

# Impaired respiration is positively correlated with decreased life span in *Caenorhabditis elegans* models of Friedreich Ataxia

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**ABSTRACT** Impaired expression of mitochondrial genes causes alterations in life span of the nematode *Caenorhabditis elegans*. Intriguingly, although some of these genes have been shown to extend life expectancy and reduce aging processes, others are known to shorten life span in the same model organism. Reduced expression of a mitochondrial protein called frataxin causes a neurodegenerative disorder named Friedreich Ataxia, which decreases life span in humans. Surprisingly, reduced expression of the *C. elegans* frataxin homologue *frh-1* has been associated with both increased as well as decreased life span by different laboratories. To further elucidate these conflicting findings, here we show that different RNA interference (RNAi) constructs directed against *frh-1* reduce *C. elegans* life span. Moreover, we show that *frh-1*-inhibiting RNAi impairs oxygen consumption and that respiratory rate is positively correlated with life span in this multicellular eukaryote ( $r=0.8566$ ), suggesting that >73% of life span variance in *C. elegans* is explained by changes in respiratory rate. Taken together, impaired mitochondrial metabolism due to RNAi-mediated inhibition of the frataxin homologue *frh-1* causes both impaired respiration as well as decreased life span in the multicellular eukaryote *C. elegans*.—Zarse, K., Schulz, T. J., Birringer, M., Ristow, M. Impaired respiration is positively correlated with decreased life span in *Caenorhabditis elegans* models of Friedreich Ataxia. *FASEB J.* 21, 1271–1275 (2007)

**Key words:** mitochondria • oxygen • frataxin • hormesis

FRIEDREICH ATAXIA IS AN INHERITED neurodegenerative disorder (1) caused by reduced expression of the mitochondrial protein frataxin, leading to premature death due to cardiac failure, diabetes mellitus, and insulin resistance, as well as impaired ATP synthesis in muscle of humans (2). Concurrently, it was shown that frataxin overexpression promotes oxidative phosphorylation and hence ATP synthesis (3). Although the primary function of frataxin is still a matter of debate (4), increasing evidence suggests that this protein directs the intramitochondrial synthesis of iron-sulfur clusters (5). Individuals suffering from Friedreich

Ataxia have a reduced life expectancy of 38 yr in average (1).

Meanwhile, several *Caenorhabditis elegans* models for Friedreich Ataxia have been published. Surprisingly, reduced expression of the *C. elegans* frataxin homologue *frh-1* has been associated with increased life span by Ventura *et al.* (6), while Vazquez-Manrique *et al.* (7) found a significant decrease in life span after impairment of *frh-1* expression. These conflicting findings have initiated discussions regarding experimental issues in determination of *C. elegans* life span in states of reduced *frh-1* expression (8), whereas the opposite effects described by two different laboratories nevertheless require further elucidation.

Primarily, we here show that different RNA interference (RNAi) constructs directed against *frh-1* reduce *C. elegans* life span, supported by findings by Vazquez-Manrique *et al.*, and reflecting the phenotype of human frataxin deficiency. Moreover, we show that RNAi constructs directed against *frh-1* reduce oxygen consumption in *C. elegans*, suggesting that life span can be positively correlated with mitochondrial respiration in a multicellular eukaryote.

## MATERIALS AND METHODS

### Plasmids and RNAi constructs

Fragments used for RNAi constructs were obtained by polymerase chain reaction (PCR) from *C. elegans* genomic DNA (gDNA) that was prepared using standard methods (9). PCR products as well as the L4440 feeding vector (pPD129.36, obtained from AddGene, Boston, MA, USA; ref 10) were digested with *Sma*I (Roche, Basel, Switzerland), and fragments were cloned into the vector by blunt end ligation following standard procedures (11). The resulting plasmids were transformed into the HT115 (DE3; ref 12) *E. coli* strain. The following primer pairs were used for PCR amplification: *frh-1-A* (5'-TCC CCC GGG GCT CAA ATA CCA CAT AAT

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doi: 10.1096/fj.06-6994com

TTC GC-3' and 5'-TCC CCC GGG AAT CCG TCA GCA CTT TTT GAT AA-3'), *frh-1-B* (5'-TCC CCC GGG ATG CTC TCC ACT ATT CTA CG-3' and 5'-TCC CCC GGG TTA GAC ATG TCG CGA GAA ATC-3'), and *frh-1-C* (5'-TCC CCC GGG GAA CGT ATG TTA TTA ACA AAC AA-3' and 5'-TCC CCC GGG TTT CCC TCC TCT TCT AAA TC-3'). *C. elegans* RNAi by HT115 feeding was performed exactly as described previously (13).

