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The Target of Rapamycin (TOR) pathway antagonizes *pha-4/FoxA* to control development and aging

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SUMMARY

BACKGROUND—FoxA factors are critical regulators of embryonic development and post-embryonic life, but little is know about the upstream pathways that modulate their activity [1]. *C. elegans pha-4* encodes a FoxA transcription factor that is required to establish the foregut in embryos, and to control growth and longevity after birth [2–5]. We previously identified the AAA+ ATPase homologue *ruvb-1* as a potent suppressor of *pha-4* mutations [6].

RESULTS—Here we show that *ruvb-1* is a component of the TOR pathway in *C. elegans* (CeTOR). Both *ruvb-1* and *let-363/TOR* control nucleolar size and promote localization of box C/D snoRNPs to nucleoli, suggesting a role in rRNA maturation. Inactivation of *let-363/TOR* or *ruvb-1* suppresses the lethality associated with reduced *pha-4* activity. The CeTOR pathway controls protein homeostasis and also contributes to adult longevity [7,8]. We find that *pha-4* is required to extend adult lifespan in response to reduced CeTOR signaling. Mutations in the predicted CeTOR target *rsks-1/S6 kinase* or in *ife-2/eIF4E* also reduce protein biosynthesis and extend lifespan [9–11], but only *rsks-1* mutations require *pha-4* for adult longevity. In addition, *rsks-1*, but not *ife-2*, can suppress the larval lethality associated with *pha-4* loss-of-function mutations.

CONCLUSION—The data suggest that *pha-4* and the CeTOR pathway antagonize one another to regulate post-embryonic development and adult longevity. We suggest a model in which nutrients promote TOR and S6 kinase signaling, which represses *pha-4/FoxA*, leading to a shorter lifespan. A similar regulatory hierarchy may function in other animals to modulate metabolism, longevity or disease.

INTRODUCTION

Members of the *FoxA* family of transcription factors encode critical regulators of development, growth and metabolism. In embryos, FoxA proteins establish the digestive tract and notochord [1,12], and they contribute to brain development [13,14]. Post-embryonically, FoxA factors control metabolism, developmental progression and lifespan in response to dietary restriction induced in liquid media or by mutation (*eat-2*) [1,3,4,15]. These functions depend on the appropriate dosage of FoxA activity, and reduced FoxA is associated with developmental abnormalities and disease. For example, animals with a lower dose of *FoxA2* in mice or *pha-4* in worms often arrest at birth [5,16–18]. *FoxA2* heterozygotes lose dopaminergic neurons

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and develop symptoms that resemble Parkinson's Disease [13,14]. The dosage sensitivity of FoxA factors may reflect the contribution of DNA binding site affinity for FoxA target gene selection [5,19]. Suboptimal DNA binding sites that associate weakly with FoxA may lose occupancy when FoxA levels are reduced.

Given the involvement of FoxA proteins in metabolism and growth, it is appealing to consider that nutrient signaling pathways might regulate FoxA. Wolfrum and colleagues suggested that insulin induces FoxA2 nuclear exclusion via Akt phosphorylation [1]. However, others have found that FoxA2 associates with target genes in liver nuclei regardless of the status of insulin signaling [1]. Thus, the relationship between FoxA and the insulin pathway is unclear. A second nutrient sensing pathway is the TOR pathway, which couples growth factors and nutrients to protein homeostasis [20]. Regulation of protein synthesis depends on substrates involved in translation including the eIF4E binding protein 4E-BP and ribosomal S6 kinase (S6K) [20]. TOR also modulates ribosome biogenesis, autophagy and transcription [20]. *C. elegans* possesses homologues of TOR complex 1 (TORC1) components, including TOR kinase (*let-363* [21]), Raptor (*daf-15*; [15]) and LST8 (http://www.wormbase.org/). Both *let-363/TOR* and *daf-15/Raptor* influence larval growth, protein synthesis, adult aging and autophagy [8,9,11,15,22]. *let-363* has been implicated in dietary restriction induced by *eat-2* or *pep-2* mutations [9,23], making it an attractive candidate to function with FoxA. However, little is known about how CeTOR controls growth and aging, or its involvement, if any, with FoxA.

To identify regulators of FoxA factors, we previously undertook a genetic screen for mutations that could suppress the lethality associated with *pha-4*, which encodes the sole *C. elegans* FoxA protein [6]. This screen identified the AAA+ ATPase homologue *ruvb-1* as a potent suppressor of *pha-4* mutations [6]. Here we show that *ruvb-1* is a component of the CeTOR pathway, which established a genetic connection between *pha-4/FoxA* and CeTOR. In larvae, both *ruvb-1* and *let-363/TOR* promote Box C/D snoRNP localization to the nucleolus, which is required for robust protein synthesis. In adults, inactivation of CeTOR or *rsks-1/S6 kinase* prolongs lifespan, and this effect requires *pha-4* activity. Another regulator of protein translation, *ife-2/eIF4E*, also modulates lifespan but is *pha-4*-independent. The data suggest that CeTOR and *rsks-1* antagonize *pha-4/FoxA* to control post-embryonic development and adult longevity. Other animals may rely on an analogous regulatory relationship to control metabolism, longevity or disease states.

RESULTS

Similarity in phenotypes between *ruvb-1*, a *pha-4* suppressor, and CeTOR mutants

We initiated our study by analysis of the loss of function phenotype associated with *ruvb-1*. Heterozygous *ruvb-1/+* animals appear wildtype and suppress loss of *pha-4* function [6]. The most striking phenotype associated with *ruvb-1* homozygous mutants is an arrest during the third larval stage (L3), as deduced by body size, perturbed vulval development and blocked gonadogenesis (Figure 1,Table S1). Previous studies had found that mutations that disrupt insulin signaling (e.g. *daf-2/insR*), and the CeTOR kinase pathway (e.g. *let-363/TOR, daf- 15/raptor*), led to a Dauer or L3 arrest [21,24], suggesting *ruvb-1* might belong to one of these pathways. Like *daf-2*, *let-363* and *daf-15*, *ruvb-1* mutants had increased intestinal lipids and an abundance of granules in the epidermis (Figure 1,Table S1). All four mutants also had small nucleoli, suggesting reduced or defective ribosome biogenesis (Figure 1,Table S1). Further inspection revealed that the *ruvb-1* phenotypes were distinct from those of *daf-2*. For example, *daf-2* mutants have cuticular ridges called alae, the buccal cavity is sealed against the external environment, the intestinal lumen constricts, and the pharynx shrinks radially and ceases to pump [24]. Mutants for *ruvb-1* lacked all of these features: they did not have alae, their digestive tract remained open and their pharynx continued to pump despite the larval arrest (Figure 1,

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Table S1). Thus, *ruvb-1* larvae lacked phenotypes typically associated with *daf-2/insR* and resembled *let-363/TOR* mutants.

As a second means to probe the relationship between *ruvb-1*, insulin and CeTOR, we examined interactions with *daf-16/FoxO*. Mutations in *daf-16* are epistatic to all components of the insulin pathway, but not to those of CeTOR [8,24]. We found that reduction of *ruvb-1* by RNAi led to an L3 arrest, high fat and epidermal granules even in combination with a null allele of *daf-16* (Figure 2A). This result indicates that *ruvb-1* functions independently of *daf-16*.

