

Lifespan extension by conditions that inhibit translation in *Caenorhabditis elegans*

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Summary

Many conditions that shift cells from states of nutrient utilization and growth to states of cell maintenance extend lifespan. We have carried out a systematic lifespan analysis of conditions that inhibit protein synthesis. We find that reducing the levels of ribosomal proteins, ribosomal-protein S6 kinase or translation-initiation factors increases the lifespan of *Caenorhabditis elegans*. These perturbations, as well as inhibition of the nutrient sensor target of rapamycin (TOR), which is known to increase lifespan, all increase thermal-stress resistance. Thus inhibiting translation may extend lifespan by shifting cells to physiological states that favor maintenance and repair. Interestingly, different types of translation inhibition lead to one of two mutually exclusive outputs, one that increases lifespan and stress resistance through the transcription factor DAF-16/FOXO, and one that increases lifespan and stress resistance independently of DAF-16. Our findings link TOR, but not *sir-2.1*, to the longevity response induced by dietary restriction (DR) in *C. elegans*, and they suggest that neither TOR inhibition nor DR extends lifespan simply by reducing protein synthesis.

Key words: aging; *C. elegans*; caloric restriction; insulin/IGF-1 signaling; TOR; translation.

Introduction

The lifespan of *Caenorhabditis elegans* can be extended by many treatments or mutations that shift cells from states favoring growth to states favoring maintenance and stress resistance. These perturbations include down-regulation of insulin/IGF-1 or target of rapamycin (TOR) signaling, dietary restriction (DR), and up-regulation of stress resistance-associated pathways activated by the FOXO transcription factor DAF-16, heat-shock factor,

sirtuins or c-Jun N-terminal kinase (JNK) (Vellai *et al.*, 2003; Kenyon, 2005; Oh *et al.*, 2005).

In this study, we investigated the effect of inhibiting protein synthesis, another process linked to growth, on aging. Protein synthesis is tightly regulated by cues that signal energy and nutrient levels. In many organisms, nutrient limitation has been found to inhibit translation, at least in part, by down-regulating the highly conserved kinase TOR (Wullschleger *et al.*, 2006). Inhibition of TOR decreases ribosome biogenesis as well as the process of translation itself. For example, when TOR activity falls, phosphorylation of ribosomal-protein S6 kinase (S6K) is reduced, which, in turn, leads to the dephosphorylation and inactivation of translation elongation factor 2 (eEF2) kinase, thereby inhibiting global translation (Wang *et al.*, 2001). TOR inhibition also reduces the phosphorylation of translation-initiation factor 4 binding proteins (eIF4-BPs) (Hay & Sonenberg, 2004; Tee & Blenis, 2005). As a consequence, eIF4-BPs bind to eIF4E, which impairs the recruitment of the 40S ribosomal subunit to the cap structure present at the 5'-end of eukaryotic mRNAs (Sonenberg & Gingras, 1998).

The pathways that influence growth and lifespan are interconnected. Like TOR, the insulin/IGF-1 signaling pathway has been shown to influence translation in some species (Proud, 2006). Nutrient limitation lowers insulin levels, which in turn reduces the activity of the Ser/Thr kinase Akt and thereby prevents Akt from phosphorylating and activating S6K. Inhibition of insulin/IGF-1 signaling extends lifespan in many animals, and, where examined, this lifespan extension requires activity of FOXO transcription factors such as *C. elegans* DAF-16 (Kenyon, 2005). In contrast, regulation of S6K by insulin/IGF-1 signaling appears to be strictly post-transcriptional.

The TOR and insulin/IGF-1 pathways cross-regulate one another. In flies and mammals, Akt is known to activate TOR by phosphorylating and inactivating the negative regulator of TOR, TSC2 (tuberous sclerosis protein, tuberlin). In addition, S6K cross-inhibits insulin signaling by phosphorylating and inactivating insulin-receptor substrate proteins (Manning, 2004).

In both yeast and flies, dietary restriction may extend lifespan, at least in part, by down-regulation of the TOR pathway. The lifespan extension of yeast carrying mutations in TOR is not increased further by dietary restriction, and the lifespans of mutant flies with low TOR activity levels are extended only modestly under conditions of nutrient limitation (Kapahi *et al.*, 2004; Kaeberlein *et al.*, 2005).

Recently, we identified two ribosomal-protein (RP) genes in a screen designed to identify *C. elegans* longevity genes (M.H. & C.K., unpublished data). Because translation plays such an important role in animal physiology and growth, this finding stimulated us to examine, in a systematic fashion, the effect that

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Accepted for publication 14 November 2006

inhibiting translation has on *C. elegans* lifespan. Here we show that reducing the levels of any of a large number of ribosomal proteins during adulthood extends lifespan. This suggests that the process of protein synthesis itself influences *C. elegans* longevity. In addition, we find that lifespan is extended by depleting the translational regulator S6K and the translation initiation factors (eIFs) eIF2 β /*iftb-1*, eIF4E/*ife-2*, and eIF4G/*ifg-1*. Finally, we find that reducing the level of TOR does not further extend the lifespan of animals subjected to DR, suggesting that, as in yeast and flies, TOR may mediate the response to DR. While our studies were in progress, Kaeberlein *et al.* reported that, in yeast, inhibiting translation by reducing the levels of several ribosomal proteins extends replicative lifespan (2005), and Kapahi *et al.* reported that, in flies, lifespan is extended by inhibiting S6K, which, in turn, inhibits translation (2004). In addition, in *C. elegans*, the translation-initiation factor eIF5A/*iff-1* was identified in a genome-wide RNA interference (RNAi) longevity screen (Hamilton *et al.*, 2005), and the Johnson lab reported their independent analysis of the role of the translation-initiation factors eIF2 β and eIF4G in lifespan determination (Henderson *et al.*, 2006).

Curiously, we find that the lifespan extension produced by depleting translation-initiation factors is completely dependent on the DAF-16/FOXO transcription factor, whereas lifespan extension caused by depleting ribosomal proteins (RP) and S6K, like depleting TOR, is DAF-16 independent. Thus, inhibition of translation-initiation factors not only engages DAF-16 but also appears to block all of the DAF-16-independent lifespan extension that would have been produced by inhibiting translation itself. To explain this, we propose a switch-like model, in which the activation of a DAF-16-dependent pathway suppresses the operation of the DAF-16-independent pathway.

Finally, our findings suggest that, in spite of their differences, all of these translation-inhibiting treatments ultimately extend lifespan by similar mechanisms, because we find that they all increase resistance to thermal stress. Thus, inhibition of translation, like many other perturbations that extend lifespan, may do so by shifting animals from states of growth and nutrient utilization to states of cell maintenance and stress resistance.

Results

Reducing the levels of ribosomal proteins extends lifespan

Because RPs are essential for translation, reducing the levels of RPs using RNAi should be a fairly direct way of asking whether the process of translation *per se* is likely to affect lifespan in *C. elegans*. The ~70 RPs associate with either the small (40S) or large (60S) subunit of the 80S ribosome. We used RNAi to inhibit the expression of genes encoding six 40S RPs (*rps-6*, *rps-10*, *rps-11*, *rps-15*, *rps-22* and *rps-26*) and five 60S RPs (*rpl-4*, *rpl-6*, *rpl-9*, *rpl-19* and *rpl-30*). Because of the high demand for translation during development, when the size of the animal increases

dramatically, we initiated the RNAi treatment in young adults, whose somatic cells are post-mitotic. These RNAi treatments reduced RP mRNA levels approximately two- to fivefold (Supplementary Fig. S1A), and, in each case, increased lifespan significantly (~10–50%; Fig. 1A and Table 1). To verify that these treatments inhibited protein synthesis, we measured the level of radioactive methionine incorporated into proteins during a 3-h period in adult animals subjected to *rps-15* and *rpl-19* RNAi. As predicted, we found that it was reduced (Fig. 2A). (We note the caveat that in these and similar experiments described below, levels of newly synthesized protein could, in principle, be reduced because of increased rates of protein degradation.)

We also examined animals subjected to RP RNAi from the time of hatching. Both of the RNAi clones we tested (*rps-15* and *rps-22*) delayed development, reduced growth and vigor substantially, and failed to extend lifespan (Table 1), consistent with the importance of translation during this period of growth.

Reducing the levels of translational regulators extends lifespan

Next, we asked whether reducing the levels of proteins that regulate translation would also influence lifespan. We found that reducing S6K (*rsk-1*) mRNA levels approximately twofold by RNAi extended the mean lifespan of animals 13–47% (Fig. 1B, Supplementary Fig. S1A and Table 1). In addition, we found that the S6K deletion mutant *rsk-1(sv31)* lived longer than wild-type animals (Fig. 1C and Table 2). The rate of accumulation of newly synthesized protein in this mutant was 35% that of wild-type (Fig. 2B).

Reducing the levels of translation-initiation factors also increased lifespan. RNAi depletion of eIF2 β (*K04G2.1/iftb-1*), a subunit of the general translation-initiation factor eIF2, and eIF4G (*ifg-1*), a scaffold protein for translation initiation factors, increased lifespan by ~30% (Fig. 1D and Table 1). These RNAi treatments reduced eIF2 β and eIF4G mRNA levels approximately two- to fivefold (Supplementary Fig. S1A). We also analyzed a deletion mutant of the eIF4E/*ife-2* gene, *ife-2(ok306)*, which encodes an mRNA cap-binding protein subunit and found that this mutant lived slightly longer than wild type (Fig. 1E and Table 2). We measured the accumulation of newly synthesized protein in animals subjected to eIF2 β or eIF4E RNAi, as well as in animals carrying eIF4E/*ife-2(ok306)* mutations, and, as expected, found that it was reduced (Fig. 2A,C).

In *C. elegans*, inhibiting insulin/IGF-1 receptor activity extends lifespan without reducing global translation rates

The insulin/IGF-1-like pathway affects lifespan in *C. elegans* through the FOXO-transcription factor DAF-16 (Kenyon *et al.*, 1993; Tatar *et al.*, 2003; Kenyon, 2005). Animals carrying partial loss-of-function alleles of the *daf-2* insulin/IGF-1-receptor gene, or mutations in genes encoding components of a downstream

