Age-dependent upregulation of Y RNAs in Caenorhabditis elegans
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Abstract

Y RNA is a conserved small non-coding RNA whose functions in aging remain unknown. Here, we sought to determine the role of C. elegans Y RNA homologs, CeY RNA (CeY) and stem-bulge RNAs (sbRNAs), in aging. We found that the levels of CeY and sbRNAs generally increased during aging. We showed that CeY was downregulated by oxidative and thermal stresses, whereas several sbRNAs were upregulated by oxidative stress. We did not observe lifespan phenotypes by mutations in CeY-coding yrn-1. Future research under various genetic and environmental conditions is required to further evaluate the role of Y RNA in C. elegans aging.

Figure 1: Age-dependent changes in C. elegans Y RNA homologs, and the effects of yrn-1 and rop-1 mutations on lifespan. (A) A schematic showing the conserved structure of C. elegans Y RNA homologs, CeY RNA (CeY) and stem-bulge RNAs (sbRNAs). Each of CeY and sbRNAs contains a conserved cytidine bulge (Van Horn et al. 1995; Deng et al. 2006; Boria et al. 2010), a characteristic of Y RNAs. Each sbRNA also contains a highly conserved UG-CA motif. (B) A heat map showing fold changes in the levels of the CeY, sbRNAs, and rop-1 mRNA in wild-type C. elegans at days 4, 7, and 11 of adulthoods compared to those in day 1 animals, which were analyzed using qRT-PCR [n=3 except for CeY (n=6), rop-1 (n=5), CeN71 (n=5), and CeN72 (n=5)]. (C and D) qRT-PCR analysis for changes in the levels of CeY (tan), sbRNAs (gray), and
rop-1 mRNA (green) under oxidative stress [7.5 mM tert-butyldihydroperoxide (t-BOOH)] [(C), n=4 except for CeN74-2 (n=3), CeN75 (n=3), CeN76 (n=3), and CeN77 (n=3)] and thermal stress (33°C) conditions [(D), n=4]. Red dotted lines indicate relative RNA levels in control conditions, which are normalized to one. Inset scatter plots show relative RNA levels of CeY (dotted box). [We noticed that a part of our data in panel D are not consistent with a previous report (Deng et al. 2006). By using northern blot analysis, Deng et al. reported that the levels of several sbRNAs, including CeN71, CeN72, CeN73-2, CeN74-1, CeN76, and CeN77, were increased with heat shock (30°C) for 3 hours. In contrast, we treated worms with 33°C for 30 minutes, which may have caused the difference.] n: the number of biological repeats. P values were calculated by using two-tailed Student’s t test (*P < 0.05, **P < 0.01). (E) A schematic showing the regions altered by yrn-1(yh84) and yrn-1(yh85) mutant alleles generated by CRISPR/Cas9-mediated genome editing. yrn-1(yh84) caused 35 base pair (bp) indel mutation (2 bp deletion and 37 bp insertion) at 4 bp upstream of yrn-1. yrn-1(yh85) caused one bp deletion at the 5‘ end of yrn-1, 51 bp deletion spanning 45 bp at the 3‘ end and 6 bp immediately downstream of yrn-1, and 2 bp insertion immediately downstream of the 45 bp deletion site. See Methods for sequence information of yrn-1(yh84) and yrn-1(yh85) mutations. (F and G) qRT-PCR analysis for the effects of yrn-1(yh84) (n=4), yrn-1(yh85) (n=2), rop-1(pk93) (n=4), and rop-1(ok2690) (n=3) on the levels of CeY (F) and rop-1 mRNA (G). Levels of CeY and rop-1 mRNA in wild-type are normalized to one. P values were calculated by using two-tailed Student’s t test (*P < 0.05, **P < 0.001). (H-K) Pooled lifespan curves of wild-type (WT) and yrn-1(yh84) (H), yrn-1(yh85) (I), rop-1(pk93) (J), and rop-1(ok2690) (K) mutant animals treated with control RNAi or daf-2 RNAi. (L) Percent animals that displayed vulval rupture phenotypes and that were subsequently censored during lifespan assays [panels H to K, n (trial number) =2]. P values were calculated by using two-tailed Student’s t test (*P < 0.05, **P < 0.01). (M and N) Statistical analysis of individual replicates of lifespan assays [panels H to K]. All percent lifespan changes and P values were calculated against WT animals within each control or daf-2 RNAi-treated groups otherwise noted with superscripts. WT: percent lifespan change or P value against WT in a control RNAi condition within replicate. P values were calculated using Mantel-Cox log-rank test. s.e.m. indicates the standard error of mean.