Next we examined the subcellular localization of DAF-16. Normally, insulin signaling promotes cytoplasmic retention of a DAF-16::GFP reporter, whereas reduced insulin signaling leads to nuclear accumulation [25–28]. DAF-16::GFP remained cytoplasmic in *ruvb-1*(*RNAi*), *pha-4 (RNAi)* or *let-363*(*RNAi*); *daf-15*(*RNAi*) animals (Figure 2B), suggesting that *ruvb-1* functions in parallel or downstream of the insulin pathway. As a control, we observed nuclear DAF-16::GFP when animals were starved, in agreement with previous studies [25,28] (Figure 2B). In sum, the constellation of phenotypes and genetic interactions we observed for *ruvb-1* was identical to those associated with *let-363/TOR* and its interacting partner *daf-15/Raptor* [15,21,29]; no other known pathway has a matching set of attributes in *C. elegans*. We conclude that *ruvb-1* is a likely component of the CeTOR pathway.

ruvb-1 and CeTOR are critical for box C/D snoRNP localization

How might *ruvb-1* contribute to the CeTOR pathway? Biochemical studies in other organisms have identified RUVB orthologues as members of several multiprotein complexes [30–32]. To assess which of these complexes could account for the role of *ruvb-1* in the CeTOR pathway, we screened members of these complexes by RNAi to determine if any were associated with *let-363/TOR*-like phenotypes (Table S2, Figure S3). Inactivation of *nol- 5*, K07C5.4 or *fib-1* each resulted in a larval arrest with excess epidermal granules and small nucleoli (Table S2). We did not observe a high-fat phenotype for *nol-5*, K07C5.4 or *fib-1*, indicating that either a separate RUVB-1-containing complex is responsible for lipid accumulation, or that inactivation of these four genes affects a common target to different extents. The similarity of phenotypes suggested that *nol-5*, K07C5.4 and *fib-1* could account for at least part of the CeTOR phenotype of *ruvb-1*.

nol-5, K07C5.4 and *fib-1* encode proteins predicted to be members of the Box C/D snoRNP [33]. Box C/D snoRNPs function in the nucleolus and methylate pre-rRNAs during ribosome maturation [33]. In other organisms, the Box C/D snoRNP complex is assembled and stabilized in the nucleus by association with multiple proteins, including RUVB [31,34]. Once stabilized, the mature box C/D snoRNP is transported into the nucleolus where it methylates rRNAs [32]. We used an antibody to the predicted box C/D snoRNP component FIB-1 to localize box C/D snoRNPs in wild-type and mutant worms. We observed robust FIB-1 in 100% of the nucleoli of wild-type animals, and this signal was lost after *fib-1(RNAi)*, indicating the stain was specific (Figure 3). FIB-1 levels were reduced, and FIB-1 failed to localize to the nucleolus in the majority of *ruvb-1* mutant larvae (Figure 3). In affected animals, we observed a faint ring of FIB-1 at the nucleolar periphery and, to a lesser degree, within the nucleoplasm. Thus, *C. elegans ruvb-1* is required for snoRNP localization and accumulation, similar to its human and yeast counterparts. Inactivation of *let-363/TOR* produced similar defects, with low levels of FIB-1, which were localized to the nucleoplasm and nucleolar periphery (Figure 3). This result is consistent with *ruvb-1* and CeTOR functioning in the same pathway. We also tested if snoRNP localization required *pha-4* activity. FIB-1 localization was unperturbed by *pha-4 (RNAi)* (Figure 3). Moreover, *pha- 4(RNAi)* did not restore FIB-1 localization to *ruvb-1* or *let-363* mutants (Figure S1). The data indicate that PHA-4 is not involved in Box C/D snoRNP localization.

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TOR is responsive to nutrient status [20], and CeTOR may be part of the dietary restriction (DR) pathway for lifespan extension [9,23], suggesting that FIB-1 localization might be regulated by nutritional status. We analyzed worms that had undergone eight hours starvation and observed that FIB-1 levels were reduced and restricted to the nucleoplasm (Figure 3). Regulation of FIB-1 was specific, since nucleolar FIB-1 was observed in *daf-2/insR, rsks-1/S6 kinase* or *ife-2/eIF4E* mutants (Figure 3). These data reveal that food and CeTOR signaling promote accumulation of FIB-1 in the nucleolus, and by extension rRNA maturation.

Given the effect of *let-363/TOR* and *ruvb-1* on snoRNP localization, we predicted that protein biosynthesis should require these factors, as well as Box C/D snoRNP components. *fib-1* or TOR pathway members were inactivated in adult worms, and ^{35}S incorporation monitored over a five-hour period. Reduction of *let-363* led to a 50% decrease in ^{35}S incorporation, in agreement with previous studies (Figure 4)[9,11]. We observed a 20% decrease in ^{35}S incorporation when we inactivated *pha-4*, genes required for the Box C/D snoRNP (*ruvb-1* or *fib-1*), or *ife-2* for translation initiation (Figure 4). An empty vector controlled that produced a small, nonspecific double-stranded RNA (dsRNA), served as a negative control (Figure 4). These data suggest that one way CeTOR controls protein biosynthesis is by modulating the accumulation and localization of Box C/D snoRNPs. In addition, *pha-4* is required for protein synthesis independent of the Box C/D snoRNP complex.

let-363/TOR and *daf-15/Raptor* antagonize *pha-4*

ruvb-1 was originally discovered as a suppressor of the lethality associated with partial inactivation of *pha-4* [6], prompting us to test whether *let-363/TOR* and *daf-15/Raptor* could also suppress *pha-4*. We examined suppression in two ways. First, we used RNAi to inactivate *let-363/TOR* and *daf-15/Raptor*, and we examined the effect of reduced CeTOR signaling on *pha-4* mutants. We engineered *pha-4* to be cold sensitive (*pha-4(ts)*;Experimental Procedures) and chose an intermediate temperature when worms die due to intermediate levels of PHA-4 protein (20°; [16]). The intermediate temperature provided a sensitive means to uncover genetic interactions between CeTOR pathway components and *pha-4*. We inactivated *let-363* and *daf-15* together, to ensure the strongest possible reduction in CeTOR signaling, and scored the proportion of *pha-4(ts)* progeny that progressed past the first larval stage (L1). We found that the fraction of survivors for *pha-4(ts)*; *let-363(RNAi); daf-15(RNAi)* was similar to that observed for *pha-4(ts); ruvb-1(RNAi)* and about two-fold higher than *pha-4(ts)* alone (Figure S2). This result suggests that inactivation of canonical CeTOR pathway components suppresses *pha-4* mutations.

Second, we examined survival of *let-363/+* or *daf-15/+* heterozygotes treated with *pha-4* dsRNA. To sensitize our ability to detect genetic interactions, *pha-4* dsRNA was diluted with GFP dsRNA to generate a partial inactivation of *pha-4* (Experimental Procedures). Alone, *let-363/+* and *daf-15/+* animals appeared superficially wild-type, which allowed us to score the number of animals that lived when subjected to *pha-4(RNAi)* [6](data not shown). This experiment revealed that approximately twice as many *let-363/+; pha- 4(RNAi)* larvae lived compared to *pha-4(RNAi)* alone (Figure S2). *daf-15/+* heterozygotes failed to rescue *pha-4 (RNAi)* to a significant extent, which may reflect distinct roles for *ruvb- 1, let-363* and *daf-15*, or dissimilar genetics such as maternal effects or genetic dominance [6]. Together, the data show that reduced CeTOR activity (i.e. *let-363/TOR* or *ruvb-1*) can suppress the lethality associated with reduced *pha-4*, and that therefore the CeTOR pathway antagonizes *pha-4* during development.

pha-4 is required for lifespan extension due to decreased CeTOR

Reduced CeTOR signaling leads to prolonged lifespan whereas reduced *pha-4* shortens life, and both genes are implicated in dietary restriction [3,8,9,11,15]. To explore the relationship

