F56F11.4 Extends Healthspan by Regulation of TOR-Related Pathway in C. elegans

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Abstract. Aging has emerged as a significant issue in human society, warranting intensified research efforts. Despite this, there is a noticeable dearth of research on genetic factors that could potentially extend lifespan. The genetic role of TOR/*let-363* and its interactome was delved into in the model organism *C. elegans*. Utilizing RNA interference (RNAi), the impact of predicted interactors within the TOR pathway were explored on aging, with a particular emphasis on healthspan. This was achieved through comprehensive lifespan and pumping rate assays. Of the 20 genes examined, only the knockdown of F56F11.4 resulted in a reduction in both lifespan and early-stage pumping rate. To elucidate the interplay between F56F11.4 and TOR/*let-363*, double knockdown and RT-qPCR assays were employed to reveal their intricate regulatory roles in the aging process. Additionally, the conserved nature of F56F11.4 and *let-363* across five well-studied organisms (*C. elegans, H. sapiens, M. musculus, R. norvegicus,* and *D. melanogaster*) was identified using bioinformatics analyses. The discovery of the influence of F56F11.4 on healthspan through the modulation of TOR/*let-363* signaling suggests a promising target for the development of novel therapeutic strategies aimed at enhancing healthy aging.

Keywords: F56F11.4, aging, TOR pathway, *C. elegans*.

1. Introduction

The global aged population (65 years old or older) reached 9% in 2020, and this number is expected to increase two-fold in 2050.[1] As life expectancy continues to rise and as aged population continues to enlarge, more people become suffering from age-related diseases while becoming older.[2] Compared to considering the lifespan, people should focus more on healthspan, the time period they live in good health. However, only few studies revealed a corner of the healthspan. To balance between healthy body function and the delay of aging process, it is crucial to discover aging and its related mechanisms, and to develop treatments to extend healthspan.

Aging and lifespan are highly regulated by genetic factors in humans. Large-scale genome-wide association studies (GWAS) have recently identified multiple loci that influence key human aging pathways, and found that there is an association between parental and offspring lifespan.[3] Many genetic diseases that severely affect lifespan, such as progeria, are also hereditary.[3] In the model organism for studying aging, *Caenorhabditis elegans*, several genes are found to be correlated with nematode lifespan. For example, a mutation on *daf-2* or *age-1* gene can dramatically extend lifespan in *C. elegans*.[4] Another disease that highly relates to aging is cancer, which shares similar signaling and regulating pathways to aging. Some cancer-related genes are also lifespan-extending genes, such as the

TOR (Target Of Rapamycin) pathway.[5] Firstly discovered in yeast, this highly conserved pathway regulates multiple eukaryotic functions, including aging and cancer.[5] Genetic inhibition of the TORC1 pathway has been shown to extend lifespan and inhibit cancer in *C. elegans*.[5] However, its function on healthspan still remains mostly unknown.

In this study, *C. elegans* was used as the model organism. *C. elegans* has been widely employed as an animal model in aging research due to various advantages, foremost being the ease of synchronization on life stage and short life cycle. In addition, *C. elegans* is the first multicellular eukaryotic organism to have its complete genome being sequenced, making it a powerful model organism in genetic researches.[6] Many lifespan-regulating genes found in *C. elegans* are evolutionarily conserved. For example, *let-363* in nematode is the ortholog of human gene mTor.[7] Multiple pathways that regulate aging were also experimentally testified in *C. elegans*, including the TOR signaling pathway, which regulates nematode lifespan by inhibiting autophagy and by promoting the accumulation of oxidative stress.[4,6,8,9,10] The proteins in TOR pathway are also highly evolutionarily conserved.[11]

In this study, RNA interference assay was performed to identify the effect of TOR complex-related genes on lifespan. The online STRING database was used to identify the predicted interactomes of TOR pathway, including *let-363* (ortholog of human gene mTor[7]) interactome genes (F56F11.4, *ppfr-4*, *epg-9*, *aakg-1*, *lmp-1*, *rho-1*, and *ddb-1*), *daf-15* (ortholog of human gene Rptor[7]) interactome genes (*lmtr-2*, *lmtr-3*, *atg-13*, *pitp-1*, and F16A11.1), *rict-1* (ortholog of human gene rictor[7]) interactome genes (*tln-1*, *mek-2*, *lat-1*, *tns-1*, and *cyd-1*), and *sinh-1* (ortholog of human gene Mapkap1[7]) interactome genes (*mlst-8*, R04A9.7, *ckc-1*, and R10H10.7). Lifespan-shortening genes were already well-studied[12-14], but not enough attention was paid to genes that extend lifespan. Therefore, we decided to focus on lifespan-extending genes after RNAi screening. After identifying F56F11.4 as the lifespan-extending gene, we applied double knockdown assay and RT-qPCR assay to examine the regulatory relationships between the identified gene F56F11.4 and its interactome *let-363*.

