for maximal longevity and the retention of replicative potential in chronologically ageing yeast. Mech Ageing Dev. 2006;127:733-740.

**37.** Cassanova N, O'Brien KM, Stahl BT, McClure T, Poyton R. Yeast flavohemoglobin, a nitric oxide oxidoreductase, is located in both the cytosol and the mitochondrial matrix: effects of respiration, anoxia, and the mitochondrial genome on its intracellular level and distribution. J Biol Chem. 2005;280:7645-7653.

**38.** Burke D, Dawson D, Stearns T (2000). Gene Replacement. In Methods in Yeast Genetics, Burke D, Dawson D, Stearns T ed. (Woodbury, USA: Cold Spring Harbor Laboratory Press), pp.55-59.

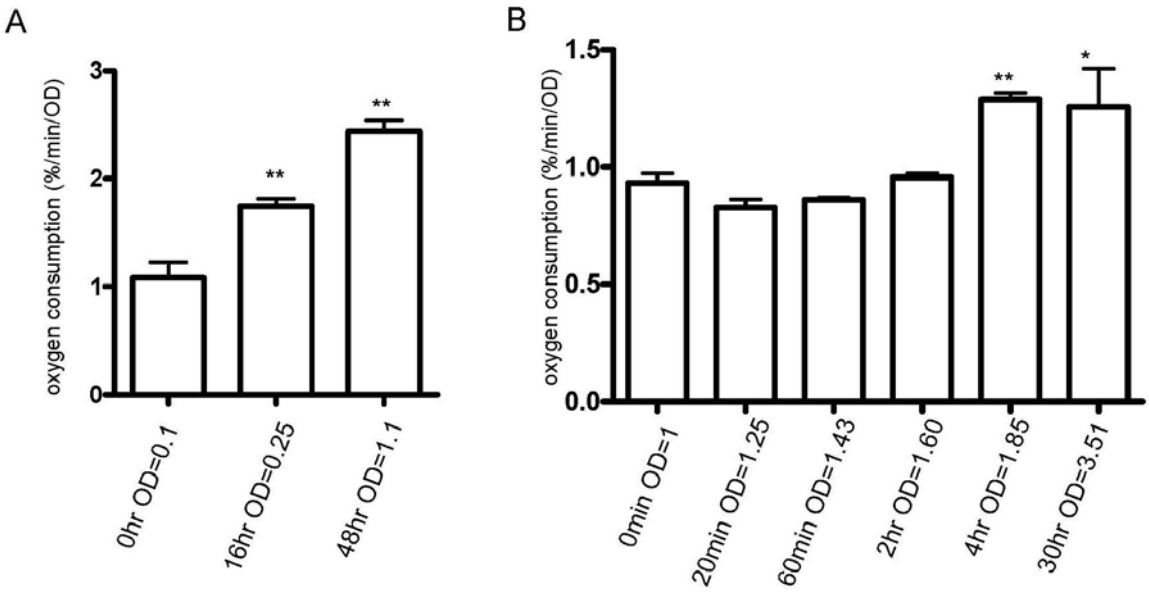
**39.** Frederick R, McCaffery J, Cunningham K, Okamoto K, Shaw J, Yeast Miro GTPase, Gem1p, regulates mitochondrial morphology via a novel pathway. J. Cell Biol. 2004;167:87–98.

**40.** Meisinger C, Pfanner N, Truscott K (2006). Isolation of Yeast Mitochodria. In Methods in Molecular Biology, vol 313: Yeast Protocols, Xiao W ed. (Totowa, USA: Humana Press), pp.33-39.

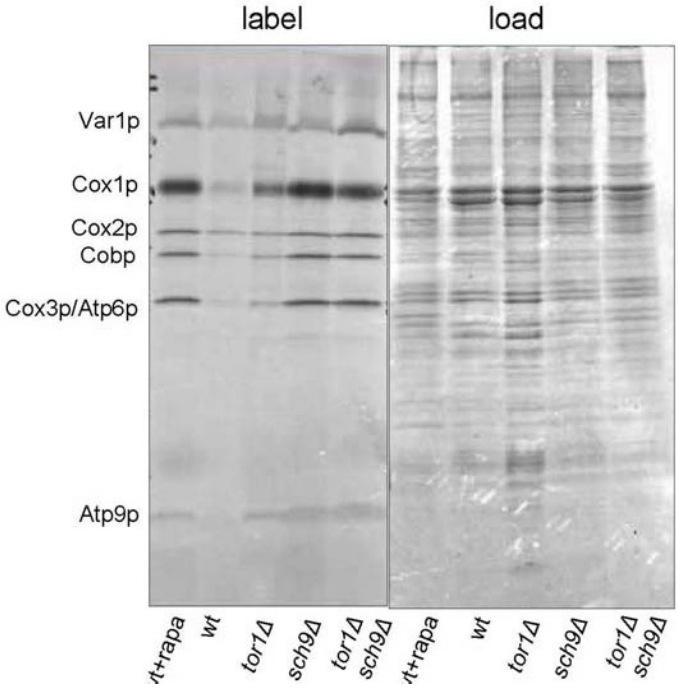
**41.** Taylor SD, Zhang H, Eaton JS, Rodeheffer MS, Lebedeva MA, O'rourke TW, Siede W, Shadel GS. The conserved Mec1/Rad53 nuclear checkpoint pathway regulates mitochondrial DNA copy number in Saccharomyces cerevisiae. Mol Biol Cell. 2005;16:3010-3018.

**42.** Lebedeva MA, Shadel GS. Cell cycle- and ribonucleotide reductase-driven changes in mtDNA copy number influence mtDNA Inheritance without compromising mitochondrial gene expression. Cell Cycle. 2007;6:2048-2057.

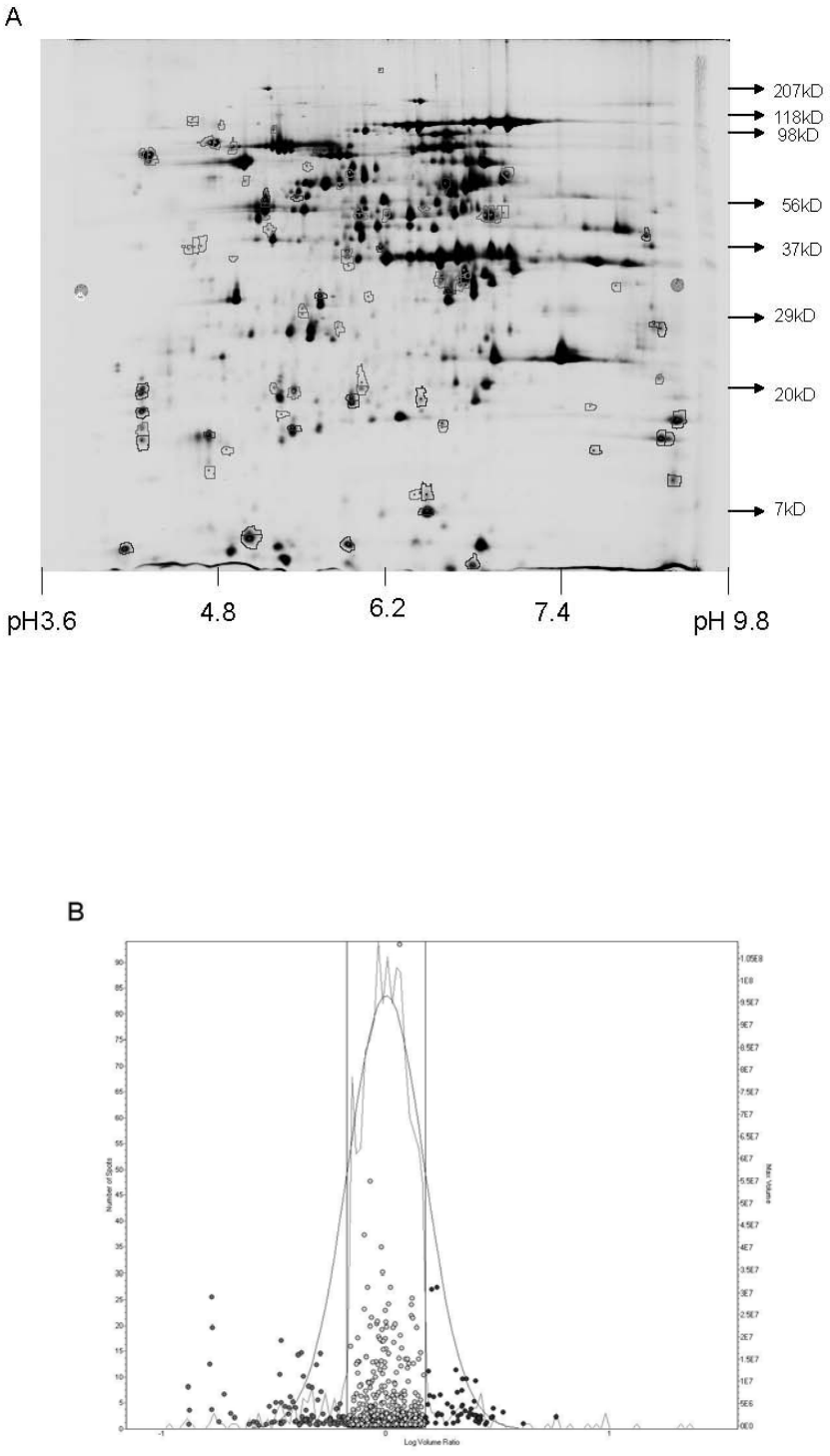
## SUPPLEMENTAL FIGURES




**Supplemental Figure 1. Rapamycin affects mitochondrial oxygen consumption differently depending on whether it is added at the beginning of the culture or during exponential growth.** (**A**) Mitochondrial oxygen consumption of a wild-type (DBY2006) culture supplemented with 200 nM rapamycin upon inoculation. Indicated on the x-axis are the time after inoculation and the OD600 at that time point. (**B**) Same as in (**A**) except rapamycin was added during active growth ($OD_{600}$ of 1) and the times indicate the time after addition and the OD600 at that time point.




**Supplement Figure 2. Newly synthesized mtDNA-encoded OXPHOS subunits have similar stability in wild-type and *tor1* strains.** The experiment shown is identical to that described in Figure 5B, except non-radioactive amino acids were added to the culture (cold chase) at 30°C for 90 minutes, instead of 10 minutes.




**Supplemental Figure 3.** 2D-DIGE analysis of changes in the mitochondrial proteome in *tor1* strains. (A) 2D gel image of *tor1* mitochondrial proteins. Wild-type (DBY2006) and *tor1* were labeled with cy3 and cy5, respectively. The indicated pI (x-axis) and molecular weight (y-axis) are approximate. (**B**) Spot distribution of differentially expressed mitochondrial proteins. The x-axis indicates the cy5/cy3 ratio (positive values indicate up-regulation in *tor1* strains; negative values down-regulation). The left y-axis shows spot frequency; the right y-axis represents the maximum spot volume of a given spot (pair). Frequency distribution of the log volume ratios (rough curve) is plotted, while the normalized model frequency (smooth curve) was fitted to the spot ratios so that the modal peak is zero. Vertical lines indicate a 1.5-fold difference cutoff in cy5/cy3 spot volume ratio.

Research perspective

# Macronutrient balance and lifespan

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***Key words:*** *caloric restriction, dietary restriction, protein, geometric framework, nutrient balance, TOR, AMPK*
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**Abstract: Dietary restriction (DR) without malnutrition is widely regarded to be a universal mechanism for prolonging lifespan. It is generally believed that the benefits of DR arise from eating fewer calories (termed caloric restriction, CR). Here we argue that, rather than calories, the key determinant of the relationship between diet and longevity is the balance of protein to non-protein energy ingested. This ratio affects not only lifespan, but also total energy intake, metabolism, immunity and the likelihood of developing obesity and associated metabolic disorders. Among various possible mechanisms linking macronutrient balance to lifespan, the nexus between the TOR and AMPK signaling pathways is emerging as a central coordinator.**

Convincingly separating the effects of CR on lifespan from more specific nutrient effects is not trivial and requires experimental designs comprising multiple dietary regimes in which energy intake and nutrient balance are considered both separately and interactively [1]. Building upon an earlier study questioning the role of CR in *Drosophila melanogaster* [2], the first study to employ a design that unequivocally disentangled CR from specific nutrient effects was that of Lee et al. [3]. Mated female flies were allowed ad libitum access to one of 28 diets, varying in the ratio and concentration of yeast to sugar. Food intake was measured for each fly and bi-coordinate intakes of protein and carbohydrate (the major macronutrients in the diets) were plotted. Response surfaces for lifespan, age of maximal mortality, rate of age-dependent increase in mortality, lifetime egg production and rate of egg production were then fitted over the array of protein-carbohydrate intake points (see Figure 1A for lifespan and lifetime egg production surfaces). Flies lived longest on a diet containing a 1:16 P:C ratio and lived progressively less long as the P:C ratio increased. The contours of the longevity surface ran almost orthogonally to lines of equal caloric intake (dotted lines in Figure 1A). Even allowing for possible differences in the relative availability of energy in protein and carbohydrate or interactions between protein and carbohydrate metabolism, the lifespan and caloric intake isoclines in Figure 1A cannot be aligned. The data therefore prove that CR could not account for the variation in lifespan. Rather, the balance of carbohydrate to protein ingested was strongly correlated with longevity.