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between these genes, we inactivated both *pha-4* and CeTOR components conditionally, beginning at the fourth larval stage and continuing through adulthood. Alone, *pha-4(ts)* animals had a slightly shortened lifespan, as had been observed previously [3]. Reduction of *let-363* and *daf-15* together led to a statistically-significant increase in lifespan in 3/4 experiments ($p<0.05$; Figure 5A,Table S4). To examine the effect of *pha-4*, we analyzed our datasets by multivariate Cox regression modelling, which allowed four experimental conditions to be compared simultaneously [35]. This analysis revealed that *pha-4* was required for CeTOR-induced lifespan extension since we observed a statistically significant decrease in longevity for *pha-4* with reduced CeTOR compared to *pha-4* alone ($p<0.0001$; Figure 5A,Table S4, Figure S5). Strikingly, *pha-4(ts)*; *let-363(RNAi)*; *daf-15(RNAi)* animals had shorter life spans than *pha-4(ts)* alone. This result suggests that *pha-4* is crucial for survival when the TOR pathway is inactivated. The effect of *pha-4* on longevity was specific since *pha-4* was not required for the pronounced lifespan extension induced by *daf-2/insR* mutations (Figure 5B,Table S4), in agreement with previous studies [3]. Moreover, inactivation of *pha-4* had only minor effects on the number of eggs laid, indicating that these worms were generally healthy and fecund (115 ± 37 eggs for *pha-4(RNAi)* mothers vs. 127 ± 33 for wild-type (n=18)). We conclude that *pha-4/FoxA* is selectively required for lifespan extension by reduced CeTOR.

To investigate how CeTOR signaling negatively regulates *pha-4*, we examined whether PHA-4 levels or localization changed in response to CeTOR inactivation, starvation or aging. By monitoring a translational PHA-4::mCherry reporter in adults, we observed strong expression in the pharynx and lower levels in the intestine under all experimental conditions (Figure S4). We also observed constitutively nuclear expression in agreement with Panowski et al. and Zhang et al., but distinct from Wolfrum et al. [1, 3]. Our data suggest that the levels of nuclear PHA-4 protein do not change in response to aging or starvation. Instead, the transcriptional activity may be altered by CeTOR signaling.

rsks-1 antagonizes *pha-4*

The TOR pathway controls protein homeostasis at many levels, including ribosome biogenesis, translation and autophagy [20]. We wondered which of these downstream pathways relied on *pha-4* for lifespan extension. One appealing candidate was *rsks-1*, which is homologous to the TOR target S6 kinase [20] and which leads to lifespan extension when inactivated in *C. elegans* [9–11]. We observed a ~10% extension in lifespan for *rsks-1(ok1255)* in 4/4 experiments, similar to previous studies (Figure 5C,Table S5)[10]. This effect was dependent on *pha-4* since *pha-4(RNAi)* caused worse survival in *rsks-1* compared to wild-type (p=0.007 by multivariate Cox modeling [35]; Figure 5C, Table S5, Figure S5).

Next we tested *ife-2*, which encodes one of five eIF4E isoforms (WS180 www.wormbase.org). In other animals, TOR promotes eIF4E activity by inactivating the eIF4E repressor 4E-BP [20]. In 3/3 experiments, we observed extended longevity for *ife-2* mutants (Figure 5D,Table S5), similar to previous work [9,11]. Lifespan decreased when *pha-4* was inactivated by RNAi (4/5 experiments, Figure 5D,Table S5). However, inactivation of *pha-4* reduced longevity to a similar extent as inactivation of *pha-4* in wild-type animals, and never returned *ife-2* lifespan to baseline (p=0.158 by multivariate Cox modeling [35], Table S5, Figure S5). These data implicate alternative processes for lifespan extension by *ife-2*. We suggest that *pha-4/FoxA* plays a critical role for lifespan extension due to decreased *let-363* and *rsks-1*, but not *ife-2*.

We were surprised that *rsks-1* and *ife-2* had different genetic interactions with *pha-4* for adult aging. To extend this finding, we tested whether *rsks-1* or *ife-2* mutations could suppress the larval lethality associated with loss of *pha-4* function. *pha-4* was partially inactivated by RNAi in wild-type, *rsks-1* or *ife-2* mutants. We observed a ≥2x suppression of *pha-4* by *rsks-1* but

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no suppression by *ife-2* (Figure 5E, Table S6). These results bolster the conclusion that *rsks-1* is a negative regulator of *pha-4*, whereas *ife-2* is not.

DISCUSSION

We have identified the *pha-4* suppressor *ruvb-1* as a new component of the *C. elegans* TOR pathway, which lead us to probe the genetic interactions between CeTOR and *pha-4*. Our findings reveal that CeTOR antagonizes *pha-4* during larval development and adult aging. *let-363/TOR* and *ruvb-1* are both needed to accumulate Box C/D snoRNPs in the nucleolus, and loss of these proteins leads to decreased protein synthesis. Downstream of CeTOR, reduced *rsks-1/S6* kinase, but not *ife-2/eIF4E*, relies on *pha-4* activity to prolong life. Moreover, *rsks-1/S6* kinase mutations, but not *ife-2/eIF4E*, can suppress *pha-4*-associated larval lethality. These data suggest that nutrients activate the CeTOR pathway and *rsks-1/S6* kinase to repress *pha-4* during larval development and adult aging (Figure 6). Reduced nutrients, for example during dietary restriction, leads to enhanced *pha-4/FoxA* activity and prolonged lifespan, via reduced TOR signaling.

CeTOR and *ruvb-1* share common phenotypes

We have shown that *ruvb-1* and *let-363/TOR* control the accumulation and localization of Box C/D snoRNPs within nucleoli, providing an explanation for *ruvb-1* function in the CeTOR pathway. Box C/D snoRNPs methylate rRNAs during maturation, and loss of Box C/D snoRNPs is predicted to reduce ribosome biogenesis. This function likely explains, at least in part, why inactivation of CeTOR [9,15,21] or *ruvb-1* (this study) leads to decreased protein biosynthesis and arrested larval development. In our hands, CeTOR had the most pronounced effect on protein synthesis rates compared to other genes, which may reflect multiple levels of regulation of protein homeostasis by CeTOR, similar to other organisms [20]. By contrast, *rsks-1/S6 kinase* and *ife-2/eIF4E* each promoted protein biosynthesis, but neither was necessary to complete larval development or to localize FIB-1 ([9–11], this study). *C. elegans* possesses five predicted eIF4E factors [36], which may explain why *ife-2/eIF4E* was associated with weaker phenotypes than *ruvb-1* or CeTOR.

CeTOR and one of the core components of the Box C/D snoRNP complex, W01B11.3/Nop58, have been implicated in lifespan extension [8,37], raising the question of whether *ruvb-1* or other snoRNP components contribute to longevity as well. Inactivation of *ruvb-1* beginning at the L4 stage did not cause a reproducible extension of lifespan (Table S3). One possibility is that *ruvb-1* and the Box C/D snoRNP may be important for lifespan extension, but pleiotropic phenotypes associated with other RUVB-1-containing complexes may mask a role in aging. Different experimental conditions may reveal an aging role.