The genetic studies on aging could possibly pinpoint new drug targets to increase human lifespan and healthspan. Therefore, this study may provide new insights intohuman aging and contribute to new anti-aging drug designs.

2. Materials and Methods

2.1. Nematode Strains and Synchronization
All *C. elegans* strains were incubated at 22.5°C on NGM media seeded with OP50 *E. coli* as described by Stiernagle.[15] N2 was used as the wild-type strain and synchronized to eggs by bleaching.

2.2. RNA Interference Assay
Bacterial feeding RNAi experiments were performed as described by Kamath et al., and the RNAi clones were derived from the Ahringer RNAi library.[16] Twenty genes related to TOR pathway were included (*cyd-1*, *mlst-8*, *tns-1*, *ncbp-2*, F16A11.1, *lat-1*, *atg-13*, *ppfr-4*, *pitp-1*, *lmp-1*, *lmtr-2*, F56F11.4, *mek-2*, R04A9.7, *ckc-1*, *ddb-1*, *lpin-1*, *rho-1*, *lmtr-3*, and *aakg-1*). The *bli-3* RNAi clone was set as positive control group for RNAi assay (Figure 1), the *age-1* RNAi clone was set as positive control group for lifespan-extending phenotype, and the *daf-16* RNAi clone was set as positive control group for lifespan-shortening phenotype, while pL4440 (EV) was the empty control group. The synchronized worms were subjected to RNAi bacteria from L4 stage, which were then used to identify the effect of RNAi on aging phenotypes, including lifespan and pharynx pumping rate. All RNAi clones were confirmed by comparing the sequencing results with NCBI-Blast, and no significant off-target was predicted.
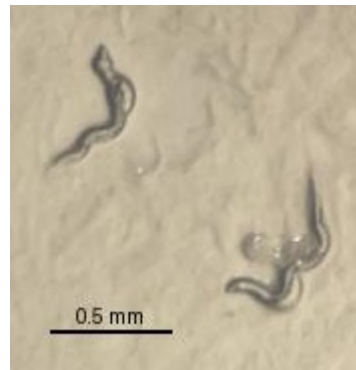
Figure 1. A group with bli-3 RNAi was carried out along with each experiment to test the effectiveness of RNAi treatment. At D3 stage, blisters were found at the surface of treated worms. Scale bar = 0.5 mm.

2.3. Lifespan Assay

For all lifespan assays in this study, age-synchronized L4 larvae were manually transferred to NGM plates, and seeded with *E. coli* with RNAi plasmids (N = 40). Worms were kept at 22.5°C and scored every two days as dead or alive based on their response to a gentle touch with a wire. Worms that presented externalization of internal organs, died because of bagging, or crawled up the wall of the dish were censored.

2.4. Pharynx Pumping Rate Assay

Pumping rate was monitored and recorded by filming worms (N = 10) at the indicated time point (D1, D5, D9, and D13 stages). Recordings of 25-30 seconds were obtained on Laika M165 microscope using the camera of iPhone 13 Pro Max. One pharyngeal contraction was defined as a cycle of backward and forward movements, and the number of contractions were accurately counted during a 20-second interval. For each strain, the pumping rate is calculated as pumping rate = average N / min.

2.5. RT-qPCR

Synchronized eggs were placed on NGM plates seeded with RNAi bacteria. The worms grew from hatching until day one of adulthood unless otherwise indicated. The worm samples were then harvested and washed with M9 buffer to remove bacteria from the samples. To separate RNA from protein and other materials, worm samples underwent centrifugation in a centrifuge. RNA was then extracted and cDNA was generated by reverse transcription of the total RNA samples. qPCR was performed and normalized to the levels of *tba-1* cDNA, with specified experiment settings in the following table (Table 1). The primer sequences for each gene are listed below (Table 2).