The response surface for lifetime egg production peaked at a higher protein content than supported maximal lifespan (1:4 P:C, Figure 1A). This demonstrates that the flies could not maximize both lifespan and egg production rate on a single diet, and raises the interesting question of what the flies themselves prioritized – extending lifespan or maximizing lifetime egg production. Lee et al. [3] answered this by offering one of 9 complementary food choices in the form of

separate yeast and sugar solutions differing in concentration. The flies mixed a diet such that they converged upon a nutrient intake trajectory of 1:4 P:C, thereby maximizing lifetime egg production and paying the price of a diminished lifespan.

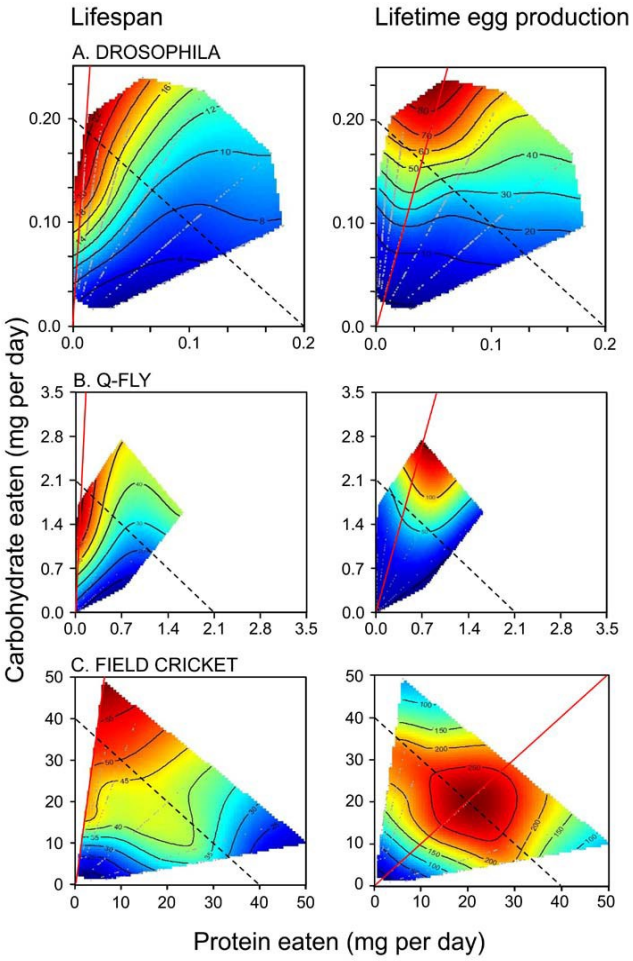


**Figure 1. How the intake of protein and carbohydrate influence longevity and lifetime egg production in adults of three insect species.** Individuals were given ad libitum access to one of 28 (*Drosophila* and the Queensland fruit fly, Q-fly) or 24 (field cricket) diets varying in the ratio and total concentration of protein to carbohydrate (P:C). Plotted onto arrays of points of nutrient intake are fitted surfaces for the two performance variable, which rise in elevation from dark blue to dark red. Unbroken red lines indicate the dietary P:C that maximized the response variable, whereas the dotted lines indicate isocaloric intakes. In each case, insects lived longest when the diet contained a low P:C, and lifespan declined as P:C rose. Female reproductive output was maximal on higher P:C diets than sustained greatest longevity, but fell as P:C rose further, even at high total energy intakes. Data are replotted from Lee et al. [3] (*Drosophila*), Maklakov et al. [11] (field crickets), and Fanson et al. [4] (Q-fly).

Lee et al. [3] compared their data against a longevity surface compiled from previously published studies, individually involving many fewer dietary treatments and no measurement of long-term food intake. The two surfaces corresponded closely, despite substantial procedural differences across studies and differences in mean lifespan between capillary-fed, singly housed flies in the study of Lee et al. [3] and flies housed in groups and fed agar-based diets in the other experiments. To further demonstrate that the nutritional associations were robust, traditional demography cage trials were run for a selection of diets without measuring intake. These flies lived longer than when housed singly and fed from capillaries, but the pattern of lifespan, egg production and egg production rate in relation to dietary P:C ratio was the same.

A parallel experiment was conducted by Fanson et al. [4] on Queensland fruit fly, *Bactrocera tryoni* (another dipteran but from a different family, Tephritidae rather than Drosophilidae) subjected to one of 28 no-choice or 25 choice diet treatments. As can be seen in Figure 1B, the results and conclusions were similar in all respects to those reported by Lee et al. [3] for *Drosophila*. Once again, dietary P:C and not energy intake was strongly associated with lifespan. The data were also consistent with those from studies on another species of tephritid, the Mexican fruit fly, *Anastrepha ludens* [5].

Recently, Ja et al. [6] confirmed that increasing the ratio of yeast to sugar (hence P:C) in the diet substantially reduced lifespan in adult *Drosophila*, to an extent that maps precisely onto the data of Lee et al. [3]. Additionally, these authors found that the more modest shortening of lifespan found on concentrated relative to dilute versions of a diet containing a 1:1 yeast to sugar ratio (the diet composition employed in many previous studies) was absent when flies had access to free water; implying that what has previously been reported as the beneficial effects of DR may instead be the obverse of the deleterious consequences of water deprivation. Providing a separate water source had no effect on the change in lifespan associated with a change in yeast:sugar. Indeed, it can now be suggested with some credence that perhaps the life-prolonging effects of DR, as traditionally conceived, do not occur in *Drosophila*. It is interesting to note how a recent study [7] denotes an increase in P:C combined with overall dilution as 'diet restriction', rather than relying on dilution of a 1:1 yeast:sugar diet as in the past.

In the studies of Lee et al. [3], Fanson et al. [4], Ja et al. [6] and others, longevity was primarily associated with the ratio of yeast to sugar eaten. Yeast is a complex food, containing micronutrients and other chemicals in

addition to protein and carbohydrate. To be sure that P:C is influencing lifespan rather than some correlated component of yeast or another confounding change in diet composition will require using chemically defined diet formulations. No fully satisfactory such diet exists as yet for *Drosophila*, although Troen et al. [8] used four chemically defined diets in which the amino acid methionine and glucose were varied. Small but significant effects of dietary methionine on lifespan were reported.

However, chemically defined diets do exist for other insect species. It is well documented that lowering P:C in chemically defined diets slows the development of juvenile insects [9, 10], and the recent work of Maklakov et al. [11,12] on adult crickets provides conclusive evidence that the ratio of protein to carbohydrate is the primary dietary determinant of lifespan in that insect (Figure 1C). Maklakov et al. fed field crickets, *Teleogryllus commodus*, one of 24 chemically defined diets and measured intake, lifespan, female lifetime egg production, daily egg production, male lifetime courtship singing effort, and singing effort per night. As for tephritids and *Drosophila*, crickets lived longest on low P:C ratio diets, and died progressively earlier as P:C ratio increased. Males but not females demonstrated a reduction in lifespan at high intakes of very low P:C diets; a result which was consistent with their greater propensity to lay down excess body fat on such diets and hence reflects the costs of obesity (a point that we consider further below). Again as for flies, female lifetime egg production was maximal at a higher P:C ratio than sustained maximal lifespan (Figure 1C). Male courtship singing attained a maximum at a lower P:C ratio than did female egg production.

The data for insects show that CR is not responsible for lifespan extension, rather, dietary P:C is critical: is the same true for mammals? It is widely held that CR, not specific nutrient effects, is responsible for lifespan extension in mammals [13,14]. However, we have argued previously [1] that it is not possible to estimate response surfaces such as those in Figure 1 without using a much larger number of diet treatments than have been employed to date in experiments on any mammal, including rodents. Without such surfaces it is simply not possible to separate CR from the effects of nutrient balance. Additionally, it has been reported over many years, notably in the early work of Morris Ross, that protein restriction, and of methionine in particular, extends lifespan in rodents [15-19]. Therefore, a study akin to that of Lee et al. [3] is required on rodents.

Whereas the experiments on insects have been able to concentrate on two macronutrient dimensions, protein and carbohydrate, a full design for rodents would need to extend to three dimensions by including variation in dietary lipid. An efficient initial design would need to include around 30 dietary treatments (e.g. 10 P:C:L ratios and 3 total concentrations), which would need to be fed to mice throughout their lives. This is challenging but by no means intractable – and would allow surfaces for lifespan and all manner of histological, biochemical and molecular variables, including those implicated in the process of aging, to be plotted onto macronutrient intake arrays.

To this point we have concentrated on evidence that increasing the ratio of protein and non-protein energy in the diet decreases lifespan; but as seen in the example from male crickets discussed above, if this ratio falls too far there is an increased risk of decreased longevity associated with obesity. The reason for this is that in omnivores and herbivores studied to date, protein intake is more strongly regulated than that of carbohydrate and fat [20]. As a result, protein appetite drives overconsumption of energy on low percent protein diets, promoting obesity and metabolic disorders with consequent effects on longevity. Overconsumption of energy on low percent protein diets has been reported for insects (e.g. [21]), fish (e.g. [22]), birds [23], rodents [24, 25], nonhuman primates [26] and humans [20, 27]. Fat deposition in response to excess ingested carbohydrate, driven by low dietary percent protein, has been shown to be labile in laboratory selection experiments in an insect - it increased in response to habitual shortage of carbohydrate across successive generations and decreased in the face of persisting carbohydrate excess in the diet [28]. One adaptive mechanism that helps counteract the risk of developing obesity on low percent protein diets is increased facultative diet-induced thermogenesis, whereby excess ingested carbohydrates are removed via wastage metabolic cycles, e.g. involving uncoupling proteins [29].

In the context of the deleterious consequences of overconsumption it is interesting to note that the major causes of increased longevity in studies on calorically restricted primates (most recently [30]) is a reduction in the incidence of diabetes, cancer and cardiovascular disease relative to ad libitum fed controls. This may not result from benefits associated with CR per se, but rather reflect the costs of nutrient imbalance when feeding ad libitum on a fixed diet. As the required balance of nutrients changes over time (with time of day, season, growth and development, and senescence), animals will be forced to overeat some nutrients to gain enough of others. Even if a fixed diet is nutritionally balanced when integrated across the entire lifespan (and

worse if it is not), changes in requirements at a finer timescale will result in accumulated damage from short-term nutrient excesses, which may be ameliorated by modest diet restriction [1].

When protein is eaten in higher then optimal quantities relative to non-protein energy it shortens lifespan - in insects certainly and perhaps too in mammals - but what might the underlying mechanisms be? There are several possibilities, including enhanced production of mitochondrial radical oxygen species [19, 31], DNA and protein oxidative modification, changes in membrane fatty acid composition and mitochondrial metabolism [19, 32], changes in the relationship between insulin/IGF and amino acid signaling pathways, including TOR [33-38], toxic effects of nitrogenous breakdown products and capacity to deal with other dietary toxins [39, 40], changes in immune function to pathogen attack [41, 42], and changed functioning of circadian systems [43]. How these various components are interrelated will begin to emerge from analyses in which multiple biomarkers and response variables are mapped onto nutrient intake surfaces such as shown in Figure 1.