Models for lifespan extension by reduced protein biosynthesis

Large-scale screens in *C. elegans* have identified a multitude of genes that can modulate adult lifespan (reviewed in [38]). An ongoing challenge is to organize these genes into defined pathways. Analysis has been complicated by the large number of genes that can impinge on aging, the complex genetic interactions between these genes, the different approaches to induce lifespan extension by DR and the need for conditional or partial inactivation of essential genes [3,39–44]. Previous studies have shown that reduction of CeTOR or the translation machinery can extend lifespan [8–11,45,46]. Given that CeTOR controls protein homeostasis and responds to nutrients [9,20,23], one might predict that inhibition of translation would extend longevity by the same pathway as reduction of CeTOR signaling. However, genetic studies have lead to seemingly contradictory conclusions regarding the relationship between TOR and protein translation, or between these factors and DR [9–11,25,45]. *pha-4* epistasis offers another approach to group genes within common pathways. Based on our analysis with *pha-4/*

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FoxA and results from other studies, we suggest two models for coupling nutrients to protein translation and aging (Figure 6). These models both place *pha-4* downstream of S6 kinase and CeTOR, but differ with regard to positioning *ife-2* and the translation initiation factors within an aging network. We recognize that the two models are not mutually exclusive, and the translation initiation factors may function in multiple contexts.

For both models, we place CeTOR and *rsks-1/S6 kinase* downstream of nutrients, based on genetic epistasis experiments in *C. elegans* [9,10,23], common effects on snoRNP localization and protein biosynthesis (this study, [9,10]), and studies with other species [20]. Inactivation of *rsks-1* and CeTOR together resembles inactivation of CeTOR alone for lifespan extension [9], consistent with these genes functioning in a common pathway. Moreover, neither gene is dependent on *daf-16/FoxO* [9,10], suggesting this pathway is parallel or downstream of insulin signaling. The genetic interactions between *pha-4/FoxA* and either CeTOR or *rsks-1*, tentatively place *pha-4* downstream of these kinases. We position *ruvb-1* and snoRNPs downstream of CeTOR but separate from *rsks-1* or *ife-2*, based on a lack of aging phenotypes for *ruvb-1* and common snoRNP phenotypes for *ruvb-1* and *let-363*, but not *ife-2* or *rsks-1*. Finally, we draw a dotted arrow between food and the insulin pathway, to reflect examples of *daf-16* regulation or function during starvation or dietary restriction induced by some means but not others [25,28,40].

In the first model, we position *ife-2/eIF4E* and presumably additional translation initiation factors downstream of CeTOR (Figure 6A). In other organisms, 4E-BP is a negative regulator of translation initiation that binds and sequesters eIF4E [20]. TOR phosphorylates 4E-BP, leading to eIF4E release. If a similar regulatory hierarchy exists in worms, CeTOR may activate translation initiation machinery. An advantage of this model is that all genes that affect protein biosynthesis (CeTOR, *rsks-1*, translation initiation factors, *pha-4* as well as food deprivation; this study, [9–11]) are consigned to one branch of the aging network.

A critical feature of the first model is a pair of feedback loops. In the first loop, *pha-4* promotes food uptake (Figure 6A, blue; [2]), which may explain why inactivation of *pha-4* leads to reduced protein synthesis. In the second feedback loop, *daf-16* negatively regulates *daf-15/Raptor* [15], to modify CeTOR activity (Figure 6A, blue). This feedback loop may explain two perplexing genetic interactions. First, there have been differing claims regarding the dependence on *daf-16/FoxO* for lifespan extension after inactivation of translation initiation factors [9–11, 37, 45]. We suggest that inactivation of *daf-16* may increase CeTOR activity, which is predicted to boost translation and thereby suppress *ife-2/eIF4E* mutations. This scenario can explain genetic interactions between *daf-16* and *ife-2* (or other translation factors) [9, 11], despite the absence of nuclear-enriched DAF-16 (Figure 2). Second, although DR and CeTOR appear largely independent of insulin signaling for aging [8, 41, 43], *let-363/TOR* and *daf-2/InsR* fail to synergize for lifespan extension when they are inactivated together [8, 9]. One possibility is that the negative feedback loop decreases CeTOR activity in *daf-2* mutants, such that *daf-2* single mutants resemble *daf-2; let-363* double mutants for aging (Figure 6A, blue). We note, however, that *daf-2* mutants do not alter protein synthesis rates [9] or suppress *pha-4* mutations (data not shown), suggesting that *daf-16* does not repress TOR completely or in all cells.

A second model separates *ife-2* and the translation initiation factors from CeTOR, where they impinge on *daf-16* more directly (Figure 6B). For example, the absence of food or inactivation of translation factors may induce a stress response that activates DAF-16. In yeast, one of the two isoforms of eIF4E is up-regulated by stress and required for the stress response [47]. *C. elegans* also possesses multiple isoforms of eIF4E [36], and future studies will determine if any of these isoforms are involved in stress. A link to stress may explain why inactivation of some translation factors impacts the nuclear localization of DAF-16 to some extent [45]. A

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stress response may also explain why food deprivation induces nuclear localization of DAF-16 [25, 28], even though dietary restriction does not [40].

Both DR and TOR are important regulators of autophagy, which cells use to survive periods of starvation by recycling macromolecules and nutrient transporters [48]. Autophagy is necessary for lifespan extension due to mutations that inactivate feeding (*eat-2*), CeTOR or insulin signaling throughout the life of the animal [22,49–52]. However, inactivation of *let-363/TOR* in adults, rather than throughout life, can prolong lifespan in the absence of an obvious autophagic response [22]. Neither *ife-2/eIF4E* nor *rsks-1/S6K* mutants have increased autophagy, yet they extend longevity [22]. Induction of autophagy in *daf-2/InsR*; *daf-16/FoxO* mutants is not sufficient for increased lifespan [22]. These three observations suggest that there must be additional processes beyond autophagy involved in lifespan extension, and that these processes depend on *pha-4/FoxA*. *pha-4* can also impinge on autophagy. Long-term reduction (>1 generation) of *pha-4* blocks the induction of autophagy in response to long-term reduction in feeding (*eat-2*) or *daf-15/Raptor* heterozygotes [22]. These effects could reflect a developmental role for these proteins, particularly *pha-4*, which is required for embryonic and larval development [4,5]. Alternatively, *pha-4* may be an acute regulator of the autophagic response. Comparison of long-term vs. short-term inactivation of these proteins will clarify their roles.

In summary, our analysis of *ruvb-1* has revealed that the activity of *pha-4* is modulated by the CeTOR pathway. This interaction could be relatively direct, for example, by PHA-4 modification. Alternatively, it could be indirect, if both *pha-4* and CeTOR impinge on common processes. An intriguing avenue for future studies will be to determine if the developmental or metabolic roles of FoxA proteins in other animals are modified by TOR signaling.

EXPERIMENTAL PROCEDURES

See Supplemental Data for additional experimental procedures.

RNA Interference

RNAi by bacterial feeding was performed essentially as described in [6]. HT115 bacteria [53] expressing dsRNA for *GFP*, *ruvb-1*, *pha-4, let-363, daf-15, dpy-1, nhr-23, fib-1, rsks-1* or *ife-2* were grown in overnight cultures and seeded onto plates containing 5mM IPTG (Sigma) and 60ug/ml Carbenicillin (Sigma) or 1mM IPTG only for lifespan analysis. All RNAi clones were derived from the Ahringer library [54] except for *pha-4* (bSEM 865) [55], *GFP* [53], *let-363* (bSEM 911), *daf-15* (bSEM 912) and *rsks-1* obtained from the *C. elegans* ORF-RNAi library v1.1 (Geneservice Ltd.). Clones were confirmed by restriction enzyme digest.

daf-16 epistasis

Wild-type or *daf-16(mu86)* [56] hermaphrodites were subjected to *ruvb-1(RNAi)* beginning at the L4 stage. Progeny were scored for phenotypes after 3–4 days incubation at 20°C. L3 larval arrest was determined by body size, gonad extension and presence/absence of a mature vulva with either wild-type or protruding morphology. Fat was detected by Nile Red staining [57] and scored if increased from average wild-type staining by visual inspection. Epidermal granules are refractile storage vesicles in the epidermis and were scored for increased abundance compared to the wild type.