Table 1. Experiment setting in RT-qPCR

Expression level	pL4440	F56F11.4 mutant	let-363 mutant
Tested genes		tba-1 F56F11.4 let-363	

Table 2. Primer sequences

Gene	Forward primer sequence	Reverse primer sequence
tba-1	5'-GTTCCATCCAGAACAGATGATCACC-3'	5'-CAGCTTCGACTTCTTTCCATAGTCAAC-3'

Table 2. (continued).

let-363	5'-CGCACTTCTGTGGAGTCGAC-3'	5'-GAATTGCTTCATCTTTGTGATCATC CTGATAC-3'
F56F11.4	5'-CGACGTGGATAAATCCATTGA TATCAACTC-3'	5'-CCAACCATCTCGTATGTTGAATCAG G-3'

2.6. Phylogenetic Tree

Phylogenetic tree was made by Neighbor-joining method in MEGA7. The detailed parameters are: Test of Phylogeny-Bootstrap method; No. of Bootstrap Replications-1000; Substitution Type-Nucleotide; Model/Method-Maximum Composite Likelihood; Substitutions to Included: Transitions + Transversions; Rates among sites-Uniform rates; Pattern among Lineages-Same (Homogeneous); Gaps/Missing Data Treatment-complete deletion.

2.7. Use of Open-Source Online Databases

We utilized the STRING database to predict the interactomes of genes *let-363*, *daf-15*, *rict-1*, and *sinh-1* by inputting their respective identifiers and selecting *C. elegans* as our organism of interest, with default confidence scores set to medium for a comprehensive view of potential interactions. NCBI-Blast was also employed to ensure the specificity of designed primer and RNAi sequences.

2.8. Statistic Analysis

Descriptive statistics for continuous variables are presented as mean ± SEM, whereas categorical variables are presented as percentages. Hazard ratios (HRs) and 95% CIs were calculated by Log-rank model to assess the association between groups.

All analyses were performed using Graphpad Prism software (version 9.5). All P-values for the tests were two-sided and P-values < 0.05 were considered statistically significant.

3. Results

3.1. Deficiency of F56F11.4 shortened lifespan in C. elegans

In lifespan assay, the EV, *daf-16*, and *age-1* controls had results consistent with former studies at 22.5°C.[12,17] Among 20 RNAi groups tested, 14 of them were significantly different from the EV control group. The only RNAi group with the phenotype of shortened lifespan was F56F11.4 (P-value < 0.01, Hazard ratio = 1.726) (Figure 2).

RNAi groups with phenotype of extended lifespan included *atg-13* (P-value < 0.01, Hazard ratio = 0.6023), *cyd-1* (P-value < 0.05, Hazard ratio = 0.7189), *tns-1* (P-value < 0.0001, Hazard ratio = 0.4841), F16A11.1 (P-value < 0.0001, Hazard ratio = 0.3178), *lat-1* (P-value < 0.01, Hazard ratio = 0.6306), *ppfr-4* (P-value < 0.05, Hazard ratio = 0.7038), *pitp-1* (P-value < 0.05, Hazard ratio = 0.6567), *mek-2* (P-value < 0.001, Hazard ratio = 0.5694), R04A9.7 (P-value < 0.0001, Hazard ratio = 0.4723), *ckc-1* (P-value < 0.0001, Hazard ratio = 0.4009), *ddb-1* (P-value < 0.01, Hazard ratio = 0.6431), *rho-1* (P-value < 0.0001, Hazard ratio = 0.3664), and *lmtr-3* (P-value < 0.05, Hazard ratio = 0.6990) (Supplementary Figure 1). Non-significant groups included *ncbp-2* (P-value = 0.1869), *mlst-8* (P-value = 0.0539), *lmp-1* (P-value = 0.5491), *lmtr-2* (P-value = 0.2644), *lpin-1* (P-value = 0.1750), and *aakg-1* (P-value = 0.5526) (Supplementary Figure 2).
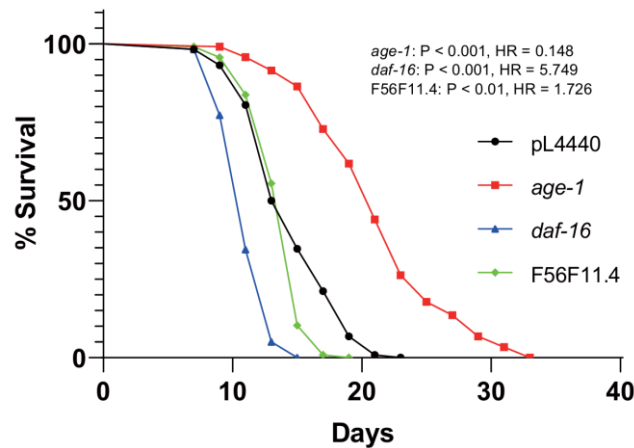
Figure 2. The effect of F56F11.4 knockdown on nematode lifespan. Survival of *C. elegans* wild-type (black) n = 119, *age-1* (red) n = 120, *daf-16* (blue) n = 120, and F56F11.4 RNAi (green) n = 120.