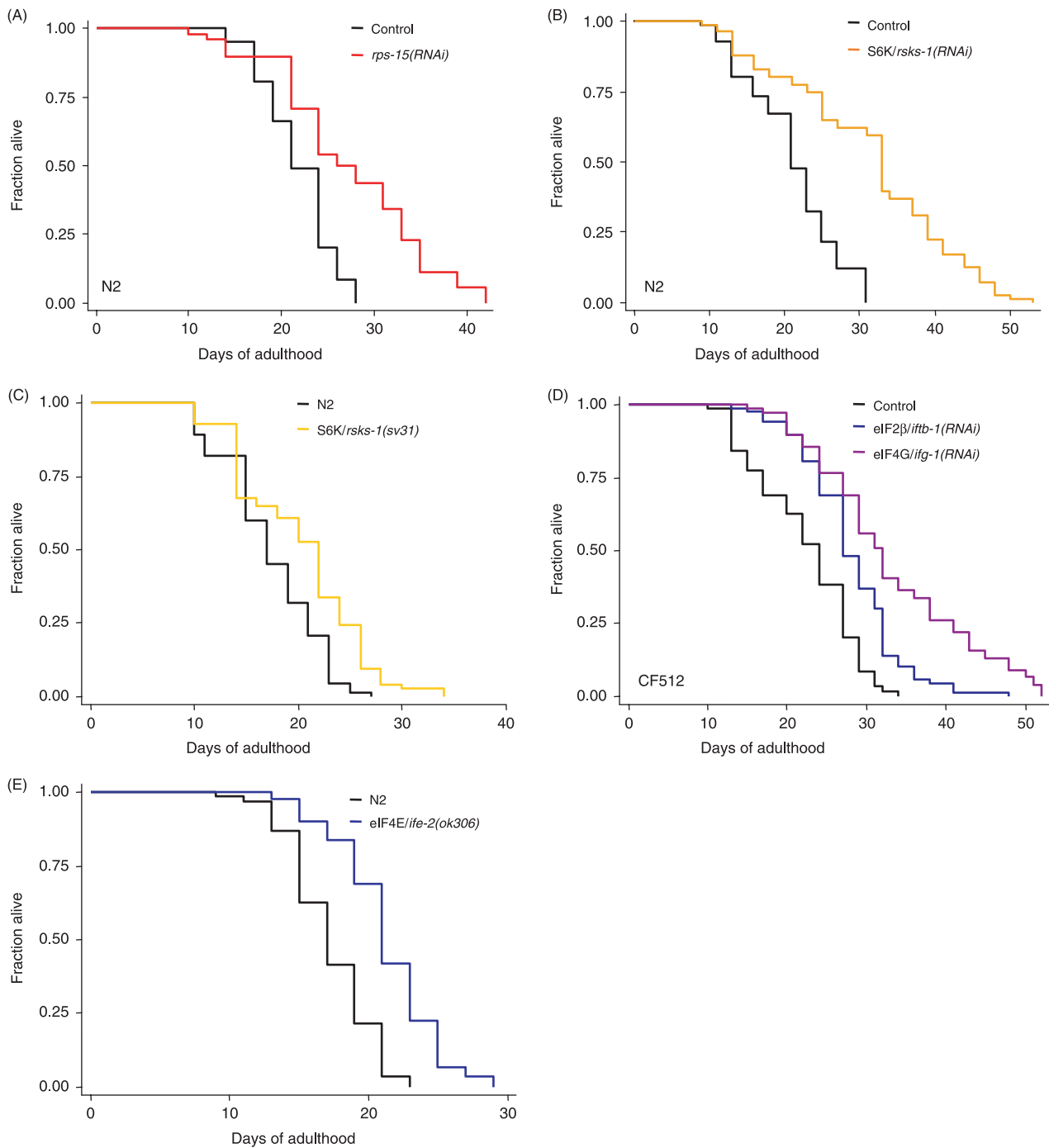


Fig. 1 Conditions that reduce translation extend lifespan. (A) Survival curves of wild-type (N2) animals fed either control bacteria (vector only) or bacteria expressing *rps-15* dsRNA during adulthood at 20 °C. Adult-only treatment with *rps-15* dsRNA resulted in lifespan extensions in five independent trials. Please see Table 1 for statistics and additional information, including trials of the other 10 ribosomal RNAi clones we tested. (B) Survival curves of N2 animals fed either control bacteria or bacteria expressing S6K (*rks-1*) dsRNA during adulthood at 20 °C. Please see Table 1 for details, and for repetitions of this experiment. (C) Survival curves of N2 vs. S6K/*rks-1(sv31)* animals at 20 °C. Please see Table 2 for details, and for a repetition of this experiment. (D) Survival curves of sterile *fer-15(b26); fem-1(hc17)* (CF512) animals fed either control bacteria or bacteria expressing eIF2 β (K04G2.1/*iftb-1*) or eIF4G (*ifg-1*) dsRNA during adulthood at 20 °C. Please see Table 1 for details, and for repetitions of this experiment. (E) Survival curves of N2 vs. eIF4E/*ife-2(ok306)* animals at 25 °C. Please see Table 2 for details, and for a repetition of this experiment. All of the conditions we tested that reduced translation also decreased brood size (see Supplementary Table S1 and data not shown).

Table 1 Mean lifespan extensions observed in wild-type and sterile *fer-15*; *fem-1* animals grown on translation-associated RNAi during adulthood

Strain	Adult-only RNAi treatment	RNAi lifespan (days)	75%	Number of RNAi animals	Control lifespan (days)	75%	Number of control animals	Percentage lifespan increase	<i>P</i> value vs. control	
N2	<i>rps-15</i>	32.4	37	73/96 (9)	21.2	25	50/91 (25)	53	< 0.0001	
	<i>rps-15</i>	24.1	30	77/100 (12)	17.3	21	71/92 (1)	40	< 0.0001	
	<i>rps-15*</i>	27.4	33	43/60 (0)	21.8	24	33/62 (6)	26	< 0.0001	
	<i>rps-15</i>	23.1	27	67/98 (25)	19.6	24	63/98 (17)	18	0.0001	
	<i>rps-15</i>	21.1	24	55/80 (3)	17.6	20	50/79 (0)	20	< 0.0001	
	<i>rps-15</i> WL	20.8	24	27/91 (64)	19.6	24	63/98 (17)	6	0.33	
	<i>rps-15</i> WL	21.9	25	36/60 (12)	21.8	24	33/62 (6)	0	0.90	
	<i>rpl-6</i>	24.5 F	24	63/89 (23)	20.4 F	22	92/107 (11)	20	< 0.0001	
	<i>rpl-6</i>	22.3 F	24	31/106 (60)	18.9 F	22	33/103 (60)	18	0.012	
	<i>S6K/rsks-1*</i>	31.2	39	73/97 (18)	21.2	25	50/91 (25)	47	< 0.0001	
	<i>S6K/rsks-1</i>	22.0	25	85/97 (2)	19.7	23	79/115 (4)	12	0.0002	
	<i>TOR/let-363</i>	23.2	26	59/83 (6)	16.1	19	64/84 (0)	44	< 0.0001	
	<i>TOR/let-363</i>	21.9	25	75/90 (6)	17.3	21	71/92 (1)	27	< 0.0001	
	<i>TOR/let-363</i>	20.1	25	35/86 (34)	17.5	19	58/87 (18)	15	0.0005	
	<i>fer-15</i> ; <i>fem-1</i>	<i>rps-6</i>	27.1	35	68/89 (13)	21.9	25	49/91 (23)	25	0.0002
		<i>rps-10</i>	18.6†	23	82/92 (3)	16.0†	18	83/99 (2)	16	< 0.0001
<i>rps-11</i>		28.0	32	60/94 (10)	22.4	27	66/93 (25)	25	< 0.0001	
<i>rps-15</i>		30.6	36	64/91 (6)	22.8	26	80/94 (2)	34	< 0.0001	
<i>rps-22</i>		30.8	36	66/87 (1)	22.8	26	80/94 (2)	35	< 0.0001	
<i>rps-22</i>		19.4†	23	82/93 (1)	16.0†	18	83/99 (2)	21	< 0.0001	
<i>rps-22</i> WL		13.8†	16	79/90 (8)	16.0†	18	83/99 (2)	-14	< 0.0001	
<i>rps-26</i>		19.2†	23	81/94 (1)	16.0†	18	83/99 (2)	20	< 0.0001	
<i>rpl-4</i>		18.2†	20	87/106 (3)	16.0†	18	83/99 (2)	14	< 0.0001	
<i>rpl-9</i>		23.3	26	66/85 (14)	21.9	25	49/91 (23)	6	0.010	
<i>rpl-19</i>		17.7†	20	86/93 (1)	16.0†	18	83/99 (2)	11	0.0016	
<i>rpl-30</i>		25.2	29	79/100 (3)	22.4	27	66/93 (25)	13	0.0064	
<i>S6K/rsks-1</i>		24.8	28	74/91 (6)	21.9	25	49/91 (23)	13	0.0005	
<i>S6K/rsks-1</i>		25.3	29	65/95 (25)	22.4	27	66/93 (25)	13	0.011	
<i>eIF2β/iftb-1</i>		28.2	35	52/72 (7)	21.9	25	49/91 (23)	29	< 0.0001	
<i>eIF2β/iftb-1</i>		28.9	33	72/94 (9)	22.8	26	80/94 (2)	27	< 0.0001	
<i>eIF2β/iftb-1*</i>		28.1	32	87/100 (9)	22.4	27	66/93 (25)	25	< 0.0001	
<i>eIF2β/iftb-1</i>		16.9†	20	85/92 (3)	16.0†	18	83/99 (2)	6	0.027	
<i>eIF4G/ifg-1*</i>		33.1	41	77/95 (7)	22.4	27	66/93 (25)	48	< 0.0001	
<i>eIF4G/ifg-1</i>		28.5	35	75/90 (2)	21.9	25	49/91 (23)	30	< 0.0001	
<i>eIF4G/ifg-1</i>	24.9	29	79/95 (9)	22.8	26	80/94 (2)	9	0.018		
<i>TOR/let-363</i>	18.2†	20	79/90 (4)	16.0†	18	83/99 (2)	14	< 0.0001		
<i>TOR/let-363</i>	24.3	28	75/89 (9)	21.9	25	49/91 (23)	11	0.0015		

RNAi lifespan, mean adult lifespan, in days, of either N2 (wild type) or *fer-15(b26)*; *fem-1(hc17)* animals grown on specific RNAi bacterial clones during adulthood. 75%, mean lifespan, in days, of the 75th percentile (the age at which the fraction of animals alive reaches 0.25).

Number of RNAi animals, number of observed deaths/total number of animals subjected to RNAi treatment. The difference between these numbers represents the number of animals censored during the experiment, which includes animals that ruptured (this number is shown in parenthesis; see Experimental procedures), bagged (i.e. exhibited internal progeny hatching), or crawled off the plates.

Control lifespan, mean adult lifespan, in days, of animals grown on control bacteria (vector only).

Number of control animals, number of observed deaths/total number of animals grown on control bacteria. *P* values were calculated by pair-wise comparisons to the control of the experiment by using the log-rank test.

Repetitions of the same experiments are listed in order.

Lifespan experiments were carried out at 20 °C (i.e. *fer-15(b26)*; *fem-1(hc17)* eggs were incubated at 25 °C until adulthood, and lifespan analysis of adult animals was performed at 20 °C) except for experiments marked †, which were carried out at 25 °C; WL, whole-life RNAi treatment; F, 2'fluoro-5'deoxyuridine (FUDR) (100 μM) was added to the plates to prevent progeny production. See also general comment on censoring rates in Experimental procedures.

*data shown in Fig. 1.

PI-3K/PDK/AKT/SGK-kinase cascade are long lived, and their lifespan extension requires the DAF-16/FOXO transcription factor. In other organisms, inhibition of insulin/IGF-1 signaling has been linked to reduced rates of protein synthesis (Tatar et al., 2003; Sharp & Bartke, 2005). To ask whether that is the case in the long-lived *daf-2(e1370)* mutant, we measured the

level of newly synthesized protein present in mutants fed radioactively labeled bacteria for 3 h. Unexpectedly, we found that newly synthesized protein levels were the same as in wild type (Fig. 2C). This finding demonstrates that the increased longevity of *daf-2* mutants is not caused by a global decrease in translation rates.

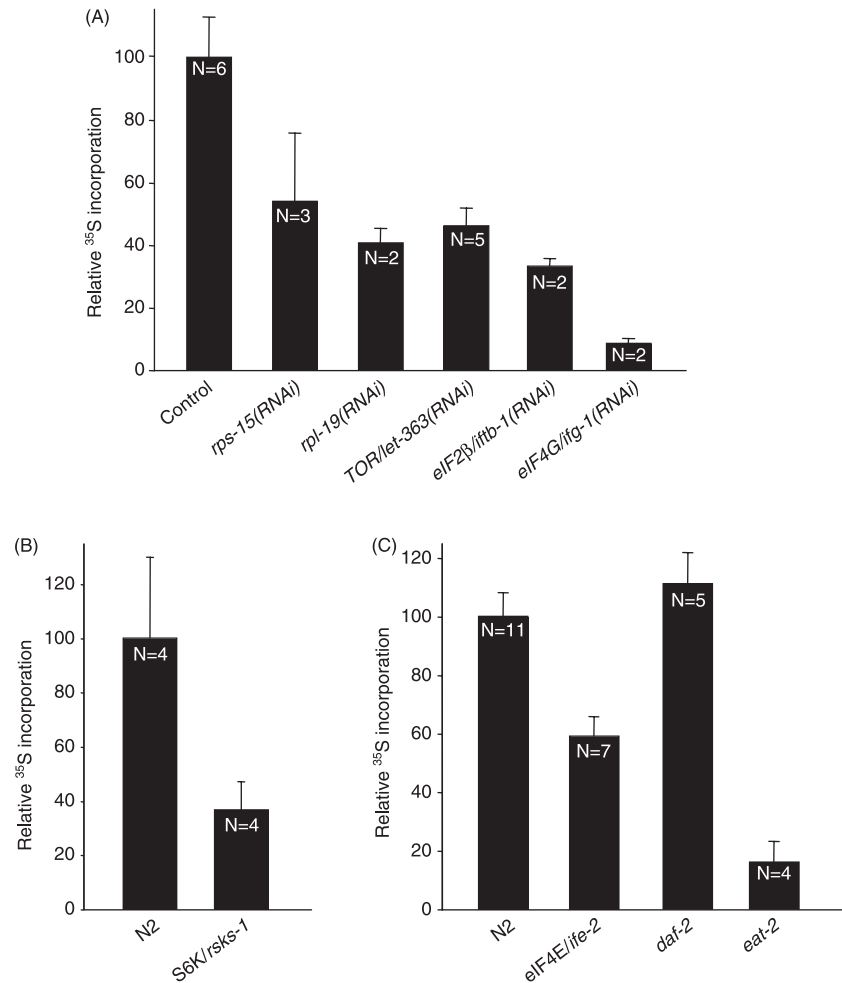


Fig. 2 Inhibition of translation-associated proteins reduces the rate of accumulation of newly synthesized protein. (A) Relative levels of ^{35}S -methionine incorporation in 3-day-old wild-type (N2) animals fed either control (vector only) or RNAi bacteria from day 1 of adulthood. $P = 0.036$ for *rps-15* RNAi, $P = 0.038$ for *rpl-19* RNAi, $P = 0.0057$ for TOR RNAi, $P = 0.016$ for *eIF2 β* RNAi, and $P = 0.0067$ for *eIF4G* RNAi, all compared to control RNAi by one-sided, paired *t*-test. (B) Relative levels of ^{35}S -methionine incorporation in 1- to 3-day-old *S6K/rsk-1(sv31)* animals compared to N2 animals. $P = 0.015$, one-sided, paired Student's *t*-test. (C) Relative levels of ^{35}S -methionine incorporation in 1- to 3-day-old *eIF4E/life-2(ok306)*, *daf-2(e1370)*, and *eat-2(ad1116)* mutants compared to N2 animals. $P = 0.0012$ for *ife-2*, $P = 0.68$ for *daf-2*, and $P = 0.0009$ for *eat-2*, all compared to N2 by one-sided, paired *t*-test. Bar graphs represent average incorporated ^{35}S -methionine normalized to total protein levels of the indicated mutant compared to N2/control animals; error bars represent SEM. N, number of measurements.