Immunostaining

Immunostaining was performed as described previously [58] with the following changes. Microscope slides were treated with a 0.1% poly-L-lysine solution overnight (Sigma-Aldrich Product #P8920). In situ antibody staining for FIB-1 was performed using a 1:200 dilution of

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α-FIB-1 mouse monoclonal antibody (EnCor BioTechnology®, Catalog #MCA-38F3) and detected using a 1:200 Cy3 conjugated α-IgG secondary antibody (Jackson ImmunoResearch Inc). Mounting medium consisted of 50% glycerol in PBS with DAPI and p-phenylenediamine.

Worms were subjected to *pha-4(RNAi), Empty Vector(RNAi)* or OP50 bacteria at the L4 or L1 stage. L1 progeny or L3 stage worms were picked off of plates, washed with water and placed in 2% paraformaldehyde and only L3 worms were cut to release gonad and intestine. Worms were fixed in 2% paraformaldehyde and permeabilized by freeze-crack method for 30 min, then submersed in ice-cold methanol for 3 min. Following methanol treatment slides were rinsed twice, 5 min each, in 2X TBST (Tris-Buffered Saline Tween). Slides were later blocked for 30 min in TNB (0.1 M Tris–HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.5 containing blocking reagent (NEN)) and 10% NGS (Normal Goat Serum) followed by overnight incubation with the primary antibody at 15°C. Following overnight incubation slides were washed 3 times in 2X TBST and secondary antibodies were added. Slides incubated with the secondary antibody at room temperature for 2 hrs. Images were captured using DeltaVision RT Deconvolution system and SoftWoRx software (Applied Precision).

Lifespan Analysis

Lifespan analysis was performed as described previously [9] with the following changes. Worms were grown for at least 2 generations at 25°C or 20°C, as indicated, before the experiment was initiated. Hermaphrodites were allowed to lay eggs for 4–8 hours on OP50, and progeny were grown to the L4 stage. In all experiments, L4 larvae were transferred to new plates with 1mM IPTG and appropriate bacteria to initiate *pha-4(RNAi)* or *ruvb-1(RNAi)* vs. a vector control. The first day of adulthood was counted as day one of the experiment. Worms were moved daily until reproduction ceased. Worms were moved every 3–4 days for the rest of the lifespan assay. Lifespan analysis was conducted with wild-type, *daf-2(e1368)* [59], *ife-2 (ok306)* [36], and *rsks-1(ok1255)* [10].

For experiments with reduced CeTOR, *smg-1(cc546ts); pha-4(zu225)* [16] and control *smg-1 (cc546ts)* (www.addgene.org/labs/Fire/Andrew/Vec97.pdf) worms were used. Worms were grown at the permissive temperature of 24°C for at least two generations prior to beginning the experiment. Hermaphrodites were allowed to lay eggs for 4–6 hours and removed. L4 progeny were moved to the non-permissive temperature of 15°C and RNAi was initiated. We used a 1:1 mixture of bacteria for *let-363(RNAi); daf-15(RNAi)*. The first day of adulthood was counted as day one of the experiment. Worms were moved every other day until reproduction ceased. Worms were moved every 5–7 days for the rest of the lifespan assay. Censoring within each experiment included animals that ruptured, crawled off the plate or exhibited progeny hatching internally. We have reported the number of ruptured animals.

Statistical Methods for Lifespan Analysis

Log rank tests were used in pair-wise comparisons of wildtype, mutant, RNAi, and control groups. To determine if *pha-4* had an effect beyond its effect on wild-type worms, we combined data from multiple experiments using the same conditions and applied multivariate Cox proportional modelling (Cox regression) using Stata Software [35]. This statistical approach enabled us to compare four experimental conditions at once, and determine if *pha-4* had a greater effect on our experimental strain relative to the wild-type control. It also included information from the censored subjects.

Suppression of *pha-4*

pha-4(*RNAi*) suppression was performed as reported previously [6] using bacteria expressing *pha-4* and *GFP* dsRNA at a ratio of 1:4 or 1:8, to give an intermediate inactivation of *pha-4*. 5–10 L4 stage wild-type, *unc-42 ruvb-1(px34)/evl-1*, *let-363(h111)/ dpy- 5* and *daf-15(m81)/*

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unc-24 worms were picked to 2–4 RNAi plates per experiment and incubated at 25°C. P_0 were allowed to lay eggs for 1 day and then removed. Progeny were counted 2 days later for the percentage of animals older than L1 (n≥100 animals/plate). P-values were determined by t-test.

pha-4(ts) suppression was performed as reported previously [6] using bacteria expressing *ruvb-1(RNAi)*, a mix of *let-363(RNAi); daf-15(RNAi)* or bacteria containing empty vector as a control. Ten L4 *pha-4(ts)* worms were picked to 2–5 RNAi plates per experiment and incubated at 20°C. P_0 were allowed to lay eggs for 1 day and then removed. Progeny were counted 2 days later for the percentage of animals older then L1 (n≥20 animals/plate). Strength of RNAi was monitored by observation of progeny for larval arrest (*ruvb-1(RNAi)*) or slow growth and sterility (*TOR(RNAi)*). P-values were determined by t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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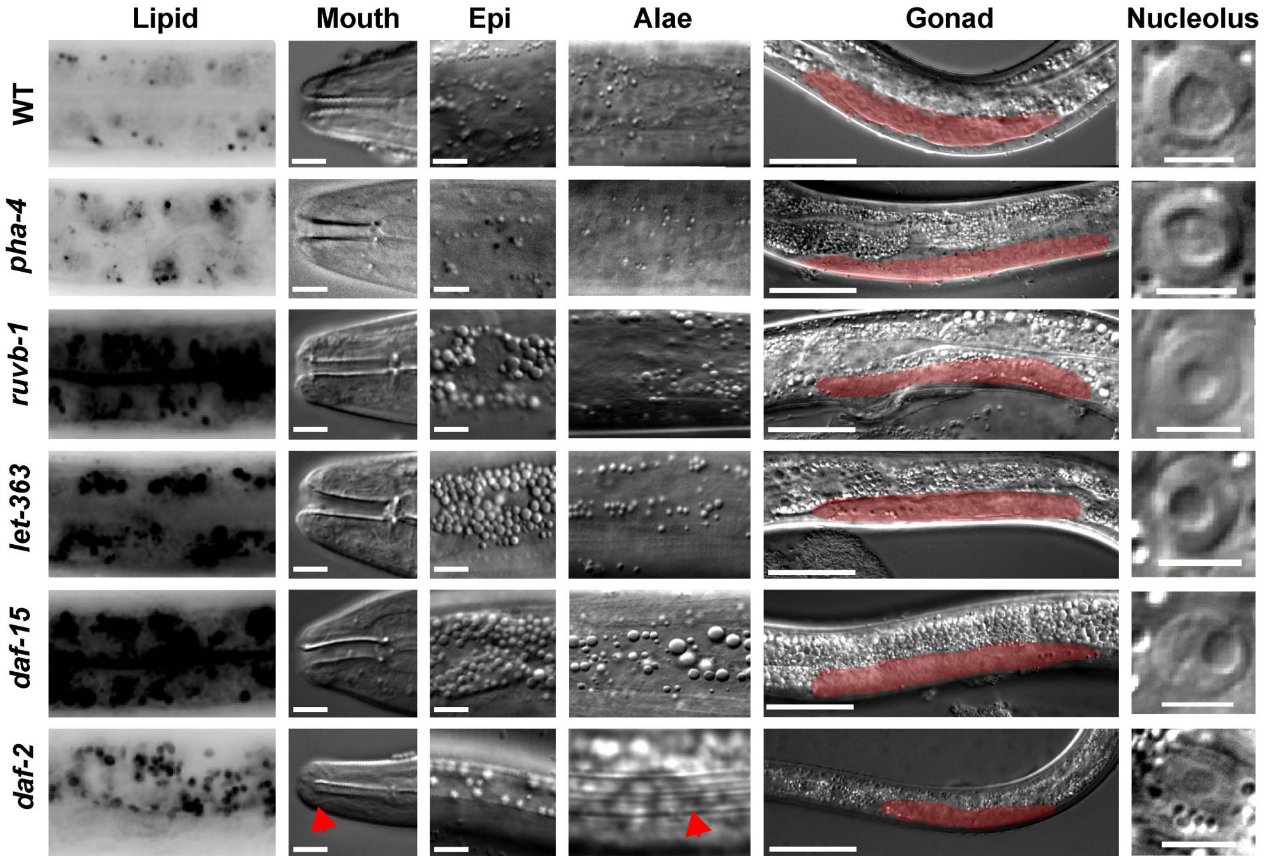