Description

Various non-coding RNAs (ncRNAs) change their levels during aging, and influence lifespan in many species, including Caenorhabditis elegans (Garg and Cohen 2014; He et al. 2018; Kim and Lee 2019, Kim et al. 2021). However, the role of Y RNAs, ncRNAs that regulate DNA replication and RNA quality control (Kowalski and Krude 2015), in aging and lifespan remains unknown. C. elegans genome encodes 19 Y RNA homologs, C. elegans Y RNA (CeY) encoded by yrn-1 and 18 stem-bulge RNAs (sbRNAs), each of which contains a conserved stem-loop structure (Fig. 1A) (Kowalski and Krude 2015). Y RNAs form a Ro60 ribonucleoprotein by binding Ro 60-kDa protein (Ro60), which is conserved from bacteria to human (Sim et al. 2020). ROP-1, the C. elegans Ro60, binds and stabilizes CeY (Van Horn et al. 1995; Labbé et al. 1999). rop-1 mRNA and ROP-1 protein levels increase during aging in C. elegans (Liang et al. 2014; Mansfeld et al. 2015; Angeles-Albores et al. 2017), suggesting the potential aging-regulatory functions of ROP-1 and its associated RNA, CeY. Here, we sought to determine the role of the Y RNA homologs in aging and lifespan.

We first tested whether the levels of C. elegans Y RNA homologs changed during aging by performing real-time quantitative reverse transcription PCR (qRT-PCR) analysis. We found that the levels of the CeY and sbRNAs were generally increased during aging (Fig. 1B). In addition, we showed that the mRNA level of rop-1 was increased during aging (Fig. 1B), consistent with previous reports (Mansfeld et al. 2015; Angeles-Albores et al. 2017).

Increased stress resistance is associated with longevity in multiple species (Epel and Lithgow 2014, Park et al. 2017), and stress-responsive factors play crucial roles in aging (Son et al. 2019). We therefore measured the effect of oxidative or thermal stresses on the levels of C. elegans Y RNA homologs. We found that the level of CeY was decreased under both oxidative (Fig. 1C) and thermal stress conditions (Fig. 1D). We also showed that oxidative stress significantly increased the levels of four sbRNAs, Ce4, Ce5, CeN75, and CeN73-1 (Fig. 1C), whereas thermal stress did not (Fig. 1D; see Figure 1D legend for discussion). In contrast to the decrease in the level of CeY under these tested stress conditions, the mRNA level of rop-1 was increased by oxidative stress (Fig. 1C) but not by thermal stress (Fig. 1D). Thus, it seems likely that these two external stresses affect the expression of various C. elegans Y RNA homologs and rop-1 differently.

