

Autophagy induction extends lifespan and reduces lipid content in response to frataxin silencing in *C. elegans*

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ABSTRACT

Severe mitochondria deficiency leads to a number of devastating degenerative disorders, yet, mild mitochondrial dysfunction in different species, including the nematode *Caenorhabditis elegans*, can have pro-longevity effects. This apparent paradox indicates that cellular adaptation to partial mitochondrial stress can induce beneficial responses, but how this is achieved is largely unknown. Complete absence of frataxin, the mitochondrial protein defective in patients with Friedreich's ataxia, is lethal in *C. elegans*, while its partial deficiency extends animal lifespan in a p53 dependent manner.

In this paper we provide further insight into frataxin control of *C. elegans* longevity by showing that a substantial reduction of frataxin protein expression is required to extend lifespan, affect sensory neurons functionality, remodel lipid metabolism and trigger autophagy. We find that Beclin and p53 genes are required to induce autophagy and concurrently reduce lipid storages and extend animal lifespan in response to frataxin suppression. Reciprocally, frataxin expression modulates autophagy in the absence of p53. Human Friedreich ataxia-derived lymphoblasts also display increased autophagy, indicating an evolutionarily conserved response to reduced frataxin expression.

In sum, we demonstrate a causal connection between induction of autophagy and lifespan extension following reduced frataxin expression, thus providing the rationale for investigating autophagy in the pathogenesis and treatment of Friedreich's ataxia and possibly other human mitochondria-associated disorders.

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1. Introduction

Mutations in genes that directly or indirectly affect the functionality of the mitochondrial respiratory chain (MRC) lead to a variety of devastating disorders in humans (Wallace, 2005). Friedreich's ataxia (FRDA), the most frequently inherited recessive ataxia, is one such disease and it is ascribed to severe deficiency of frataxin, a nuclear-encoded mitochondrial protein involved in the biogenesis of iron-sulfur cluster (ISC) containing proteins (Campuzano et al., 1996; Puccio et al., 2001). Residual levels of frataxin are critical for survival and inversely

correlate with disease onset, progression and severity (McDaniel et al., 2001). Symptoms only appear when levels of frataxin are severely decreased, and non-pathological levels of frataxin deficiency are associated with alterations in gene expression profiles (Haugen et al., 2010; Huang et al., 2009). These observations suggest that animals attempt to cope with partial frataxin deficiency by inducing adaptive responses, which, if characterized, may reveal novel therapeutic strategies to prevent or postpone the established disease in humans.

In the nematode *Caenorhabditis elegans* (*C. elegans*), complete knock-out of the frataxin ortholog (*frh-1*) or severe deficiency of other nuclear-encoded MRC proteins leads to pathological phenotypes such as arrested development or short lifespan (Rea et al., 2007; Ventura and Rea, 2007). On the other hand, partial suppression of the same genes, including *frh-1* (Ventura et al., 2005), consistent with the induction of beneficial adaptive responses under these conditions, translates

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into life extension (Ni and Lee, 2010). Nevertheless, after these initial findings, contrasting results were published on lifespan regulation following *frh-1* silencing in *C. elegans* (Vazquez-Manrique et al., 2006; Zarse et al., 2007). Although some explanations (Ventura et al., 2006) could be evoked to reconcile the opposite lifespan outcomes observed in response to frataxin suppression, these conflicting data still await compelling experimental clarification, which are required to gain insight into the biology of aging and to help establish an appropriate *C. elegans* model to study Friedreich's ataxia.

The extended longevity of animals with reduced expression of genes directly or indirectly involved in regulating MRC functionality is associated with the induction of different stress responses and it is regulated among others by the p53 *C. elegans* homolog *cep-1* (Torgovnick et al., 2010; Ventura et al., 2009). P53 integrates several intrinsic and extrinsic stress signals to modulate intracellular responses and its activation is impaired in frataxin-deficient mammalian cells (Guccini et al., 2011; Palomo et al., 2011). Besides its classical function in response to DNA damage, p53 controls mitochondrial energy metabolism, antioxidant defenses, and autophagy (Maddocks and Vousden, 2011). Macroautophagy (hereafter referred to as autophagy) is a fundamental housekeeping program responsible for recycling of cellular components and necessary for tissue homeostasis; it is required for normal growth, development, proper energy metabolism and aging, and can be stimulated in response to a variety of different stressors, such as oxidative stress, energy deprivation, hypoxia, and mitochondria or DNA damage (Kroemer et al., 2010). The autophagic process is finely regulated by several proteins, which cooperate to coordinate the nucleation, elongation and degradation of autophagosomes in the lysosomes (Klionsky et al., 2010), and are very well conserved between humans and nematodes. Excessive or hindered activation of this finely regulated program can lead to cell death and has been associated with many diseases in humans, including neurodegenerative disorders (Levine and Kroemer, 2008).

Here we show that a substantial amount of frataxin protein expression must be reduced to trigger autophagy, in turn extending lifespan and reducing lipid content in *C. elegans*. Interestingly, we found that a reciprocal interplay exists in regulating autophagy between levels of frataxin expression and p53/*cep-1*. Collectively, we demonstrate a causal connection between induction of autophagy and lifespan extension following reduced frataxin expression, and provide a rationale for investigating autophagy in the pathogenesis and treatment of FRDA and possibly other human mitochondrial-associated disorders (HMAD).

