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SHORT TAKE

Reduced expression of frataxin extends the lifespan of *Caenorhabditis elegans*

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Summary

Defects in the expression of the mitochondrial protein frataxin cause Friedreich's ataxia, an hereditary neurodegenerative syndrome characterized by progressive ataxia and associated with reduced life expectancy in humans. Homozygous inactivation of the frataxin gene results in embryonic lethality in mice, suggesting that frataxin is required for organismic survival. Intriguingly, the inactivation of many mitochondrial genes in the nematode *Caenorhabditis elegans* by RNAi extends lifespan. We therefore investigated whether inactivation of frataxin by RNAi-mediated suppression of the frataxin homolog gene (*frh-1*) would also prolong lifespan in the nematode. Frataxin-deficient animals have a small body size, reduced fertility and altered responses to oxidative stress. Importantly, frataxin suppression by RNAi significantly extends lifespan in *C. elegans*.

Key words: aging; Frataxin; Friedreich's Ataxia; mitochondria.

Defective frataxin expression in humans causes Friedreich's ataxia, the most common of inherited ataxias, with an estimated prevalence of ~1 : 40 000 and an estimated carrier rate of ~1 : 90. The disease is characterized by gait and limb ataxia, dysartria, high incidence of diabetes, cardiomyopathy and premature death. Symptoms directly correlate with the extent of decrease in the levels of the frataxin protein and are mainly ascribed to alterations in mitochondrial metabolism (Pandolfo, 2001; Puccio & Koenig, 2002). In the nematode *Caenorhabditis elegans*, inhibition of a number of mitochondrial genes increases lifespan (Feng *et al*., 2001; Dillin *et al*., 2002; Lee *et al*., 2003; Rea & Johnson, 2003), suggesting that mitochondrial metabolism may affect aging. To investigate the possible role of frataxin in controlling lifespan, we selectively interfered with the expression of the *C. elegans* frataxin ortholog (*frh-1*), and found that reducing the expression of frataxin increases longevity in nematodes.

We examined the progeny of wild-type (N2) animals injected with either double-stranded RNA to *frh-1* (*frh-1* RNAi) or control RNA (pTRI-Xref, Ambion Inc, *Xenopus laevis* elongation factor 1), each at ~1 mg mL^{-1}. *frh-1* RNAi animals were viable and fertile, yet small and pale compared with control RNAi animals (Fig. 1A). *frh-1* RNAi animals laid eggs at a much slower rate and had reduced total brood sizes compared with control RNAi animals (control RNAi 312 ± 34 vs. *frh-1* RNAi 212 ± 64). Fertility was extended in *frh-1* RNAi animals; the last day of progeny production was day 17 for *frh-1* RNAi animals vs. day 8 for control RNAi animals (Fig. 1B). Larval development was largely unaffected, as both control and *frh-1* RNAi animals began producing progeny on the same day (day 3 at 20 °C). Importantly, inhibition of *frh-1* increased mean lifespan by about 25% (< 0.0001) (Fig. 1C) over three replications. To investigate whether the suppression of *frh-1* conferred resistance to oxidative stress, animals were exposed for 8 h to 10 mM of hydrogen peroxide and for 2 h to 250 μM juglone, a superoxide generator (de Castro *et al*., 2004). We found that *frh-1* RNAi animals were relatively resistant to hydrogen peroxide, but were more sensitive to juglone, as compared with control RNAi animals (Fig. 1D).

Injection of RNAi constructs is labor intensive; to further investigate the role of *frh-1* in aging, we generated a large population of *frh-1* RNAi animals by feeding the N2 strain with HT115, a competent *Escherichia coli* strain, transformed with either empty L4440 vector or an *frh-1* RNAi construct (Fraser *et al*., 2000), so that *frh-1* expression could be quantitated. In the initial generation of worms, exposed to these bacteria from hatching, there was little effect, but after two generations of ingesting these bacteria, worms displayed a phenotype almost identical with the injected animals (pale and smaller body, reduced egg laying) and lifespan was extended by ~25% (Fig. 2A). In these animals, quantitative RT-PCR analysis indicated that *frh-1* expression was reduced 30–70% (Fig. 2B). Finally, we obtained a genomic knock-out (KO): *frh-1*(ok610) in strain VC389 (genotype: *frh-1*(*ok610*)/*mIn1*[*mIs14 dpy-10*(*e128*)]II). VC389 heterozygotes are wild-type (WT) with semidominant green fluorescent protein (GFP) expression in the pharynx. They segregate WT GFP heterozygotes, Dpy GFP homozygotes and GFP-negative ok610 homozygotes (*frh-1* KO). We isolated GFP-negative animals and confirmed them to be the *frh-1* KO

