A Long Noncoding RNA on the Ribosome Is Required for Lifespan Extension

Highlights

- The long noncoding RNA tts-1 is found on C. elegans daf-2 mutant ribosomes
- Depletion of tts-1 restores ribosome levels in daf-2 mutants
- The extended lifespans of daf-2 and clk-1 mutants are dependent on tts-1

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In Brief
Essers et al. find a long noncoding RNA, transcribed telomeric sequence 1 (tts-1), on ribosomes of C. elegans carrying the life-extending daf-2 insulin receptor mutation as well as the clk-1 mitochondrial mutant. They then demonstrate that this RNA is required for the life-extension phenotypes and that its depletion results in increasing ribosome levels.
A Long Noncoding RNA on the Ribosome Is Required for Lifespan Extension

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SUMMARY

The biogenesis of ribosomes and their coordination of protein translation consume an enormous amount of cellular energy. As such, it has been established that the inhibition of either process can extend eukaryotic lifespan. Here, we used next-generation sequencing to compare ribosome-associated RNAs from normal strains of Caenorhabditis elegans to those carrying the life-extending daf-2 mutation. We found a long noncoding RNA (lncRNA), transcribed telomeric sequence 1 (tts-1), on ribosomes of the daf-2 mutant. Depleting tts-1 in daf-2 mutants increases ribosome levels and significantly shortens their extended lifespan. We find tts-1 is also required for the longer lifespan of the mitochondrial clk-1 mutants but not the feeding-defective eat-2 mutants. In line with this, the clk-1 mutants express more tts-1 and fewer ribosomes than the eat-2 mutants. Our results suggest that the expression of tts-1 functions in different longevity pathways to reduce ribosome levels in a way that promotes life extension.

RESULTS

We hypothesized that the daf-2 reduction of protein synthesis would be reflected by changes in ribosome-associated RNAs. In order to compare these RNA subsets, we took a nonbiased approach by performing next-generation sequencing on the RNAs isolated from the monosomal or polysomal fractions (hereafter referred to as “ribosomal”) of N2, daf-2, and daf-2;daf-16 strains normalized to total protein amounts and separated over sucrose density gradients (experimental setup shown in Figure S1A). The expected reduction of the ribosome profile peaks specifically in the daf-2 mutants compared to wild-type levels was consistent with our previous findings (Stout et al., 2013). Figure S1B illustrates the relative proportion of different subsets of
RNA that we found in the ribosomal fractions of N2, daf-2, and daf-2;daf-16 strains. Tables S1 and S2 reveal that the mRNAs found in the ribosomal fractions of the N2 and daf-2;daf-16 strains largely code for proteins important for biological processes such as growth, development, the cell cycle, and reproduction, while the same fractions of the daf-2 mutants reveal mRNAs that largely code for proteins involved in aging and stress response. Gene Ontology (GO) analysis of gene functions using Database Annotation, Visualization, and Integrated Discovery (DAVID) in Figure S1C confirmed these biological functions to be consistent with other studies measuring transcriptional changes in the daf-2 mutant (Halaschek-Wiener et al., 2005; Murphy, 2006).

In addition to the differential enrichment of many mRNAs on daf-2 ribosomes, we also found a long noncoding RNA (lncRNA), transcribed telomeric sequence 1 (tts-1), highly expressed in ribosomal fractions of daf-2 cells, but not in those of N2 or daf-2;daf-16. The tts-1 lncRNA is transcribed to two different isoforms of 711 or 659 bp long from a gene found on chromosome X that is not conserved in any other species and has almost no homology with other C. elegans genes. The G/C content of the tts-1 transcript is very low, calculated to be 34% for the total length of the transcript and dropping to less than 15% at the final 150 bp of the 3’ end. A low-affinity cyclic-AMP-responsive element (TGATGTCA) lies 728 nt upstream of the tts-1 transcription start site. Figure S1D illustrates the location of the RNA-sequencing mapped read densities with an Integrative Genomics Viewer (Robinson et al., 2011). Serial analysis of gene expression (SAGE) previously found that compared to wild-type strains, tts-1 is one of the most upregulated transcripts in the daf-2 mutants as well as in the developmentally arrested and longer-lived C. elegans in dauer formation (a type of stasis resulting from unfavorable environments that permits survival under harsh conditions) (Halaschek-Wiener et al., 2005; Jones et al., 2001). The expression of tts-1 is also significantly upregulated in C. elegans subjected to attack by Gram-positive bacterial pathogens, a situation that slows the growth and increases constipation of the worms (O’Rourke et al., 2006). However, the function of the tts-1 lncRNA and its role in the longevity and immunity programs of C. elegans are, to date, entirely unknown.