### Life span assays and maintenance of *C. elegans*

The wild-type (WT) strain used for all experiments was Bristol N2. Nematodes were grown and maintained on NGM agar plates as described previously (14) except for *E. coli* HT115, which were used as a nutrient source. All life span experiments were performed at 20°C and in the absence of 5-fluoro-2'-deoxyuridine.

Life span analysis was carried out according to standard protocols (15). Briefly, to obtain an age-synchronously growing population, eggs were prepared by treating a population of *C. elegans* with hypochlorite/NaOH solution and transferring the resulting eggs to plates covered with the corresponding RNAi-bacteria, or control HT115 carrying the empty plasmid, respectively. As fertile young adults, ~150 nematodes were transferred to fresh plates, which also represents the first day of life span analysis. During progeny production period, nematodes were transferred to fresh plates daily and after that every second to third day but monitored daily for dead animals.

Animals that did not respond to gentle prodding and displayed no pharyngeal pumping were scored as dead. Animals that crawled off the plate or died due to internal hatching or due to protrusion of the gonads through the vulva were censored. Censoring describes an event where partial information on the life span of an individual animal is lost as a consequence of premature death. Thus, censored animals were included into statistical analysis only until the day of the censoring event.

### Oxygen consumption assays

Oxygen consumption rates were measured using a DW1/AD Clark-type oxygen electrode (Hansatech, Norfolk, England, GB) as described previously (16). Adult worms that were maintained on NGM agar plates containing 5-fluoro-2'-deoxyuridine (Sigma-Aldrich, St. Louis, MO, USA) were washed three times with M9-buffer and resuspended in 2 ml of M9-buffer. One milliliter aliquots were transferred into the chamber, and respiration was measured at 20°C for at least 10 min. Samples were carefully recovered from the chamber and homogenized in a dounce-type Homgen-Plus homogenizer (Schütt Labor Technik, Göttingen, Germany) for 30 s at 3000 rpm. After centrifugation at 8000 *g* for 5 min, the supernatant was removed and used for protein quantification by using the Bradford method (17).

### Statistical analyses

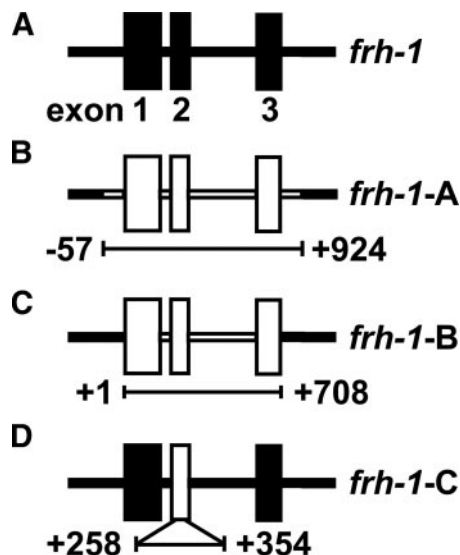
Statistical analyses for the oxygen consumption was performed by Student's *t* test. For comparing significant distributions between different groups in the life span assays, statistic calculations were carried out using the log-rank test. All calculations were performed with Statistical Packages for the Social Sciences (SPSS) Version 13.0 as described previously (16).

## RESULTS

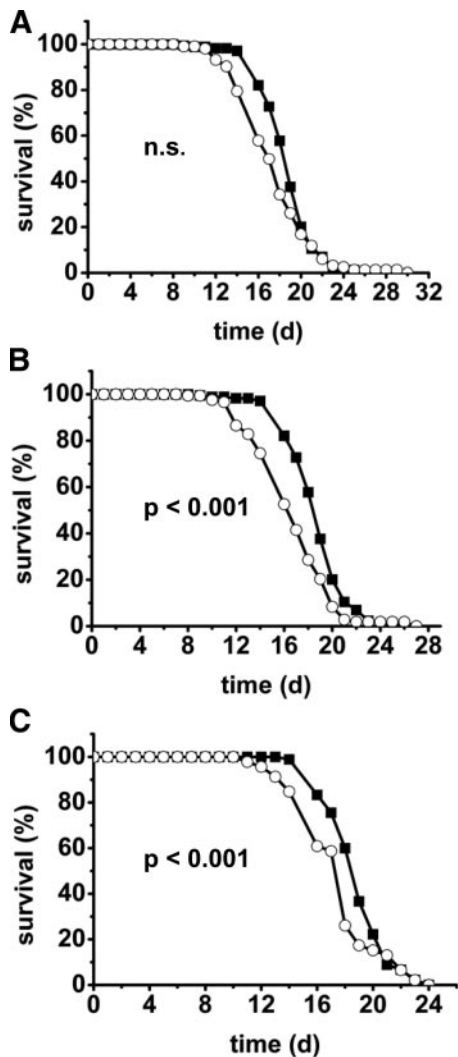
Given the apparent inconsistencies in previous publications regarding the effects of RNAi-based *frh-1* knock-down, we have used three different constructs to generate RNAi-vectors against *C. elegans frh-1* (Fig. 1). The genomic structure of *C. elegans frh-1* consists of three exons (Fig. 1A). Constructs for RNAi used for the following life span experiments were generated to cover all three exons including intermittent introns, the proximal promoter region, and the adjacent 3' end (construct *frh-1-A*, Fig. 1B), exactly all three exons including intermittent introns (construct *frh-1-B*, Fig. 1C), or exon 2 only (construct *frh-1-C*; Fig. 1D). It should be noted that exon 2 encodes major parts of the highly conserved region of human frataxin and its homologues, as previously shown (18).