Table 2 Lifespan analysis of *S6K/rsk-1(sv31)* and *eIF4E/life-2(ok306)* deletion mutants

Strain	Mean lifespan (days)	75%	Number of animals	Percentage lifespan increase	<i>P</i> value vs. control
N2*	17.5	21	94/109 (6)		
<i>S6K/rsk-1(sv31)*</i>	20.2	24	77/102 (15)	15	0.0001
N2	18.9	21	93/118 (1)		
<i>S6K/rsk-1(sv31)</i>	21.8	25	59/95 (14)	15	0.0035
N2	18.4 F	21	75/105 (21)		
<i>eIF4E/life-2(ok306)</i>	20.4 F	23	62/102 (37)	11	0.0009
N2*	17.2 F	19	86/102 (5)		
<i>eIF4E/life-2(ok306)*</i>	21.3 F	23	90/102 (10)	23	< 0.0001

See Table 1 for explanations.

Lifespan experiments were carried out at 20 °C for *S6K* mutants, and at 25 °C for *eIF4E* mutants.

F, 2'fluoro-5'deoxyuridine (FUDR) (100 μM) was added to the plates to prevent reproduction. *data shown in Fig. 1.

Lowering the levels of some but not all regulators of translation engages DAF-16/FOXO

Inhibiting TOR (*let-363*) extends lifespan in a *daf-16*-independent fashion (Vellai et al., 2003), suggesting that TOR acts in a pathway that is distinct from the insulin/IGF-1 pathway. However, it is possible that the two pathways converge downstream of

DAF-16, because TOR RNAi does not further extend the lifespan of *daf-2* mutants [Vellai et al. (2003), and confirmed by us, Supplementary Table S3].

Because inhibiting TOR reduces rates of translation [as was confirmed by us directly (Fig. 2A)], it seemed likely that reducing translation by any method would mimic TOR inhibition, extending the lifespan of *daf-16* but not *daf-2* mutants. We performed

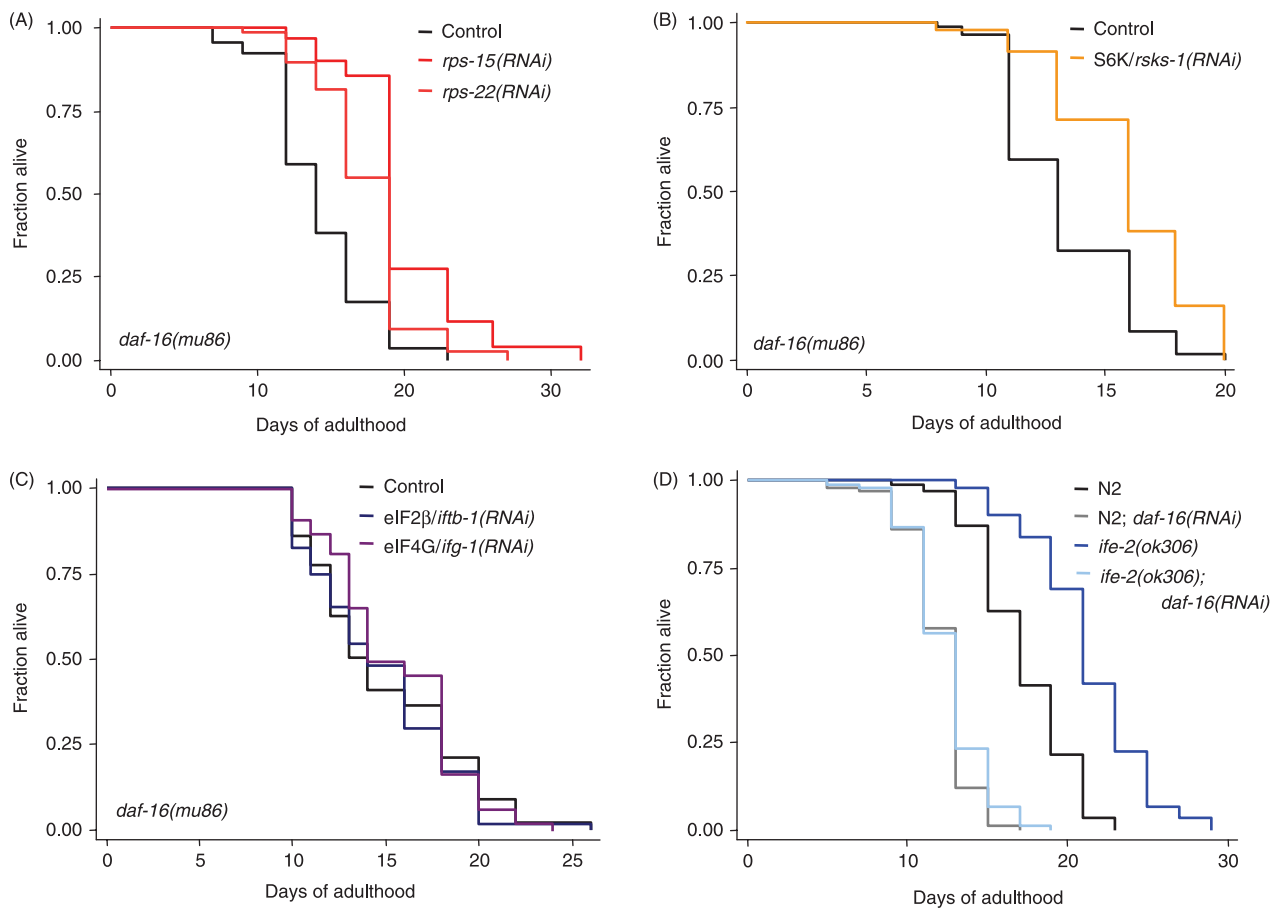


Fig. 3 Effects of translation-associated RNAi on the lifespan of *daf-16* mutants. (A) Survival curves of *daf-16(mu86)* mutants fed either control bacteria (vector only) or bacteria expressing either *rps-15* or *rps-22* dsRNA during adulthood at 20 °C. Mean lifespan was 14.3 days for control and 20.0 days for *rps-15* and 17.5 days for *rps-22* RNAi. $P < 0.0001$ for each treatment vs. control, log-rank test. Adult-only treatment with these RNAi clones resulted in lifespan extensions in each of three to five independent trials. Please see Supplementary Table S2 for additional information, including trials of *rpl-19* and *rpl-4* RNAi clones which both extend the lifespan of *daf-16(mu86)* mutants. (B) Survival curves of *daf-16(mu86)* mutants fed either control bacteria or bacteria expressing S6K (*rsk-1*) dsRNA during adulthood at 20 °C. Mean lifespan was 13.3 days for control and 16 days for S6K RNAi. $P < 0.0001$, log-rank test. Adult-only treatment of *daf-16(mu86)* mutants with S6K RNAi induced significant lifespan extensions in two different trials. Please see Supplementary Table S2 for additional information. (C) Survival curves of *daf-16(mu86)* mutants fed either control bacteria or bacteria expressing either eIF2 β (*iftb-1*) or eIF4G (*ifg-1*) RNAi during adulthood at 20 °C. Mean lifespan was 15.2 days for control and 14.8 days for eIF2 β RNAi, $P = 0.56$, and 15.6 days for eIF4G RNAi, $P = 0.62$, log-rank test. Adult-only treatment with either eIF2 β or eIF4G RNAi failed to cause any lifespan extensions in each of three independent trials. Please see Supplementary Table S2 for additional information. (D) Survival curves of wild-type (N2) vs. outcrossed eIF4E/*ife-2(ok306)* animals fed control bacteria or bacteria expressing *daf-16* dsRNA at 25 °C. Mean lifespan of strains fed control RNAi are in Table 2, mean lifespan of N2 subjected to *daf-16* RNAi was 12.4 days and of eIF4E/*ife-2(ok306)* subjected to *daf-16* RNAi was 12.0 days, $P = 0.18$, log-rank test. Please see Supplementary Table S2 for a repetition of this experiment and additional information.

this experiment by feeding RNAi bacteria to *daf-16(null)* mutants or *daf-2* reduction-of-function mutants during adulthood and measuring their lifespans. We found that RNAi of RPs and S6K produced effects similar to RNAi of TOR in both tests, extending the lifespan of *daf-16* mutants (Fig. 3A,B and Supplementary Table S2) but not those of *daf-2* mutants (Fig. 4A,B and Supplementary Table S3). These findings suggest that TOR, S6K and RPs affect lifespan in a similar way.

Because *daf-2* exerts its effects on lifespan by changing patterns of transcription, we wondered whether *daf-2* mutants might have reduced levels of TOR, RP or S6K gene expression. We found that mRNA levels of all of the genes we tested were similar in *daf-2(mu150)* mutants and *daf-2(+)* control animals (Supplementary Fig. S1B). [We note that in a previous study,

adult *daf-2(m41)* mutants subjected to serial analysis of gene expression (SAGE) were found to have slightly increased levels of RP gene expression (Halaschek-Wiener et al., 2005).]

Next, we asked whether the lifespan extension produced by decreasing translation-initiation factor levels was also *daf-16* independent. Remarkably, unlike reducing TOR, RP or S6K levels, we found, in multiple trials, that reducing the levels of eIF2 β or eIF4G did not extend the lifespan of *daf-16* mutants at all (Fig. 3C and Supplementary Table S2). Likewise, the eIF4E/*ife-2(ok306)* mutation did not extend the lifespan of *daf-16(RNAi)* animals (Fig. 3D and Supplementary Table S2).

eIF2 β and eIF4G RNAi slightly shortened the lifespan of *daf-2* mutants (Fig. 4C and Supplementary Table S3). Thus, the eIFs have opposing effects in *daf-2* mutants (in which eIF RNAi