Figure 1. Similarity of phenotypes associated with *ruvb-1* and CeTOR

The phenotypes of wild-type (WT), *pha-4(RNAi), ruvb-1(px34), let-363(h111)/TOR*, *daf-15 (m81)/Raptor* and *daf-2(e1370)/insR* mutants were compared at the third larval stage (L3). *daf-2* mutants were examined as Dauer larvae except for lipid accumulation, which was examined in arrested larvae [60]. *pha-4(RNAi)* was initiated at the L1 stage and analyzed at the L3 stage. Lipid accumulation was visualized by Nile Red staining [57]. Mouth opening (arrowhead, scale bar=5µm), epidermal granules (Epi, scale bar=5µm), cuticular alae (arrowhead), arrested gonad development (red, scale bar=50µm) and epidermal nucleoli (scale bar=5.12µm) were examined by light microscopy. Larvae were monitored at 20°C except for *daf-2*, which was temperature-sensitive and therefore examined at 25°.

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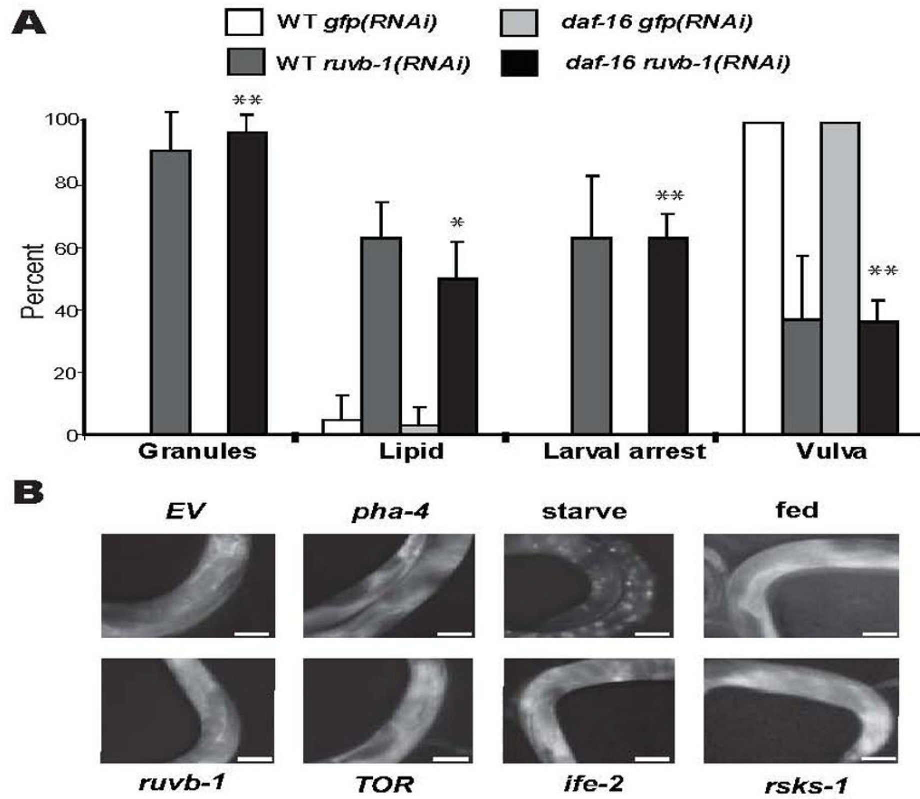

Figure 2. Genetic interactions between Fox factors and *ruvb-1* or CeTOR

A) *ruvb-1* phenotypes do not depend on *daf-16/FoxO*. RNAi against *GFP* or *ruvb-1* was induced in either wild-type (WT) or *daf-16(mu86)* hermaphrodites, and their progeny scored by light microscopy for larval arrest, epidermal granules (Granules) or a mature vulva. Lipid accumulation of L3 progeny was determined by Nile Red staining [57] (20°, 3 experiments, n≥24 animals for each condition, error bars denote standard deviation, **p<0.0002, *p=0.0038). B) DAF-16 localization. DAF-16 localization was monitored with *daf-16p::daf-16*::GFP [25]. Worms subjected to OP50 (fed), empty vector (EV), *ruvb-1(RNAi), pha- 4 (RNAi), ife-2(RNAi), rsks-1(RNAi)* or *let-363(RNAi); daf-15(RNAi) (TOR)* show cytoplasmic localization compared to worms starved for 24 hrs, which display nuclear localization (n≥9 worms for each condition, scale bar=50μm).

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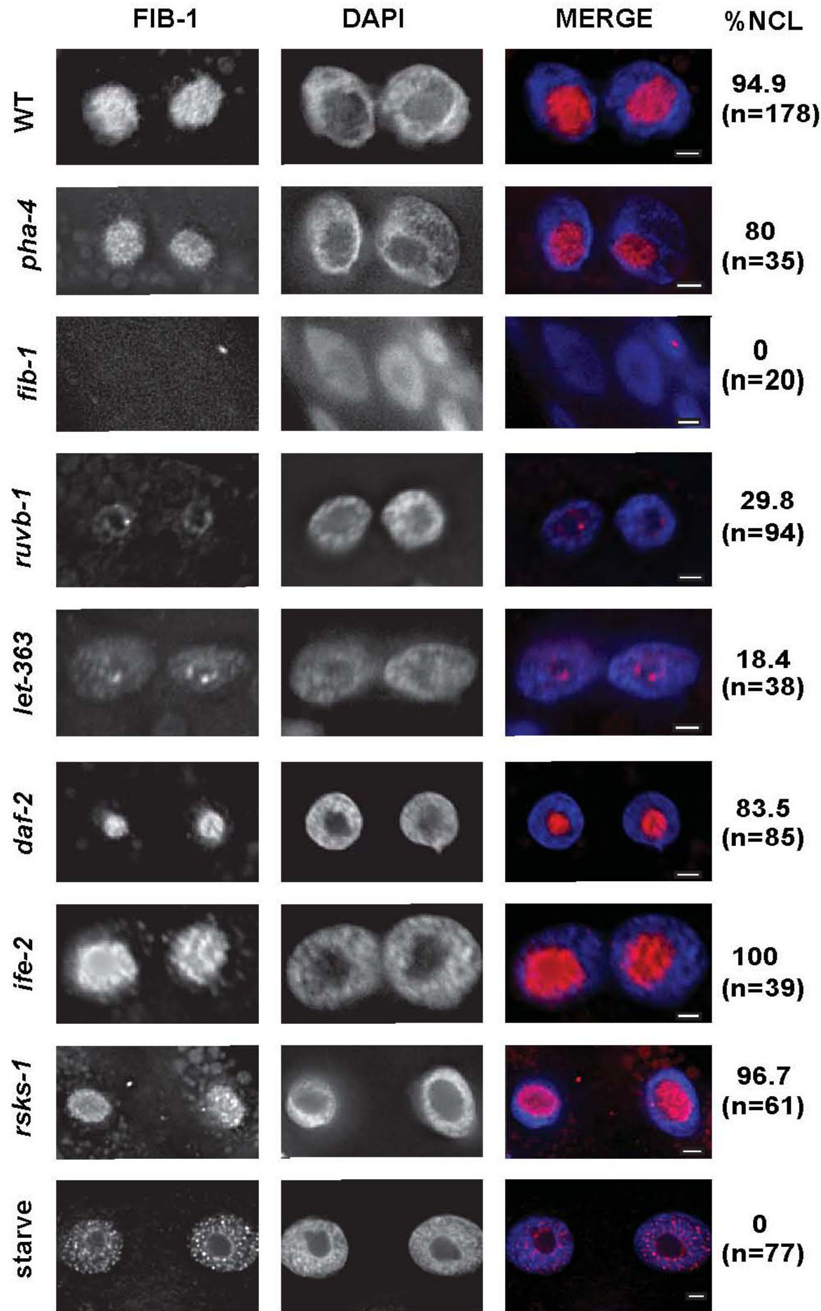