We then focused our functional analysis on CeY by using CRISPR/Cas9 gene targeting, because the potential functional redundancy of 18 sbRNAs may hinder characterizing the roles of individual sbRNAs in aging. We generated two mutant alleles, yh84 and yh85, in yrn-1 gene that encodes CeY (Fig. 1E). We showed that yh84 or yh85 mutation substantially reduced the level of CeY (Fig. 1F). In addition to these yrn-1 mutants, we characterized two different rop-1 mutant alleles, rop-1(pk93) and rop-1(ok2690), both of which substantially reduced the level of CeY and rop-1 mRNA (Fig. 1F, G) (Labbé et al. 1999). We then measured the lifespan of these mutants, and found that yrn-1(yh84) or yrn-1(yh85) mutation did not affect the lifespan of wild-type animals at 20°C (Fig. 1H, I, M, N). Reducing insulin/IGF-1 signaling via inhibition of daf-2/insulin/IGF-1 receptor doubles lifespan (Kenyon et al. 1993), and is one of the most studied longevity-promoting interventions in C. elegans...
A major function of insulin/IGF-1 signaling in *C. elegans* is to regulate the proper formation of dauer, an alternative hibernation-like developmental stage (Hu 2007). *rop-1(pk93)* mutations decrease dauer formation in a wild-type background, and enhance or suppress the increased dauer formation by *daf-2(-)* mutants in an allele-specific manner (Labbé et al. 2000). These findings raise the possibility that *rop-1* and/or CeY affects physiological processes regulated by insulin/IGF-1 signaling. However, *yrn-1(yh84)* or *yrn-1(yh85)* mutation had little effect on the lifespan of *daf-2* RNAi-treated worms (Fig. 1H, I, M, N). Therefore, CeY appears to be dispensable for maintaining normal lifespan and promoting longevity by reduced insulin/IGF-1 signaling. We also found that *rop-1(pk93)* mutations decreased the lifespan of both control RNAi- and *daf-2* RNAi-treated worms (Fig. 1J, M, N), whereas *rop-1(ok2690)* mutation did not (Fig. 1K, M, N). A previous study reported that *rop-1(pk93)* mutations do not affect lifespan (Labbé et al. 1999). We found that *rop-1(pk93)* mutations substantially increased vulval rupturing during adulthood (Fig. 1L), which reduces lifespan (Leiser et al. 2016). Thus, mutations in *rop-1* do not appear to directly affect aging per se but may cause early deaths during adulthood by increasing vulval rupture phenotypes in our experimental conditions. Together, we did not find apparent lifespan phenotypes in *yrn-1* or *rop-1* mutants, both of which displayed substantially reduced CeY levels.

Our study points to the possibility that CeY may not directly participate in the regulation of lifespan. CeY alone may not be functionally equivalent to the vertebrate Y RNA, as CeY does not affect DNA replication, likely because of the lack of conserved motif in the upper stem (Gardiner et al. 2009; Boria et al. 2010; Kowalski et al. 2015). In contrast, sRNAs contain a conserved UG-CA motif (Fig. 1A) and participate in DNA replication (Kowalski et al. 2015). Thus, it will be important to test whether the inhibition of sRNAs affects lifespan. In particular, investigating the roles of four oxidative stress-induced sRNAs, Ce4, Ce5, CeN75, and CeN73-1, which were also age-dependently upregulated, in aging and lifespan will be crucial for future research.

**Methods**

**qRT-PCR analysis.** qRT-PCR analysis was performed as previously described (Park et al. 2020). For qRT-PCR analysis using aged worms, wild-type (N2) animals were synchronized at L1 stage by embryo bleaching (Stiernagle 2006) followed by incubation in S-basal medium at 20°C with a gentle rotation for 16 h. Approximately 4,800 to 9,000 synchronized L1 animals were placed and cultured on nematode growth medium (NGM) plates seeded with *E. coli* OP50. L4 larvae were then treated with 50 µM 5-fluoro-2′-deoxyuridine (FUDR; Sigma-Aldrich, MO, USA) to prevent progeny from hatching. Fertile adult animals at days 1, 4, 7, or 11 were harvested and used for total RNA extraction. Total RNA was extracted by using RNAiso plus (Takara, Shiga, Japan), and cDNA was synthesized by using ImProm-II Reverse Transcriptase (Promega, WI, USA) following manufacturer’s instruction. Real-time quantitative PCR was performed using Power SYBR Green PCR master mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and StepOne real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Relative quantity was calculated by comparative C_T method using *pmp-3* and small nucleolar RNA sn2343 as endogenous controls for *mRNA* and CeY/sbRNAs, respectively. *P* values were calculated by two-tailed Student’s *t* test. For qRT-PCR analysis using animals under oxidative or thermal stress conditions, approximately 5,600 stage-synchronized wild-type animals were grown on NGM plates seeded with OP50 at 20°C until they reached L4 stage. The L4 larvae were then exposed to 7.5 mM tert-butyl hydroperoxide (t-BOOH) for 4 h for eliciting oxidative stress (Goh et al. 2018) or placed in a 33°C incubator for 30 min for thermal stress (Brunquell et al. 2016). The animals were then harvested and washed three times with M9 buffer, and stored at -80°C before extracting total RNA for qRT-PCR analysis.