2. Materials and methods

2.1. Nematode strains and maintenance

We employed standard nematode culture conditions (Stiernagle, 2006). All strains were maintained at 20 °C on Nematode Growth Media agar supplemented with *Escherichia coli* (OP50 or transformed HT115). The complete list of strains utilized in this work can be found in Supplemental Information (SI).

2.2. RNAi feeding

The different dsRNA constructs against *frh-1* (I, II, III, IV) were generated using pL440 vector through standard molecular cloning techniques and utilized to transform HT115(DE3) *E. coli* for RNAi feeding. Unless otherwise indicated, all the experiments in this study were carried out in animals fed for three consecutive generations with bacteria expressing *frh-1IV* dsRNA, which targets the entire *frh-1* CDS. See SI.

2.3. Lifespan and statistical analysis

Survival curves and statistical analyses were carried out as previously described (Ventura et al., 2009). Data from survival assays are

summarized in Supplementary Tables. Figures display survival curves of pooled populations utilized for statistical analysis.

2.4. Quantitative RT-PCR

Quantification of mRNA in *frh-1* RNAi-treated animals relative to vector-only treated animals was undertaken from three different biological samples. cDNA from each biological sample was run in triplicate, using specific primer pairs (available upon request). Different methodologies were utilized for different subset of genes, to design primers and to quantify gene expression. See SI. Regardless, fold change in gene expression in *frh-1* (RNAi) animals was considered significantly different from empty-vector treated animals when a two-tailed Student's t-test resulted in $p < 0.05$.

2.5. Western blot analysis

Protocols for western blot analysis in both nematodes and mammalian cells are described in SI. Generation of a specific antibody against *C. elegans* FRH-1 is carefully described in SI. Briefly, Frataxin cDNA was utilized to synthesize recombinant frataxin protein (GeneScript) and to immunize Balb/c mice. Somatic cell hybrids were prepared using the mouse myeloma cells lines NS-1 and hybridoma supernatants were screened for immunoreactivity to recombinant frataxin. Positive hybridoma cell lines were cloned and one MAb was selected and designated 1D2.

2.6. Autophagy quantification

2.6.1. Nematodes

The level of autophagy (autophagosomal accumulation) was assessed by quantifying the number of GFP positive foci in embryos and in seam cells of L3 larvae, using the translational reporter strain GFP::LGG-1. See SI.

2.6.2. Mammalian cells

The basal level of autophagy (LC3 processing) in mammalian cells was assessed by western blot quantification of LC3II accumulation normalized to the level of tubulin in the same cellular extract. Autophagic flux was similarly assessed by LC3II quantification after treating cells for the indicated time with the lysosome inhibitor NH₄Cl.

2.7. Lipid and lysosomes content quantification

Lysosome related organelles (LROs) were quantified by vital Nile Red staining, while fat content was quantified by fixing animals and staining them with Oil Red O. See SI.

2.8. Quantification of ATP, reactive oxygen species and GSSG/GSH content

ATP and ROS measurements were carried out as carefully described in SI. The ratio between oxidized and reduced pools of glutathione was assessed through the *Prp17::Grx1-roGFP2* strain. All assays were carried out in one-day old adults in, at least, triplicate. See SI.

2.9. Behavioral assays

Behavioral assays were carried out in one-day old adults in, at least, triplicate, each time by two independent operators. See SI.

Pumping rate was calculated as the number of pharyngeal pumps per minute in 15 animals. **Defecation** was scored by averaging the time between two consecutive pBoc contractions in 15 animals. **Locomotion** was assessed by counting the changes in the body bend at the mid-body point per minute of 10 animals placed on solid agar plates with no bacteria. **Mechanosensation** was calculated by touching 10 animals with an eyelash alternating 6 touches on the head (backward

movement) and 6 touches on the tail (forward movement). **Chemo-taxis** was calculated as carefully described in SI. Briefly, a population of 80–100 age-synchronized animals was spotted in the centre of the testing plate and the number of worms at attractant and control was counted every 15 minutes for two hours to calculate the Chemotaxis Index (CI). $CI = (A - B) / (A + B + C)$, where *A* is the number of worms at attractant, *B* is the number of worms at control and *C* is the number of animals which did not reach any of the two spots at the end of the two hours.

2.10. Statistical analysis

Unless otherwise specified all statistical analysis were calculated with the unpaired Student's *t*-test and considered significantly different from control condition when $p < 0.05$.