3.2. Deficiency of F56F11.4 failed to maintain nematode healthspan in early and late adulthood

After pharyngeal pumping rate assay was done on control groups and all 20 RNAi groups, we compared the pumping rate of every RNAi group with pL4440, at D1, D5, D9, and D13 stages, at which the control group was in a healthy state. Among the above stages, D1 was the baseline, D5 represented early healthspan, D9 represented middle healthspan, and D13 represented late healthspan. For our gene of interest, the knockdown of F56F11.4 significantly decreased nematode pumping rate at early and late healthspan (p < 0.001, p = 0.0105, respectively), as shown in Figure 3. This suggests that F56F11.4 is necessary in maintaining the healthspan of *C. elegans*.

For other genes, deficiencies of *lmtr-2*, *lat-1*, *ckc-1*, *rho-1*, *lmtr-3*, *ncbp-2*, and *lmp-1* all have effect on pumping rate at different periods of adulthood (Supplementary Table 1).
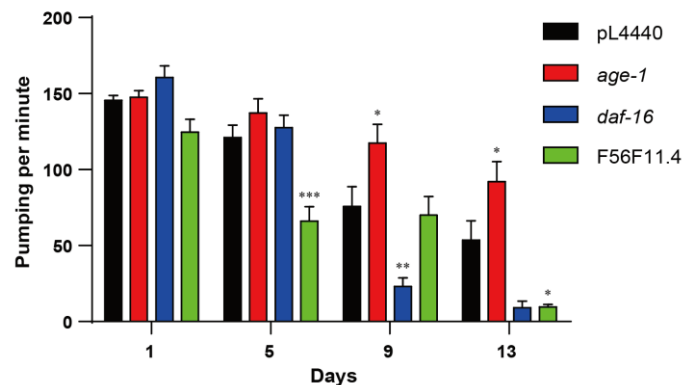
Figure 3. The effect of F56F11.4 knockdown on nematode pumping rate. The pharyngeal pumping rate (average number of contractions per minute) of WT (black), *age-1* (red), *daf-16* (blue), and F56F11.4 (green) RNAi were recorded on days 1, 5, 9 and 13 of adulthood. *P < 0.05, **P < 0.01, ***P < 0.001.

3.3. Double knockdown of F56F11.4 and let-363 balanced out the phenotype

To examine the regulatory relationships between F56F11.4 and *let-363*, we used the double knockdown assay to test the two genes. The results of F56F11.4 and *let-363* knockdown were consistent with the former single knockdown experiment in this study and previous studies (Figure 4).[12] The deficiencies of both *let-363* and F56F11.4 genes contributed to a lifespan longer than F56F11.4 single-knockdown (P-value < 0.001, Hazard ratio = 0.3512) but shorter than *let-363* single-knockdown (P-value < 0.001, Hazard ratio = 1.960), as shown in Figure 4.
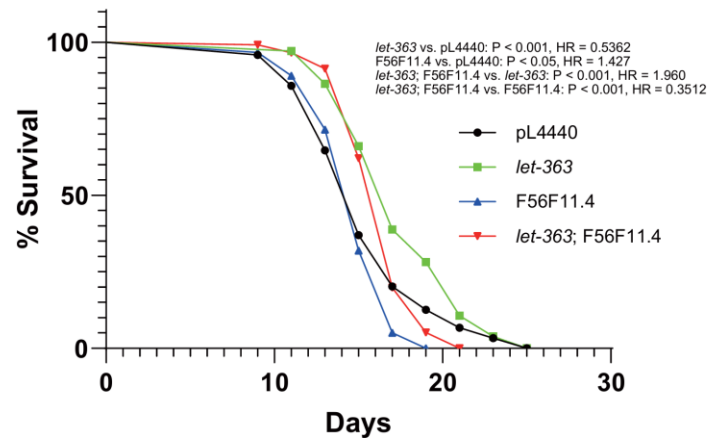
Figure 4. Effect of *let-363*; F56F11.4 double knockdown on nematode lifespan. Survival of *C. elegans* wild-type (black) n = 119, *let-363* (green) n = 120, F56F11.4 (blue) n = 103, and *let-363*; F56F11.4 RNAi (red) n = 116.

3.4. Interaction between F56F11.4 and let-363 regulates nematode lifespan

To analyze the regulatory mechanisms between F56F11.4 and *let-363*, we used RT-qPCR to monitor the expression level of the two genes when one was knocked down. *tba-1* was chosen as a constitutively expressed gene for normalization. As shown in Figure 5, when F56F11.4 was subject to RNAi, it showed an 18% reduction in expression level; when *let-363* was subject to RNAi, it showed a 53% reduction in expression level. When *let-363* was knocked-down, the expression level of F56F11.4 increased 40%; when F56F11.4 was knocked-down, on the contrary, the expression level of *let-363* decreased 40% (Figure 5). The 18% knockdown efficiency of F45F11.4 RNAi was enough to increase *let-363* expression by 40%, showing that F56F11.4 is a crucial gene, whose little change could lead to a huge alteration in other genes' expression levels.