With the use of these constructs to obtain HT115 *E. coli* producing the corresponding RNAi (see Materials and Methods), life span assays were performed in triplicate for each construct. Whereas construct *frh-1-A* did not significantly affect life span (Fig. 2A; Table 1), both other constructs *frh-1-B* and *frh-1-C* caused a significant reduction of mean life span (Fig. 2B, C; Table 1). The reason for a lack of significant effect of *frh-1-A* on life span might be due to a comparably high degree of variation in life span assays, in contrast to findings with the other two constructs. Maximum life span was not affected by neither RNAi construct (Table 1), possibly due to the fact that RNAi-treated nematodes appear to reduce food uptake and hence RNAi incorporation after long-term exposure to RNAi-containing bacteria, suggesting that efficiency of knock-down may decrease after long-term exposure to RNAi-containing bacteria.

Worms maintained on corresponding RNAi-producing bacteria (constructs *frh-1-A*, *frh-1-B*, and *frh-1-C*),



**Figure 1.** Genomic regions covered by RNAi constructs. A) Genomic structure of *C. elegans frh-1*. B, C, D) Corresponding regions covered (open rectangles) by 3 different RNAi constructs used for expression in *E. coli*.



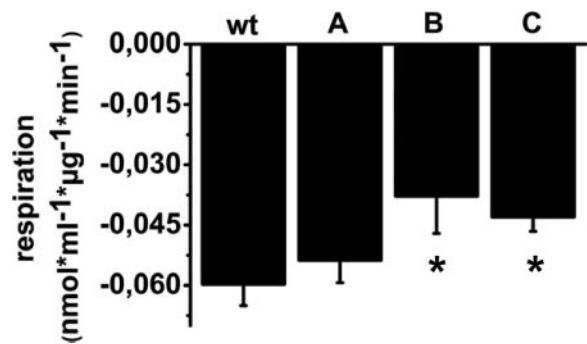
**Figure 2.** Life span assays based on three different *frh-1* RNAi constructs. A) Survival in *C. elegans* exposed to RNAi construct *frh-1-A*. B) Construct for *frh-1-B*. C) Construct for *frh-1-C*. Open circles = RNAi-treated worms; filled squares reflect control nematodes.

where then transferred into a Clark-type electrode to determine oxygen consumption for each knock-down as described in Materials and Methods. Whereas WT worms showed the highest rate of respiration (Fig. 3), knock-down worms had reduced oxygen consumption (Fig. 3). Reduction of respiration was significantly different from WT worms for constructs *frh-1-B* and *frh-1-C* only (Fig. 3).

TABLE 1. Table 1. Mean and maximum lifespan

	Mean Lifespan	Maximum Lifespan
Control	18.3175 ± 0.0845	25.0 ± 1.0
<i>frh-1-A</i>	16.606 ± 1.643	26.5 ± 3.5
<i>frh-1-B</i>	16.2785 ± 0.3385*	24.0 ± 3.0
<i>frh-1-C</i>	16.399 ± 0.44*	24.0 ± 0.5

\* $P < 0.05$ .

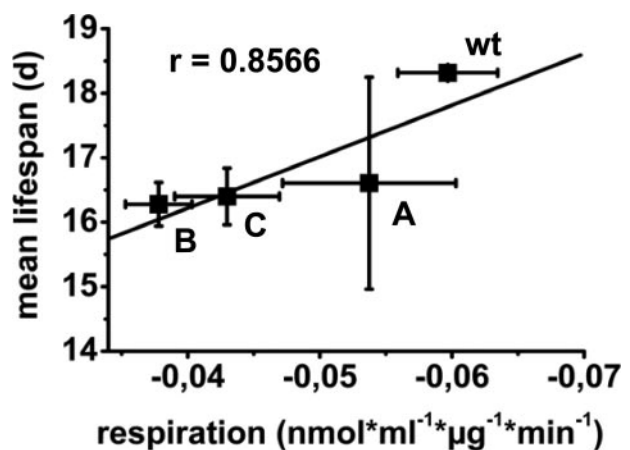


**Figure 3.** Oxygen consumption in *C. elegans* exposed to *frh-1* RNAi treatment. Respiration rates of *C. elegans* WT (bar wt) and after exposure to *frh-1-A* (bar A), *frh-1-B* (bar B) and *frh-1-C* (bar C). Error bars show SE means, stars indicate  $P < 0.05$  in comparison to WT worms.