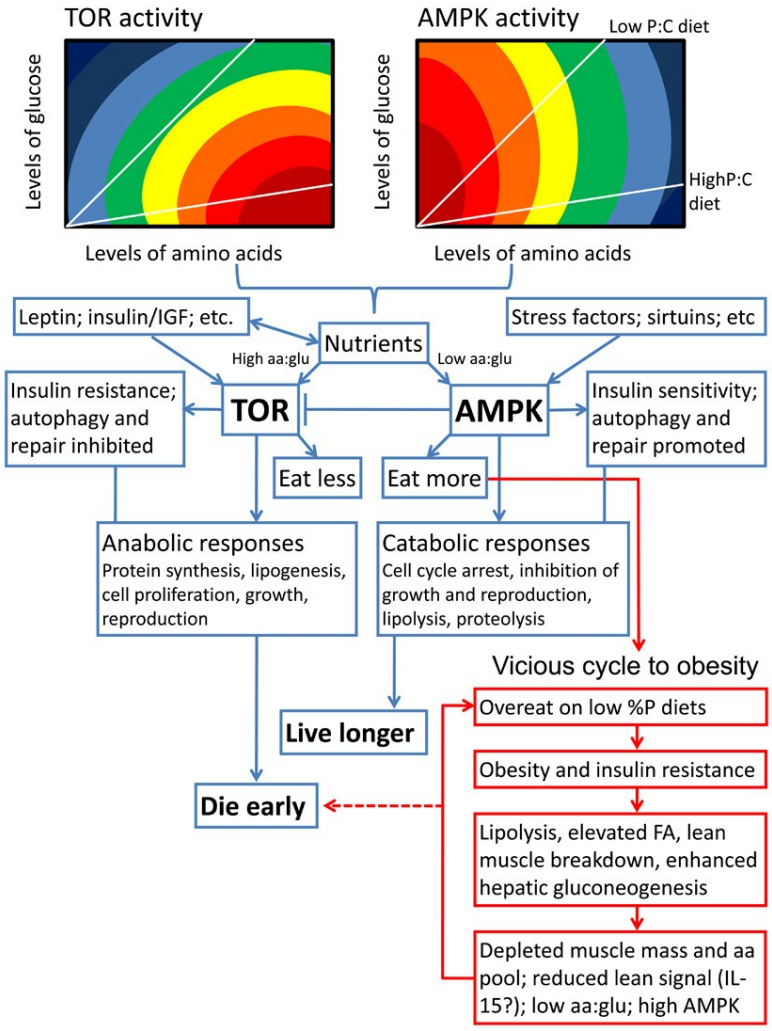


**Figure 2. Schematic summarizing our hypothesis for how diet balance might affect lifespan via the TOR and AMPK signaling pathways.** We propose that both TOR and AMPK respond not only to the concentration of circulating nutrients (with TOR activity stimulated and AMPK depressed either directly or indirectly by increasing concentrations), but also to nutrient balance. We show hypothetical response surfaces for TOR and AMPK in relation to circulating concentrations and ratios of amino acids (aa) and glucose (glu), with responses rising from dark blue to deep red. The red boxes indicate what we have termed the vicious cycle to obesity, in which chronic exposure to a low percent protein diet can drive overconsumption, metabolic disorders and shortened lifespan unless excess ingested energy is dissipated (see [20], and further supporting evidence from rodents in [52,53]). Otherwise, low percent protein diets are life extending via the normal actions of AMPK, whereas high percent protein diets shorten lifespan and encourage aging via the TOR pathway.

If we were to propose one candidate for the hub linking nutrient balance and other inputs to longevity it would be the interplay between the TOR and AMPK signaling pathways. Both TOR and AMPK serve as nutrient sensors and are linked to nutrient intake and metabolism. Factors that directly or indirectly increase TOR signaling, including elevated nutrients such a branch chain amino acids, glucose and fatty acids, are broadly anabolic and life-shortening. In contrast low levels of nutrients, declining ATP:AMP, and other influences that stimulate AMPK signaling are catabolic and life-extending [34; 38; 44-48] (Figure 2); – except when overconsumption, obesity and insulin resistance are driven by protein shortage on a habitually low percent protein diet [20] (see Figure 2). Although it is not yet establish whether TOR and AMPK are nutrient *balance* detectors, there are suggestions that they may well be. For example, glucose activates TOR in an amino acid-dependent manner [49] and elevated percent protein diet stimulates TOR and inhibits AMPK (e.g. [50, 51]). We predict that mapping the responses of both TOR and AMPK onto nutrient intake arrays will provide fundamental new insights not only into aging, but also a whole range of interlinked metabolic phenomena, including obesity, type 2 diabetes, cancer risk and cardiovascular disease. To illustrate this point, we have predicted response surfaces in Figure 2 and linked aspects of nutrient balance, aging and obesity within a single schema.

## CONFLICT OF INTERESTS STATEMENT

The authors have no conflict of interests to declare.

## REFERENCES


**1.** Simpson SJ, Raubenheimer D. Caloric restriction and aging revisited: the need for a geometric analysis of the nutritional bases of aging. J Gerontol A Biol Sci Med Sci 2007; 62:707-713.

**2.** Mair W, Piper MDW, Partridge L. Calories do not explain extension of life span by dietary restriction in *Drosophila*. PLoS Biol 2005; 3:1305-1311.

**3.** Lee KP, Simpson SJ, Clissold FJ, Brooks R, Ballard JWO, Taylor PW, Soran N, Raubenheimer, D. Lifespan and reproduction in *Drosophila*: New insights from nutritional geometry. PNAS 2008; 105:2498-2503.

**4.** Fanson BG, Weldon CW, Pérez-Staples D, Simpson, SJ, Taylor PW. Nutrients, not caloric restriction, extend lifespan in Queensland fruit flies (*Bactrocera tryoni*). Aging Cell 2009; 8:514-523.

**5.** Carey JR, Harshman LG, Liedo P, Müller H-G, Wang J-L, Zhang Z. Longevity-fertility trade-offs in the tephritid fruit fly, *Anastrepha ludens*, across dietary-restriction gradients. Aging Cell 2008; 7:470-477.

**6.** Ja WW, Carvalho GB, Zid BM, Mak EM, Brummel T, Benzer S. Dual modes of lifespan extension by dietary restriction in *Drosophila*. PNAS 2009; (in press).

**7.** Wong R, Piper MDW, Wertheim B, Partridge L. Quantification of food intake in *Drosophila*. PLoS ONE 2009; 4:1-10.

**8.** Troen AM, French EE, Roberts JF, Selhub J, Ordovas JM, Parnell LD, Lai CQ. Lifespan modification by glucose and methionine in *Drosophila melanogaster* fed a chemically defined diet. Age 2007; 29:29-39.

**9.** Raubenheimer D, Simpson SJ. The geometry of compensatory feeding in the locust. Anim Behav 1993; 45:953-964.

**10.** Lee KP, Behmer ST, Simpson SJ, Raubenheimer D. A geometric analysis of nutrient regulation in the generalist caterpillar *Spodoptera littoralis* (Boisduval). J Insect Physiol 2002; 48:655-665.

**11.** Maklakov AA, Simpson SJ, Zajitschek F, Hall MD, Dessmann J, Clissold F, Raubenheimer D, Bonduriansky R, Brooks RC. Sex-specific fitness effects of nutrient intake on reproduction and lifespan. Curr Biol 2008; 18:1062-1066.

**12.** Maklakov AA, Hall MD, Simpson SJ, Dessmann J, Clissold F, Zajitschek F, Lailvaux SP, Raubenheimer D, Bonduriansky R, Brooks RC. Sex differences in nutrient-dependent reproductive ageing. Aging Cell 2009; 8:324-330.

**13.** Weindruch R, Walford RL. The retardation of aging and disease by dietary restriction. Charles C Thomas, Springfield, IL. 1988.

**14.** Masoro EJ. Caloric restriction and aging: Controversial issues. J Gerontol A Biol Sci Med Sci 2006; 61:14-19.

**15.** Ross MH. Length of life and nutrition in the rat. J Nutr 1961; 75:197-210.

**16.** Orentreich N, Matias JR, DeFelice A, Zimmerman JA. Low methionine ingestion by rats extends life span. J Nutr 1993; 123:269-274.

**17.** Miller RA, Buehner G, Chang Y, Harper JM, Sigler R, Smith-Wheelock M. Methionine-deficient diet extends mouse lifespan, slows immune and lens aging, alters glucose, T4, IGF-I and insulin levels, and increases hepatocyte MIF levels and stress resistance. Aging Cell 2005; 4:119-125.

**18.** Zimmerman JA, Malloy V, Krajcik R, Orentreich N. Nutritional control of aging. Exp Gerontol 2003; 38:47-52.

**19.** Ayala V, Naudí A, Sanz A, Caro P, Portero-Otin M, Barja G, Pamplona, R. Dietary protein restriction decreases oxidative protein damage, Peroxidizability Index, and mitochondrial complex I content in rat liver. J Gerontol A Biol Sci Med Sci 2007; 62:352-360.

**20.** Simpson SJ, Raubenheimer D. Obesity: the protein leverage hypothesis. Obesity Rev 2005; 6:133-142.

**21.** Simpson SJ, Sibly RM, Lee KP, Behmer ST, Raubenheimer D. Optimal foraging when regulating intake of multiple nutrients. Anim Behav 2004; 68:1299-1311.

**22.** Ruohonen K, Simpson SJ, Raubenheimer D. A new approach to diet optimisation: A reanalysis using European whitefish (*Coregonus lavaretus*). Aquaculture 2007; 267:147-156.

**23.** Raubenheimer D, Simpson SJ. Integrative models of nutrient balancing: Application to insects and vertebrates. Nutr Res Rev 1997; 10:151-179.

**24.** Simpson SJ, Raubenheimer D. The geometric analysis of feeding and nutrition in the rat. Appetite 1997; 28:201-213.

**25.** Sørensen A, Mayntz D, Raubenheimer D, Simpson SJ. Protein-leverage in mice: The geometry of macronutrient balancing and consequences for fat deposition. Obesity 2008; 16:566-571.

**26.** Felton AM, Felton A, Raubenheimer D, Simpson SJ, Foley WJ, Wood JT, Wallis IR, Lindenmayer, DB. Protein content of diets

dictates the daily energy intake of a free-ranging primate. Behav Ecol 2009; 20:685-690.

**27.** Simpson SJ, Batley R, Raubenheimer D. Geometric analysis of macronutrient intake in humans: the power of protein? Appetite 2003; 41:123-140.

**28.** Warbrick-Smith J, Behmer ST, Lee KP, Raubenheimer D, Simpson SJ. Evolving resistance to obesity in an insect. PNAS 2006; 103:14045-14049.

**29.** Stock MJ. Gluttony and thermogenesis revisited. Int J Obesity 1999; 23:1105-1117.

**30.** Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, Allison DB, Cruzen C, Simmons HA, Kemnitz JW, Weindruch R. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science 2009; 325:201-204.

**31.** Sanz A, Caro P, Barja G. Protein restriction without strong caloric restriction decreases mitochondrial oxygen radical production and oxidative DNA damage in rat liver. J Bioenerg Biomembr 2004; 36:545-552.

**32.** Toden S, Bird AR, Topping, DL, Conlon MA. High red meat diets induce greater numbers of colonic DNA double-strand breaks than white meat in rats: attenuation by high-amylose maize starch. Carcinogenesis 2007; 28:2355-2362.

**33.** Kapahi P, Zid BM. TOR pathway: linking nutrient sensing to life span. SAGE KE 2004; 36:pe34.

**34.** Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S. Regulation of lifespan in *Drosophila* by modulation of genes in the TOR signaling pathway. Curr Biol 2004; 14:885-890. [See also Curr Biol 2004; 14:1789].

**35.** Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller RA. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature 2009; 460:392-395.

**36.** Powers III RW, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S. Extension of chronological life span in yeast by decreased TOR pathway signaling. Gene Dev 2009; 20:174-184.

**37.** Blagosklonny MV. Aging: ROS or TOR. Cell Cycle 2008; 7:3344-3354.

**38.** Blagosklonny MV, Hall MN. Growth and aging: a common molecular mechanism. Aging 2009; 1:357-362.

**39.** Simpson SJ, Raubenheimer D. The geometric analysis of nutrient-allelochemical interactions: A case study using locusts. Ecology 2001; 82:422-439.

**40.** Raubenheimer D, Simpson SJ. Nutritional PharmEcology: Doses, nutrients, toxins, and medicines. Integr Comp Biol 2009; 49:329-337.

**41.** Lee KP, Cory JS, Wilson K, Raubenheimer D, Simpson SJ. Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. Proc R Soc B Biol Sci 2006; 273:823–829.

**42.** Povey S, Cotter SC, Simpson SJ, Lee K-P, Wilson K. Can the protein costs of bacterial resistance be offset by altered feeding behaviour? J Anim Ecol 2009; 78:437-446.

**43.** Hirao A, Tahara Y, Kimura I, Shibata S. A balanced diet is necessary for proper entrainment signals of the mouse liver clock. PLoS ONE 4:1-10.

**44.** Cota D, Proulx K, Seeley RJ. The role of CNS fuel sensing in energy and glucose regulation. Gastroenterol 2007; 132:2158-2168.

**45.** Meijer AJ, Codogno P. Nutrient sensing: TOR's ragtime. Nature Cell Biol 2008; 10:881-883.

**46.** Minokoshi Y, Shiuchi T, Lee, S, Suzuki A, Okamoto S. Role of hypothalamic AMP-kinase in food intake regulation. Nutrition 2008; 24:786-790.

**47.** Levine AJ, Feng Z, Mak TW, You H, Jin S. Coordination and communication between the p53 and IGF-1–AKT–TOR signal transduction pathways. Gen Dev 2009; 20:267-275.

**48.** Steinberg GR, Kemp BE. AMPK in health and disease. Physiol Rev 2009; 89:1025-1078.

**49.** Kwon G, Marshall CA, Pappan KL, Remedi MS, McDaniel ML. Signaling elements involved in the metabolic regulation of mTOR by nutrients, incretins, and growth factors in islets. Diabetes 2004; 53:S225-S232.

**50.** Ropelle ER, Pauli JR, Fernandes MFA, Rocco SA, Marin RM, Morari J, Souza KK, Dias MM, Gomes-Marcondes MC, Gontijo JAR, Franchini KG, Velloso LA, Saad MJA, Carvalheira, JBC. A central role for neuronal AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) in high-protein diet–induced weight loss. Diabetes 2008; 57:594-605.