When combining the results of RT-qPCR with the previous double knockdown experiment, we inferred about the direction of regulation between *let-363* and F56F11.4. Since they have different phenotypes on survival when knocked down, one gene should be inhibiting another if they interact directly. Because *let-363* RNAi increased F56F11.4 expression, *let-363* should be the upstream inhibitor of F56F11.4.
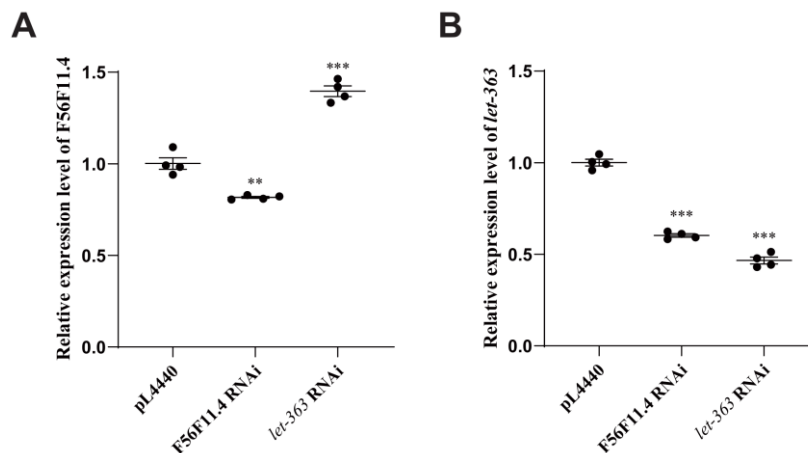

Figure 5. RT-qPCR of F56F11.4 and *let-363* expression levels. **A**, Relative expression level of F56F11.4. **B**, Relative expression level of *let-363*. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

3.5. F56F11.4 and let-363 are highly conserved lifespan regulators

To explore the potential of F56F11.4 in human drug design, we then analyzed the homology of *C. elegans* genes *let-363* and F56F11.4 in other organisms, and a phylogenetic tree was made for each gene (Figure 6).
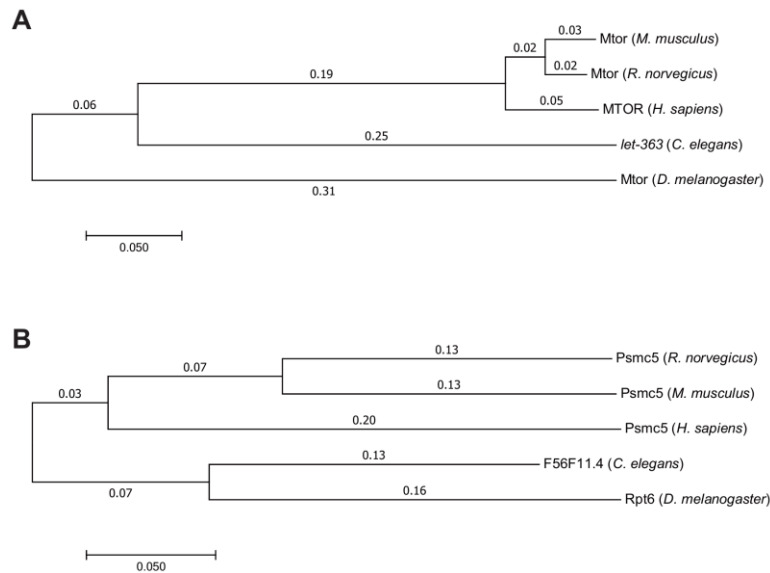

Figure 6. Homologs of C. elegans genes let-363 (**A**) and F56F11.4 (**B**) in H. sapiens, M. musculus, R. norvegicus, and D. melanogaster.

For both genes, the evolutionary relationships between *H. sapiens*, *M. musculus*, and *R. norvegicus* were identified to be the closest among all 5 homologs, as those 3 species are all mammals. However, the *C. elegans* gene *let-363* is evolutionarily closer to the mammals' homologs than to the homolog of *D. melanogaster*, while F56F11.4 is evolutionarily closer to the homolog of *D. melanogaster* than to the mammals' homologs. This might be because mTOR complex and related genes (including *let-363*) are more evolutionarily conserved than F56F11.4. [8]

4. Discussion

In this work, we aimed to identify the lifespan-extending gene and analyze its mechanism in regulating lifespan. Lifespan assay and pumping rate assay showed that the deficiency of gene F56F11.4 decreased nematode lifespan and healthspan. Further, double knockdown and RT-qPCR uncovered a more precise interaction between this gene and TOR signaling pathway.