**CRISPR/Cas9 genome editing.** *yrn-1* mutants were generated by using CRISPR/Cas9 genome editing methods as previously described with modifications (Ghanta and Mello 2020). Day 1 adult N2 animals were injected with mixture of Alt-R CRISPR-Cas9 tracrRNA (15 µM; Integrated DNA Technologies (IDT), Coralville, IA, USA), Alt-R CRISPR-Cas9 crRNAs [dpy-10 crRNA and two different *yrn-1* crRNAs (5 µM each); IDT], and Alt-R S. pyogenes HiFi Cas9 Nuclease V3 (0.5 µg/µl; IDT). Individual animals were then placed on different plates, and allowed to lay eggs. F_1 progeny that displayed dumpy and/or roller phenotypes were individually transferred to new plates and subsequently genotyped using PCR to identify *yrn-1* mutations. *yrn-1* mutations were confirmed with Sanger sequencing (Solgent, Daejeon, South Korea). The sequences of the mutant alleles generated here are listed below. Capital letters indicate *yrn-1*, and lowercase letters indicate upstream and downstream sequences of *yrn-1*. 

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Wild-type: ttaaacatttGGGCTCGGTCCGAGTTTCATGGTCTCCAATGTGTGTGTGTGTGGTTTTCTTTAGGAACCCGGTTCAACCTCATCTTGAACCTTACTTTTGAACGCCTCTtttttgttgattt

yrn-1(yh84): ttaataggagaataagacaataagagaataattttGGGCTCGGTCCGAGTTTCATGGTCTCCAATGTGTGTGTGTGTGTGTTTTCTTTAGGAACCTCGGTTCCAACCTCATCTTGAAACTACTTTGACCGCTCCttttggattt

yrn-1(yh85): ttaaacatttGGGCTCGGTCCGAGTTTCATGGTCTCCAATGTGTGTGTGTGTGTGTTTTCTTTAGGAACCTCGGTTCCAACCTCATCTTGAAACTACTTTGACCGCTCCttttggattt

Lifespan analysis. Lifespan assays were performed as previously described with some modifications (Park et al. 2021). Plates for feeding RNAi induction were prepared by growing HT115 RNAi bacteria containing empty vector (pAD12; control RNAi) or daf-2 double-stranded RNA-expressing vector (pAD48; daf-2 RNAi) on NGM plates containing ampicillin (50 μg/ml; USB, Santa Clara, CA, USA) and isopropyl-β-D-thiogalactoside (1 mM; Gold Biotechnology, St. Louis, MO, USA) at 37°C. Stage-synchronized wild-type, outcrossed yrn-1(yh84) and yrn-1(yh85) mutants, and rop-1(pk93) and rop-1(ok2690) mutants were placed on the plates and fed with control or daf-2 RNAi bacteria at 20°C until reaching a prefertile adult stage. The animals were then transferred to new plates containing 5 μM FUDR. Animals that did not respond to a gentle touch using a platinum wire were counted as dead. Animals that ruptured, bagged, crawled off or burrowed the plates were censored but included in the statistical analysis. Lifespan assays were performed as duplicates by two independent researchers double-blindly. The lifespan data from two replicates were pooled to generate lifespan curves shown in Fig. 1H-K, but the statistical analysis was performed separately and shown in Fig. 1M and 1N. Statistical analysis of lifespan data was performed using OASIS2 (http://sbi.postech.ac.kr/oasis2) (Han et al. 2016), and P values were calculated by Log-rank (Mantel-Cox) test.

Reagents

Following C. elegans strains were used in this study: wild-type Bristol N2, IJ1991 yrn-1(yh84) IV, IJ1992 yrn-1(yh85) IV, IJ1832 yrn-1(yh84) IV (IJ1991 outcrossed 4 times to Lee laboratory N2), IJ1851 yrn-1(yh85) IV (IJ1992 outcrossed 4 times to Lee laboratory N2), MQ470 rop-1(pk93) V, RB2032 rop-1(ok2690) V, IJ1876 rop-1(pk93) V (MQ470 outcrossed 4 times to Lee laboratory N2), IJ1887 rop-1(ok2690) V (RB2032 outcrossed 4 times to Lee laboratory N2). MQ470 and RB2032 were provided by CGC. HT115 RNAi bacteria containing pAD12 and pAD48, gifts from Cynthia Kenyon (Addgene plasmid # 34832 and #34834), were used as control RNAi and daf-2 RNAi bacteria, respectively (Dillin et al. 2002).