3. Results

3.1. Substantial suppression of frataxin protein expression is required to extend lifespan in *C. elegans*

Residual levels of frataxin expression are crucial to maintain cell survival in FRDA disease, but how this is achieved is largely unknown. Moreover, contrasting evidence has been reported following frataxin suppression in modulating *C. elegans* lifespan (Vazquez-Manrique et al., 2006; Ventura et al., 2005; Zarse et al., 2007). To reconcile discrepancies in the field, and to shed light on mechanistic aspects of *frh-1*-suppression control of longevity, we generated four dsRNA constructs targeting different portions of the *frh-1* gene and a specific antibody against *C. elegans* FRH-1. The four different dsRNA displayed different interference potency as revealed by western blotting of total *C. elegans* extracts (Figs. 1A–B; S1A–B). The anti-FRH-1 antibody

revealed one or two bands (depending on protein extraction method) at the expected molecular weight of 15 kDa in wild-type lysate, likely corresponding, as in mammalian cells, to different forms of the protein (Fig. S1A–B). Lifespan extension was only achieved with the *frh-1* IV dsRNA construct – referred to as *frh-1* hereafter unless otherwise specified – which reduced FRH-1 expression to more than 50% of its wild-type level, without completely abolishing its expression (Fig. 1B–C; Table 1). Our results unambiguously demonstrated that a critical amount of frataxin protein must be suppressed to induce significant life-extending responses in *C. elegans* thus providing us as with a great tool to untangle protective pathways activated to compensate for frataxin suppression at the organismal level.

3.2. Reduced expression of frataxin affects sensory neurons and mitochondria functionality in *C. elegans*

To shed light on mechanistic aspects of frataxin control of longevity, which can also be relevant for the human disease, we characterized in more detail mitochondria and neuronal functions in response to *frh-1* RNAi. As seen in mammals, reduced expression of frataxin protein in *C. elegans* specifically affected sensory neurons functionality: the reduced sensitivity of *frh-1*(RNAi) animals to specific chemicals, namely their attraction to pyrazine and to sodium acetate, reveals alteration of specific sensory neurons, AWA and ASE respectively (Figs. 2A; S1C–D). Interestingly the AWA pair of neurons participate in the regulation of *C. elegans* lifespan (Apfeld and Kenyon, 1999). These sensory defects are not ascribed to altered moving ability since *frh-1*(RNAi) worms have normal response to touch and locomotion activity (Fig. 2B–C), and they actually move slightly faster than control animals (Fig. 2D).

FRDA cells are more sensitive to oxidative stress (Condo et al., 2006) while long-lived *frh-1*(RNAi) animals, similar to other Mit

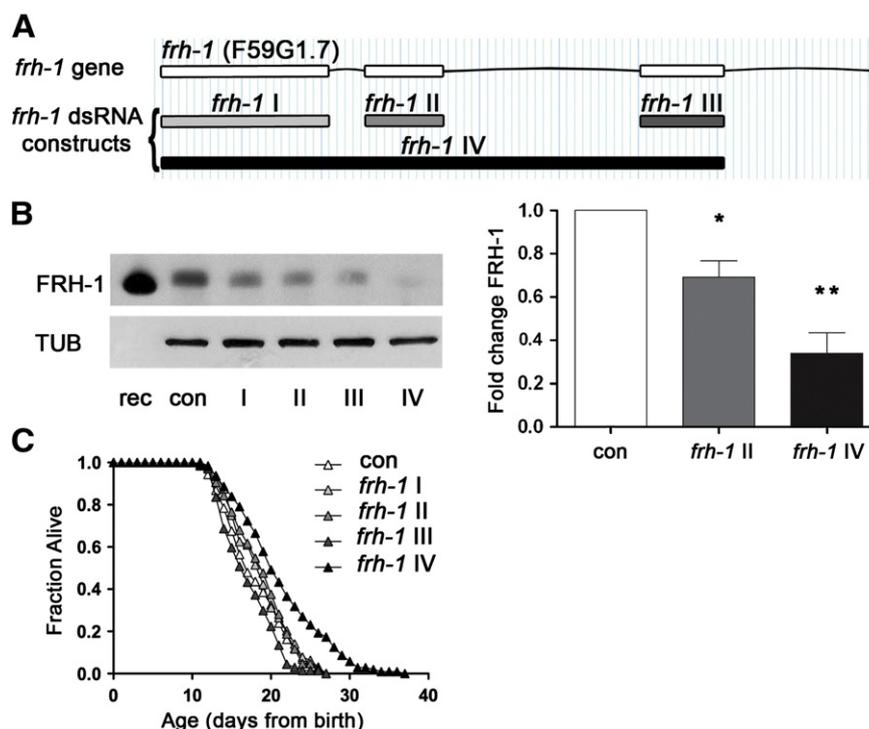


Fig. 1. Substantial frataxin suppression is required to extend *C. elegans* lifespan. A) *frh-1* gene and dsRNA interference constructs. One construct against the entire frataxin genomic CDS (*frh-1*IV), and three additional constructs targeting the three single exons, respectively named *frh-1*I, *frh-1* II and *frh-1*III, were generated in this work. B) Frataxin protein (FRH-1) expression was quantified in 1-day-old adult wild-type (WT) animals fed bacteria expressing either empty-vector (con) or one of the four different *frh-1* dsRNA. In the left panel FRH-1 expression was revealed through western blot with a specific antibody anti Ce-FRH-1 and normalized to tubulin (TUB). In the first lane 10 ng of FRH-1 recombinant protein (rec) were loaded. FRH-1 appears at the expected molecular weight ~15 kDa. In the right panel, bars and errors indicate mean FRH-1 expression \pm standard error of the mean (SEM) after densitometric analysis from two independent experiments. * $p < 0.05$, ** $p < 0.001$ *t*-test. C) Survival analysis of wild-type animals fed bacteria expressing either empty-vector (con) or the different *frh-1* dsRNA.