We first validated the increase of tts-1 expression in daf-2 mutants compared to the levels in N2s and daf-2;daf-16 mutants using qPCR analysis of cDNA generated from isolated total RNA. The results of tts-1 expression in these mutants and also suggest that tts-1 is enriched over 12-fold in the monosomal fraction, with again almost no detectable expression in the polysomal fraction, with again almost no detectable tts-1 found in either fraction of the N2 strain (Figure 1B). These data suggest that tts-1 is not only expressed at much higher levels in daf-2 mutants but also preferentially

![Figure 1. tts-1 lncRNA Expression Is High in the Ribosomal Fractions of daf-2 Mutants](image-url)
enriched on ribosomes. Fluorescent in situ hybridization analysis (FISH) with 11 different probes against tts-1 confirmed the substantially higher expression of tts-1 in daf-2 mutants compared to N2 (Figure 1C). Moreover, it reveals that tts-1 is uniformly expressed in the cytoplasm and nuclei of cells in the intestine of the C. elegans intestine in the regulation of insulin/IGF-1 lifespan (Libina et al., 2003). To confirm that tts-1 is in fact ribosome bound and not merely contaminating the polysomal fractions of the sucrose gradients, we measured levels of tts-1 in the polysomal fractions of untreated daf-2 mutants compared to those treated with puromycin. The puromycin-induced dissociation of polysomes is confirmed by profiles revealing a reduction of polysome peaks and a widening of the monosome peak (Figure S1E). This dissociation of polysomes results in a reduction of both pmp-3 and tts-1 levels in the polysomal fraction, indicating that the expression of tts-1 in the polysomal fractions is not merely a contaminant (Figure S1F).

To understand the function of the tts-1 lncRNA in the context of lifespan extension, we designed a double-stranded small interfering RNA (siRNA) construct against tts-1 in the L4440 vector that we expressed in HT115 E. coli and then fed to the worms. Compared to daf-2 mutants fed bacteria expressing the empty L4440 vector, the bacteria expressing the tts-1 siRNA were successful at reducing the levels of tts-1 siRNA when lysate is normalized to total protein levels (Figure S2A). This siRNA of tts-1 significantly shortened the extended lifespan of the daf-2 mutants (Figures 2A and 2B). Importantly, as opposed to inducing toxicity, tts-1 is regulating daf-2 lifespan specifically, as neither wild-type nor daf-2;daf-16 nematodes reveal any changes in lifespan upon exposure to the tts-1 siRNA (Figures 2A and 2B). BLAST results of the siRNA sequence revealed no significant homology between the construct and any other gene in the C. elegans genome except for tts-1 itself, suggesting a low probability of any off-target effect of the siRNA that may negatively affect daf-2 lifespan (Kamath et al., 2001, 2003). These results reveal a necessary role of tts-1 in the extension of the daf-2 mutant lifespan.

We next examined the effect of tts-1 depletion on the polysome profiles of daf-2 mutants normalizing the lysate on the gradients to total protein levels. Consistent with what we previously reported (Stout et al., 2013), the profiles of the daf-2 mutants reveal low levels of ribosomes (Figure 3A). These results are also in line with the reduction of the total number of ribosomal proteins that both proteomic studies of the daf-2 mutants revealed earlier (Depuydt et al., 2014; Stout et al., 2013). We found...
that knocking down the expression of \textit{tts-1} in the \textit{daf-2} mutants results in the polysome peak sizes returning to more wild-type levels, suggesting an increase of ribosome levels (Figures 3 B and 3C). In order to evaluate if \textit{tts-1} loss shifted the location of ribosomes from polysomal to nonpolysomal fractions in the density gradient, which would give an indication about the overall level of protein translation, we then normalized the lysates to cytoplasmic rRNA levels. Here, we detect no difference in the polysome peak sizes (Figure S3A). Moreover, we do not observe any differences in the area under the curves of the polysomal compared to the nonpolysomal fractions (Figure S3B). Taken together, these data suggest that while \textit{tts-1} loss in the \textit{daf-2} mutants increases ribosome levels, it does not affect the overall rate at which these ribosomes translate protein.

In order to assess the stoichiometry of the \textit{tts-1} lncRNA relative to the number of ribosomes, we isolated monosomal RNA from \textit{daf-2} mutants and compared the expression of \textit{tts-1} to 18S rRNA by qPCR analysis. We calculated that for every \textit{tts-1} transcript in the monosomal fraction, there are $116,000 \pm 10,000$ (n = 3) 18S rRNA transcripts. This suggests that while \textit{tts-1} may be highly expressed in the \textit{daf-2} mutants, it is not acting simply to compete with all other mRNAs for occupancy on the ribosome.