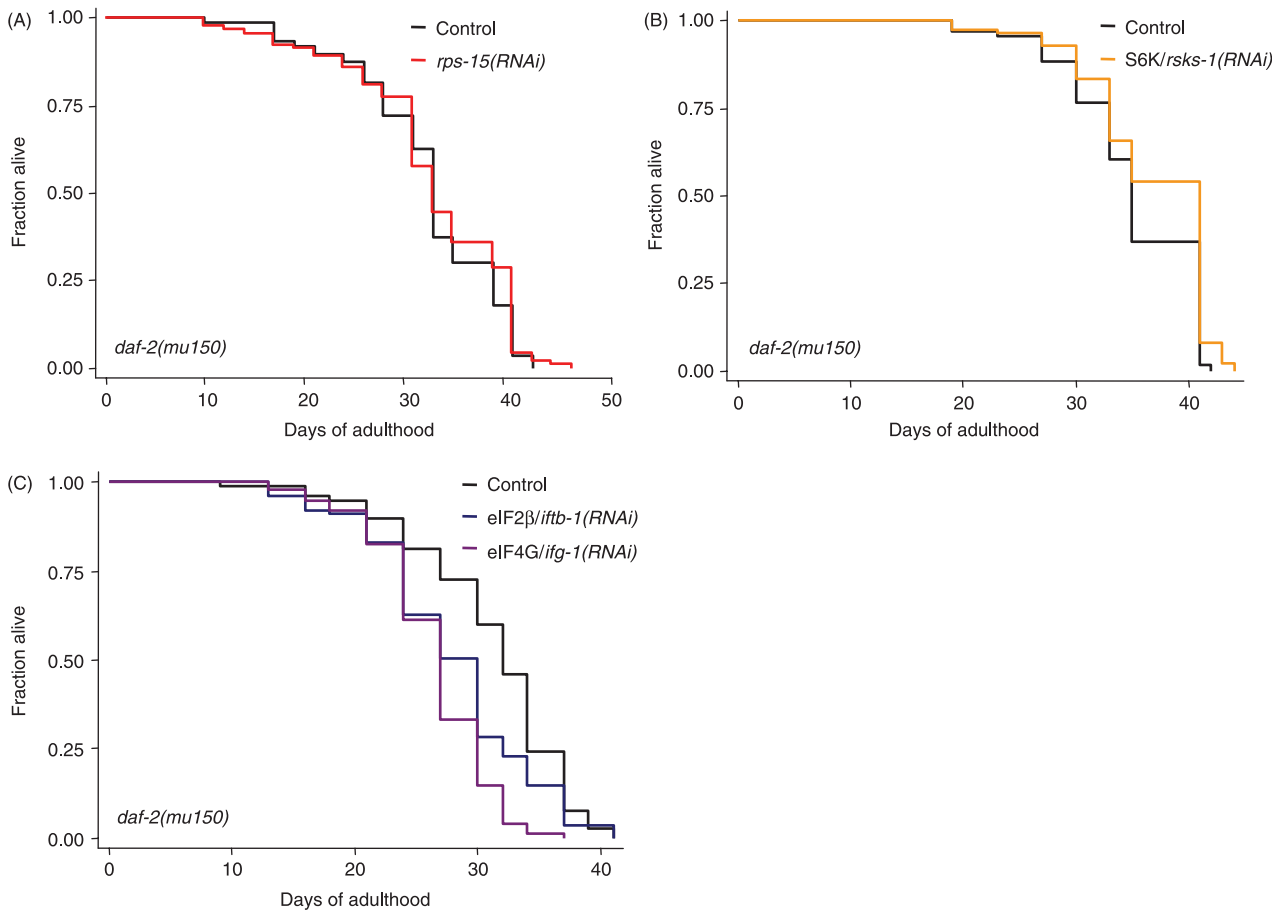


Fig. 4 Effects of translation-associated RNAi on the lifespan of *daf-2* mutants. (A) Survival curves of sterile *fer-15(b26); daf-2(mu150); fem-1(hc17)* mutants fed either control bacteria (vector only) or bacteria expressing *rps-15* dsRNA during adulthood at 25 °C. Mean lifespan was 32.5 days for control and 33.0 days for *rps-15* RNAi. $P = 0.25$, log-rank test. Adult-only treatments of *fer-15(b26); daf-2(mu150); fem-1(hc17)* or *daf-2(e1370)* mutants with *rps-15* or *rps-22* RNAi were done a total of six times with similar results. Please see Supplementary Table S3 for additional information. (B) Survival curves of sterile *fer-15(b26); daf-2(mu150); fem-1(hc17)* mutants fed either control bacteria or bacteria expressing S6K (*rsk-1*) dsRNA during adulthood at 25 °C. Mean lifespan was 35.1 days for control and 36.8 days for S6K RNAi, $P = 0.015$. Please see Supplementary Table S3 for a repetition of this experiment and additional information. (C) Survival curves of sterile *fer-15(b26); daf-2(mu150); fem-1(hc17)* mutants fed either control bacteria or bacteria expressing either eIF2 β (*iftb-1*) or eIF4G (*ifg-1*) dsRNA during adulthood at 25 °C. Mean lifespan was 31.1 days for control and 28.0 days for eIF2 β RNAi, $P < 0.0001$, and 26.3 days for eIF4G RNAi. $P < 0.0001$, log-rank test. Adult-only treatment with eIF2 β or eIF4G RNAi resulted in small lifespan shortenings in a total of five independent trials. Please see Supplementary Table S3 for additional information.

shortens lifespan) and wild-type animals (in which eIF RNAi increases lifespan).

Conditions that reduce translation, including food limitation, extend lifespan independently of SIR-2.1

Overexpressing the SIR-2.1 deacetylase extends *C. elegans* lifespan (Tissenbaum & Guarente, 2001). Therefore, we asked whether the lifespan extension produced by inhibiting translation was dependent on *sir-2.1*. To do this, we subjected a *sir-2.1* deletion mutant, *sir-2.1(ok434)*, to RP, S6K, TOR or eIF RNAi. In each case, lifespan was extended (Fig. 5A,B and Supplementary Table S4), as was the case for eIF5A/*iff-1*, examined previously (Hamilton et al., 2005). Thus, in *C. elegans*, SIR-2.1 is not required for the inhibition of translation, apparently by any mechanism, to increase lifespan.

In yeast and flies, SIR-2 activity has been associated with the response to dietary restriction (Guarente & Picard, 2005). Dietary restriction (DR) extends the lifespan of *C. elegans* in a *daf-16*-independent manner (Lakowski & Hekimi, 1998; Houthoofd et al., 2003), suggesting that *sir-2.1* is not involved in the response to DR in *C. elegans*. We tested this directly, and found that the extended lifespan of *eat-2(ad1116)* animals, which have impaired feeding behavior and represent a genetic model for DR (Raizen et al., 1995; Lakowski & Hekimi, 1998), was *sir-2.1*-independent (Fig. 5C and Supplementary Table S4). Direct food limitation (Klass, 1977) also increased the lifespan of the *sir-2.1*-deletion mutant (data not shown). Thus, in *C. elegans*, we find that *sir-2.1* is not required for dietary restriction to extend lifespan. [We note that Heidi Tissenbaum's group has shown that mutants carrying other *eat-2* alleles require *sir-2* for their long lifespan (Wang & Tissenbaum, 2006). However, David

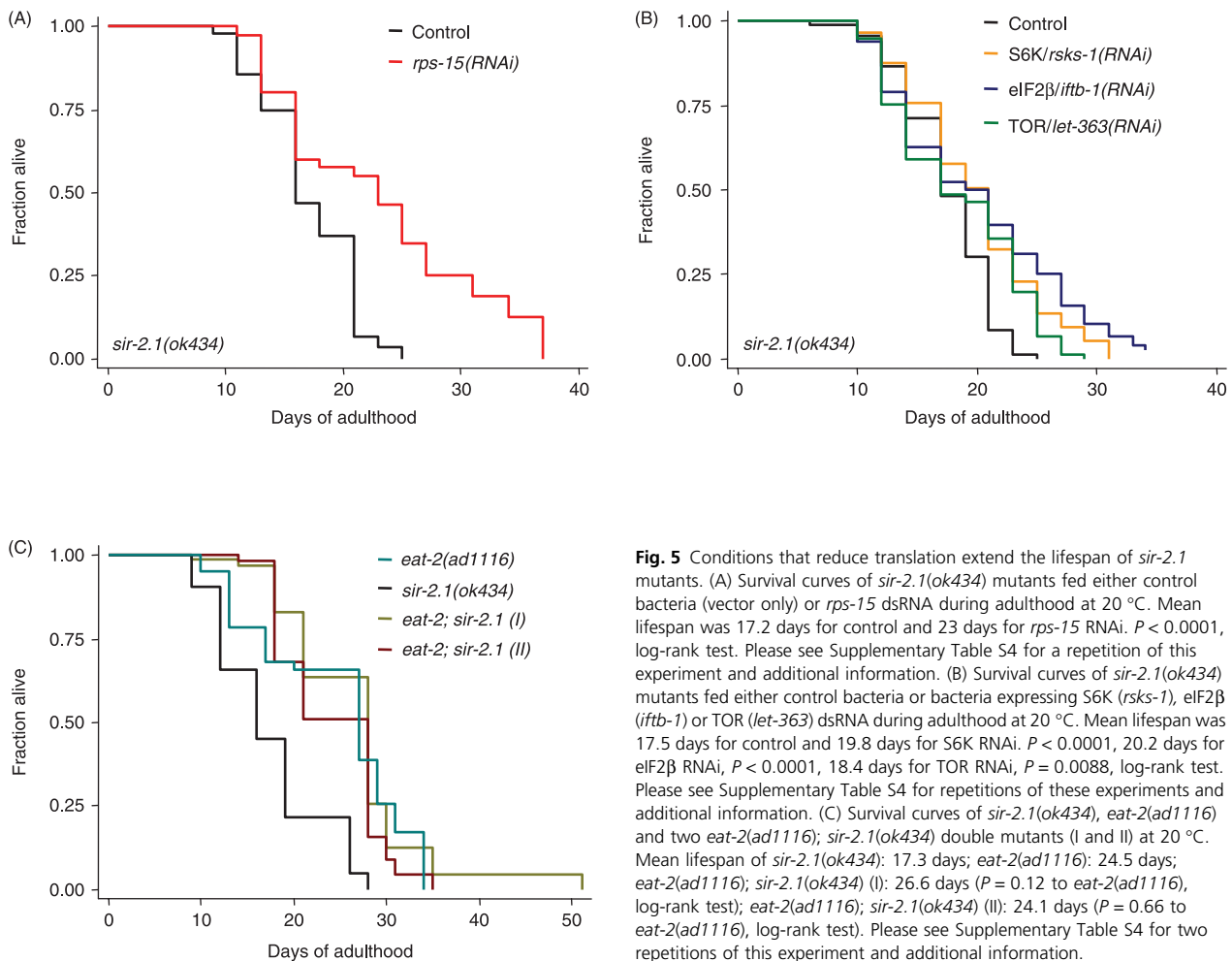


Fig. 5 Conditions that reduce translation extend the lifespan of *sir-2.1* mutants. (A) Survival curves of *sir-2.1(ok434)* mutants fed either control bacteria (vector only) or *rps-15* dsRNA during adulthood at 20 °C. Mean lifespan was 17.2 days for control and 23 days for *rps-15* RNAi. $P < 0.0001$, log-rank test. Please see Supplementary Table S4 for a repetition of this experiment and additional information. (B) Survival curves of *sir-2.1(ok434)* mutants fed either control bacteria or bacteria expressing S6K (*rks-1*), eIF2 β (*iftb-1*) or TOR (*let-363*) dsRNA during adulthood at 20 °C. Mean lifespan was 17.5 days for control and 19.8 days for S6K RNAi. $P < 0.0001$, 20.2 days for eIF2 β RNAi, $P < 0.0001$, 18.4 days for TOR RNAi, $P = 0.0088$, log-rank test. Please see Supplementary Table S4 for repetitions of these experiments and additional information. (C) Survival curves of *sir-2.1(ok434)*, *eat-2(ad1116)* and two *eat-2(ad1116); sir-2.1(ok434)* double mutants (I and II) at 20 °C. Mean lifespan of *sir-2.1(ok434)*: 17.3 days; *eat-2(ad1116)*: 24.5 days; *eat-2(ad1116); sir-2.1(ok434)* (I): 26.6 days ($P = 0.12$ to *eat-2(ad1116)*, log-rank test); *eat-2(ad1116); sir-2.1(ok434)* (II): 24.1 days ($P = 0.66$ to *eat-2(ad1116)*, log-rank test). Please see Supplementary Table S4 for two repetitions of this experiment and additional information.

Sinclair's group (with 'data not shown'), Matt Kaerberlein's group, and Sige Zou's group have all reported that direct food limitation extends the lifespan of *sir-2* mutants (Lamming et al., 2005; Kaerberlein et al., 2006; Lee et al., 2006).]

A relationship between TOR inhibition and dietary restriction

TOR inhibition and DR both alter many aspects of cellular metabolism in response to nutrient limitation. As described above, we found that TOR inhibition decreases the rate of accumulation of newly synthesized proteins in *C. elegans*. To ask whether DR also has this effect, we measured the rate of accumulation of newly synthesized proteins in *eat-2* mutants, which ingest bacteria at a much slower rate than wild type (Raizen et al., 1995). We found that *eat-2* mutants incorporated only ~15% as much ^{35}S -labeled methionine into proteins as did control animals after feeding on ^{35}S -labeled bacteria for 3 h (Fig. 2C). One could imagine that protein synthesis rates are actually normal in *eat-2* mutants, and that the reduced level of labeled protein in this experiment was due to reduced uptake of labeled bacteria. However, in this case, *eat-2* mutants would

have to be synthesizing proteins at a normal rate from stored pools of unlabeled amino acids, an *ad hoc* idea that seems unlikely. Thus, we conclude that the global rate of protein synthesis is low in *eat-2* mutants. Indeed, it is possible that *eat-2* mutants transcriptionally down-regulate the protein-synthesis machinery, as they exhibit lower relative mRNA levels of several RP genes (Supplementary Fig. S1C).

In both yeast and flies, reducing TOR activity results in little or no lifespan increase under conditions of nutrient limitation. This suggests that dietary restriction may extend lifespan by down-regulating TOR activity in these animals. To investigate the situation in *C. elegans*, we asked whether the longevity produced by TOR RNAi could be increased further by dietary restriction. TOR is required for *C. elegans* development, as loss of TOR causes larval arrest (Long et al., 2002). For this reason, we initiated TOR RNAi in young *eat-2(ad1116)* adults, reducing TOR mRNA levels by approximately threefold (Supplementary Fig. S1A). In an experiment in which TOR RNAi extended the mean lifespan of wild-type animals ~27%, we found that TOR RNAi did not further extend the lifespan of the *eat-2(ad1116)* mutant (Fig. 6A,B). This finding was reproducible in each of six experiments (Supplementary Table S5).