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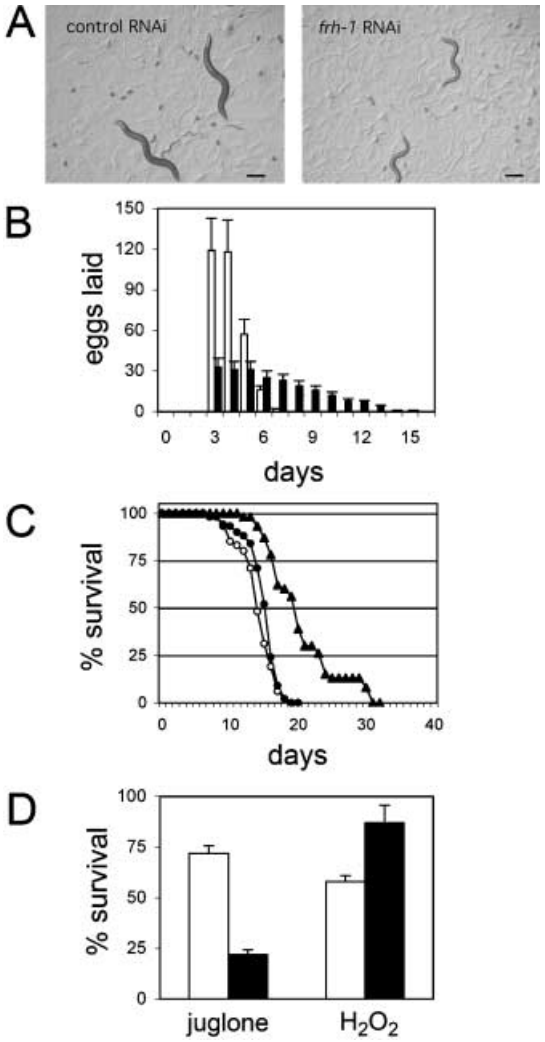

Fig. 1 (A) Photomicrographs of worms injected with *frh-1* RNAi. Five-day-old terminal-size adult animals injected with control dsRNA or *frh-1* dsRNA. Scale bars = 100 μm. (B) Histogram showing the number of eggs laid per day by control RNAi animals (open bars) and *frh-1* RNAi animals (closed bars). Egg lay values are means ± 1 SD from five animals at 20 °C. Egg lay is the number of eggs laid per day during the fertile period, and brood size is the total number of offspring. (C) Survival plot of injected animals. Lifespan was investigated at 25 °C. Open circles, uninjected animals; closed circles, control RNAi animals; closed triangles, *frh-1* RNAi animals. One representative survival plot is shown. Mean lifespan for *frh-1* RNAi animals was 20.6 ± 1.0 days (140 animals) vs. 16.5 ± 0.4 days (139 animals) for control RNAi animals (P = 0.00017). Mean lifespan of uninjected animals (16.1 ± 0.8 days, 126 animals) is similar to that of control RNAi animals (P = 0.376). Each survival analysis was replicated at least three times. Lifespan is defined as the time elapsed from when eggs hatched to when worms were scored as dead. Animals were scored as dead if no pharyngeal pumping was evident and they failed to respond to repeated gentle prodding. Statistical analyses were carried out using Statsoft Statistica 5.0 software package (Statistica for Windows, StatSoft, Inc., Tulsa, OH, USA). (D) Sensitivity to oxidative stress. Control RNAi and *frh-1* RNAi animals were exposed to 10 mM hydrogen peroxide or 250 μM juglone. One representative experiment, in which survival was assessed after 8 and 2 h, respectively, from the beginning of treatment, is shown; six were performed with similar results. Means of duplicates ± 1 SD are shown.

by PCR (data not shown). These animals arrested at the L2/L3 stage, and had an extended lifespan in this stage (data not shown), similar to other mitochondrial mutations (Tsang *et al.*, 2001).