4.1. Lifespan is regulated by the conserved TOR pathway

Our experiment showed that the deficiency of *let-363*, the ortholog of human mTor in *C. elegans*, extends nematode lifespan, and that this gene is highly evolutionarily conserved among many species, including humans.

In mammals, mTOR functions in regulation of lifespan. Previous experiments in mice, marmosets, and dogs all reported statistically significant results in lifespan or healthspan when treated with rapamycin, an inhibitor of mTOR.[18-20] In humans, due to the moral concerns, there were only clinical trials for small doses of rapamycin or similar mTOR inhibitors.[20] Nonetheless, previous researches have found that those drugs can slow down age-related conditions in multiple organs, including brain, heart, muscle, skin, etc.[20]

The mechanism of TOR pathway regulating lifespan and aging is rather complex. Previous studies reported that TOR shortens yeast lifespan through increasing mitochondrial electron transport chain activity and therefore promoting the accumulation of oxidative stress.[6,9] Another downstream

pathway of TOR complex is autophagy, especially mitophagy, the type of autophagy that targets mitochondrion.[21] In this pathway, HLH-30 is the transcription factor regulated by *let-363* in *C. elegans*, and its activated form can increase the expression of downstream autophagy-related genes.[6,10]

4.2. Mechanism of F56F11.4 and rpt-/rpn- families extending lifespan in C. elegans and other species

In identifying the exact mechanism of gene F56F11.4 affecting lifespan, we used the STRING database to look for interactomes of this gene. Among its top 20 predicted interactomes, there are 4 (*rpt-1*, *rpt-2*, *rpt-3*, and *rpt-5*) in *rpt-* family and 8 (*rpn-1*, *rpn-3*, *rpn-5*, *rpn-6.2*, *rpn-7*, *rpn-10*, *rpn-11*, and *rpn-13*) in *rpn-* family.

The *rpt-* and *rpn-* families both belong to the components of the proteasomal complex. The proteasome maintains cellular homeostasis by degrading oxidized and damaged proteins, a process known to slow down the progress of aging.[22] A former study reveals that upregulation of *rpn-6* expression can directly increase lifespan by mitigating oxidative pressure, and this is one of the pathways in which DAF-16/FOXO regulates lifespan.[23] In another study, 4 genes from *rpt-* and *rpn-* families were tested and compared on their effect on lifespan, and the deficiency of every tested gene resulted in lifespan reduction.[24]

The *rpt-* and *rpn-* families are also closely related to *let-363*/mTOR pathway. Starvation and inactivation of mTOR complex were previously identified to enhance the activity of autophagy and proteasomal complexes, in which the *rpt-* and *rpn-* families locate and function.[25]

Apart from their effect on nematode lifespan, *rpt-* and *rpn-* families also regulate aging in other model organisms. In *Saccharomyces cerevisiae*, the increased expression level of protein Rpn4 can result in increased replicative lifespan.[26] In *Drosophila melanogaster*, overexpression of Rpn11 also upregulates the lifespan of flies by suppressing aging-dependent reduction in proteasomal activity.[27]

For mammals, there were no previous studies about the direct influence of *rpt-*/*rpn-* families on animal aging, but some researches focused on senescence on the cellular level. One study revealed that the high constitutive expression of *rpt-* and *rpn-* families contributes to the immortality of embryonic stem cells (ESCs).[28] Mouse ESCs are highly correlated with high expression of Psmd14/Rpn11, a deubiquitinating enzyme in 19S regulatory component of proteasome.[29] Similarly, in human, PSMD11/Rpn6 is highly expressed in hESCs, keeping the cells free from senescence.[30,31]

4.3. The regulatory relationship between TOR pathway and rpt-/rpn- families

In our double knockdown and RT-qPCR experiments, we found that TOR complex and F56F11.4 have a regulatory relationship, in which *let-363* inhibits F56F11.4. While this is the first time the interaction between TOR complex and F56F11.4 is identified since F56F11.4 is highly related and interacting with *rpt-*/*rpn-* families, past studies had also found that TOR pathway inhibits proteasomal function and activities of *rpt-*/*rpn-* families in multiple ways.[32]

First, TOR complex regulates the assembly of regulatory components of proteasome.[33] In yeast, inhibition of TORC1 can cause activation of Adc17, a suppressor for proteasomal dysfunctioning, therefore increasing proteasomal activity.[32,34] Another mechanism is that TORC1 controls proteasome biogenesis, by sensing the signal of the amino acid amount and regulating the catabolic/anabolic pathways.[32] One study used starvation to inhibit TORC1, which led to higher proteasomal function and higher autophagy.[32]