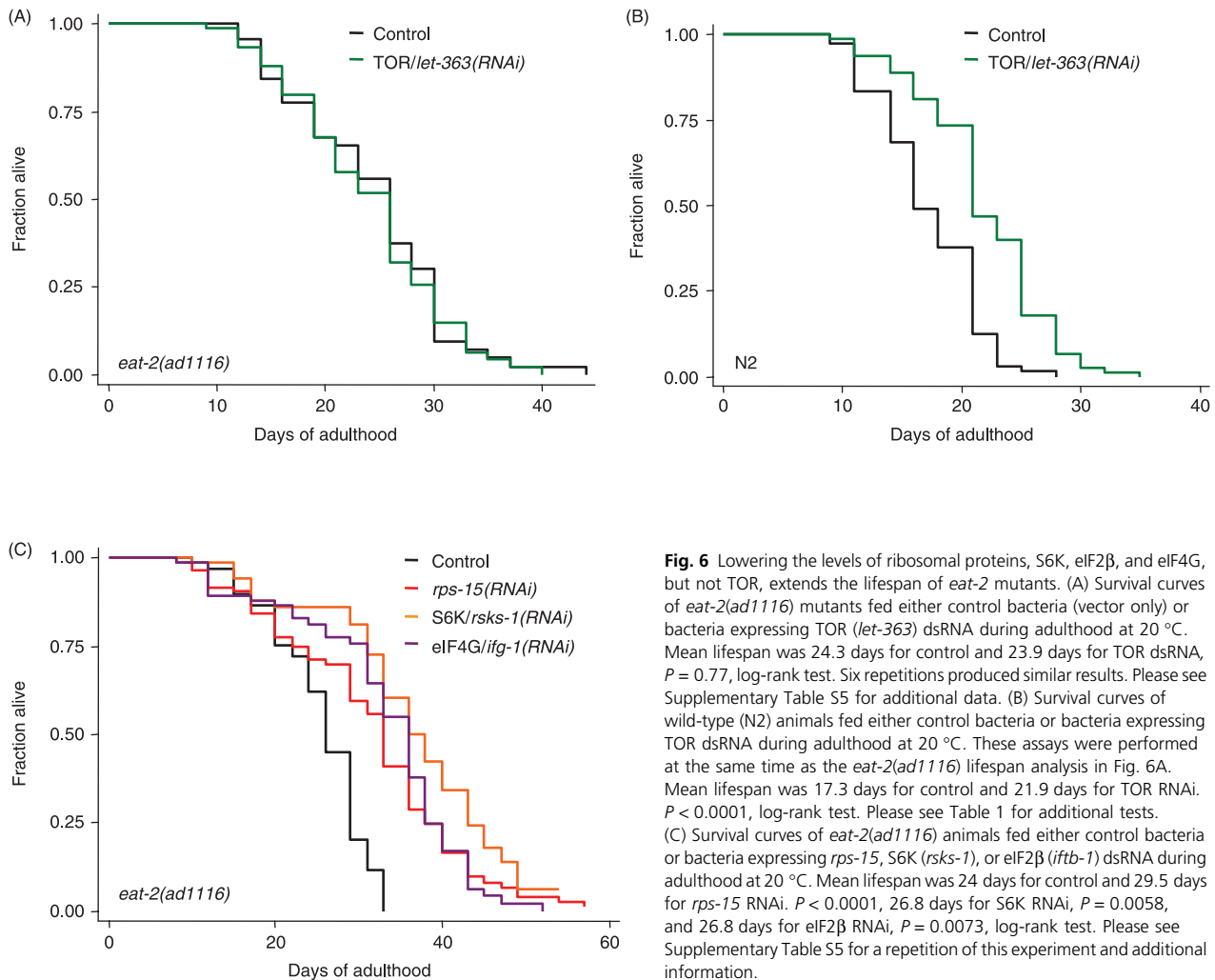


Fig. 6 Lowering the levels of ribosomal proteins, S6K, eIF2 β , and eIF4G, but not TOR, extends the lifespan of *eat-2* mutants. (A) Survival curves of *eat-2(ad1116)* mutants fed either control bacteria (vector only) or bacteria expressing TOR (*let-363*) dsRNA during adulthood at 20 °C. Mean lifespan was 24.3 days for control and 23.9 days for TOR dsRNA, $P = 0.77$, log-rank test. Six repetitions produced similar results. Please see Supplementary Table S5 for additional data. (B) Survival curves of wild-type (N2) animals fed either control bacteria or bacteria expressing TOR dsRNA during adulthood at 20 °C. These assays were performed at the same time as the *eat-2(ad1116)* lifespan analysis in Fig. 6A. Mean lifespan was 17.3 days for control and 21.9 days for TOR RNAi. $P < 0.0001$, log-rank test. Please see Table 1 for additional tests. (C) Survival curves of *eat-2(ad1116)* animals fed either control bacteria or bacteria expressing *rps-15*, S6K (*rsks-1*), or eIF2 β (*iftb-1*) dsRNA during adulthood at 20 °C. Mean lifespan was 24 days for control and 29.5 days for *rps-15* RNAi. $P < 0.0001$, 26.8 days for S6K RNAi, $P = 0.0058$, and 26.8 days for eIF2 β RNAi, $P = 0.0073$, log-rank test. Please see Supplementary Table S5 for a repetition of this experiment and additional information.

Previously, we showed that mRNA levels of several genes that appear to function in the response to DR are decreased by DR (Hansen et al., 2005). In contrast, TOR mRNA levels were similar in adult *eat-2(ad1116)* mutants and wild-type animals (Supplementary Fig. S1C). This is consistent with the TOR signaling pathway's being post-translationally regulated by nutrient limitation in *C. elegans*, as in other organisms (Wullschlegel et al., 2006).

Since both TOR RNAi and DR appear to reduce translation, it would seem likely that subjecting animals to both TOR RNAi and DR would reduce translation even more. If so, given the findings described above, we reasoned that reducing translation by other means in animals subjected to either DR or TOR RNAi would probably not further extend lifespan. However, we found that this was not the case. We subjected *eat-2(ad1116)* mutants to RNAi of genes encoding RPS-15, RPS-22, S6K, eIF2 β , or eIF4G, and found that their lifespans were further extended (Fig. 6C and Supplementary Table S5). In addition, the lifespans of *S6K/rsks-1(sv31)* and *eIF4E/ife-2(ok306)* mutants subjected to TOR RNAi were longer than the lifespans of either the S6K or eIF4E mutant alone (Fig. 7A,C and Supplementary Table S6). In no case did we find the effect that we saw when we subjected

TOR RNAi animals to DR, i.e. no lifespan increase. These findings suggest that either the combination of DR and TOR RNAi does not further reduce translation, or if it does, then other factors must prevent increased longevity. To distinguish between these possibilities, we measured the rate of accumulation of newly synthesized proteins in *eat-2(ad1116)* mutants subjected to TOR RNAi. We found that TOR RNAi reduced the low translation rate of *eat-2(ad1116)* mutants by 49% ($\pm 24\%$ SEM; $P = 0.053$, one-sided *t*-test, Supplementary Fig. S2). This finding suggests that TOR RNAi has another, unknown, effect that prevents the reduction in protein synthesis it produces from further increasing the lifespan of *eat-2* mutants.

Lowering translation further extends the lifespan of animals with reduced respiration

Mitochondrial respiration, like translation, is a major cellular process linked to growth, and reducing respiration in *C. elegans* extends lifespan (Feng et al., 2001; Dillin et al., 2002; Lee et al., 2003). We found that treating the respiratory mutant *isp-1(qm150)* with *rps-15* or *S6K/rsks-1* RNAi further extended its lifespan

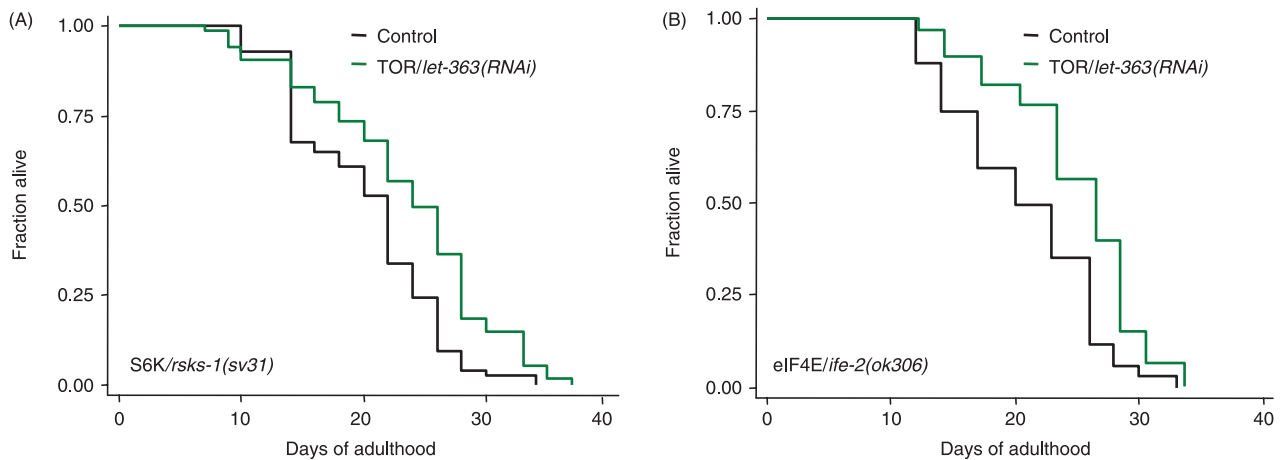


Fig. 7 Lowering TOR levels extends the lifespan of *S6K/rsk-1* and *eIF4E/ife-2* mutants. (A) Survival curves of *S6K/rsk-1(sv31)* mutants fed either control bacteria (vector only) or bacteria expressing TOR dsRNA during adulthood at 20 °C. Mean lifespan was 20.8 days for control and 24.4 days for TOR dsRNA, $P = 0.00020$, log-rank test. Please see Supplementary Table S6 for additional information. (B) Survival curves of *eIF4E/ife-2(ok306)* mutants fed either control bacteria (vector only) or bacteria expressing TOR dsRNA during adulthood at 20 °C. Mean lifespan was 20.2 days for control and 23.4 days for TOR dsRNA, $P = 0.0003$, log-rank test. Please see Supplementary Table S6 for repetitions and additional information.

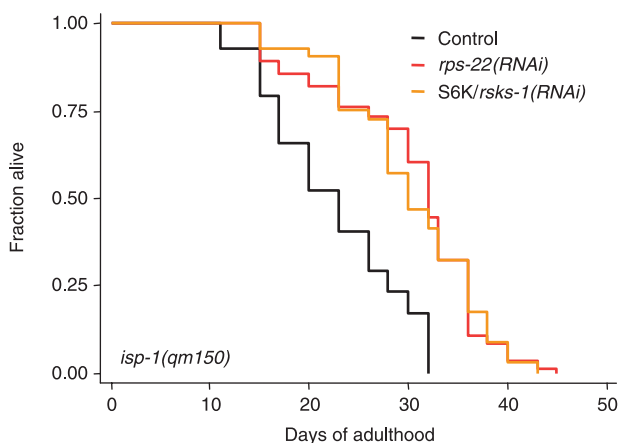


Fig. 8 Lowering the levels of ribosomal proteins and S6K extends the lifespan of *isp-1* mutants. Survival curves of *isp-1(qm150)* animals fed either control bacteria (vector only) or bacteria expressing *rps-22* or S6K (*rsk-1*) RNAi during adulthood at 20 °C. Mean lifespan was 22.5 days for control and 30.1 days for *rps-22* dsRNA, $P < 0.0001$, and 30.2 days for S6K dsRNA, $P < 0.0001$, log-rank test. RNAi of *rps-15* was found to give similar results. Please see Supplementary Table S7 for additional information.

(Fig. 8 and Supplementary Table S7). Previously, we found that reducing respiration rates during adulthood does not extend lifespan (Dillin et al., 2002), but reducing translation during adulthood does extend lifespan. Taken together, these findings suggest that translation and respiration inhibition probably extend lifespan in different ways.

Inhibiting translation increases thermotolerance

Many long-lived mutants are resistant to heat and other types of stress. Because increased resistance to oxidative and other forms of damage could potentially increase cellular fitness and

longevity, we asked whether conditions that inhibit translation elicit thermotolerance, a stress-resistance phenotype often observed among long-lived mutants. We tested animals in which we had used RNAi to reduce the levels of TOR, S6K, several RPs, eIF2 β , eIF4G or eIF4E, as well as animals carrying the deletion mutation *eIF4E/ife-2(ok306)*, and found that each of these treatments significantly increased thermotolerance (Fig. 9A–C and Supplementary Table S8).

Because the lifespan extension caused by inhibiting S6K and RPs was *daf-16* independent, we asked whether the stress resistance caused by these perturbations was also *daf-16* independent. We found that it was: RNAi of S6K and RPs increased thermotolerance in *daf-16(null)* mutants, as in wild-type animals (Supplementary Table S8). Conversely, we found that depletion of the translation-initiation factors eIF2 β and eIF4G, which increased lifespan in a *daf-16*-dependent fashion, also increased stress resistance in a *daf-16*-dependent fashion (Table S8). Together these findings genetically link the stress resistance of these animals to their longevity.

Finally, because of the association between translation inhibition and the longevity response to dietary restriction, we also asked whether dietary restriction increased thermotolerance in *C. elegans*, as it does in many other species (Munoz, 2003). We found that it did; *eat-2(ad1116)* mutants were more thermotolerant than were wild-type animals (Fig. 9D).