Frataxin is required for the proper assembly of the Fe–S cluster, which is necessary for proper function of key components of the electron transport chain (ETC) (Huynen *et al.*, 2001; Muhlenhoff *et al.*, 2002; Tan *et al.*, 2003); thus, reducing the level of frataxin may cause defective ETC function. In *C. elegans,* preventing the synthesis of major subunits of the ETC, during development by feeding RNAi, results in animals that can live significantly longer than their wild-type controls, but are generally smaller than normal, have reduced fertility and a slow phenotype (Dillin *et al.*, 2002; Lee *et al.*, 2003). Moreover, worms with defects in ETC components have been reported to display lower oxygen consumption and ATP production and altered responses to oxidative stress (Feng *et al.*, 2001; Dillin *et al.*, 2002; Lee *et al.*, 2003). Different phenotypes, including uncoordinated movement, slow growth, a thin body and limited embryonic lethality, have been reported in other *frh-1* RNAi experiments (http://www.wormbase.org).

We report, for the first time, information about the longevity of *frh-1* RNAi *C. elegans*. Similar to other reports on RNAi of ETC components in the worm, RNAi treatment to reduce *frh-1* results in increased longevity. In these animals frataxin expression is not completely eliminated, as is similarly observed in Friedreich's ataxia patients. Total loss of frataxin appears to have a more dramatic effect on development, because *frh-1* KO animals show an arrested phenotype, and homozygous gene inactivation of frataxin is lethal in embryonic mice (Cossee *et al.*, 2000). Nevertheless, assuming that human frataxin and *C. elegans* frataxin have similar functions, as suggested by the remarkable sequence conservation of frataxins across phyla and by genetic complementation in yeast (Cavadini *et al.*, 2000; Cho *et al.*, 2000), our data highlight a fundamental difference in frataxin requirement between mammals and nematodes and suggest differences in the determination of lifespan. The possibility of utilizing alternative metabolic pathways under anaerobic or semi-anaerobic conditions, such as fermentative malate dismutation, may allow ETC-defective nematodes to survive and actually live longer because of the lower production of reactive oxygen species (ROS) due to reduced oxidative phosphorylation (Rea & Johnson, 2003). Humans and other mammals are unable to 'reverse' the TCA cycle productively; in the presence of ETC defects, such as in Friedreich's ataxia, mammalian cells 'force' the defective respiratory chain in an attempt to generate more ATP. This is inevitably associated with higher ROS production and cellular damage. Interestingly, whereas YFH1-defective yeast and Friedreich's ataxia cells display higher sensitivity to hydrogen peroxide (Foury & Cazzalini, 1997; Wong *et al.*, 1999), *frh-1* RNAi worms are more resistant to hydrogen peroxide, similar to the *lrs-1* mitochondrial mutants of *C. elegans* (Lee *et al.*, 2003). Preliminary evidence also indicates that *frh-1* RNAi animals are relatively resistant to iron toxicity, given that chronic iron exposure does not shorten their lifespan compared with the wild-type. By contrast, *frh-1* RNAi worms appear relatively sensitive to the superoxide-generator juglone. Although the action of juglone on YFH1-defective yeast or Friedreich's ataxia cells has not been reported, the latter are known to be sensitive