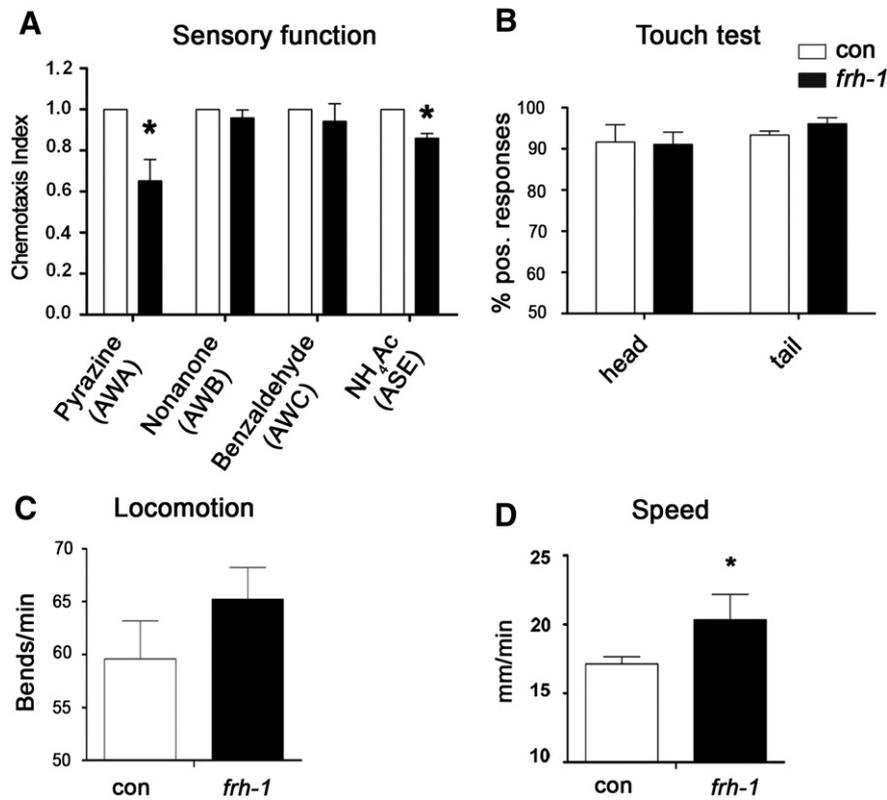


Fig. 2. Characterization of neuronal features. A) Sensory function (chemotaxis index), B) proprioception (touch test) and C–D) locomotion activity (body bends and speed), in wild-type (1 day-old adults) animals fed empty-vector (con) or *frh-1IV* dsRNA (*frh-1*) expressing bacteria. Bars and errors indicate mean values \pm SEM from three independent replicates. * $p < 0.05$ vs control, *t*-test.

mutants, are differentially sensitive to different pro-oxidants (Lee et al., 2003; Ventura et al., 2005). Here we found that *frh-1* RNAi significantly decreased the levels of reactive oxygen species (ROS), of the oxidized pool of glutathione, and of ATP compared to control animals (Figs. 3A–C; S2A–B). Our data indicate that frataxin suppression in *C. elegans* recapitulates some of the features observed in the human disease (i.e. altered sensory neuron functionality and ATP production), while other features (i.e. reduced ROS and oxidized glutathione) may result from the activation of specific compensatory pathways.

3.3. Reduced frataxin expression specifically remodels lipid metabolism in *C. elegans*

Long-lived *frh-1*(RNAi) worms, similar to other Mit mutants, are smaller and paler than wild-type animals. These animals, as previously observed but with a different *frh-1* dsRNA construct (Vazquez-Manrique et al., 2006), have reduced pumping and defecation rate (Fig. S1E), and we found that they display a reduced content

of lipid and of intestine-specific lysosome-related organelles (LROs) compared to control animals (Figs. 3D–E; S3A–B). The expression of genes involved in lipid metabolism was also selectively reduced upon *frh-1* RNAi: specifically, the expression of enzymes regulating both lipid catabolism (*gpdh-2/K11H3.1*, *lip1-4/K04A8.5* and another predicted lipase/F28H7.3) and lipid anabolism (*acs-17/C46F4.2* and *fasn-1/F32H2.5*) were significantly reduced (Fig. 3F; Table SI). The transcript levels of key enzymes regulating other major metabolic pathways (glycolysis, gluconeogenesis, TCA cycle, anaerobic, glyoxylate and pentose phosphate pathways) were not significantly affected (Fig. 3G–I; Table SII), except for *pdhk-2/ZK370.5* (Fig. 3G) and Y24D9A.8 (Fig. 3H).

Interestingly, the *frh-1* II dsRNA construct that did not extend animal lifespan, only marginally affected animal metabolic parameters (Figs S2C–D; S3A–C; Table SII) and the expression of antioxidant genes (Fig. S3D–E) previously shown to be induced upon life-lengthening *frh-1* RNAi (Torgovnick et al., 2010). Taken together, these observations indicate that a critical threshold (50%) of FRH-1 suppression must be surpassed to concurrently extend lifespan and significantly alter mitochondria and lipid metabolism in *C. elegans*.