4.4. Lifespan-shortening genes and their mechanisms

Lifespan-shortening genes identified in our knockdown experiment (*atg-13*, *cyd-1*, *tns-1*, F16A11.1, *lat-1*, *ppfr-4*, *pitp-1*, *mek-2*, R04A9.7, *ckc-1*, *ddb-1*, *rho-1*, and *lmtr-3*) have different mechanisms in regulating lifespan. The most common way among them is through regulating protein ubiquitination and proteasomal complex, including genes F16A11.1, *lat-1*, and *ddb-1*. This is also the pathway in which the identified lifespan-extending gene F56F11.4 regulates lifespan. Since one main function of mTOR complex is inhibiting the proteasome function[11], this result is consistent with previously identified pathways. Another key mechanism of shortening lifespan is through inhibiting autophagy, which was

embodied in the gene *atg-13*, the interactome of *daf-15* gene in the mTOR pathway. Since mTOR pathway also has the downstream effect of inhibiting autophagy[8], the position of *atg-13* in mTOR pathway was verified.

Other lifespan-shortening genes also use TOR-related pathways to reduce lifespan. They included regulating cell cycle (*cyd-1*), regulating axon regeneration (*tns-1*), regulating protein kinase or phosphatase (*mek-2*, R04A9.7, *ckc-1*, and *ppfr-4*), regulating lipid transfer (*pitp-1*), regulating scaffold protein binding (*mek-2*), and regulating G protein-coupled receptor (*rho-1*).[7]

4.5. The value of this study

Potential lifespan-extending drugs are currently being discovered, and many of them are based on TOR pathway. In 2009, rapamycin was verified to extend lifespan in *M. musculus*, functioning by binding with FKBP12, binding to mTORC1, and inhibiting mTOR pathway.[35] A recently emerged class of senolytic drugs was first discovered in 2015 that could extend lifespan in *M. musculus*.[36] This type of drug also functions by inhibiting mTOR pathway, and many of those drugs are now in the process of clinical phase 2 trials.[36,37] These drugs demonstrate the potential of aging-related studies based on TOR pathway: to identify new drug targets for clinical use.

5. Conclusion

In this work, we screened out and demonstrated F56F11.4 as a lifespan-extending gene and analyzed its mechanism in regulating lifespan. Double knockdown experiment and RT-qPCR uncovered a more precise regulation between this gene and the TOR signaling pathway. With more and more research being done in the field of human aging, we are optimistic that safe and effective drugs can extend human healthspan in the future.

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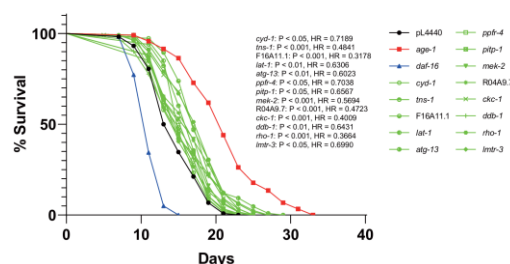
Appendices

Figure A1. Effect of deficiency of lifespan-shortening genes on lifespan. Survival of *C. elegans* wild-type (black) n = 119, *age-1* (red) n = 120, *daf-16* (blue) n = 120, and all other RNAi groups that resulted in lifespan extension (green).
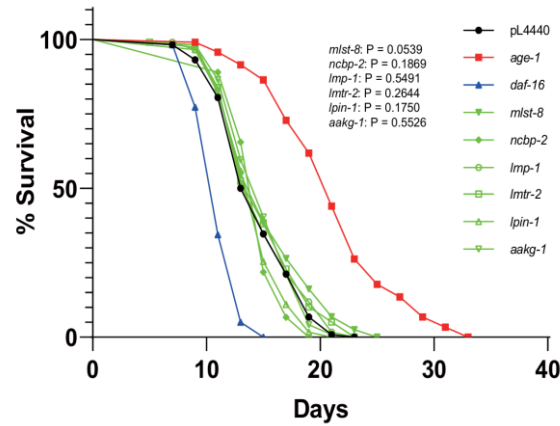
Figure A2. Effect of deficiency of other genes. Survival of *C. elegans* wild-type (black) n = 119, *age-1* (red) n = 120, *daf-16* (blue) n = 120, and all other RNAi groups (green).