Discussion

Over the last fifteen years, mutations in a wide variety of genes have been found to extend lifespan. Many of these mutations inhibit pathways normally associated with growth (insulin/IGF-1 and TOR signaling, activity of the electron transport chain) and/or activate pathways associated with stress resistance (increased DAF-16/FOXO, heat-shock factor, sirtuin or JNK

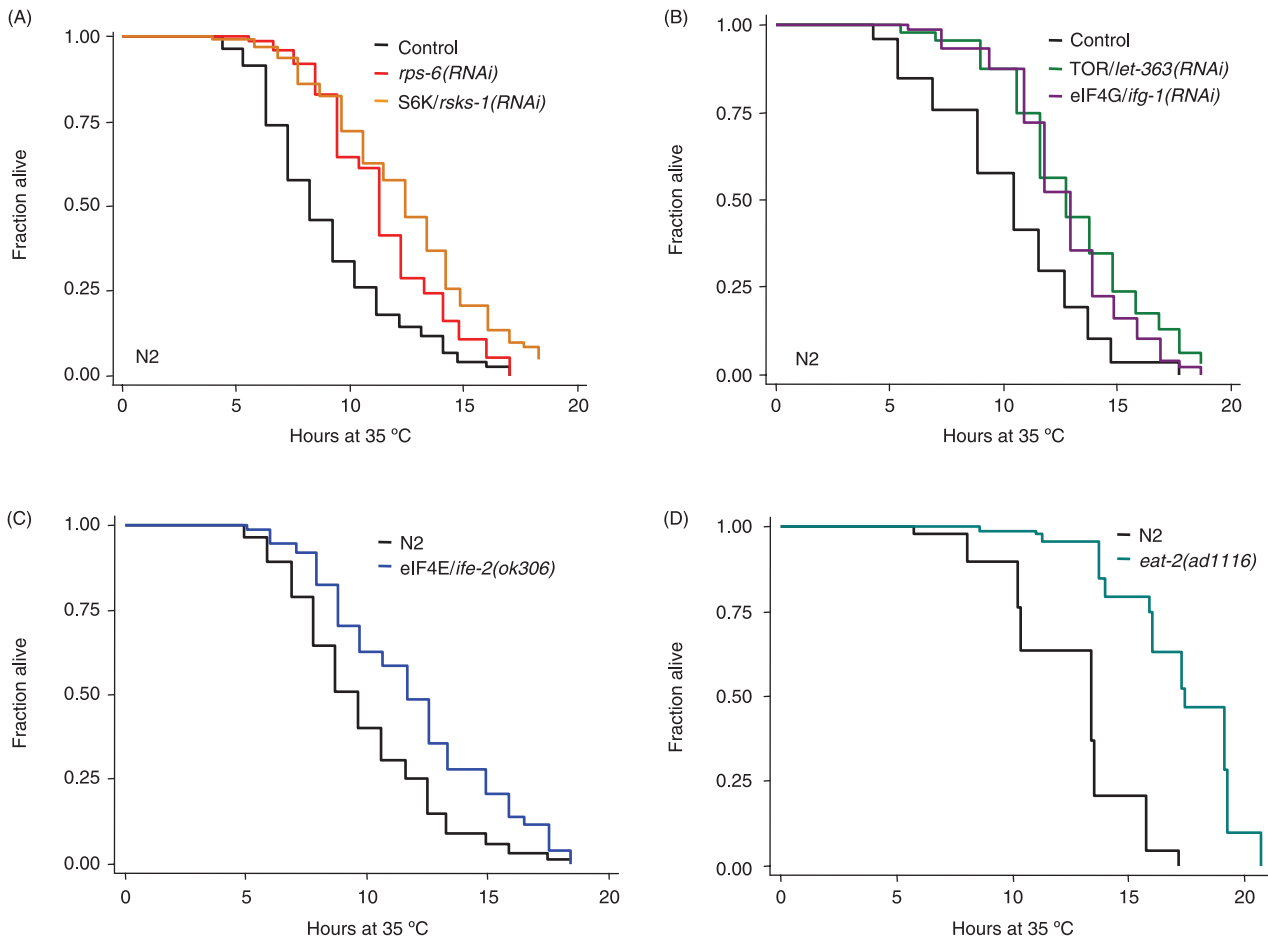


Fig. 9 Conditions that reduce translation increase thermotolerance. (A) Survival curves of wild-type (N2) animals fed either control bacteria (vector only) or bacteria expressing *rps-6* or S6K (*rsk-1*) dsRNA during adulthood following exposure to 35 °C at day 4 of adulthood. Mean survival time was 9.1 h for control and 11.5 h for *rps-6* RNAi, $P < 0.0001$, and 12.7 h for S6K RNAi $P < 0.0001$, log-rank test. These RNAi clones also produced significant thermotolerance effects in *daf-16(mu86)* mutants. Please see Supplementary Table S8 for a repetition of this experiment and for *daf-16(mu86)* data. (B) Survival curves of N2 animals fed either control bacteria or bacteria expressing TOR (*let-363*) or eIF4G (*ifg-1*) dsRNA during adulthood following exposure to 35 °C at day 4 of adulthood. Mean survival time was 10.2 h for control and 13.3 h for TOR RNAi, $P < 0.0001$, and 12.7 h for eIF4G RNAi, $P < 0.0001$, log-rank test. These RNAi clones produced significant thermotolerance effects in at least two experiments. TOR RNAi also induced thermotolerance in *daf-16(mu86)* mutants. Please see Supplementary Table S8 for a repetition of this experiment and for *daf-16(mu86)* data. (C) Survival curves of N2 vs. outcrossed eIF4E/*ife-2(ok306)* animals following exposure to 35 °C at day 4 of adulthood. Mean survival time was 9.9 h for N2 and 11.9 h for eIF4E/*ife-2(ok306)*, $P < 0.0001$, log-rank test. Please see Supplementary Table S7 for a repetition of this experiment. Adult-only treatment with eIF2 β , eIF4G, or eIF4E RNAi failed to increase thermotolerance in *daf-16(mu86)* animals in each of six different trials (Supplementary Table S8). (D) Survival curves of N2 vs. *eat-2(ad1116)* animals following exposure to 35 °C at day 3 of adulthood. Mean survival time was 12.6 h for N2 and 17.2 h for *eat-2(ad1116)*, $P < 0.0001$, log-rank test. See Supplementary Table S8 for additional information.

activity). Because of the importance of protein synthesis during cell growth, and because we identified two ribosomal protein genes in a longevity-pathway screen, we were interested in learning more about how inhibiting translation affects aging.

We reasoned that reducing ribosome biogenesis by inhibiting the synthesis of RPs was a fairly direct way to inhibit translation. One concern was that several RPs have been shown to participate in processes other than translation; for instance the ribosomal protein L26 plays a role in the DNA damage response (Takagi et al., 2005), and L22 and L6 affect transcription by interacting with chromatin (Ni et al., 2006). However, no common function for RPs besides their role in translation has been reported. We observed lifespan extension upon depletion of each of the six

40S and five 60S RPs that we tested. While we cannot rule out the possibility that all of these proteins function together in another process, the simplest interpretation of these findings is that lifespan extension is due to reduced ribosomal function itself. While these studies were in progress, a report was published showing that inhibiting RPs also extends lifespan in yeast (Kaeberlein et al., 2005), although only a subset of RPs produced this effect. Thus, it is not clear whether the underlying mechanisms are the same.

We also tested the effects of inhibiting positive regulators of translation. We found that reducing the levels of S6K and the translation initiation factors eIF2 β , eIF4G, and eIF4E all significantly extended lifespan. It is possible that particular

translational regulators have additional, unknown targets that influence lifespan. However, the fact that inhibition of so many translation regulators extends lifespan makes a strong consistency argument that the effects we see are directly related to translation.

How might reducing translation affect lifespan? Adult-only RNAi of TOR, RP, S6K, and eIF genes reduced brood size (Supplementary Table S1 and data not shown). Reduced fecundity alone cannot explain the lifespan extensions we observed, since we saw significant lifespan extension when we treated sterile animals with RP RNAi [*CF512* animals; *fer-15(b26)III*; *fem-1(hc17)III*] (Table 1). Nevertheless, this finding suggested a trivial explanation: When germline cell division is inhibited in animals that have somatic reproductive tissues, even during adulthood, lifespan is extended (Hsin & Kenyon, 1999; Arantes-Oliveira *et al.*, 2002). Is it possible that inhibiting translation extends lifespan because it inhibits germ cell growth? The finding that none of these RNAi treatments further increased the lifespan of *daf-2* mutants makes this very unlikely, since removing the germ cells doubles the already long lifespan of *daf-2* mutants (Hsin & Kenyon, 1999). In addition, lifespan extension following germline removal is *daf-16* dependent (Hsin & Kenyon, 1999), whereas the lifespan extensions produced by at least some translation-inhibiting treatments are *daf-16* independent.

Another possibility is that reducing translation extends lifespan because the normal process of translation causes some type of damage that accelerates aging. Although this is possible, it seems unlikely, because the selective pressure for the beneficial aspects of protein synthesis would seem to be so strong. Alternatively, it is possible that the act of *reducing* translation triggers a cellular response that extends lifespan. This idea seems likely to be correct, because all of the perturbations that we used to inhibit protein synthesis increased the thermotolerance of the animal. Thus, animals with reduced rates of translation may live longer because they mount a stress response that improves their ability to prevent or repair damage that normally accelerates aging (Syntichaki & Tavernarakis, 2006).

Since animals in nature are not likely to encounter RP-RNAi bacteria, we considered the possibility that our perturbations create an unnatural, stressful state that is not likely to be relevant to the normal biology of the animal. To address this, we examined the stress resistance of animals with defects further up the regulatory hierarchy. Inhibiting the central regulator TOR by nutrient limitation, a condition commonly encountered in nature, down-regulates protein synthesis, and we found that depletion of TOR increases thermotolerance. Moreover, we found that animals subjected to dietary restriction have increased thermotolerance in *C. elegans*, as in other organisms (Munoz, 2003). Thus it seems likely that the stress resistance caused by inhibiting translation in the laboratory is a phenomenon that is also induced by harsh conditions in nature.

To learn more about how translation inhibition exerts its effects on lifespan, we asked whether the functions of known lifespan genes were required. Surprisingly, we found that not all translation perturbations exerted their effects on lifespan and

stress resistance in the same way. The deacetylase *sir-2.1* was not required for the lifespan extension produced by any mode of translation inhibition; however, *daf-16* was required for some but not other modes of translation inhibition to extend lifespan. We found that reducing the level of TOR, S6K or RPs extended lifespan independently of *daf-16*. This was known previously for TOR (Vellai *et al.*, 2003), and shown here for S6K and RPs. Likewise, we found that the thermotolerance caused by inhibiting TOR, S6K or RPs does not require *daf-16*. These findings suggest that TOR inhibition exerts its effects on lifespan, at least in part, through reduced S6K activity and reduced translation (however, see Discussion below).

Remarkably, we found that the lifespan extension and stress resistance produced by depleting any of three translation initiation factors during adulthood requires *daf-16*. Working concurrently and independently, the Johnson lab reported similar findings for two of these initiation factors (Henderson *et al.*, 2006). In addition, a fourth translation initiation factor was identified previously in a genome-wide longevity screen; its inhibition was found to increase lifespan in a *daf-16*-dependent fashion (Hamilton *et al.*, 2005). Why might this be? Several types of environmental stress reduce global translation rates by perturbing the activities of translation-initiation factors (Ptushkina *et al.*, 2004; Connolly *et al.*, 2006). Moreover, DAF-16 regulates many stress-response genes (Honda & Honda, 1999; McElwee *et al.*, 2003; Murphy *et al.*, 2003) and DAF-16 is known to be activated by environmental stress (Henderson & Johnson, 2001; Lin *et al.*, 2001). Perhaps artificial down-regulation of translation initiation factors engages a *daf-16*-dependent stress-response network that is not engaged by down-regulating the nutrient sensor TOR or its effectors.

The finding that inhibiting translation initiation factors extends lifespan in a *daf-16*-dependent fashion leads to an apparent paradox: inhibiting factors that activate translation would be expected to inhibit translation itself, which we have shown extends lifespan independently of *daf-16*. Yet we detected no evidence of any *daf-16*-independent component of the lifespan extension caused by inhibiting these translation initiation factors. This leads to two interesting conclusions: first, inhibiting translation initiation factors cannot produce the exact same effect on translation that is produced by inhibiting RPs and S6K; second, the signal that extends lifespan and stress resistance in response to inhibiting translation itself (which acts independently of *daf-16*) must somehow be suppressed or converted to a *daf-16*-dependent signal when translation initiation factors are inhibited and DAF-16 activity is engaged.