**51.** Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, Haqq AM, Shah SH, Arlotto M, Slentz CA, Rochon J, Gallup D, Ilkayeva O, Wenner BR, Yancy WS, Eisenson H, Musante G, Surwit RS, Millington DS, Butler MD, Svetkey LP. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab 2009; 9:311-326.

**52.** Zhou Q, Du J, Hu Z, Walsh K, Wang XH. Evidence for adipose-muscle cross talk: Opposing regulation of muscle proteolysis by adiponectin and fatty acids. Endocrinol 2007; 148:5696-5705.

**53.** Quinn LS. Interleukin-15: A muscle-derived cytokine regulating fat-to-lean body composition. J Anim Sci 2008; 86:E75-E83.

Research paper

# The mitochondrial ribosomal protein of the large subunit, Afo1p, determines cellular longevity through mitochondrial back-signaling via TOR1

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***Running title:*** *Yeast AFO1 and TOR1 in replicative aging*
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**Abstract: Yeast mother cell-specific aging constitutes a model of replicative aging as it occurs in stem cell populations of higher eukaryotes. Here, we present a new long-lived yeast deletion mutation, *afo1* (for aging factor one), that confers a 60% increase in replicative lifespan. *AFO1/MRPL25* codes for a protein that is contained in the large subunit of the mitochondrial ribosome. Double mutant experiments indicate that the longevity-increasing action of the *afo1* mutation is independent of mitochondrial translation, yet involves the cytoplasmic Tor1p as well as the growth-controlling transcription factor Sfp1p. In their final cell cycle, the long-lived mutant cells do show the phenotypes of yeast apoptosis indicating that the longevity of the mutant is not caused by an inability to undergo programmed cell death. Furthermore, the afo1 mutation displays high resistance against oxidants. Despite the respiratory deficiency the mutant has paradoxical increase in growth rate compared to generic petite mutants. A comparison of the single and double mutant strains for *afo1* and *fob1* shows that the longevity phenotype of *afo1* is independent of the formation of ERCs (ribosomal DNA minicircles). *AFO1/MRPL25* function establishes a new connection between mitochondria, metabolism and aging.**

## INTRODUCTION

Yeast (*Saccharomyces cerevisiae*) mother cell-specific aging has been shown to be based on the asymmetric distribution of damaged cellular material including oxidized proteins [1]. The mother cell progressively accumulates this material and ages depending on the number of cell division cycles, while the daughter "rejuvenates" and enjoys a full lifespan. Young daughter cells and old (senescent) mother cells can be efficiently separated based on their different size, by elutriation centrifugation [2].

At least some biochemical and genetic mechanisms of aging are conserved throughout the evolution of eukaryotes. A prominent hypothesis postulates that the progressive deterioration of mitochondrial metabolism leads to the production of reactive oxygen species (ROS) that oxidize vulnerable cellular proteins and lipids, while damaging the genome. The cell's genetic response to this oxidative stress may appear as a "genetic program of aging". In this light, some of the current aging theories could well be interrelated and compatible among each other (for review, see [3, 4]).

The TOR signaling pathway is highly conserved from yeast to human cells [5]. It regulates nutrient responses by modulating the nucleo-cytoplasmic shuttling of transcription factors including Sfp1p, which governs ribosome biosynthesis [6]. Down-regulation of TOR kinase induces entry into stationary phase and stimulates autophagy, a process that is vital for survival in conditions of starvation [7]. TOR kinase activity may also be involved in the retrograde response of cells that adapt their nuclear transcriptome to defects in mitochondrial respiration [8]. Yeast possesses two closely related proteins, Tor1p and Tor2p, forming two "TOR complexes" among which only one, TORC1 (containing either Tor1p or Tor2p and active in growth control), is inhibited by rapamycin. Deletion of *TOR2* is lethal due to its essential function in TORC2 (acting on determination of cell polarity). Deletion of *TOR1* leads to an increase in mitochondrial respiration and protein density [9, 10] and to a 15% increase in replicative lifespan, thus establishing a link between nutrition, metabolism, and longevity [11].

In this paper we are presenting a novel long-lived mutant of yeast that establishes a new connection between mitochondria, metabolism and aging. The life-prolonging mutation affects a gene encoding a mitochondrial ribosomal protein, leads to respiratory deficiency, and relies on *TOR1* to confer longevity.

## RESULTS

### A novel yeast mutant with reduced replicative aging

We compared the transcriptome of senescent yeast mother cells (fraction V) with young daughter cells (fraction II) after separating them by elutriation centrifugation [2]. Senescent cells were found to upregulate 39 genes and to down-regulate 53 transcripts. Deletion mutants [12] corresponding to these 92 genes were tested for their resistance or hypersensitivity to five different oxidants (hydrogen peroxide, *tert*-butyl hydroperoxide (t-BHP), diamide, cumene hydroperoxide, and menadione). Only two mutants were found to be consistently resistant against more than one oxidant (and not hypersensitive to any other oxidant). Among these two mutants only one, deleted for YGR076C/*MRPL25* (later termed *AFO1*, see below) caused a mother cell-specific lifespan expansion on the standard media used by us (SC + 2% glucose) (Figure 1). This deletion mutation conferred resistance to diamide and t-BHP and a somewhat weaker resistance to hydrogen peroxide, as well as a 50% reduced ROS production (as compared to the BY4741 $\rho^0$ mutant). ROS production was measured by quantitation of fluorescence signals obtained after dihydroethidium (DHE) staining. The mutant displayed a 60% increase in the median and a 71% increase in the maximum lifespan (Figure 1). The mutant only grew on media containing fermentable carbon sources and hence is respiration deficient. We therefore asked if the respiratory deficiency caused the increased replicative life span. However, a *bona fide* BY4741 $\rho^0$ mutant did not show any extension in replicative life span (as compared to BY4741 WT cells), meaning that lack of respiration is not sufficient to confer longevity to mother cells (Figure 1). We also tested if *afo1*Δ cells displayed the retrograde response [3, 13] by measuring *CIT2* transcription and no effect of the *afo1*Δ mutation could be discerned (see supplementary Figure S1). We conclude that the elongation of lifespan observed here is not caused by respiratory deficiency and is independent of the retrograde response as defined by Jazwinski [14] and Butow [15].

### *AFO1* codes for a mitochondrial ribosomal protein

*AFO1* (YGR076C) codes for MrpL25p, identified by proteomic analysis as a component of the large subunit of the mitochondrial ribosome [16]. Because of its remarkable longevity phenotype, we re-named the gene *AFO1* (for aging factor one). A recombinant construct in which Afo1p was fused in its C-terminus with GFP (Afo1-GFP) was transfected into a heterozygous *afo1Δ* strain. Tetrad dissection revealed that the Afo1-GFP

could replace endogenous Afo1p to enable growth on a non-fermentable carbon source. Confocal fluorescence microscopy confirmed that the protein is located in mitochondria irrespective of the cellular age and the genetic background (supplementary material, Figure S2). The deletion mutant *afo1Δ* exhibited a $\rho^0$ petite phenotype, meaning that it failed to grow on glycerol media and lacked DAPI-detectable mitochondrial DNA. The mutant also showed negligible oxygen consumption when growing on glucose (data not shown). However, in contrast to a *bona fide* BY4741 $\rho^0$ petite mutant, which grew much more slowly than WT cells on standard media with 2% glucose as carbon source, the *afo1*Δ mutant grew as rapidly as WT cells. The growth properties of the mutant and its metabolic implications will be published in detail elsewhere. The average size of the *afo1*Δ mutant cells in exponential phase was equal to that of WT cells, while cells of the $\rho^0$ strain were about 20% larger. Importantly, disruption of the *AFO1* gene in $\rho^0$ cells restored rapid growth, hence reversing the growth defect induced by the absence of mitochondrial DNA.

To obtain definite genetic proof that the *AFO1* deletion caused the resistance against oxidative stress and the extension of the life span described above, we performed co-segregation tests in meiotic tetrads after out-crossing the *afo1*Δ strain in an isogenic cross. In 10 unselected tetrads, which all revealed a regular 2:2 segregation, we observed strict co-segregation of G418 resistance indicating the presence of the gene deletion, respiratory deficiency, and resistance against hydrogen peroxide stress (Figure 2A). We also tested the lifespan of all four haploid progeny of one tetrad and found consistent co-segregation of the deletion allele of *afo1* with extended lifespan (Figure 2B). Furthermore, we also tested if the phenotype of the mutant might result from changes of the expression of the two neighboring genes of *AFO1*. No such effect was apparent (Figure 2C). We conclude that lack of *AFO1* results in long lifespan and the oxidative stress resistance.

We next addressed the possibility that the deletion of other mitochondrial ribosomal genes might also lead to an increase in replicative life span. For this, we investigated the lifespan, growth properties and oxidative stress resistance of two additional deletion mutants in the genes *MRP17* and *PPE1*, encoding mitochondrial ribosomal proteins of yeast. YKL003CΔ (*mrp17Δ*) was found to be resistant against diamide, t-BHP and juglone, but was hypersensitive to hydrogen peroxide and had a normal lifespan. YHR075CΔ (*ppe1Δ*) was resistant against diamide, yet had a normal lifespan (Figure 3). Therefore, the effect of the *afo1*Δ deletion mutant on lifespan is gene-specific.

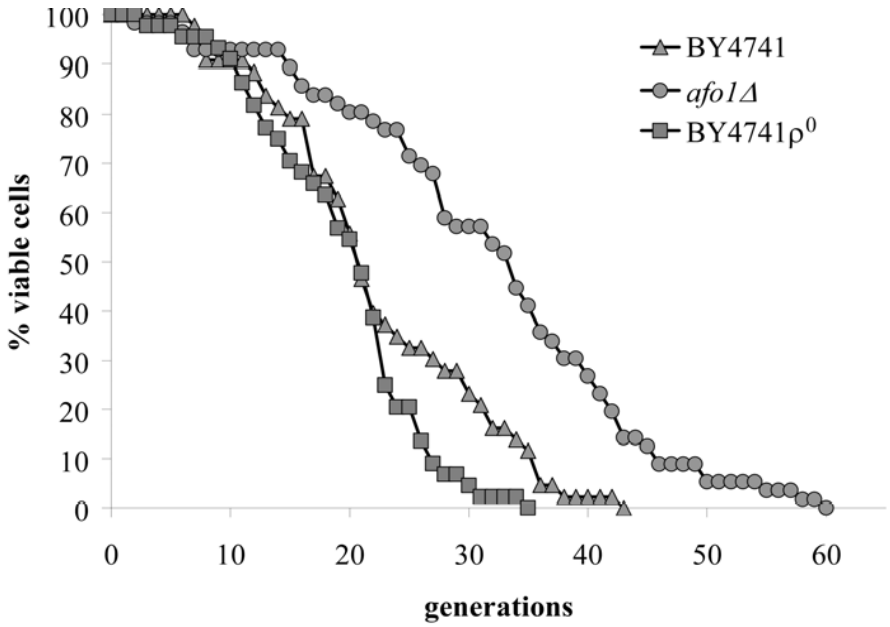


**Figure 1.** Lifespans of isogenic strains *afo1Δ*, wild type BY4741 and BY4741 $\rho^0$. Lifespans were determined as described previously [2] by micromanipulating daughter cells and counting generations of at least 45 yeast mother cells on synthetic complete (SC) media with 2% glucose as carbon source.

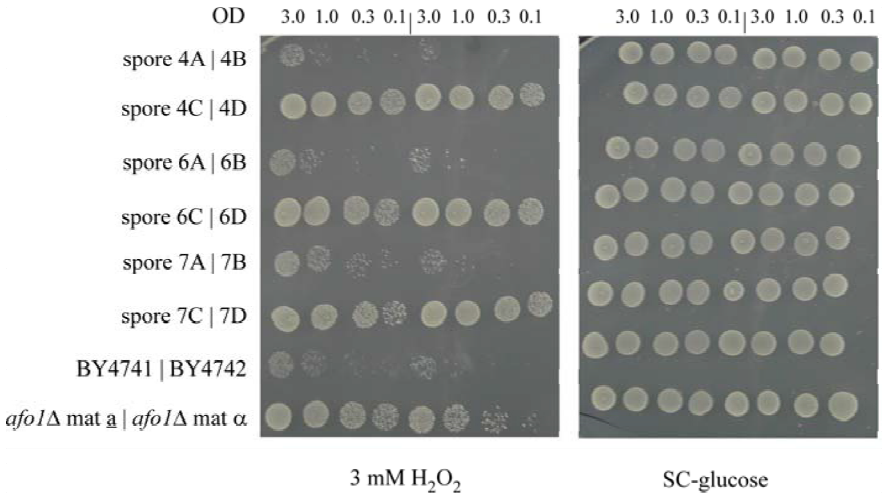


**Figure 2A.** Segregation of the mutant phenotypes of *afo1*Δ in meiotic tetrads after outcrossing and influence of the genes adjacent to *AFO1*. 10µl aliquots of the cultured strains were spotted on SC-glucose and on SC-glucose + oxidants, as indicated in the figure. Cultures were grown to $OD_{600}$ = 3.0 and diluted as indicated. Three out of ten tetrads tested are shown together with two wild type and two *afo1* deletion strains.