3.4. Reduced frataxin expression extends lifespan independently of AMP-kinases

AMP-activated protein kinases (AMPKs) respond to variation in cellular ATP/AMP content and cooperate with p53 to modulate energy metabolism (Jones et al., 2005; Maddocks and Vousden, 2011; Mandal et al., 2005; Thoreen and Sabatini, 2005). AMPk and p53/*cep-1* are in part required for the lifespan extension of *C. elegans* in response to mitochondrial disruption (Curtis et al., 2006; Ventura et al., 2009). We hypothesized that AMPKs are activated in response to *frh-1* RNAi in turn modulating animal metabolism and longevity. Contrary to our expectations, none of the kinases involved in energy

Table 1
Lifespan analyses *frh-1* dsRNA constructs – summary statistics.

Genotype	RNAi ^a	Mean Lifespan	\pm SEM	<i>p</i> vs con ^b	Sample size (censored) ^c	N of replicates
Wild-type (N2)	con	17.6	0.4		140(22)	2
	<i>frh-1 I</i>	18.3	0.5	0.45750	130(10)	2
	<i>frh-1 II</i>	17.2	0.3	0.37669	140(12)	2
	<i>frh-1 III</i>	16.7	0.4	0.61293	130(6)	2
	<i>frh-1 IV</i>	20.9	0.5	0.00001	140(36)	2

^a Unless otherwise specified, all lifespan analyses in the paper were performed on 3rd generation worms cultured continuously on *frh-1* RNAi.

^b *p*-values were calculated using the log-rank test between pooled populations.

^c Pooled worms from N replicate experiments (censored individuals were included in the log-rank analysis).