The lifespan extension produced by TOR inhibition in yeast and flies has been linked to the response to DR (Kapahi *et al.*, 2004; Kaeberlein *et al.*, 2005). Recently, the Johnson lab reported that TOR RNAi further extended the lifespan of DR animals; however, the significance of this is unclear because in their experiments *eat-2(ad465)* mutants were not long lived (Henderson *et al.*, 2006). In contrast, in each of six trials, we found that TOR RNAi did not further increase the lifespans of animals subjected to DR. Thus in *C. elegans*, as in yeast and flies,

DR may extend lifespan, at least in part, by down-regulating TOR. This is a significant finding, because only a few of the many *C. elegans* mutations or RNAi clones that extend lifespan fail to further extend the lifespan of DR animals. Therefore, TOR inhibition may constitute a major mechanism by which DR extends lifespan in this organism.

Why, at the molecular level, doesn't TOR RNAi further extend the lifespan of DR animals? One might imagine that for some reason TOR RNAi does not further inhibit protein synthesis in DR animals. However, we found that subjecting *eat-2* mutants to TOR RNAi further inhibited protein synthesis. Such an additional inhibition would be predicted to further extend the lifespan of DR animals, because RNAi depleting either S6K or RPs further extends the lifespan of DR animals. This suggests that TOR RNAi has a second output that prevents the further reduction in protein synthesis it produces from further extending the lifespan of DR animals. [Conversely, we note that DR must do more to extend lifespan than simply to inhibit TOR, because DR, but not TOR knockdown, further extends the lifespan of insulin/IGF-1 pathway mutants (Lakowski & Hekimi, 1998; Houthoofd *et al.*, 2003).]

While our findings imply a special, still unclear, relationship between TOR inhibition and DR, they do not answer the question of whether the low rates of protein synthesis seen in *eat-2(ad1116)* mutants contribute to their longevity. We favor this model, since direct inhibition of protein synthesis increases lifespan. We found that inhibiting translation in *eat-2* mutants further extends lifespan. However, this need not mean that inhibition of translation and DR affect lifespan in different ways, since direct food limitation further increases the lifespan of *eat-2(ad1116)* mutants (data not shown). Interestingly, DR has been reported to increase rates of protein synthesis in mammals (Tavernarakis & Driscoll, 2002). Whether this reflects a fundamental difference in mechanism is not clear.

Like the lifespan extension produced by DR, the lifespan extension caused by the *daf-2(e1370)* mutation is not further extended by TOR RNAi (Vellai *et al.*, 2003). However, unlike the lifespan of DR animals, the lifespan of *daf-2* mutants could not be further extended by any depletion of translation-related proteins. Inhibiting RPs or S6K had no effect on the lifespan of *daf-2* mutants, and inhibiting translation initiation factors actually shortened the *daf-2* mutant lifespan. This is particularly intriguing because rates of protein synthesis are not reduced in *daf-2(e1370)* mutants. The simplest (although not the only) interpretation of this finding is that *daf-2* mutations and conditions that inhibit translation ultimately converge on the same downstream targets that mediate stress resistance and longevity.

Inhibiting respiration also extends lifespan in *C. elegans* (Feng *et al.*, 2001; Dillin *et al.*, 2002; Lee *et al.*, 2003). None of our findings suggest that inhibition of translation and inhibition of respiration extend lifespan in the same way. First, our previous RNAi timing experiments indicated that respiration inhibition during development is required for lifespan extension (Dillin *et al.*, 2002), whereas we find that adult-only inhibition of translation is very effective. Second, RNAi of several genes involved

in respiration did not significantly increase thermotolerance (Lee *et al.*, 2003; A. Dillin & C. Kenyon, unpublished data); in contrast, RNAi against all of our translation-related proteins did. Finally, we found that inhibition of translation by any means; whether by lowering the levels of RPs or S6K further increased the lifespan of *isp-1(qm150)* mutants.

In summary, a wide variety of conditions that inhibit translation increase stress resistance and longevity in *C. elegans*. Thus translation inhibition appears to be a powerful way to increase lifespan in this organism, and it may do so by activating cellular protective mechanisms. In addition, our findings raise many fascinating questions about why different conditions that inhibit translation can, in different mutant strains, have such differential effects on lifespan. These questions can be addressed by future biochemical and molecular experiments.

Experimental procedures

Strains

All strains were maintained as described previously (Brenner, 1974). CF1037: *daf-16(mu86)*, DA1116: *eat-2(ad1116)*II, CF1041: *daf-2(e1370)*III, CF512: *fer-15(b26)*II; *fem-1(hc17)*III. CF1844: *fer-15(b26)*II; *daf-2(mu150)*III; *fem-1(hc17)*IV. VB633: *rsk-1(sv31)*III outcrossed eight times to N2 from Simon Tuck's lab, University of Umeå. This strain is developmentally delayed and animals often rupture during early adulthood. Adult worms also appear smaller and darker and have reduced progeny (J. Friberg & S. Tuck, in preparation). VC199.4x: *sir-2.1(ok434)*IV outcrossed four times to David Sinclair's N2. CF2254 and CF2255 (different strain isolates from the cross between *eat-2* and *sir-2.1* mutants): *eat-2(ad1116)*II; *sir-2.1(ok434)*VI. The *sir-2* deletion generally caused relatively high levels of censoring due to bagging (i.e. internal progeny hatching) and/or missing worms. CF2412: *ife-2(ok343)*X outcrossed three times to Kenyon-lab N2. This strain has a slightly increased incidence of rupturing compared to wild-type.

RNAi clone analysis

The identity of all RNAi clones was verified by sequencing the inserts using the M13-forward primer. The TOR RNAi clone was obtained from Dr Xiaomeng Long, Massachusetts General Hospital. All other clones were from Julie Ahringer's RNAi library (Kamath *et al.*, 2003) except for the *rsk-1* RNAi clone, which was from Marc Vidal's RNAi library (Rual *et al.*, 2004).

Lifespan analysis

Lifespan analysis was conducted at 20 °C as described previously (Hansen *et al.*, 2005) unless otherwise stated. RNAi treatments were either performed as whole-life treatments or adult-only treatments. In the whole-life analysis, eggs were added to plates seeded with the RNAi bacteria of interest. In the adult-only analysis, eggs were added to plates seeded with control (vector

only) RNAi bacteria, and adult animals were transferred to gene-specific RNAi bacterial plates. In some experiments, the chemical 2'fluoro-5'deoxyuridine (FUDR, Sigma, St Louis, MO, USA) was added to adult worms (100 μM) to prevent their progeny from developing. At least 80 worms were tested in each experiment. Strains were grown at 20 °C at optimal growth conditions for at least two generations before lifespan analysis. During the analysis of large numbers of RNAi clones, CF512 or N2 controls were performed either concurrently or in overlapping time frames. In all experiments, the pre-fertile period of adulthood was used as $t = 0$ for lifespan analysis. Censoring in the lifespan analysis included animals that ruptured, bagged (i.e. exhibited internal progeny hatching), or crawled off the plates. We note that ruptured animals were usually censored before they died. We have reported the number of ruptured animals in each experiment in our tables, since this group generally constitutes the majority of censored animals. The translation-associated RNAi clones, in particular TOR RNAi, often induced a slightly higher level of rupturing when fed to adult animals. This effect was largely independent of the genetic background of the worms to which the RNAi bacteria were fed. We note that, had we not censored any animals, the fraction alive during early adulthood in our lifespan analyses would decrease; however, since events leading to censoring almost always occurred during early adulthood, failure to censor would not change the maximum lifespan (75th percentile, see Tables), which was almost always increased by conditions that inhibit translation. STATA software was used for statistical analysis and to determine means and percentiles. In all cases, P values were calculated using the log-rank (Mantel-Cox) method.

Thermotolerance assay

Synchronized N2 animals were transferred to gene-specific RNAi plates on the first day of adulthood and then shifted to 35 °C as 3- or 4-day-old adults. Survival was scored periodically after the temperature shift. STATA software was used for statistical analysis and P values were calculated using the log-rank (Mantel-Cox) method.

³⁵S-methionine incorporation

OP50 bacteria were cultured in LB (1 mL per worm sample) containing ³⁵S-methionine (10–15 μCi per worm sample, Perkin-Elmer, Boston, MA, USA; #NED709A001MC) for 12 h and then concentrated 10-fold in Eppendorf tubes. Days 1–3 adult worm samples (~1000–2000 worms) treated with 100 μM FUDR were mixed with the bacteria and incubated for 3 h at room temperature with shaking. RNAi treatments were initiated on day 1 of adulthood. At the end of this incubation period, negative controls were produced by mixing worm samples with radioactive bacteria for < 1 min ($t = 0$). All worm samples were subsequently washed twice with S-basal and incubated in non-radioactive OP50 (10-fold concentrated) for 30 min to purge undigested ³⁵S-methionine-labeled bacteria out of the intestines

of the worms. We then measured ³⁵S radioactivity levels in protein extracts from these worm samples to determine the levels of newly synthesized proteins. Worm samples were washed twice with S-basal and flash frozen twice in liquid nitrogen. The samples were then boiled in 100 μL 1% SDS, centrifuged for 20 min at 16 000 g , and the supernatants were precipitated by adding 100 μL 10% TCA and incubating on ice for ~1 h. Protein concentrations were measured using the detergent compatible (DC) protein assay (Bio-Rad Laboratories, Hercules, CA, USA) and ³⁵S radioactivity was measured after application to glass microfiber filters (Whatman, Middlesex, UK) by liquid scintillation (Beckman, Fullerton, CA, USA; L1801). ³⁵S incorporation levels were calculated by normalizing ³⁵S counts per min, corrected for unspecific background ($t = 0$), to total protein levels. Statistical analysis was done as one-sided, paired Student's t -test on the ³⁵S incorporation levels. The relative ³⁵S incorporation (used for plotting) was calculated by normalizing the ³⁵S incorporation levels of a given mutant to the ³⁵S incorporation levels of the control/wild type, which was set to 100.

Brood-size assay

Eggs were incubated at 20 °C on control plates and 16 late-L4 stage worms were picked for each treatment and transferred to fresh RNAi or OP50 plates every 12 h for 4–5 days. After this period, the worms were transferred every 24 h. Worms that crawled off the plates, bagged or ruptured were censored. All progeny plates were incubated at 20 °C for about 2 days following transfer of the parental worms and then held at 4 °C. The number of worms that developed was determined at the end of the experiment.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

For all experiments, ~5000 eggs were seeded. For measuring mRNA levels in RNAi-treated animals, CF512 [*fer-15(b26)*; *fem-1(hc17)*] eggs were placed on control plates and incubated at 25 °C (to make them sterile). Animals were then transferred to RNAi plates at the first day of adulthood and incubated at 20 °C. Total RNA was isolated from 3-day-old adults. Sterile CF1844 (*fer-15(b26)*; *daf-2(mu150)*; *fem-1(hc17)*) mutants were incubated as CF512 and collected on day 3. DA1116 (*eat-2(ad1116)*) mutants were incubated at 20 °C and harvested on day 1, 8–18 h after N2, due to their delayed development (these samples were kindly provided by Laura Mitic). Isolation, purification, and reverse transcription of *C. elegans* RNA were carried out as described (Taubert *et al.*, 2006). Quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) was performed in an Opticon 2 DNA Engine (MJ Research, Waltham, MA, USA) or a 7300 real-time PCR System (Applied Biosystems, Foster City, CA, USA) and analyzed using the Ct method (Applied Biosystems Prism 7700 Users Bulletin No. 2; <http://docs.appliedbiosystems.com/pebi/docs/04303859.pdf>); mRNA levels of *act-1* was used for normalization. Primer sequences are available upon request.

Acknowledgments

We thank Marta Gaglia for the initial characterization of the KX15/*ife-2(ok306)* strain. We thank Dr Xiaomeng Long, Massachusetts General Hospital, for providing the TOR (*let-363*) RNAi clone. We thank Dr Simon Tuck, University of Umeå, for providing the unpublished, outcrossed *rsk-2(sv31)* deletion mutant and Dr David Sinclair, Harvard Medical School, for providing the outcrossed *sir-2.1(ok434)* strain. We thank the Caenorhabditis Genetics Center for providing the KX15 (*ife-2(ok306)*) strain. We thank Dr Pankaj Kapahi, The Buck Institute, for sharing his lab's ³⁵S-methionine incorporation protocol with us.

Stefan Taubert carried out the qRT-PCR analysis, Douglas Crawford examined *eat-2* mutants subjected to thermal stress and direct food limitation; Douglas Crawford and Nataliya Libina tested the *sir-2.1* dependence of DR, and Seung-Jae Lee outcrossed KX15 (*ife-2(ok306)*) and performed the lifespan analysis of the outcrossed strain CF2412. All other experiments were performed by Malene Hansen.