We next asked if the increase of \textit{tts-1} expression and reduction of ribosomes is unique to the insulin/IGF-1 longevity pathway. For these experiments, we used \textit{clk-1} mutants (which carry a mitochondrial pathway mutation that reduces respiration and decreases ubiquinone biosynthesis) and \textit{eat-2} mutants (models of dietary restriction with impaired pharynxes and defective feeding behavior) (Jonassen et al., 1998; Lakowski and Hekimi, 1996, 1998; Wong et al., 1995). Both of these mutants have an established longer lifespan (Lakowski and Hekimi, 1996, 1998). qPCR analysis relative to \textit{pmp-3} expression on the total RNA isolated from these mutants reveals an increase of \textit{tts-1} expression in both strains compared to N2, with much higher levels of \textit{tts-1} found in the \textit{clk-1} mutants compared to \textit{eat-2} (Figure 4A). Correlating with these results, the profiles of both mutants compared to N2 strains reveal a far more dramatic reduction of ribosome levels in the \textit{clk-1} mutants (Figures S4A and S4B). Further in line with this, we find that the depletion of \textit{tts-1} in the \textit{clk-1} mutants results in a substantial shortening of their longer lifespan ($p < 0.0001$) and a marginal yet not nearly as significant shortening of the \textit{eat-2} mutant lifespan ($p = 0.02$) (Figures 4B and 4C). The difference between the effects of \textit{tts-1} depletion on the lifespans of the \textit{clk-1} (and the \textit{daf-2}) versus the \textit{eat-2} mutants moreover supports the specificity of the RNAi and suggests that the changes in lifespan are not due to off-target effects. All of the controls and statistical analysis for this assay are shown in Figures S4C and S4D.

Although we did attempt to construct a \textit{C. elegans} strain over-expressing \textit{tts-1} in the N2 genetic background, we were unsuccessful in establishing an integrated line that expressed \textit{tts-1} at physiological levels or that did not reveal toxicity (data not shown). Thus, whether \textit{tts-1} is sufficient to extend lifespan remains to be determined.

**DISCUSSION**

Long noncoding RNAs were until recently thought to exist and function predominantly in the nucleus. It is now fast becoming realized that they effusively associate with cytosolic ribosomes (van Heesch et al., 2014; Wilson and Masel, 2011). Several functions for short noncoding RNAs (<20 bp) bound to ribosomes...
have been described, such as those that derive from both mRNAs and tRNAs and function as stress-induced inhibitors of protein translation (Ivanov et al., 2011; Pircher et al., 2014; Sobala and Huvtagnar, 2013). Also recently a function for the ribosome-bound long intergenic noncoding RNA p21 (lincRNA-p21) was found to selectively repress the translation of JUNB and CTTNB1 mRNAs (Yoon et al., 2012). It is thus becoming clear that ncRNAs, both short and long, are playing roles in protein translation that are only beginning to be fully appreciated.

We are unable to definitively state that the tts-1 IncRNA does not code for protein. We do not find any protein sequences in http://www.wormbase.org or the NCBI database that corresponded to potential open reading frames of tts-1. Moreover, we do not detect any corresponding peptides in our proteomics study (Stout et al., 2013). It may of course be that any synthesized peptides are too small in size or short in half-life to be detected by current proteomic methods. Thus, it remains an open question as to the protein-coding potential of IncRNAs on the ribosome, reflected by a number of conflicting recent reports (Guttman et al., 2013; Niazi and Valadkhan, 2012; Smith et al., 2014).

The strong effect of tts-1 depletion on the longevity phenotype of the daf-2 and clk-1 mutants, but not the eat-2 mutants, is curious, especially given that the clk-1 mutation is known to be daf-16 independent (Lakowski and Hekimi, 1996). It is known that AAK-2, the C. elegans homolog of AMP-activated kinase subunit α, functions as a sensor of energy levels and is activated in conditions with high AMP:ATP ratios (Apfeld et al., 2004). Both clk-1 and daf-2-extended lifespans are dependent on AAK-2 in a pathway that is not shared by eat-2 (Curtis et al., 2006). Recently, it was shown that CRTC-1 (the sole C. elegans cyclic-AMP response element binding protein [CREB]-regulated transcriptional coactivator) interacts with the CREB homolog-1 transcription factor (CRH-1) and is directly activated by AAK-2 (Mair et al., 2011). As mentioned previously, a low-affinity cyclic-AMP responsive element lies 728 nt upstream of the tts-1 transcription start site. It is therefore possible that AMP:ATP levels are a driver of tts-1 transcription, which would also account for the high tts-1 expression in animals attacked by Gram-positive pathogens as they respond to the invasive stress (Hardie, 2011).