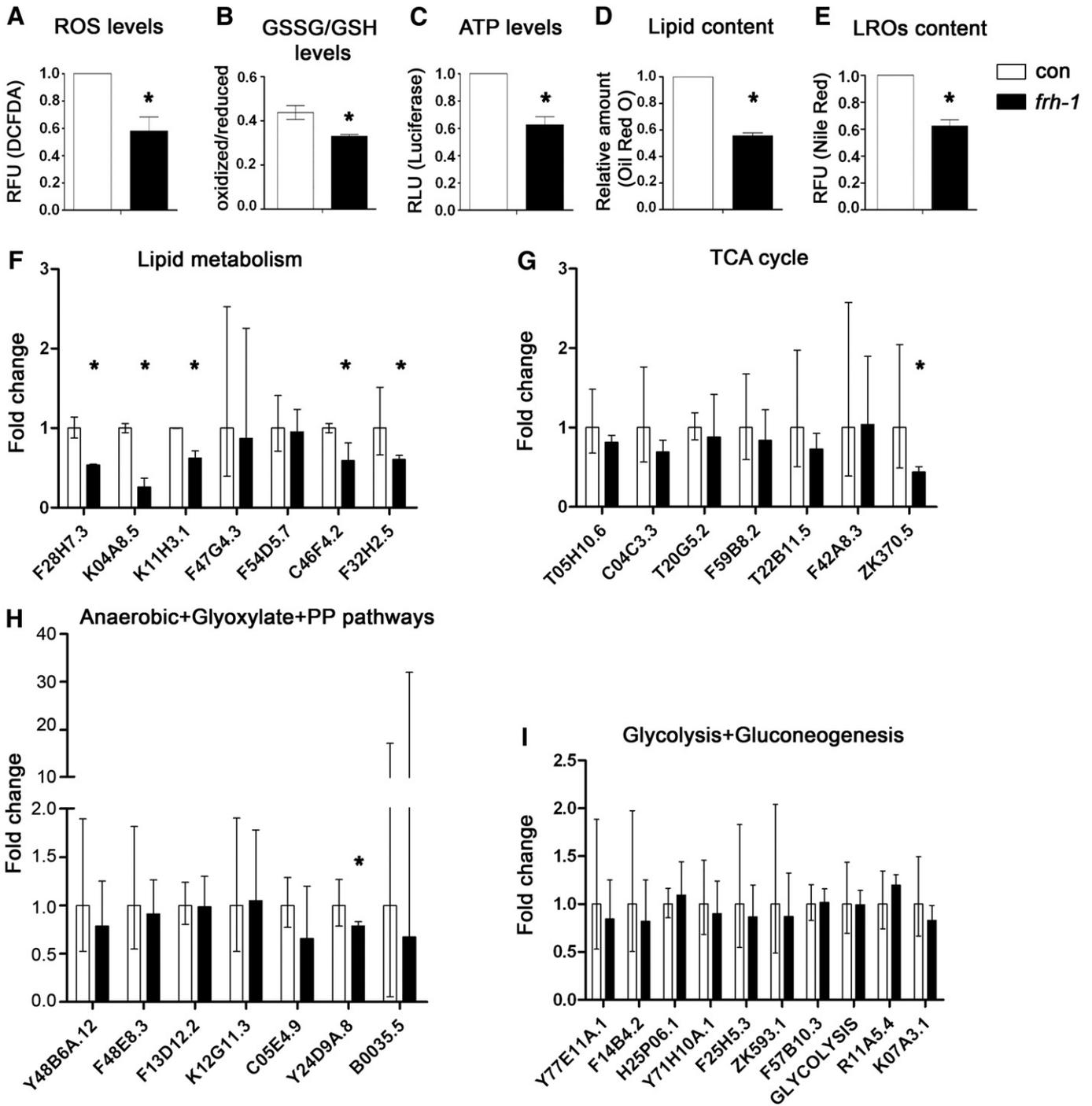


Fig. 3. Characterization of mitochondria and metabolic features. Levels of A) reactive oxygen species (ROS), B) oxidized/reduced glutathione (GSSG/GSH), C) ATP, D) lipid content and E) lysosome-related organelles (LROs) in 1-day-old adult WT animals fed bacteria expressing either empty-vector (con) or life-lengthening *frh-1* dsRNA (*frh-1*). RFU, Relative Fluorescence Unit. RLU, Relative Luminescence Unit. F–I) Transcript expression of key metabolic enzymes in animals treated as in A. Bars and errors indicate mean fold changes and the 95% confidence interval of the mean, respectively, of cumulative data from three independent experiments. **p*<0.05 of each single gene vs control, *t*-test.

metabolism that we tested (*aak-1*, *aak-2*, *par-4*, *sad-1*, *kin-29*, or *mml-1*) affected lifespan in *frh-1*(RNAi) animals (Fig. 4A–F; Table 2) or in the majority of the RNAi-mediated Mit mutants analyzed (Fig. S4A–D; Table SIII) which also displayed reduced lipid accumulation (not shown). This is nevertheless consistent with a non-linear relationship between ATP levels and extension of lifespan in different RNAi-mediated Mit mutants (Fig. S2E and Dillin et al., 2002).

Moreover, consistent with notion that *frh-1* RNAi acts in parallel to caloric-restriction (CR), it further extends lifespan (Ventura and Rea,

2007) and decreased LROs content and pumping rate in the *eat-2* mutant, a genetically defined CR model (Fig. 4G–H). Of note, reduced intestinal LROs content in *frh-1*(RNAi) worms and in *eat-2* mutants was very similar; on the other hand the pumping rate was more severely affected in *eat-2* mutants indicating that reduced LROs accumulation in *frh-1*(RNAi) animals is ascribed to mechanisms other than reduced food intake. Collectively, these findings indicate that *frh-1* RNAi extends lifespan in parallel to CR-related pathways, independently of AMPKs, major regulators of energy metabolism.