Malene Hansen was supported by a fellowship from the Ellison/American Federation for Aging Research Foundation. Stefan Taubert was supported by a fellowship from the American Heart Association. Douglas Crawford was supported by a Howard Hughes graduate fellowship, and Seung-Jae Lee is an Ellison Medical Foundation Fellow of the Life Sciences Research Foundation. This work was supported by grants from the Ellison Medical Foundation and the NIH to Cynthia Kenyon, who is the director of the UCSF Hillblom Center for the Biology of Aging, an American Cancer Society Professor, and a co-founder and director of the biotechnology company Elixir Pharmaceuticals.

References

- Arantes-Oliveira N, Apfeld J, Dillin A, Kenyon C (2002) Regulation of life-span by germ-line stem cells in *Caenorhabditis elegans*. *Science* **295**, 502–505.
- Brenner S (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71–94.
- Connolly E, Braunstein S, Formenti S, Schneider RJ (2006) Hypoxia inhibits protein synthesis through a 4E-BP1 and elongation factor 2 kinase pathway controlled by mTOR and uncoupled in breast cancer cells. *Mol. Cell. Biol.* **26**, 3955–3965.
- Dillin A, Hsu AL, Arantes-Oliveira N, Lehrer-Graiwer J, Hsin H, Fraser AG, Kamath RS, Ahringer J, Kenyon C (2002) Rates of behavior and aging specified by mitochondrial function during development. *Science* **298**, 2398–2401.
- Feng J, Bussiere F, Hekimi S (2001) Mitochondrial electron transport is a key determinant of life span in *Caenorhabditis elegans*. *Dev. Cell.* **1**, 663–644.
- Guarente L, Picard F (2005) Calorie restriction – the SIR2 connection. *Cell* **120**, 473–482.
- Halaschek-Wiener J, Khattri JS, McKay S, Pouzyrev A, Stott JM, Yang GS, Holt RA, Jones SJ, Marra MA, Brooks-Wilson AR, Riddle DL (2005) Analysis of long-lived *C. elegans* *daf-2* mutants using serial analysis of gene expression. *Genome Res.* **15**, 603–615.
- Hamilton B, Dong Y, Shindo M, Liu W, Odell I, Ruvkun G, Lee SS (2005) A systematic RNAi screen for longevity genes in *C. elegans*. *Genes Dev.* **19**, 1544–1555.
- Hansen M, Hsu AL, Dillin A, Kenyon C (2005) New genes tied to endocrine, metabolic, and dietary regulation of lifespan from a *Caenorhabditis elegans* genomic RNAi screen. *PLoS Genet.* **1**, 119–128.
- Hay N, Sonenberg N (2004) Upstream and downstream of mTOR. *Genes Dev.* **18**, 1926–1945.
- Henderson ST, Bonafe M, Johnson TE (2006) *daf-16* protects the nematode *Caenorhabditis elegans* during food deprivation. *J. Gerontol. A Biol. Sci. Med. Sci.* **61**, 444–460.
- Henderson ST, Johnson TE (2001) *daf-16* integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*. *Curr. Biol.* **11**, 1975–1980.
- Honda Y, Honda S (1999) The *daf-2* gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*. *FASEB J.* **13**, 1385–1393.
- Houthoofd K, Braeckman BP, Johnson TE, Vanfleteren JR (2003) Life extension via dietary restriction is independent of the Ins/IGF-1 signalling pathway in *Caenorhabditis elegans*. *Exp. Gerontol.* **38**, 947–954.
- Hsin H, Kenyon C (1999) Signals from the reproductive system regulate the lifespan of *C. elegans* [see comments]. *Nature* **399**, 362–366.
- Kaeberlein M, Powers RW 3rd, Steffen KK, Westman EA, Hu D, Dang N, Kerr EO, Kirkland KT, Fields S, Kennedy BK (2005) Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. *Science* **310**, 1193–1196.
- Kaeberlein TL, Smith ED, Tsuchiya M, Welton KL, Thomas JH, Fields S, Kennedy B, Kaeberlein M (2006) Lifespan extension in *Caenorhabditis elegans* by complete removal of food. *Aging Cell* **5**, 487–494.
- Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, Le Bot N, Moreno S, Sohrmann M, Welchman DP, Zipperlen P, Ahringer J (2003) Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* **421**, 231–237.
- Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S (2004) Regulation of lifespan in *Drosophila* by modulation of genes in the TOR signaling pathway. *Curr. Biol.* **14**, 885–890.
- Kenyon C (2005) The plasticity of aging: insights from long-lived mutants. *Cell* **120**, 449–460.
- Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R (1993) A *C. elegans* mutant that lives twice as long as wild type [see comments]. *Nature* **366**, 461–464.
- Klass MR (1977) Aging in the nematode *Caenorhabditis elegans*: major biological and environmental factors influencing life span. *Mech. Ageing Dev.* **6**, 413–429.
- Lakowski B, Hekimi S (1998) The genetics of caloric restriction in *Caenorhabditis elegans*. *Proc. Natl Acad. Sci. USA* **95**, 13091–13096.
- Lamming DW, Latorre-Esteves M, Medvedik O, Wong SN, Tsang FA, Wang C, Lin SJ, Sinclair DA (2005) HST2 mediates SIR2-independent life-span extension by calorie restriction. *Science* **309**, 1861–1864.
- Lee SS, Lee RY, Fraser AG, Kamath RS, Ahringer J, Ruvkun G (2003) A systematic RNAi screen identifies a critical role for mitochondria in *C. elegans* longevity. *Nat. Genet.* **33**, 40–48.
- Lee GD, Wilson MA, Zhu M, Wolkow CA, de Cabo R, Ingram D, Zou S (2006) Dietary deprivation extends lifespan in *Caenorhabditis elegans*. *Aging Cell* **5**, 515–524.
- Lin K, Hsin H, Libina N, Kenyon C (2001) Regulation of the *Caenorhabditis elegans* longevity protein DAF-16 by insulin/IGF-1 and germline signaling. *Nat. Genet.* **28**, 139–145.
- Long X, Spycher C, Han ZS, Rose AM, Muller F, Avruch J (2002) TOR deficiency in *C. elegans* causes developmental arrest and intestinal atrophy by inhibition of mRNA translation. *Curr. Biol.* **12**, 1448–1461.
- Manning BD (2004) Balancing Akt with S6K: implications for both metabolic diseases and tumorigenesis. *J. Cell. Biol.* **167**, 399–403.

- McElwee J, Bubbs K, Thomas JH (2003) Transcriptional outputs of the *Caenorhabditis elegans* forkhead protein DAF-16. *Aging Cell* **2**, 111–121.
- Munoz MJ (2003) Longevity and heat stress regulation in *Caenorhabditis elegans*. *Mech. Ageing Dev.* **124**, 43–48.
- Murphy CT, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, Ahringer J, Li H, Kenyon C (2003) Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. *Nature* **424**, 277–283.
- Ni JQ, Liu LP, Hess D, Rietdorf J, Sun FL (2006) *Drosophila* ribosomal proteins are associated with linker histone H1 and suppress gene transcription. *Genes Dev.* **20**, 1959–1973.
- Oh SW, Mukhopadhyay A, Srivizkapa N, Jiang F, Davis RJ, Tissenbaum HA (2005) JNK regulates lifespan in *Caenorhabditis elegans* by modulating nuclear translocation of forkhead transcription factor/DAF-16. *Proc. Natl Acad. Sci. USA* **102**, 4494–4499.
- Proud CG (2006) Regulation of protein synthesis by insulin. *Biochem. Soc. Trans.* **34**, 213–216.
- Ptushkina M, Malys N, McCarthy JE (2004) eIF4E isoform 2 in *Schizosaccharomyces pombe* is a novel stress-response factor. *EMBO Rep.* **5**, 311–316.
- Raizen DM, Lee RY, Avery L (1995) Interacting genes required for pharyngeal excitation by motor neuron MC in *Caenorhabditis elegans*. *Genetics* **141**, 1365–1382.
- Rual JF, Ceron J, Koreth J, Hao T, Nicot AS, Hirozane-Kishikawa T, Vandenhaute J, Orkin SH, Hill DE, van den Heuvel S, Vidal M (2004) Toward improving *Caenorhabditis elegans* phenome mapping with an ORFeome-based RNAi library. *Genome Res.* **14**, 2162–2168.
- Sharp ZD, Bartke A (2005) Evidence for down-regulation of phosphoinositide 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR)-dependent translation regulatory signaling pathways in Ames dwarf mice. *J. Gerontol. A Biol. Sci. Med. Sci.* **60**, 293–300.
- Sonenberg N, Gingras AC (1998) The mRNA 5' cap-binding protein eIF4E and control of cell growth. *Curr. Opin. Cell Biol.* **10**, 268–275.
- Syntichaki P, Tavernarakis N (2006) Signaling pathways regulating protein synthesis during ageing. *Exp. Gerontol.* **41**, 1020–1025.
- Takagi M, Absalon MJ, McLure KG, Kastan MB (2005) Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell* **123**, 49–63.
- Tatar M, Bartke A, Antebi A (2003) The endocrine regulation of aging by insulin-like signals. *Science* **299**, 1346–1351.
- Taubert S, Van Gilst MR, Hansen M, Yamamoto KR (2006) A Mediator subunit, MDT-15, integrates regulation of fatty acid metabolism by NHR-49-dependent and -independent pathways in *C. elegans*. *Genes Dev.* **20**, 1137–1149.
- Tavernarakis N, Driscoll M (2002) Caloric restriction and lifespan: a role for protein turnover? *Mech. Ageing Dev.* **123**, 215–229.
- Tee AR, Blenis J (2005) mTOR, translational control and human disease. *Semin. Cell Dev. Biol.* **16**, 29–37.
- Tissenbaum HA, Guarente L (2001) Increased dosage of a sir-2 gene extends lifespan in *Caenorhabditis elegans*. *Nature* **410**, 227–230.
- Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L, Muller F (2003) Genetics: influence of TOR kinase on lifespan in *C. elegans*. *Nature* **426**, 620.
- Wang X, Li W, Williams M, Terada N, Alessi DR, Proud CG (2001) Regulation of elongation factor 2 kinase by p90 (RSK1) and p70, S6 kinase. *EMBO J.* **20**, 4370–4379.
- Wang Y, Tissenbaum HA (2006) Overlapping and distinct functions for a *Caenorhabditis elegans* SIR2 and DAF-16/FOXO. *Mech. Ageing Dev.* **127**, 48–56.
- Wullschlegler S, Loewith R, Hall MN (2006) TOR signaling in growth and metabolism. *Cell* **124**, 471–484.

Supplementary Material

The following supplementary material is available for this article:

Fig. S1 Relative mRNA levels of translation-associated genes. mRNA levels in animals subjected to RNAi were determined by qRT-PCR in (A) CF512 (*fer-15(b26); fem-1(hc17)*) worms grown on different RNAi bacteria, (B) CF512 and CF1844 (*fer-15(b26); daf-2(mu150); fem-1(hc17)*) worms, and (C) N2 and *eat-2(ad1116)* worms. * $P < 0.01$, Student's *t*-test. Each bar graph represents average relative mRNA level of the indicated gene (top) from three independent biological replicates; error bars represent SEM. Relative mRNA levels of each gene are indicated in arbitrary units, normalized to *act-1*. Animals in (A) and (B) were incubated at 25 °C until adulthood (to make them sterile), and then incubated for 2 days at 20 °C before harvest; animals in (C) were grown at 20 °C until harvest at the first day of adulthood.

Fig. S2 Inhibition of translation-associated proteins further reduces the low rate of accumulation of newly synthesized proteins in *eat-2* mutants. Relative levels of ^{35}S -methionine incorporation in 4-day-old *eat-2(ad1116)* animals fed either control (vector only) or RNAi bacteria from day 1 of adulthood. $P = 0.052$ for *rps-15* RNAi, $P = 0.053$ for TOR RNAi, and $P = 0.0016$ for eIF4G RNAi, all compared to control RNAi by one-sided, paired Student's *t*-test. Error bars represent SEM. *N*, number of measurements. Please note that the low rate of accumulation of newly synthesized proteins in *eat-2* mutants (~15% compared to that of wild-type) leads to a relative high level of variation.

Table S1 Average brood size of wild-type (N2) animals grown on translation-associated RNAi during adulthood.

Table S2 Lifespan analysis of *daf-16* mutants grown on translation-associated RNAi during adulthood, and of eIF4E mutants grown on *daf-16* RNAi.

Table S3 Lifespan analysis of *daf-2* mutants grown on translation-associated RNAi during adulthood.

Table S4 (A) Lifespan analysis of *sir-2.1* mutants grown on translation-associated RNAi during adulthood. (B) Lifespan analysis of *eat-2; sir-2.1* double mutants.

Table S5 Lifespan analysis of *eat-2* mutants grown on translation-associated RNAi during adulthood.

Table S6 Lifespan analysis of translation-associated mutants on specific RNAi clones.

Table S7 Lifespan analysis of *isp-1* mutants grown on translation-associated RNAi during adulthood.

Table S8 (A) Mean survival time at 35 °C of N2 and *daf-16*-mutant animals grown on translation-associated RNAi during adulthood. (B) Mean survival time of eIF4E and *eat-2* animals at 35 °C.

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