The precise mechanism of the tts-1 IncRNA remains to be determined. One intriguing possibility is that it is specifically regulating the translation of ribosomal protein mRNAs. This notion is the observation that despite the marked reduction of ribosomal proteins in the daf-2 mutant proteome, expression levels of ribosomal protein mRNAs in the daf-2 mutants are actually higher than in wild-types (Depuydt et al., 2014; Halaschek-Wiener et al., 2005). This suggests that a specific block of ribosomal protein gene expression at the level of translation is imposed in mutants undergoing lifespan extension, and we believe this will be an interesting area of future study.

In sum, we propose that the tts-1 IncRNA is able to reduce ribosome levels in a manner that is necessary for lifespan extension. Since many recent reports demonstrate that both genetic and pharmacological manipulations of the translation machinery can extend longevity in eukaryotes, our study puts IncRNAs forward as a compelling area in the field of aging research.

**EXPERIMENTAL PROCEDURES**

**Nematode Strains**

All strains were maintained expanded as previously described (Brenner, 1974). Briefly, nematodes were maintained on NGM OP50 plates at 15°C. The Bristol N2 strain was used as wild-type. Mutant alleles and transgenes used in this study are CB1370 daf-2(e1370), CF1038 daf-16(mu86), CF1515 daf-16(mu86); daf-2(e1370), MQ130 clk-1(qm30), and DA465 eat-2(ad465) (obtained from the Caenorhabditis Genetics Center). The extrachromosomal tts-1–overexpressing line in Bristol N2 background was huEx645[Phs::tts-1::SL2::mCherry; Pmyo2::GFP]. An integrated line was created by irradiation of huEx645[Phs::tts-1::SL2::mCherry; Pmyo2::GFP] with 40 Gy using a 137Cs source, and the obtained line was backcrossed two times with the Bristol N2 wild-type strain to generate huls164[Phs::tts-1::SL2::mCherry; Pmyo2::GFP].

**Puromycin Treatment**

daf-2 nematodes were synchronized and grown on NGM OP50 plates at 15°C until the L4 stage. They were collected and incubated overnight at 25°C in liquid NGM medium including OP50 bacteria, 0.1% Triton X-100, and 50 μg/ml puromycin (Sigma Aldrich).

**RNAi**

The last exon of tts-1 was synthesized by Eurofins with BglII and Ncol restriction enzymes sites on the 5’ or 3’ end, respectively, and cloned into the L4440 vector (Addgene) using the same enzymes. This vector was transformed into HT115 E. coli. Positive clones were selected by Sanger sequencing and grown overnight at 37°C in Luria broth with 50 μg/ml ampicillin and 0.2 M isopropyl β-D-1-thiogalactopyranoside (IPTG) before seeding.

**Synchronization**

For experiments, nematodes were synchronized by bleeding and allowed to hatch overnight in M9 buffer (Eisenmann, 2005). The L1 arrested larvae were plated onto NGM OP50 plates or plates inoculated with tts-1-specific RNAi bacteria and grown at 15°C until L4 stage, at which point they were shifted to 25°C overnight (for daf-2, daf-2;daf-16, and controls only).

**Lifespan Analysis**

Lifespan analysis was performed as previously described (Stout et al., 2013) on 35 mm NGM plates including FLiDr (Sigma-Aldrich), 50 μg/ml ampicillin, and 0.2 M IPTG inoculated with the gene-specific siRNA bacteria of interest (Hansen et al., 2005). Lifespan curves and the associated statistics were analyzed using GraphPad Prism software and a Mantel-Haenszel test. The N2 and daf-2±tts-1 siRNA lifespan curves were performed in biological duplicate.

**Peak Calculations**

The polysome peak heights and monosomal peak widths were measured from the lower left most point of the peak curve using ImageJ software. Statistics were performed using a Student’s t test. The area under the polysome peaks was calculated using R software (http://www.r-project.org/).

**Polysome Profiling**

Polysome profiling was performed as previously described (Pereboom et al., 2011). The concentration of protein in the lysates was measured with a Bradford reagent (Bio-Rad), and the cytoplasmic ribosome particles were measured by 260 nm optical density readings. Either an equal amount of total protein or equal levels of cytoplasmic rRNA were loaded onto the sucrose gradients for every experiment. Fractions were collected using a Foxy Jr Fracton Collector (Teledyne ISCO).