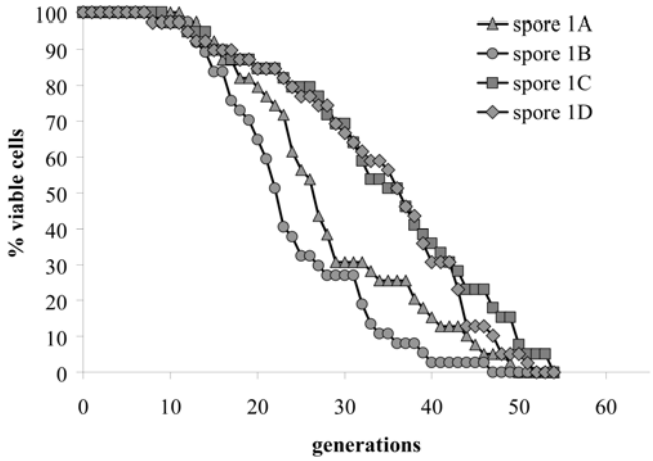


**Figure 2B.** Replicative lifespans of the four haploid segregants of one meiotic tetrad were determined.

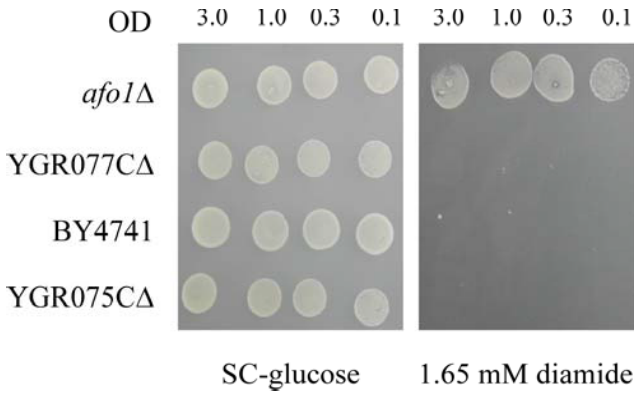


**Figure 2C.** Deletion strains corresponding to the two genes adjacent to *AFO1* are shown. These deletions have no influence on the resistance to oxidants.

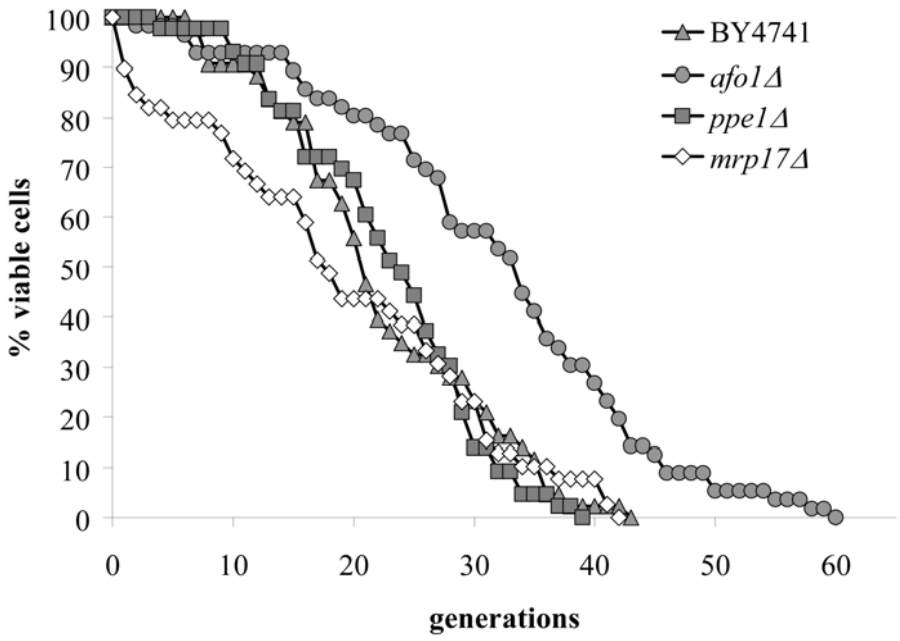


**Figure 3.** Lifespans of the strains deleted for *ppe1* and *mrp17*. The single deletion strains for YKL003C (encoding for Mrp17p) and YHR075C (encoding for Ppe1p), both of the mitochondrial ribosomal small subunit, were tested for their lifespan. The strains were constructed in the BY4741 background. The measured lifespans were not significantly different from wild type ($p<0.02$).

**Longevity mediated by the *afo1* deletion is mediated by the *TOR1* pathway**

Two independent lines of evidence revealed that the *afo1* deletion confers longevity and oxidative stress resistance through the *TOR1* signaling pathway. First, we chromosomally integrated a C-terminally GFP-labeled version of the transcription factor, Sfp1p, at the *SFP1* locus under the control of the native promotor in strains *afo1Δ,* BY4741 WT and BY4741 $\rho^0$. Sfp1p is activated by the TOR1 and PKA pathways and is regulated by shuttling between the nucleus in its active form and the cytoplasm upon deactivation. Sfp1p is a major regulator of cytoplasmic ribosome synthesis and, consequently, of cellular growth [6]. As expected, addition of the Tor1p inhibitor rapamycin to WT cells induced the translocation of Sfp1p from the nucleus to the cytoplasm. In the *bona fide* BY4741 $\rho^0$ strain, Sfp1p was found constitutively in the cytoplasm, even in the absence of rapamycin. In stark contrast, in the *afo1*Δ mutant, Sfp1p was constitutively present in the nucleus, and rapamycin failed to induce the nucleo-cytoplasmic translocation of Sfp1p (Figure 4A). Similar results were obtained with an alternative Tor1p inhibitor, arsenite [17]. Arsenite induced the nucleocyto-plasmic translocation of Sfp1p in WT cells, while Sfp1p stayed in the cytoplasm of $\rho^0$ cells and in the nuclei of *afo1* mutant cells, irrespective of the addition of arsenite (Figure 4B). Rapamycin failed to inhibit the growth of *afo1* mutant cells [18]. Altogether, these data suggested that *TOR1* signaling might govern the longevity of *afo1* cells. The relation between *TOR1* and *AFO1* was further explored by epistasis experiments using double mutants (Figure 5). The lifespan of the double deletion strain (*afo1*Δ, *tor1Δ*) was similar to the lifespan of the *tor1* deletion strain, i.e. about 15% longer than wild type (in good agreement with [11]). However, the double mutant *afo1*Δ, *tor1Δ* strain aged more rapidly than the single mutant *afo1*Δ strain (Figure 5). We conclude that a functional *TOR1* gene is needed for exerting the lifespan-prolonging effect of *afo1*Δ.

We constructed single and double knockout *afo1*Δ, *sfp1*Δ mutant strains and tested their mother cell-specific lifespan and oxidative stress resistance. The median lifespan of *sfp1*Δ cells was shortened considerably as compared to WT cells, and the lifespan of the double *afo1*Δ, *sfp1*Δ mutant was longer than that of the *sfp*1Δ mutant, yet shorter than WT and *afo1*Δ (Figure 6A). Hence, the very short lifespan of *sfp1*Δ

mutant cells is partially rescued by the *afo1* mutation. Like the double *afo1*Δ, *sfp1*Δ mutant, *sfp1Δ* cells displayed a major growth defect. When the *sfp1*Δ strain was made $\rho^0$ with ethidium bromide, cell growth was not further inhibited (data not shown). Comparison of the strains on plates containing 1.6 mM t-BHP revealed that *afo1Δ* is moderately resistant, while single *sfp1Δ* and double *afo1*Δ, *sfp1*Δ mutants exhibited a similar degree of high resistance (Figure 6B). Taken together, these results show that the lifespan-extending effect of *afo1Δ* is most likely independent of the presence of *SFP1*.

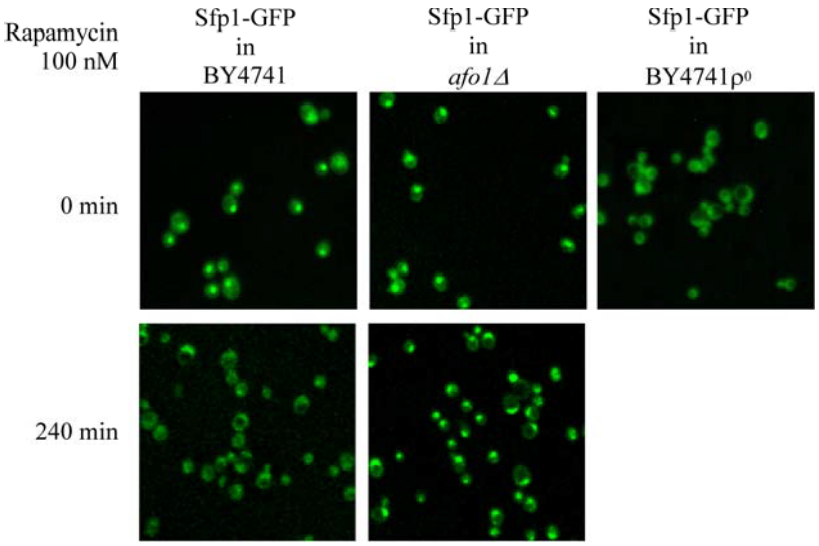


**Figure 4A.** Influence of rapamycin on subcellular localization of the transcription factor, Sfp1p. Strains were grown in liquid SC+2% glucose at 28°C until early logarithmic phase and rapamycin was added to a final concentration of 100 nM. This concentration is growth inhibitory for the wild type strain [6]. Confocal images were taken at time zero (before addition of rapamycin) and at 4 h. The chromosomally integrated *SFP1-GFP-HIS3* construct [37] was present in the wild type strain BY4741, was PCR cloned, sequenced and chromosomally integrated at the *SFP1* locus in strains *afo1Δ* and BY4741 $\rho^0$, respectively.

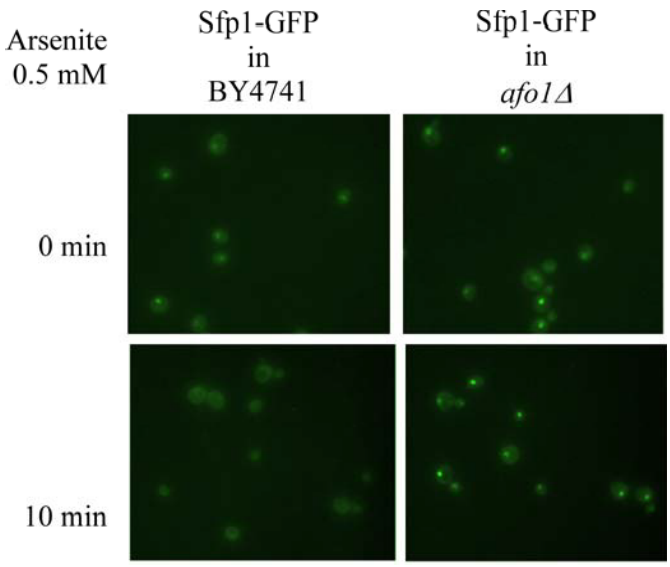


**Figure 4B.** The same strains as in A were treated with 0.5 mM arsenite for 10 min.

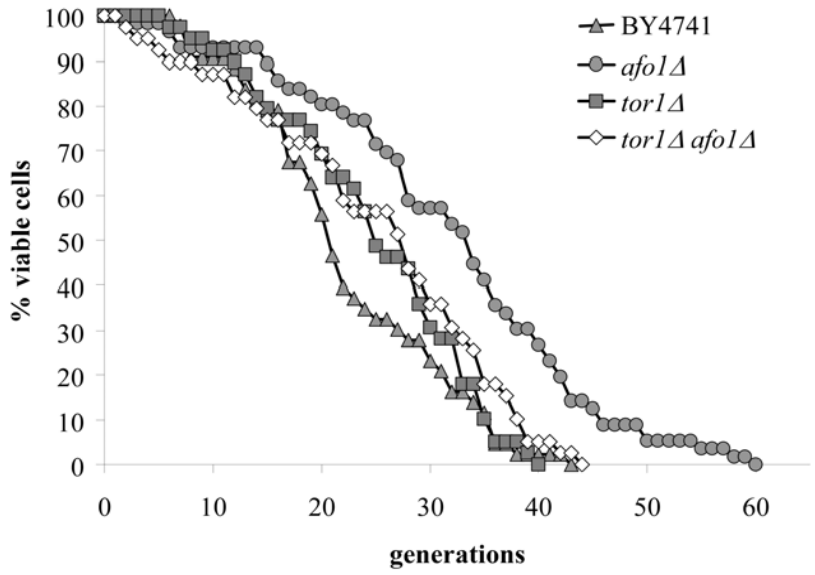


**Figure 5.** Double mutant experiments of *afo1Δ* and *tor1Δ*. The *TOR1* gene is involved in nutrient sensing and lifespan determination in yeast [5]. The double mutant was constructed in an isogenic cross between the two single mutants in the BY background. Lifespans of the wild type, both single mutants and the double mutant were determined by micromanipulation. The experiment shows that an intact *TOR1* gene is needed for the lifespan elongation observed in the *afo1Δ* strain as the lifespan of the *afo1Δ, tor1Δ* double mutant strain is not significantly different ($p<0.02$) from the lifespan of the *tor1Δ* single mutant strain.