Table A1. Pumping rate statistics. Light blue, decreased pumping rate with $P < 0.05$; blue, decreased pumping rate with $P < 0.01$; dark blue, decreased pumping rate with $P < 0.001$; Light red, increased pumping rate with $P < 0.05$; red, increased pumping rate with $P < 0.01$; dark red, increased pumping rate with $P < 0.001$.

Mean ± SEM, P-value	D1	D5	D9	D13
pL4440	145.55 ± 0.59, 1.0000	121.00 ± 1.49, 1.0000	75.70 ± 2.33, 1.0000	53.57 ± 2.70, 1.0000
age-1	147.60 ± 0.78, 0.9919	137.20 ± 1.69, 0.4324	117.40 ± 2.21, 0.0269	92.14 ± 2.78, 0.0289
daf-16	160.50 ± 1.39, 0.2385	127.50 ± 1.48, 0.9191	23.00 ± 1.04, 0.0035	9.00 ± 1.91, 0.2233
cyd-1	152.40 ± 1.37, 0.7680	107.60 ± 1.89, 0.6025	101.90 ± 2.14, 0.2337	24.14 ± 2.19, 0.2087
mlst-8	160.00 ± 1.36, 0.2298	102.60 ± 2.05, 0.3777	46.45 ± 1.77, 0.1356	51.29 ± 3.01, 0.9988
tns-1	120.10 ± 2.28, 0.0688	93.30 ± 2.10, 0.1062	76.50 ± 1.95, 0.9999	49.43 ± 2.82, 0.9925
ncbp-2	171.40 ± 0.58, 0.0012	103.20 ± 1.44, 0.3217	61.00 ± 1.88, 0.6505	6.00 ± 0.33, 0.0051
F16A11.1	134.70 ± 1.87, 0.5694	92.30 ± 1.85, 0.0721	92.80 ± 2.16, 0.5682	49.43 ± 2.80, 0.9925
lat-1	159.70 ± 1.18, 0.2101	96.70 ± 2.36, 0.2090	133.76 ± 2.20, 0.0011	27.14 ± 2.20, 0.2891
atg-13	165.00 ± 0.92, 0.0337	132.60 ± 1.60, 0.6688	93.90 ± 2.33, 0.5384	80.00 ± 2.69, 0.3351
ppfr-4	152.40 ± 1.17, 0.7380	140.70 ± 2.08, 0.3276	97.90 ± 2.28, 0.3751	57.71 ± 2.84, 0.9926
pitp-1	155.40 ± 1.08, 0.4661	139.30 ± 1.77, 0.3426	111.00 ± 1.90, 0.0592	51.57 ± 2.55, 0.9990
lmp-1	144.60 ± 1.27, 0.9990	109.10 ± 1.91, 0.6857	75.50 ± 2.40, 1.0000	7.71 ± 1.39, 0.0120
lmtr-2	134.60 ± 1.38, 0.4486	86.00 ± 2.20, 0.0324	84.90 ± 2.36, 0.8959	36.00 ± 2.86, 0.6696

Table A1. (continued).

Mean ± SEM, P-value	D1	D5	D9	D13
F56F11.4	124.60 ± 1.52, 0.0590	66.10 ± 1.71, 0.0001	70.00 ± 2.21, 0.9698	9.57 ± 0.35, 0.0105
mek-2	140.38 ± 1.52, 0.8914	98.60 ± 1.53, 0.1685	109.20 ± 2.11, 0.0892	70.57 ± 3.18, 0.7154
R04A9.7	140.00 ± 1.17, 0.8369	97.70 ± 2.02, 0.1960	96.20 ± 2.50, 0.4642	73.71 ± 3.08, 0.5928
ckc-1	157.20 ± 1.07, 0.3314	107.40 ± 1.92, 0.5961	117.80 ± 1.93, 0.0192	59.57 ± 2.74, 0.9776
ddb-1	135.62 ± 1.22, 0.4888	98.10 ± 2.09, 0.2163	100.90 ± 1.91, 0.2401	64.29 ± 2.76, 0.8917
lpin-1	139.00 ± 1.28, 0.7774	102.50 ± 2.11, 0.3811	95.00 ± 1.97, 0.4543	18.71 ± 1.40, 0.0744
rho-1	139.40 ± 1.50, 0.8339	122.80 ± 1.99, 0.9982	135.10 ± 1.71, 0.0003	60.29 ± 2.48, 0.9664
lmtr-3	154.10 ± 1.36, 0.6326	129.60 ± 2.21, 0.8665	127.50 ± 1.89, 0.0027	36.71 ± 2.13, 0.6393
aakg-1	153.90 ± 1.38, 0.6518	114.10 ± 2.33, 0.9282	93.50 ± 2.21, 0.5428	54.00 ± 2.94, 1.0000