**RNA-Sequencing Analysis**

Monosomal and polysomal fractions from two experiments were pooled and RNA was extracted using TRIzol LS (Invitrogen) according to the manufacturer’s protocol. For each condition, two libraries were constructed from RNA isolated from two separate experiments using the SOLiD Total RNA-Seq Kit (Life Technologies) and analyzed on the SOLID platform. The sequence reads were mapped against the genome assembly WBcel215. Using cufflinks,
we identified any possible new transcripts through reference annotated base transcript (RABT) assembly (Trapnell et al., 2013). Subsequently differential expression of transcripts was determined using cuffdiff across all pairs (Trapnell et al., 2013).

GO Term Cluster Analysis

GO analysis was performed by DAVID analysis (Huang et al., 2009a, 2009b).

qPCR Analysis

Total, monosomal, and polysomal RNA was isolated using TRIzol LS (Invitrogen) according to manufacturer’s protocol, and cDNA was made using iScript (Bio-Rad). qPCR reactions were run using IQ SYBR Green Supermix (Bio-Rad) on a myIQ iCycler (Bio-Rad), and the expression of the RNAs were calculated using the ∆CT method. The following primers were used for total RNA measurements: 

| Primer     | Sequence                        |
|------------|---------------------------------|
| tts-1 fw   | 5'-CGCAGACGTTCAGACACA-3'         |
| tts-1 rv   | 5'-GGTTTACCCATTGACTCAACC-3'      |
| 18S rRNA fw| 5'-TCTGGTATGAGCACCAGC-3'         |
| 18S rRNA rv| 5'-CTCGTCGGAATCCACGG-3'          |

For 18S rRNA, fw: 5'-GGTTTACCCATTGACTCAACC-3' and rv: 5'-GGAGGATTGAGGAAAATTG-3'.

MyoIQ iCycler (Bio-Rad), and the expression of the RNAs were calculated using the Ct method. The following primers were used for total RNA measurements:

| Primer     | Sequence                        |
|------------|---------------------------------|
| #1         | 5'-AGCTTACAGAAAAAAGACTCG-3'      |
| #2         | 5'-CGTATAGGAACTCAATTTC-3'        |
| #3         | 5'-GGCAAAGGATATTATACCG-3'        |
| #4         | 5'-TAAGGTTTCTTTGAGTCTC-3'        |
| #5         | 5'-GGTTGAGTACTGTAAGAAGTCT-3'     |
| #6         | 5'-CCGTGTCCTTGGGACATTGT-3'       |
| #7         | 5'-GCGAAGAAGCCTTGGGACATTGT-3'    |

FISH was performed according to FISH probe designer, using the highest level of masking (BioSearch Technologies). The probe sequences are as follows: #1: 5'-AGTCTTACAGAAAAAAGACTCG-3'; #2: 5'-CGTATAGGAACTCAATTTC-3'; #3: 5'-GGCAAAGGATATTATACCG-3'; #4: 5'-TAAGGTTTCTTTGAGTCTC-3'; #5: 5'-GGTTGAGTACTGTAAGAAGTCT-3'; #6: 5'-CCGTGTCCTTGGGACATTGT-3'; #7: 5'-GCGAAGAAGCCTTGGGACATTGT-3'.

For 18S rRNA, fw: 5'-GGTTTACCCATTGACTCAACC-3' and rv: 5'-GGAGGATTGAGGAAAATTG-3'.

Statistical analysis was performed with a Student’s t test.

Fluorescent In Situ Hybridization

A set of 11 short probes targeting tts-1 was designed with the Stellaris RNA FISH probe designer, using the highest level of masking (BioSearch Technologies). The probe sequences are as follows: #1: 5'-AGTCTTACAGAAAAAAGACTCG-3'; #2: 5'-CGTATAGGAACTCAATTTC-3'; #3: 5'-GGCAAAGGATATTATACCG-3'; #4: 5'-TAAGGTTTCTTTGAGTCTC-3'; #5: 5'-GGTTGAGTACTGTAAGAAGTCT-3'; #6: 5'-CCGTGTCCTTGGGACATTGT-3'; #7: 5'-GCGAAGAAGCCTTGGGACATTGT-3'.

For mono- and polysomal RNA, tts-1 fw: 5'-GAACCTAATGGCAGTGTTAC-3' and tts-1 rev: 5'-GGAGGATTGAGGAAAATTG-3'.

Effectiveness of specific RNA-mediated interference through in vivo experiments

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