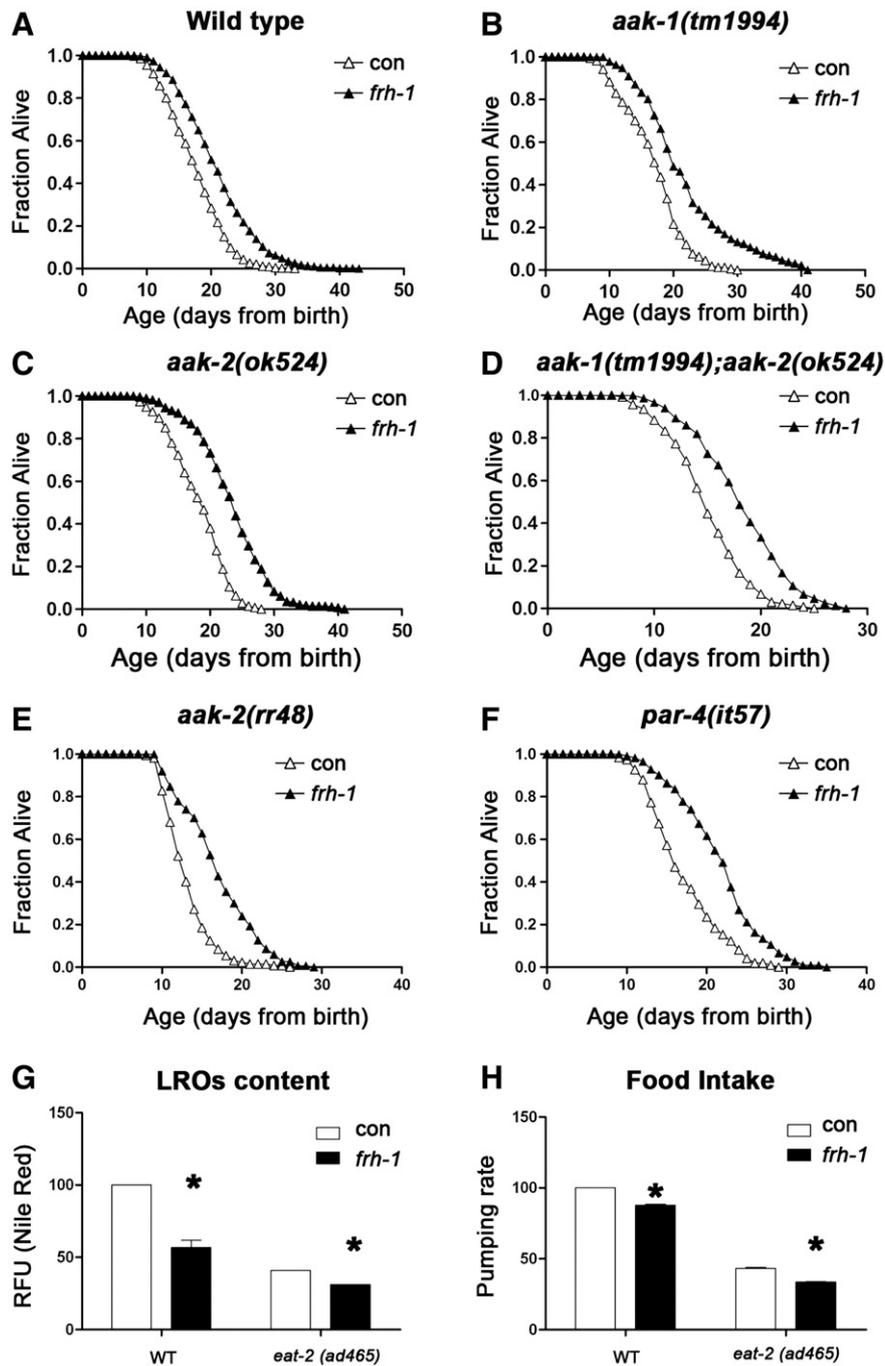


Fig. 4. Frataxin suppression acts independently of the AMP kinases, in parallel to CR. A–F) Survival analysis of strains carrying mutant alleles of different AMP regulatory kinases fed bacteria expressing either empty-vector (con) or *frh-1* dsRNA (*frh-1*). G–H) Lysosome-related organelles staining and food intake (normalized pumping rate) in WT and *eat-2(ad465)* mutants fed bacteria expressing either empty-vector (con) or *frh-1* dsRNA (*frh-1*). Bars and errors indicate mean values \pm SEM from different replica. * $p < 0.05$ vs control, *t*-test.

3.5. Autophagy is induced by reduced expression of frataxin in *C. elegans* and in mammalian cells

We next sought to investigate whether autophagy is induced in response to frataxin suppression as an adaptive process to mitochondrial damage (Lemasters, 2005). This could modulate lipid metabolism and lifespan (Lapierre et al., 2011), and suggest a critical pathogenetic target to prevent or postpone the established disease in humans. The gene *lgg-1* encodes the *C. elegans* ortholog of Atg8/LC3, an ubiquitin-like, microtubule-associated protein required for autophagic vesicle growth. The change of Atg8/LC3/LGG-1 expression's

pattern from diffuse to punctate represents a well-established marker for autophagosomes accumulation (Melendez et al., 2008). Taking advantage of a translational reporter strain expressing GFP::LGG-1 (Melendez et al., 2003) we found that *frh-1* RNAi increases the number of GFP::LGG-1 positive foci in embryos (Figs. 5A; S5A) and in hypodermal seam cells in larvae (Figs. 5B; S5B). Moreover, the overall amount of GFP::LGG-1 expression in embryos of *frh-1* (RNAi) animals was decreased (Fig. 5C), a likely indication of an intact autophagic flux (Mizushima et al., 2010). The early steps of autophagosome formation involve, among others, Atg1/ULK1, Atg6/Beclin and the class III phosphoinositide 3-kinase (PI3K) Vps34. The transcriptional

Table 2
Lifespan analyses *frh-1* RNAi on different kinase mutants – summary statistics.

Genotype	RNAi	Mean Lifespan	±SEM	<i>p</i> vs con*	<i>p</i> vs N2*	Sample size (censored) ^a	N of replicates
Wild-type (N2)	<i>con</i>	17.2	0.2			460 (40)	7
	<i>frh-1</i>	20.6	0.3	4.9E−17		468(100)	7
<i>aak-2(rr48)</i>	<i>con</i>	12.6	0.3		1.06E−26	198 (61)	3
	<i>frh-1</i>	16.3	0.4	1.5E−12	0.17044	197 (43)	3
<i>aak-2(ok524)</i>	<i>con</i>	17.9	0.3		0.20326	319 (53)	5
	<i>frh-1</i>	23.1	0.3	1.5E−27	1.39E−34	330 (54)	5
<i>aak-1(tm1994)</i>	<i>con</i>	16.5	0.4		0.33077	190 (28)	3
	<i>frh-1</i>	21.5	0.6	3.6E−08	1.06E−10	191 (61)	3
<i>aak-1(tm1994); aak-2(ok524)</i>	<i>con</i>	14.6	0.3		3.85E−01	191 (57)	3
	<i>frh-1</i>	17.9	0.3	6.9E−10	0.38536	188 (38)	3
<i>par-4(it57)</i>	<i>con</i>	16.6	0.5		0.40960	140 (40)	2
	<i>frh-1</i>	21.2	0.5	1.6E−08	2.44E−10	140 (35)	2
Wild-type (N2)	<i>con</i>	16.4	0.3			200(10)	3
	<i>frh-1</i>	19.8	0.4	1.2E−08	1.17E−08	200(32)	3
<i>sad-1(ky289)</i>	<i>con</i>	15.8	0.6		0.78698	120(46)	2
	<i>frh-1</i>	20.8	1.0	0.00003	0.00001	121(40)	2
<i>kin-29(gk270)</i>	<i>con</i>	12.1	0.2		1.29E−18	139(22)	2
	<i>frh-1</i>	16.1	0.4	5.1E−12	0.72663	141(30)	2
<i>mml-1(ok849)</i>	<i>con</i>	15.2	0.4		0.23012	200(40)	3
	<i>frh-1</i>	19.6	0.4	1.5E−09	7.96E−08	199(30)	3

* *p*-values were calculated using the log-rank test between pooled populations.