Figure 3. Localization of the Box C/D snoRNP complex requires food, *ruvb-1* and *let 363/TOR*
Fed (*WT*) or starved (8 hours, *starve*) wild-type, *pha-4(RNAi), ruvb-1(px34), let- 363(h111)/ TOR, daf-2(e1368ts)/InsR, rsks-1(ok1255)* or *ife-2(ok306)/eIF4E* larvae were stained for FIB-1 (pink) and DNA (DAPI, blue) at the L3 stage (scale bar=2 μm). *pha-4(RNAi)* was initiated at the L1 stage and analyzed at the L3 stage. *fib-1(RNAi)* was initiated at the L4 stage and L1 progeny were analyzed. Data were quantified for percent nucleolar (% NCL) and number of nuclei (n).

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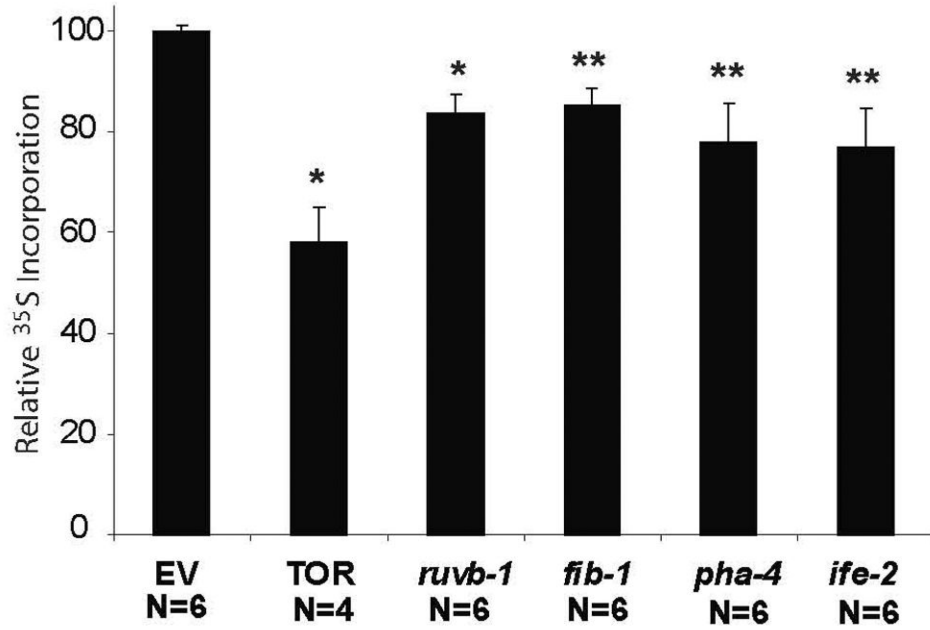

Figure 4. Inhibition of CeTOR or Box C/D snoRNPs decreases the rate of newly synthesized proteins

Relative levels of ^{35}S-methionine incorporation in 2-day-old *fog-1(q253ts)* adult animals treated with either empty vector (EV), *let-363(RNAi); daf-15(RNAi) (TOR), ruvb-1(RNAi), fib-1(RNAi), pha-4(RNAi)* or *ife-2(RNAi)*. RNAi was initiated at the L4 stage ("day 0" of adulthood) at 25°C. Bar graphs represent average ^{35}S-methionine incorporation normalized to total protein levels for different RNAi treatments compared to EV(RNAi) (**$p<0.006$, *$p<0.0002$, one-sided paired *t*-test. error bars represent SEM, N, number of measurements).

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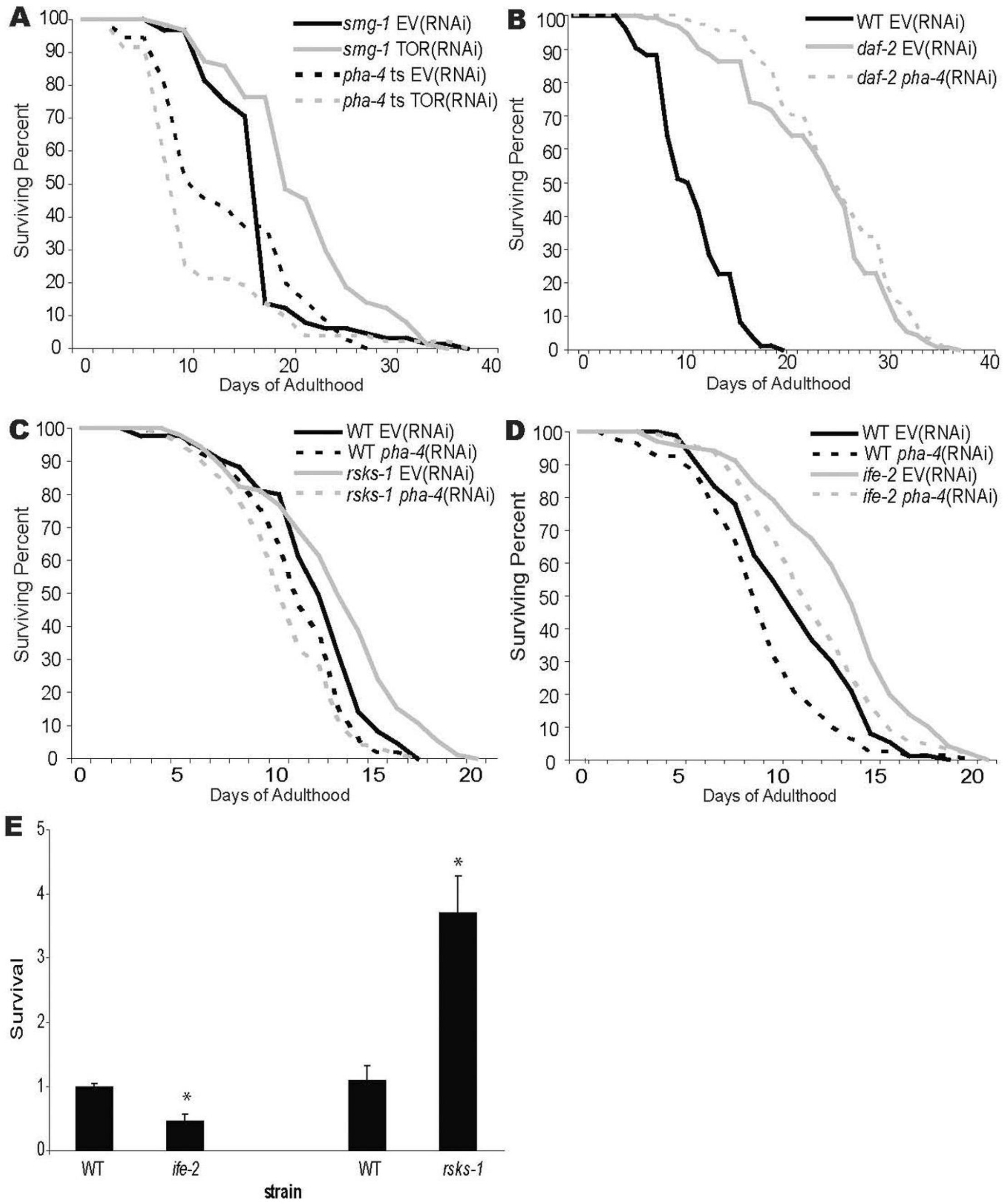