Next, we addressed the question as to whether the longevity phenotype of the *afo1* mutation might originate from suppressing the yeast apoptosis pathway. As shown previously [2], old mother cells of the wild type display all of the known markers of yeast apoptosis while these markers are absent from young cells. To tackle this problem, we isolated young (fraction II) and old cells (fraction V) from WT and *afo1*Δ cells by elutriation centrifugation and tested several markers of apoptosis such as externalization of phosphatidyl serine and DNA strand breaks (Figure 7). Our data clearly indicated that *afo1*Δ cells did not lose the ability to undergo apoptosis. In spite of a 60% longer median lifespan, senescent mother cells finally succumbed to apoptosis. We conclude that the components of the programmed cell death pathway that a yeast cell has at its disposal, do not cause replicative aging, but that *vice versa* replicative aging finally leads to cell death via apoptosis.

We also investigated whether the longevity phenotype of the *afo1Δ* mutant might be mechanistically related to the production of extrachromosomal rDNA minicircles (ERCs) [19, 20]. *FOB1* encodes a protein required for the unidirectional replication fork block in rDNA replication. We analyzed the influence of the *fob1* mutation on longevity, growth, and ERC content of WT and *afo1Δ* cells. In our analysis the *fob1Δ* mutation in the BY4741 strain leads to an increase of the replicative lifespan by about 5 generations, in good agreement with previous reports [11, 21].

However, we observed a similar median life span of the *fob1Δ, afo1*Δ double mutant and the *afo1*Δ mutant cells (Figure 8A). As an internal control, both the *fob1*Δ single mutant and the *fob1Δ, afo1*Δ double mutant exhibited the absence of ERCs even in fraction IV and V old cells, while a continuous age-dependent increase in ERCs was found, in particular in fraction IV and V senescent mother cells from WT and *afo1*Δ cells (Figure 8B). Thus, the lifespan-extension observed in the *afo1*Δ strain occurs in the presence of ERCs and is not further increased when ERCs are absent, consequently ERCs do not influence longevity in the *afo1Δ* strain.

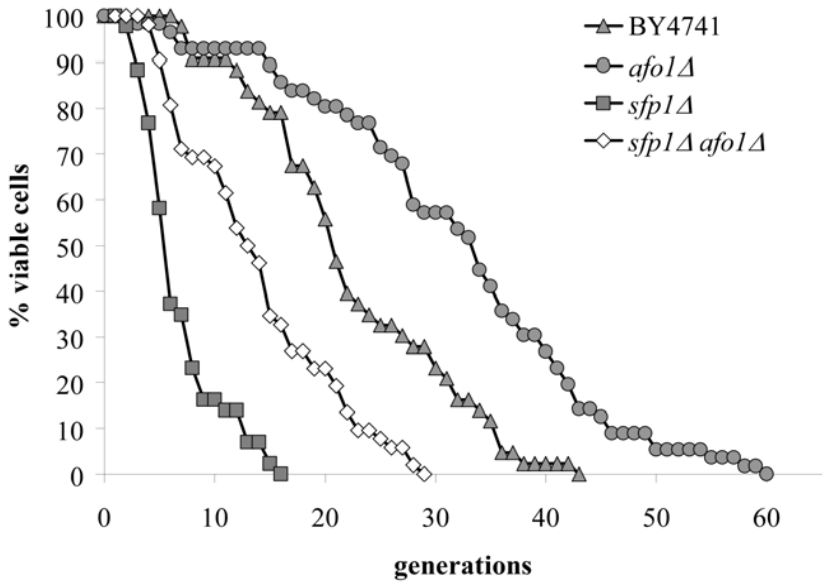


**Figure 6A.** The double mutant strain, *sfp1Δ*, *afo1Δ* was constructed as described in the Materials and Methods section, tested for lifespan, and compared with both single mutant strains and the wild type. The *sfp1Δ* strain grows very slowly although it is respiratory-competent, is highly resistant to t-BHP and is very short-lived. The short lifespan of *sfp1Δ* is partially rescued by *afo1Δ*.

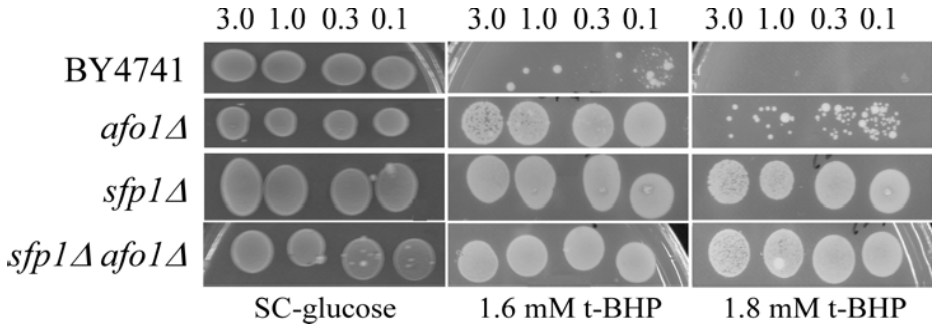


**Figure 6B.** The same strains as in A were tested for resistance against oxidative stress induced by 1.6 mM and 1.8 mM t-BHP. The strong resistance of the *sfp1Δ* mutant strain is not rescued by the *afo1* mutation.

## DISCUSSION

### *AFO1*, the retrograde response and mitochondrial back-signaling

The retrograde response (as defined by Jazwinski [14] and Butow [15]) of non-respiring cells is transmitted through the transcription factor Rtg1/Rtg3p and allows for the transactivation of genes involved in peroxisome synthesis that compensate for the deficient amino acid biosynthesis of cells that lack a complete citrate cycle. As an indicator of the retrograde response, expression of the peroxisomal Cit2p citrate synthase is usually measured [14, 15]. Yeast strains displaying a strong retrograde response increase their replicative lifespan as $\rho^0$ strains over that of the corresponding $\rho^+$ strain. The retrograde response is generally suppressed in 2% glucose but strong on raffinose as sole carbon source [13]. We have measured *CIT2* transcription under the conditions used in this study and found no increase in the transcript of this gene (Figure S1), explaining why the $\rho^0$ strain in the BY4741 series shows the same lifespan as wild type. When raffinose was used as a carbon source, *CIT2* transcription was increased, and as expected, an increase in the lifespan of the *bona fide* BY4741 $\rho^0$ strain was observed (unpublished observation). However, during growth on 2% glucose when the retrograde response is absent in our strain background, we do observe the increase in lifespan described in the present paper. We therefore conclude that the mechanism leading to this increase must be different from the retrograde response.

The lifespan elongation described for the *afo1*Δ mutant strain depends on a signal transmitted from mitochondria to the nucleo-cytoplasmic protein synthesis system and has a strong influence on replicative aging, vegetative growth, and oxidative stress resistance (see below). We propose to call this regulatory signaling interaction „mitochondrial back-signaling“ to distinguish it from the retrograde response described by Jazwinski [14] and Butow [15].

### Evidence for involvement of the TOR1 pathway in longevity of the *afo1* deletion strain

The nature of the signal created by Afo1p is unknown, especially since we found this ribosomal protein to be located in mitochondria in all physiological situations tested, including senescent yeast mother cells. Nonetheless, two independent lines of evidence support the notion that increased activity of *TOR1* determines the longevity of the *afo1* deletion mutant. First, in the double mutant deleted for both *TOR1* and *AFO1*, a life-span is observed that is only moderately longer than that of the wild type and is identical with the lifespan of the *tor1*Δ single mutant (Figure 5). Thus, paradoxically, the relatively small but significant elongation of the lifespan of $\rho^+$ respiring *tor1*Δ cells depends on inactivation of Tor1p, while the large increase in lifespan in the non-respiring *afo1Δ* cells depends on activity of the Tor1p. Second, rapamycin fails to abolish the nuclear location of the transcription factor, Sfp1p, an indicator of Tor1p activity, in *afo1Δ* cells (Figure 4A). Likewise, arsenite, another inhibitor of Tor1p [17], fails to abolish the nuclear location of the transcription factor, Sfp1p, in *afo1*Δ cells (Figure 4B). Sfp1p is well known to be one of the major metabolic regulators of growth and ribosome biosynthesis, which is limiting for growth [6]. The data presented here seem to indicate that Sfp1p activity in the nucleus could be crucial for longevity. The double mutant experiments shown in Figure 5 indicate that the *sfp1*Δ and the *afo1*Δ mutations exert their influence on longevity independently of each other. Moreover, we tested TORC1 kinase activity in WT, $\rho^0$ and in *afo*1Δ cells (data not shown) and found that the long-lived mutant, like the $\rho^0$ strain displayed only very weak TORC1 kinase activity. These results

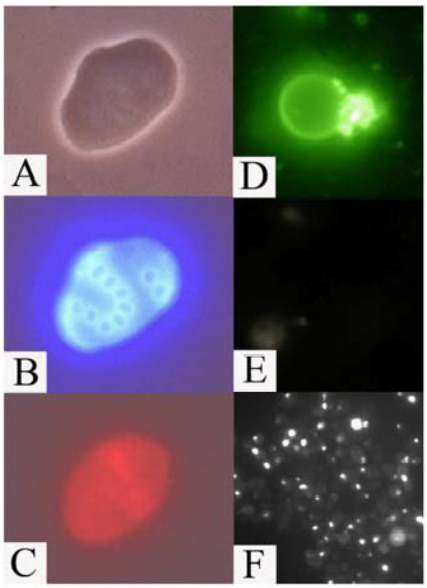


**Figure 7.** Apoptotic markers in old mother cells (fraction V) of the mutant *afo1Δ* strain. (**A**) phase contrast; (**B**) same cell as in A stained with Calcofluor White M2R; (**C**) the same cell stained with DHE indicating a high level of ROS; (**D**) an old mother cell stained with FITC-annexin V revealing inversion of the plasma membrane; (**E**) the same cell as in (**D**) shows absence of staining with propidium iodide revealing intact plasma membrane; (**F**) TUNEL staining of old *afo1Δ* cells.

indicate that the Tor1p activity needed for longevity in the mutant might be feedback-regulated by Sfp1p and/or may be independent of TORC1 kinase activity.

A tentative scheme describing the genetic interactions of mitochondrial back-signaling that we are discussing here, is presented in Figure 9.

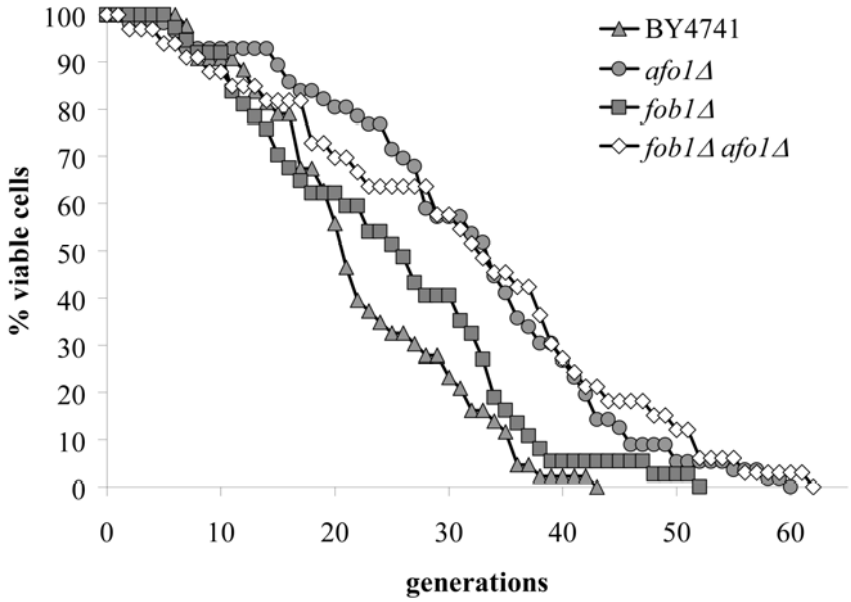


**Figure 8A.** The two mutations, *fob1Δ* and *afo1Δ* were combined in a haploid strain from a meiotic tetrad obtained from an isogenic cross. Wild type, the two single mutants and the double mutant were tested for mother cell-specific lifespan. The *fob1* mutation does not further increase the lifespan of the *afo1* mutant strain ($p<0.02$).