Following Alt-R CRISPR-Cas9 crRNAs were obtained from Integrated DNA Technologies (IDT, Coralville, IA, USA) and used for generating yrn-1(yh84) and yrn-1(yh85) mutants:

dpy-10 crRNA (Ce.Cas9.DPY-10.AQ): GCUACCAUGGCACCACGAGGUUUUAGAGCUAUGCU

yrn-1 crRNA-5' #1: UUAGUUUAAUGUUUAAACAUUUGUUUAGACCUAUGCU (for generating yh84)

yrn-1 crRNA-3' #1: ACUACUUUUGCCGUCCUUUGUUUAGACCUAUGCU (for generating yh84)

yrn-1 crRNA-5' #2: UUAGUUUAAACAUUUGGGCCUUUUGUUUAGACCUAUGCU (for generating yh85)

yrn-1 crRNA-3' #2: ACUCGGAAAUCCCCAAGGAGGUUUUAGACCUAUGCU (for generating yh85)

Following primers were used for qRT-PCR analysis:
pmp-3 forward: GTTCCTGTTGCTATCATCTCAT

pmp-3 reverse: ACACCGTCGAGGAAAGCCTGTAAGA

sn2343 forward: CCGCCTGTGATGTTCTATTAG

sn2343 reverse: CGGCTCAGCCTTTTCCAAG

CeN71 forward: GAATCCGTGTGCTTCAAGTC

CeN71 reverse: AATTCCGTTGCTGTGTTGATG

CeN72 forward: ATCATCGGTCTGGGTGTTGATG

CeN72 reverse: AACATCGGTCAATTTGTTGATG

CeN73-1 forward: GAGAGTGTGAGGTTACCAGATTA

CeN73-1 reverse: GGTGTGATGGGGAAGAGG

CeN73-2 forward: AGTGTGATGGTTACCAGTC

CeN73-2 reverse: GGTGTGATGGGAGAAGT

CeN74-1 forward: GCCGTCACTGGGTATTACGT

CeN74-1 reverse: ACACCAAGGCAAGT

CeN74-2 forward: TCTAAAATAAATATATTTTATAACGTCAGTGGTTATCAAG

CeN74-2 reverse: AGCAACGCTCAAGT

CeN75 forward: TACCGTCCGGAGGTCGTTG
CeN75 reverse: TTTTCATCGGTCAAGTTGGTGTC
CeN76 forward: AGACAGGCTGGTCCGG
CeN76 reverse: CAGGAACGGTCAAGTTGGTG
CeN77 forward: AATTCGGTCCGGAGTCAATG
CeN77 reverse: AACGCCGGTCAAGTTGTTG
CeN133 forward: ATCGGTCCGAAGTTGATGGG
CeN133 reverse: ATCAGGCAATGGTCGGAG
CeN135 forward: ATCAGGCAATGGTCGTCAAGTTGGTG
CeN135 reverse: AACAGGAAGTCAAGTTGTTGCC
Ce1 forward: CGACCACCGGTCCGGAG
Ce1 reverse: ACCCCCGGTCAAGGGATG
Ce2 forward: CTCGTCGCGGGTCAGTG
Ce2 reverse: ATCGGTCCGGCGTCAGTG
yrn-1 forward: TCGGTCCGAGTTTCATGGTC
yrn-1 reverse #1: AGCGGTCAAAGTAGTTTCAAGGT (for Fig. 1B-D)
yrn-1 reverse #2: GTTCCTAAAGAAAACACACACAC (for Fig. 1F)
rop-1 forward #1: ATGAGACTCTCTAACGTCTGC (for Fig. 1B-D)
rop-1 reverse #1: GACTTTCTCCATTTGACGAGG (for Fig. 1B-D)
rop-1 forward #2: ACTACGTTAACAACCTTGATTTCG (for Fig. 1G)
rop-1 reverse #2: CAGCCCAGGTATCGTTGTC (for Fig. 1G)

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