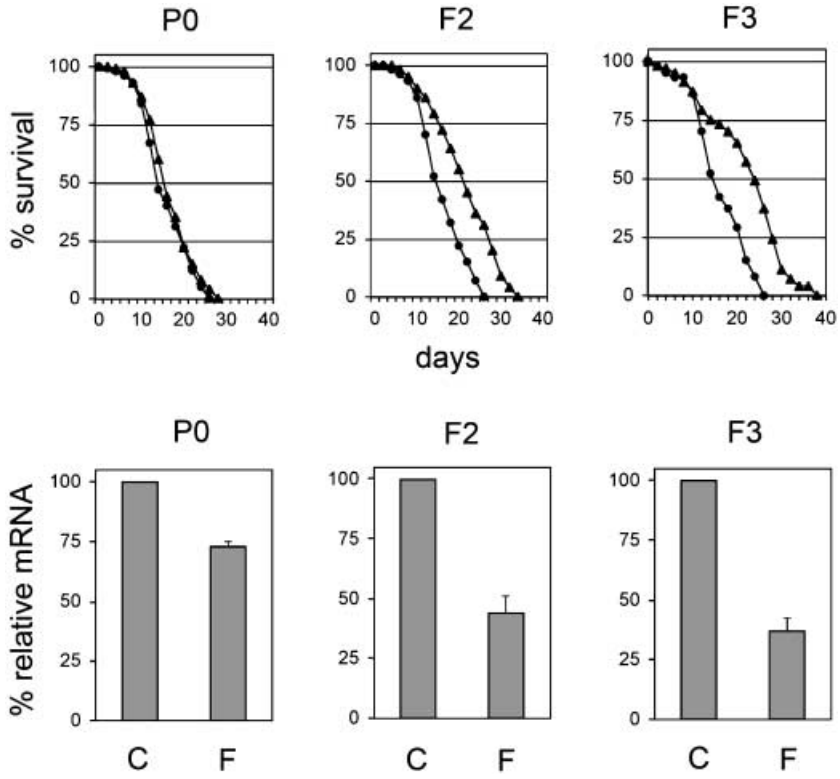

Fig. 2 (A) Survival plot of animals fed *frh-1* RNAi at 20 °C. A starting generation (P0) of wild-type N2 animals was placed, as eggs, on frataxin (triangles) or empty vector (circles) bacterial RNAi feeding lawns and maintained for three consecutive generations. Representative survival plots for synchronized populations of three of these generations are shown (P0, F2 and F3). RNAi feeding bacteria and treatment methods were as described (Fraser *et al*., 2000). Mean lifespan for *frh-1* RNAi-treated animals vs. control-treated animals was 14.6 ± 0.5 days (n = 91) vs. 13.6 ± 0.6 days (n = 88; P < 0.157), 18.8 ± 0.8 days vs. 13.8 ± 0.3 days (n = 105; P < 0.0001) and 21.9 ± 0.8 days (n = 119) vs. 16.2 ± 0.5 days (n = 119; P < 0.00001), for the P0, F2 and F3 generations, respectively. Each survival analysis was performed in duplicate. (B) Quantitation of frataxin mRNA levels in animals fed *frh-1* RNAi. Quantitative reverse transcription-PCR (Q-PCR) was used to assess the degree of loss of *frh-1* mRNA in the three generations shown in A. Bars indicate the relative amount of frataxin mRNA detected in animals fed either the *frh-1* RNAi (F) or empty vector control (C). It is apparent that with each successive generation, lower levels of frataxin mRNA are observed. Total RNA was extracted from 5000 young adult worms (Rneasy, Invitrogen). Oligo(T) primed cDNA was synthesized using Superscript reverse transcriptase, according to the manufacturer's instructions (Invitrogen). Gene-specific primers against *frh-1* and a non-variable control gene (*F23B2.13*) were designed using Primer Express (ABI Prism). SYBR green-based Q-PCR was undertaken on an ABI Prism 7000 instrument (Applied Biosystems). Data were analysed according to the manufacturer's instructions. All determinations were replicated at least three times.

to paraquat (Bradley *et al*., 2004), a free-radical generator mechanistically similar to juglone.

The molecular pathways that account for the different oxidative stress responses between frataxin-deficient mammals and nematodes clearly require further investigation. Mitochondria from oxygen-dependent organisms may have a more limited degree of flexibility in regulating metabolic rates and hence less effect on lifespan and other aspects of the aging processes. Work linking reduced mitochondrial function with slowed aging in invertebrates may therefore not transfer directly to mammals. Nevertheless, mitochondrial efficiency can play a general role in the specification of lifespan and aging.

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