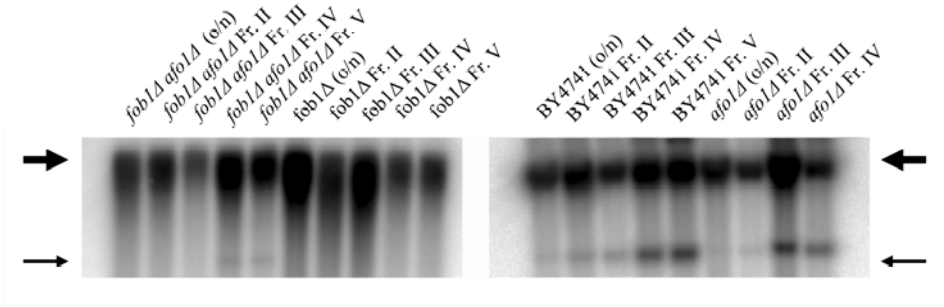


**Figure 8B.** Old and young cells of the same strains as in A were isolated by elutriation centrifugation and ERCs were analyzed by gel electrophoresis and Southern blotting with an rDNA-specific probe as described in [19]. Thick arrow: chromosomal rDNA repeats; Thin arrow: ERCs (minicircles). Taken together, the results presented in this figure indicate that longevity in the *afo1Δ* strain is not influenced by the *fob1*-deletion or the presence of ERCs.

**Has the function of *AFO1* been conserved in evolution**?

In this paper we are presenting evidence for a possibly indirect interaction of the mitochondrial ribosomal protein, Afo1p, with the *TOR1* signaling system of yeast. This interaction is independent of the primary function of Afo1p in translation. A reduction of *TOR1* signaling in yeast [7], rodent and human cells [22] suppresses cellular aging in cell culture [22], and increases longevity in mice [23], worms [24] and fruit flies [25, 26]. These effects were shown to be non-additive with caloric restriction suggesting that the TOR pathway in these organisms is crucial for transmitting the caloric restriction signal. In metazoa, cellular life, **but not organismic life** is possible in the absence of mitochondrial respiration. It is therefore difficult to draw conclusions as to the generality of the *afo1* mutant-based longevity described in the present paper.

The protein complement of mitochondrial ribosomes of both yeast and human cells has been studied [16 and the literature cited therein, 27-30] and the non-translational or extra-ribosomal functions (mostly in transcriptional regulation) of ribosomal proteins have been extensively studied [31, 32]. The published extraribosomal functions mostly concern cytoplasmic, not mito-chondrial ribosomes. Possible extraribosomal functions have to date been found for three of the yeast mitochondrial ribosomal proteins only. Mrps17p and Mrpl37p may play a special role in yeast sporulation [33]. The mitochondrial ribosomal protein of the small subunit, yDAP-3 [34] is well conserved between yeast and human cells and besides its translational role has a distinct function in apoptosis. Its role in the aging process has not been studied yet.

Afo1p is a protein of *S. cerevisae* for which an obvious homolog is known in *Neurospora crassa* [16], but which has no easily apparent counterparts in other eukaryotes (or in *E. coli*) as judged by sequence similarity alone. It is therefore impossible presently to draw conclusions about possible functions of homologs of this protein in aging of higher eukaryotes. However, this may change when the three-dimensional structure of mitochondrial ribosomes will be determined and structural and functional homologs of Afo1p in higher eukaryotes may be found.

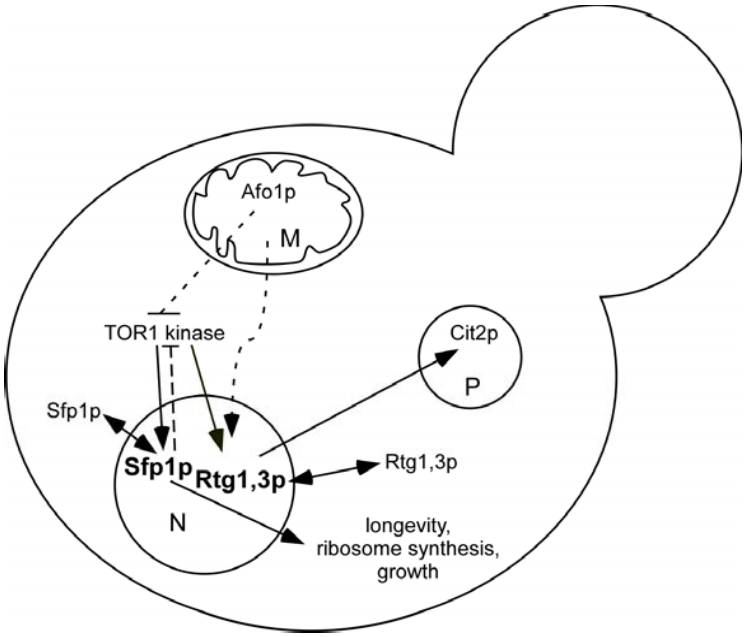


**Figure 9.** Schematic diagram of genetic interactions involving *AFO1* based on the results presented in this paper. Dashed arrows: genetic interactions for which a molecular mechanism has not been determined. Both Sfp1p and Rtg1,3p shuttle to the cytoplasm when Tor1p is inhibited by rapamycin. They are indicated in bold in the nucleus, where they are active. An activating influence of the *TOR1* kinase complex on the transcription factor *Rtg1*/*Rtg3* has been postulated by Dann [5]. Feedback inhibition of Tor1p by nuclear Sfp1p is indicated. The RAS/cAMP and *SCH9* components are omitted for clarity. Their interaction with the TOR pathway is complex. M, mitochondrion; N, nucleus; P, peroxisome.

## CONCLUSION

In conclusion, we have shown that deletion of a gene coding for a mitochondrial ribosomal protein of yeast (*AFO1*, systematic name: YGR076C) leads not only to respiratory deficiency (as expected), but also to oxidative stress resistance, very low internal production of ROS and a substantial (60%) increase in the mother cell-specific lifespan of the strain. This was unexpected because a *bona fide* $\rho^0$ strain derived from the same parental yeast displayed no increase in lifespan. The lifespan effect of the mutant depends on the presence of a functional *TOR1* gene. The relatively large effect on lifespan which *afo1Δ* confers is, however, independent of the presence or absence of ERCs in the aging mother cells. These experimental results show once again that replicative aging is multifactorial and that the limiting factor for the determination of the replicative lifespan may be very different for different strains and for different growth conditions. The physiological characterization of the long-lived mutant shows a relationship of the yeast replicative aging process to two cellular processes that have also been found to determine aging in higher organisms: i) nutritional signaling through the highly conserved TOR pathway, and ii) generation of and defense against internally generated oxidative stress molecules (ROS).

## MATERIALS AND METHODS

Media. The following media were used in this study: complex medium (YPD) containing 1% yeast extract, 2% (w/v) peptone and 2% (w/v) D-glucose; synthetic complete glucose medium (SC-glucose) containing 2% (w/v) D-glucose, 0.17% yeast nitrogen base without amino acids and ammonium sulphate, 0.5% ammonium sulphate and 10 mL complete dropout; synthetic complete raffinose (SC-raffinose), synthetic complete glycerol medium (SC-glycerol) or synthetic complete lactate medium (SC-lactate), containing the same ingredients as SC-glucose, except that 2% (w/v) D-glucose is replaced by 2% (w/v) raffinose, 2% (v/v) glycerol or 3% (w/v) lactate as a carbon source; synthetic minimal medium (SD) containing 2% (w/v) D-glucose, 0.17% yeast nitrogen base without amino acids and ammonium sulphate and 0.5% ammonium sulphate. Complete dropout contains: 0.2% Arg, 0.1%, His, 0.6% Ile, 0.6% Leu, 0.4% Lys, 0.1% Met, 0.6% Phe, 0.5% Thr, 0.4% Trp, 0.1% Ade, 0.4% Ura, 0.5% Tyr. Agar plates were made by adding 2% (w/v) agar to the media.

Strains. *S. cerevisiae* strains BY4741 and BY4742 (EUROSCARF) were used. For experiments with deletion strains we used the EUROSCARF deletion mutant collection (http://www.rz.unifrankfurt.de/FB/fb16/mikro/euroscarf/index.html). Other strains were obtained from the "Yeast-GFP clone collection" (Invitrogen Cooporation, Carlsbad, California, USA) or the "$TetO_7$ promoter collection" (Open Biosystems, Huntsville, AL, USA). Double mutants were constructed by isogenic crossing of two single mutants of opposing mating type in the BY4741 background followed by sporulation of the obtained zygote and dissection of meiotic tetrads. A *bona fide* $\rho^0$ petites strain was made from the BY4741 wild type -strain as described in [35]. Briefly the strain was grown from a small inoculum to saturation in synthetic minimal medium (SD) containing the auxotrophic requirements plus 25μg/mL ethidium bromide. A second culture was inoculated from the first in the same medium and grown to saturation. This culture was streaked out for single colonies on YPD plates and checked for petite character by growth on YPG (complex medium containing 2% (v/v) glycerol as sole carbon source). To transfer the *SFP1-GFP-HIS3* chromosomal integrated GFP construct into the *afo1Δ* and *bona fide* BY4741 $\rho^0$ strain, the *SFP1-GFP-HIS3* construct was PCR cloned and chromosomally integrated at the *SFP1* locus of the *afo1*Δ and BY4741 $\rho^0$ strains, respectively.

Elutriation. Cells were separated according to their diameter using the Beckman elutriation system and rotor JE-6B with a standard elutriation chamber. Before the separation, the cells were grown in 100 mL of YPD medium at 28°C on a rotary shaker for 24 h. Then, the cells were harvested at 3000 rpm and resuspended in 1X PBS buffer (8 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4$, 0.24 g of $KH_2PO_4$, pH 7.4, in a total volume of 1 L) at 4°C. The elutriation chamber was loaded with 4.2 mL of cell suspension corresponding to about 109 cells. To separate cell fractions with different diameters, the chamber was loaded at a flow rate of 10 mL/min and a rotor speed of 3200 rpm. Cells with a diameter <5 μm were elutriated (fraction I). To collect fraction II (diameter 5-7 μm), the flow rate was set to 15 mL/min and a rotor speed to 2700 rpm. Fraction III (diameter 7-8.5 μm) was elutriated at 2400 rpm., fraction IV (diameter 8.5-10 μm) at 2000 rpm. and, finally, fraction V (diameter 10-15 μm) at 1350 rpm. The quality of separation of particular fractions was verified microscopically. Note that in the separation of the slightly smaller *afo1Δ* mutant cells no significant amount of fraction V cells could be isolated. Therefore, fraction IV was used for ERC determination.

Replicative lifespan. The replicative lifespan measurements were performed as described previously [2]. All lifespans were determined on defined SC-glucose media for a cohort of at least 45 cells. Standard deviations of

the median lifespans were calculated according to Kaplan–Meier statistics [36]. Median lifespan is the best-suited single parameter to describe a lifespan distribution. To determine whether two given lifespan distributions are significantly different at the 98% confidence level, Breslow, Tarone-Ware and log-rank statistics were used. All statistical calculations were performed using the software package SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Sensitivity to oxidants. Plate tests for sensitivity to oxidants were performed by spotting cell cultures onto SC-glucose plates containing various concentrations of $H_2O_2$ (2–4 mM) and t-BHP (0.8–2 mM). Cells were grown to stationary phase in liquid SC-glucose, serially diluted to $OD_{600}$ values of 3.0; 1.0; 0.3; 0.1 and 10 µL aliquots were spotted onto the appropriate plates. Sensitivity was determined by comparison of growth with that of the wild-type strain after incubation at 28°C for three days. RNA preparation and Northern analysis RNA was prepared from log-phase cells in SC-glucose and SC-raffinose with the RNeasy Midi Kit (Qiagen, Vienna, Austria). Heat-denatured RNA samples (10 µg) were separated by electrophoresis (5 h, 5 V/cm) in a 1.3% (w/v) agarose gel containing 0.6 M formaldehyde, transferred to a nylon membrane, and immobilized by irradiation with UV light (UV Stratalinker 1800, Stratagene, La Jolla, CA). Membranes were pre-hybridized for 2 h at 60°C in 10 mL Church Gilbert solution (0.5 M $Na_2HPO_4$, 1 mM EDTA, 7% (w/v) SDS, 1% BSA) and 100 µl (10 mg/mL stock solution) single-stranded denatured salmon sperm DNA, and then probed under the same conditions for 16 h with *CIT2* and *ACT1* probes which were labelled with $^{32}$P-dCTP by random oligonucleotide priming. After hybridisation, the filters were washed two times for 15 min with 2xSSC/0.1% SDS at room temperature, followed by two 15-min washes with 0.2xSSC/0.1% SDS at 56°C. Blots were wrapped in Saran Wrap and exposed for 15 min to an imaging cassette (Fujifilm BAS cassette 2325). Images were scanned in a Phosphoimager (Fujifilm BAS 1800II) using the BASreader 2.26 software.