Figure 5. Longevity due to reduced CeTOR or *rsks-1* requires *pha-4*

A–D) Survival curves for A) *pha-4(zu225);smg-1(cc546ts)* (*pha-4(ts)*) or *smg-1(cc546ts)* grown at 24°C and shifted to 15°C beginning at the L4 stage. Worms were subjected to *let-363 (RNAi)*; *daf-15(RNAi) (TOR(RNAi))* to inactivate TOR signaling compared to empty vector control (EV). Mean lifespan was 18.1 days for *smg-1*; *EV(RNAi)* (control), 21.3 days for *smg-1; TOR(RNAi)* (p=0.0077 vs. control), 13.8 days for *pha-4(ts); EV(RNAi)* (p=0.0017 vs. control), and 12.2 days for *pha-4(ts); TOR RNAi* (p=<0.0001 vs. control) B) *daf-2(e1368ts)* worms were grown at 15°C and shifted to 25°C beginning at the L4 stage to inactivate insulin signaling. Worms were subjected to *pha-4*(*RNAi*) or an empty vector control (EV). Wild-type (WT) worms were grown at 25°C. Mean lifespan was 11.6 days for *WT; EV(RNAi)* (control), 23.6

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days for *daf-2; EV(RNAi)* (p<0.0001 vs. control) and 25.2 days for *daf-2; pha-4(RNAi)* (p<0.0001 vs. control). C) Wild-type (WT) and *rsks-1(ok1255)* worms were grown at 25°C. Worms were subjected to *pha-4*(*RNAi*) or an empty vector control (EV). Mean lifespan was 11.96 days for WT; *EV(RNAi)* (control), 11.1 days for WT; *pha-4(RNAi)* (p=0.0075 vs. control), 12.98 days for *rsks-1; EV(RNAi)* (p=0.0011 vs. control), 10.4 days for *rsks-1; pha-4 (RNAi)* (p=0.0001 vs. control). D) Wild-type (WT) and *ife-2(ok306)* worms were grown at 25° C. Worms were subjected to *pha-4*(*RNAi*) or an empty vector control (EV). Mean lifespan was 11.16 days for WT; EV(RNAi) (control), 9.5 days for WT; *pha-4(RNAi)* (p=0.0032 vs. control), 13.6 days for *ife-2; EV(RNAi)* (p=<0.0001 vs. control), 12.15 days for *ife-2; pha-4(RNAi)* (p=0.0664 vs. control). In all experiments, RNAi was initiated at the L4 stage [61]. E) Suppression of the lethality associated with reduced *pha-4* by *rsks-1, not ife-2*. Wildtype animals and animals mutant *for ife-2(ok306) or rsks-1(ok1255)* were subjected to weak *pha-4 (RNAi)* (Experimental Procedures). The proportion of mutant animals that survived beyond the L1 stage was counted and normalized against wildtype worms also subjected to *pha-4 (RNAi)* (25°, 2 experiments, n≥800 animals for each strain, error bars denote standard error, *p=0.001).

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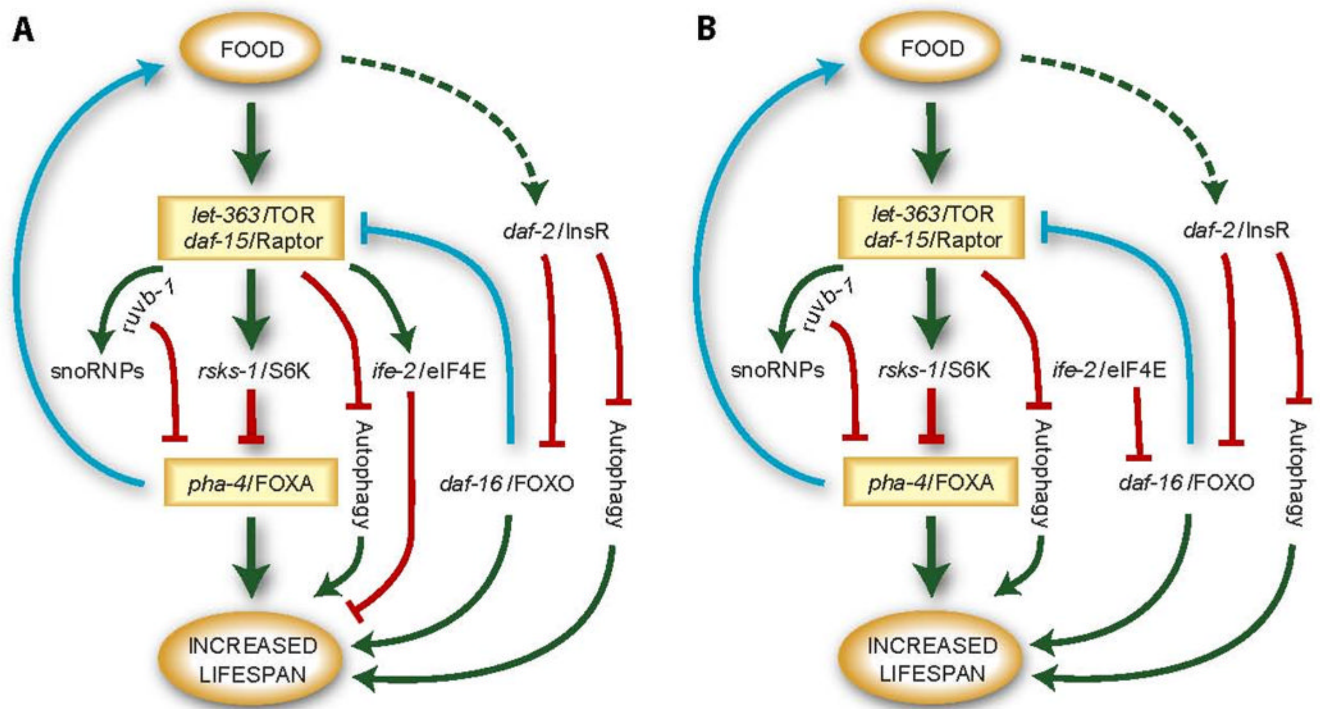

Figure 6. Two models for lifespan extension due to decreased protein synthesis
See the Discussion for an explanation of these models.