Finally, and since significant differences were observed with regard to life span and respiration for the same two constructs, we asked whether a correlation between life span and respiratory rate might exist. We found a positive correlation between respiratory rate and mean life span (Fig. 4;  $r = 0.8566$ ). Since  $r^2$  accordingly equals 0.7338, these findings suggest that >73% of life span variation in this particular model might be contributed to changes in respiratory rate only, suggesting that mitochondrial respiration is a major determinant of life span.

## DISCUSSION

It has been known for several years that impaired expression of mitochondrial genes, as well as of nuclear genes encoding proteins targeted to the mitochondria, may cause alterations in life span of the nematode *C. elegans* (19). Intriguingly, while some of these genes have been shown to extend life expectancy and reduce



**Figure 4.** Correlation of respiration and life span in *C. elegans*. Respiration rate and mean life span are correlated in *C. elegans* exposed to different *frh-1* RNAi. Error bars = SE means. Letters = RNAi constructs as defined in Fig. 1.

aging processes, others are known to shorten life span in the same model organism.

Not surprisingly, the underlying hypotheses are conflicting: one line of evidence suggests that down-regulation of mitochondrial metabolism causes decreased formation of reactive oxygen species (ROS), a mandatory by-product of mitochondrial electron transfer (20, 21). This hypothesis is essentially a modernized version of the rate-of-living theory (22), which in later years was focused on detrimental effects of ROS by Harman (23). The other and conflicting line of evidence suggests that induction of mitochondrial metabolism might induce a positive response to increased formation of ROS and other stressors, leading to a secondary increase in stress defense following primary induction of stress, cumulating in reduced net stress levels (24–27). The process has been named hormesis (28). Whether it applies to processes extending life span is currently a matter of fierce debate (26).

Moreover, a potential role of increased respiration to extend life span in eukaryotes has been suggested for the unicellular eukaryote *S. cerevisiae* in states of caloric restriction (29), a known regimen to extend life span in eukaryotes including mammals (30, 31). Recent evidence has questioned the role of increased respiration in regards to *S. cerevisiae* (32). It should be noted though that these observations are conflicting, but mechanistically not at all mutually exclusive, since both pathways (sirtuin-activation *vs.* mTOR) may coexist independently of each other.

We here have shown that life span in the multicellular eukaryote *C. elegans* is positively correlated to respiratory activity. Since increased respiration may cause increased formation of ROS, we tentatively assume that decreased life span due to reduced levels of respiration reflects a reduction of hormetic responses to systemic stressors. This assumption is supported by findings in fibroblasts where frataxin was overexpressed: these cells show increased respiration and increased oxidative phosphorylation (3), while formation and accumulation of ROS in these cells are decreased due to induction of antioxidant defense capacity (33). Similar findings have been reported for shc66 (34), another stress response protein known to cause decreased life span in mice when subjected to targeted disruption (35). Accordingly, systemic disruption of frataxin causes embryonic lethality in mice (36), while hepatocyte-specific disruption leads to decreased life span and increased ROS formation in mice (16), where respiratory activity of hepatocytes is decreased (16). Lastly and most importantly, humans with decreased expression of frataxin, *i.e.*, suffering from Friedreich's Ataxia have a significantly reduced life span (1).

Given the conflicting results from different laboratories with regard to effects of impaired *frh-1* expression on life span in worms, it should be noted that we here essentially confirm the findings of Vazquez-Manrique *et al.* (7), while our findings are in conflict with findings from Ventura *et al.* (6), as well as, at least in part, with those of Lee *et al.* (19). Nevertheless, it has correctly

been stated that experimental differences in regards to application of RNAi (microinjection *vs.* HT115 feeding) and genomic regions covered by the RNAi constructs may have a significant influence on the phenotype observed (8). This may, at least in part, also explain contradictory findings of different mitochondrial impairments on *C. elegans* life span in general, *i.e.*, the fact that reduced expression of some mitochondrial proteins apparently extends life span while others reduce life expectancy.

Hence, although it remains to be shown whether reduction of mitochondrial respiration commonly causes a decrease in life expectancy, our unambiguous findings suggest that at least for states of reduced *frh-1* expression, *C. elegans* life span is positively correlated with respiration rate, suggesting a life-extending role of increased mitochondrial metabolism in specific eukaryotic model organisms. EJ

We thank the Caenorhabditis Genetics Center for providing strains used in this study. This study was funded by the Deutsche Forschungsgemeinschaft and the Wilhelm-Sander-Stiftung (both to M. Ristow).

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*Received for publication August 4, 2006.  
Accepted for publication October 31, 2006.*