Sfp1-GFP localisation experiment. Strains of interest were grown overnight from a small inoculum to saturation in SC-glucose medium. These cultures were taken to inoculate 25 mL of SC-glucose medium in such a way that cultures were in the early exponential growth phase on the next morning. A sample was taken for confocal microscopy (cells without rapamycin treatment). The rest of the culture was treated for 4 hours with 100 nM rapamycin (LC Laboratories, Woburn, MA, USA) and further inoculated at 28°C. Before cells were used for confocal microscopy, cells were harvested by centrifugation and resuspended in fresh SC-glucose medium. For the arsenite inhibition experiments, the cells were grown in synthetic medium to an $OD_{600}$ of 1. $As_2O_3$ was added to the cells to a final concentration of 0.5 mM. Live fluorescence pictures were taken after 10 minutes incubation at 30°C. Markers of apoptosis were determined as described in [2]. ERCs were determined as described in [19].

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## CONFLICT OF INTERESTS STATEMENT

The authors in this manuscript have no conflict of interests to declare.

## REFERENCES

**1.** Aguilaniu H, Gustafsson L, Rigoulet M, Nystrom T. Asymmetric inheritance of oxidatively damaged proteins during cytokinesis. Science. 2003; 299: 1751-1753.

**2.** Laun P, Pichova A, Madeo F, Fuchs J, Ellinger A, Kohlwein S, Dawes I, Frohlich K U, Breitenbach M. Aged mother cells of Saccharomyces cerevisiae show markers of oxidative stress and apoptosis. Mol Microbiol. 2001; 39: 1166-1173.

**3.** Jazwinski S M. The retrograde response links metabolism with stress responses, chromatindependent gene activation, and genome stability in yeast aging. Gene. 2005; 354: 22-27.

**4.** Laun P, Heeren G, Rinnerthaler M, Rid R, Kossler S, Koller L, Breitenbach M. Senescence and apoptosis in yeast mother cell-specific aging and in higher cells: a short review. Biochim Biophys Acta. 2008; 1783: 1328-1334.

**5.** Dann S G, Thomas G. The amino acid sensitive TOR pathway from yeast to mammals. FEBS Lett. 2006; 580: 2821-2829.

**6.** Marion R M, Regev A, Segal E, Barash Y, Koller D, Friedman N, O'Shea E K. Sfp1 is a stress- and nutrient-sensitive regulator of ribosomal protein gene expression. Proc Natl Acad Sci U S A. 2004; 101: 14315-14322.

**7.** Bonawitz N D, Chatenay-Lapointe M, Pan Y, Shadel G S. Reduced TOR signaling extends chronological life span via increased respiration and upregulation of mitochondrial gene expression. Cell Metab. 2007; 5: 265-277.

**8.** Dilova I, Aronova S, Chen J C, Powers T. Tor signaling and nutrient-based signals converge on Mks1p phosphorylation to regulate expression of Rtg1.Rtg3p-dependent target genes. J Biol Chem. 2004; 279: 46527-46535.

**9.** Finkel T. Breathing lessons: Tor tackles the mitochondria. Aging. 2009; 1: 9-11.

**10.** Pan Y, Shadel G S. Extension of chronological life span by reduced TOR signaling requires down-regulation of Sch9p and involves increased mitochondrial OXPHOS complex density. Aging. 2009; 1: 131-145.

**11.** Kaeberlein M, Powers R W, 3rd, Steffen K K, Westman E A, Hu D, Dang N, Kerr E O, Kirkland K T, Fields S, Kennedy B K. Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. Science. 2005; 310: 1193-1196.

**12.** Winzeler E A, Shoemaker D D, Astromoff A, Liang H, Anderson K, Andre B, Bangham R, Benito R, Boeke J D, Bussey H, Chu A M, Connelly C, Davis K, et al. Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science. 1999; 285: 901-906.

**13.** Kirchman P A, Kim S, Lai C Y, Jazwinski S M. Interorganelle signaling is a determinant of longevity in Saccharomyces cerevisiae. Genetics. 1999; 152: 179-190.

**14.** Jazwinski S M. Rtg2 protein: at the nexus of yeast longevity and aging. FEMS Yeast Res. 2005; 5: 1253-1259.

**15.** Butow R A, Avadhani N G. Mitochondrial signaling: the retrograde response. Mol Cell. 2004; 14: 1-15.

**16.** Smits P, Smeitink J A, van den Heuvel L P, Huynen M A, Ettema T J. Reconstructing the evolution of the mitochondrial ribosomal proteome. Nucleic Acids Res. 2007; 35: 4686-4703.

**17.** Hosiner D, Lempiainen H, Reiter W, Urban J, Loewith R, Ammerer G, Schweyen R, Shore D, Schuller C. Arsenic toxicity to Saccharomyces cerevisiae is a consequence of inhibition of the TORC1 kinase combined with a chronic stress response. Mol Biol Cell. 2009; 20:1048-1057.

**18.** Xie M W, Jin F, Hwang H, Hwang S, Anand V, Duncan M C, Huang J. Insights into TOR function and rapamycin response: chemical genomic profiling by using a high-density cell array method. Proc Natl Acad Sci U S A. 2005; 102: 7215-7220.

**19.** Borghouts C, Benguria A, Wawryn J, Jazwinski S M. Rtg2 protein links metabolism and genome stability in yeast longevity. Genetics. 2004; 166: 765-777.

**20.** Sinclair D A, Guarente L. Extrachromosomal rDNA circles--a cause of aging in yeast. Cell. 1997; 91: 1033-1042.

**21.** Defossez P A, Prusty R, Kaeberlein M, Lin S J, Ferrigno P, Silver P A, Keil R L, Guarente L. Elimination of replication block protein Fob1 extends the life span of yeast mother cells. Mol Cell. 1999; 3: 447-455.

**22.** Demidenko Z N, Zubova S G, Bukreeva E I, Pospelov V A, Pospelova T V, Blagosklonny M V. Rapamycin decelerates cellular senescence. Cell Cycle. 2009; 8: 1888-1895.

**23.** Harrison D E, Strong R, Sharp Z D, Nelson J F, Astle C M, Flurkey K, Nadon N L, Wilkinson J E, Frenkel K, Carter C S, Pahor M, Javors M A, Fernandez E, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009; doi:10.1038/nature08221.

**24.** Vellai T, Takacs-Vellai K, Zhang Y, Kovacs A L, Orosz L, Muller F. Genetics: influence of TOR kinase on lifespan in C. elegans. Nature. 2003; 426: 620.

**25.** Kapahi P, Zid B. TOR pathway: linking nutrient sensing to life span. Sci Aging Knowledge Environ. 2004; 2004: PE34.

**26.** Kapahi P, Zid B M, Harper T, Koslover D, Sapin V, Benzer S. Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. Curr Biol. 2004; 14: 885- 890.

**27.** Gan X, Kitakawa M, Yoshino K, Oshiro N, Yonezawa K, Isono K. Tag-mediated isolation of yeast mitochondrial ribosome and mass spectrometric identification of its new components. Eur J Biochem. 2002; 269: 5203-5214.

**28.** Graack H R, Wittmann-Liebold B. Mitochondrial ribosomal proteins (MRPs) of yeast. Biochem J. 1998; 329 ( Pt 3): 433-448.

**29.** Grohmann L, Graack H R, Kruft V, Choli T, Goldschmidt-Reisin S, Kitakawa M. Extended N-terminal sequencing of proteins of the large ribosomal subunit from yeast mitochondria. FEBS Lett. 1991; 284: 51-56.

**30.** Saveanu C, Fromont-Racine M, Harington A, Ricard F, Namane A, Jacquier A. Identification of 12 new yeast mitochondrial ribosomal proteins including 6 that have no prokaryotic homologues. J Biol Chem. 2001; 276: 15861-15867.

**31.** Naora H. Involvement of ribosomal proteins in regulating cell growth and apoptosis: translational modulation or recruitment for extraribosomal activity? Immunol Cell Biol. 1999; 77: 197-205.

**32.** Wool I G. Extraribosomal functions of ribosomal proteins. Trends Biochem Sci. 1996; 21: 164-165.

**33.** Hanlon S E, Xu Z, Norris D N, Vershon A K. Analysis of the meiotic role of the mitochondrial ribosomal proteins Mrps17 and Mrpl37 in Saccharomyces cerevisiae. Yeast. 2004; 21: 1241-1252.

**34.** Berger T, Brigl M, Herrmann J M, Vielhauer V, Luckow B, Schlondorff D, Kretzler M. The apoptosis mediator mDAP-3 is a novel member of a conserved family of mitochondrial proteins. J Cell Sci. 2000; 113 ( Pt 20): 3603-3612.

**35.** Fox T D, Folley L S, Mulero J J, McMullin T W, Thorsness P E, Hedin L O, Costanzo M C. Analysis and manipulation of yeast mitochondrial genes. Methods Enzymol. 1991; 194: 149-165.

**36.** Kaplan E L, Meier P. Nonparametric estimation from incomplete observations. Am Stat Assoc J 1958; 53: :457-481.
**37.** Huh W K, Falvo J V, Gerke L C, Carroll A S, Howson R W, Weissman J S, O'Shea E K. Global analysis of protein localization in budding yeast. Nature. 2003; 425:686-691.
**38.** Bogengruber E, Briza P, Doppler E, Wimmer H, Koller L, Fasiolo F, Senger B, Hegemann J H, Breitenbach M. Functional analysis in yeast of the Brix protein superfamily involved in the biogenesis of ribosomes. FEMS Yeast Res. 2003; 3: 35-43.
**39.** Pichova A, Vondrakova D, Breitenbach M. Mutants in the Saccharomyces cerevisiae RAS2 gene influence life span, cytoskeleton, and regulation of mitosis. Can J Microbiol. 1997; 43:774-781.

## SUPPLEMENTARY FIGURES

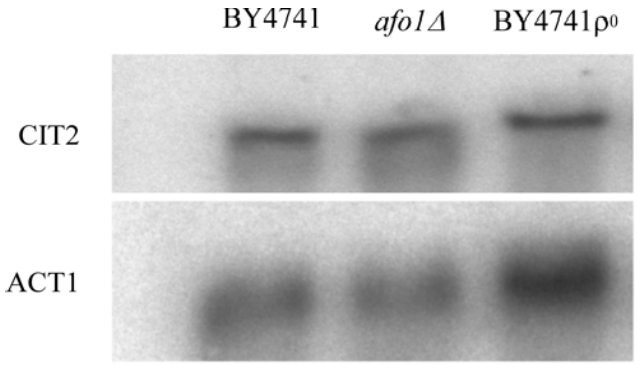


**Figure S1.** Northern blots (see also experimental procedures) of *CIT2* showing the absence of the retrograde response [15] in the *afo1Δ* strain grown on 2% glucose.

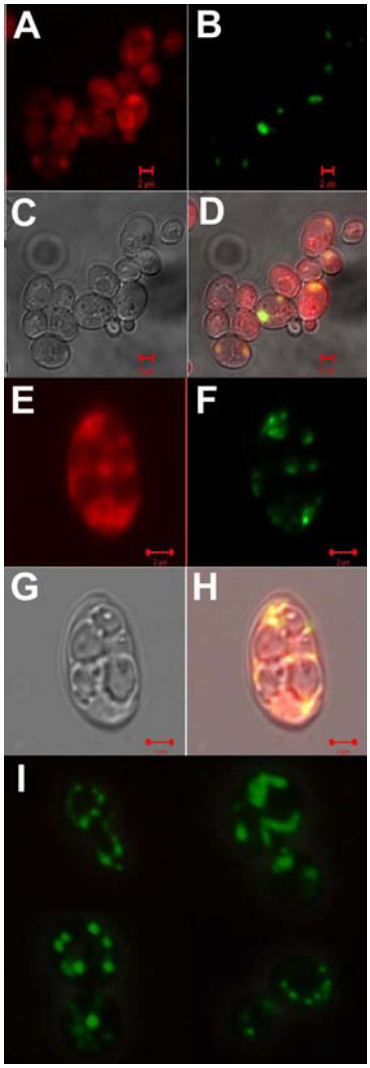


**Figure S2.** Subcellular localization of *AFO1*-GFP. Exponentially growing cells of strain YUG37 [38] transformed with the Afo1p-GFP construct in pMR2 under a tetracyclin-regulatable promoter were induced with doxycyclin, stained with Mitotracker deep red and analyzed with a Leica confocal microscope. (**A**) Cells stained with Mitotracker deep red; (**B**) the same cells as in (**A**) stained with Afo1p-GFP; (**C**) the same cells in phase contrast; (**D**) overlay of (**A**) and (**B**). (**E-H**) The same technique as in (**A**) to (**D**) was applied to a senescent mother cell (fraction V) of the same strain. (**I**) Strain JC 482 [39] transformed with plasmid pUG35 containing Afo1p-GFP under control of the *MET25* promoter and grown to mid-log phase on SC-lactate was observed by confocal microscopy to reveal the mitochondrial localization of the protein.