^a Pooled worms from N replicate experiments (censored individuals were included in the log rank analysis).

expression of *vps-34*, *bec-1* and *lgg-1* did not significantly change (Fig. 4D; Table SIV), suggesting *frh-1* RNAi induces autophagy at the post-transcriptional level.

To corroborate our findings in *C. elegans*, we assessed whether autophagy is affected in mammalian cells with reduced frataxin expression. Steady-state autophagy measurements indicated that the basal amount of autophagosomes accumulation is increased in lymphoblasts derived from FRDA patients compared to their healthy matched cells (Fig. 5E). This can be ascribed either to an increase in autophagosomes formation or to a block of lysosomal degradation. To discriminate between these two possibilities we treated FRDA cells with ammonium chloride (NH₄Cl), which inhibits lysosome acidification, and found that the amount of autophagosomes is further increased (Fig. 5F), suggesting that the autophagic flux is not impaired. Collectively these findings suggest that induction of an intact autophagic flux is an evolutionarily conserved response to frataxin suppression, a notion with critical implication for the human disease.

3.6. Autophagy is required to extend longevity and reduce lipid accumulation in *frh-1*(RNAi) animals

Autophagic genes are required to extend lifespan in different *C. elegans* genetic backgrounds but their role in controlling longevity in response to mitochondrial disruption has not been clearly elucidated (Hansen et al., 2008; Toth et al., 2008). We found that two core autophagy-regulating genes, *unc-51* (the *C. elegans* ortholog of ULK1) and *bec-1* are respectively required for survival and longevity of *frh-1*(RNAi) animals. Specifically, *unc-51* mutants and *frh-1*(RNAi) animals had a reduced but viable number of progeny (Fig. 6A). On the other hand, *unc-51* mutants fed *frh-1* RNAi laid fewer and 100% non viable embryos (a mix of unhatched eggs and arrested larvae) (Figs. 6A; S5C). RNAi or genetic suppression of *bec-1* shortened animal lifespan, and completely abolished the lifespan extension induced by *frh-1* RNAi (Figs. 6B; S5D; Table 3). The lack of *frh-1* RNAi pro-longevity effect was also observed in the *bec-1* heterozygotes (unpublished observation). Of note, we found that *frh-1* RNAi was not able to induce autophagy in *bec-1*(RNAi) animals (Fig. 6C). Interestingly, in support of a protective role for autophagy in response to frataxin suppression, the *frh-1III* dsRNA construct that did not extend lifespan was unable to trigger autophagy (Fig. S5A).

P53/*cep-1* regulates autophagy and is required for the extended longevity of different Mit mutants including *frh-1*(RNAi) animals

(Ventura et al., 2009). Consistent with work on a different *cep-1* mutant allele (Tasdemir et al., 2008), we found that the accumulation of autophagosomes in *cep-1*(*lg12501*) mutants was significantly increased compared to wild-type (Fig. 6D). Notably, *frh-1* RNAi did not increase the number of autophagosomes in the *cep-1* mutant but rather reduced it (Fig. 6D), suggesting a reciprocal functional interplay between the two genes in regulating autophagosomes accumulation.

Autophagy regulates lipid metabolism in different biological contexts (Lapierre et al., 2011; Singh et al., 2009). We found that *bec-1* RNAi significantly rescued the reduced content of lipids and of LROs in *frh-1*(RNAi) animals (Fig. 6E–F). Lipid and LROs content were similarly rescued in *bec-1* heterozygotes and in the *cep-1* mutants, which like *bec-1* RNAi, also reverted *frh-1*(RNAi) animals small size (Fig S5E and F title legend should be corrected to Lipid content. LROs content should be the title of panels G–H). Our data indicate that autophagy and *cep-1* cooperate during animal development to exert beneficial effects in response to reduced frataxin levels.

4. Discussion

In this paper we unambiguously show that a substantial level of frataxin protein expression (below 50% of its wild-type level) must be reduced to extend *C. elegans* lifespan and remodel different aspects of animal metabolism. Importantly, we propose that this is achieved by inducing an adaptive response regulated by *cep-1*, namely autophagy, a fundamental cellular recycling program.

Different experimental conditions coupled with dose-dependent effects may therefore help to reconcile the opposite lifespan outcomes reported by other groups (Vazquez-Manrique et al., 2006; Zarse et al., 2007) in response to frataxin silencing in *C. elegans*. On the one hand, these studies might have not reached the threshold of frataxin suppression necessary to extend lifespan, and on the other hand the short lifespan could be ascribed to experimental conditions which lead to an overestimation of dead animals upon *frh-1* RNAi compared to control. Namely, while Vazquez-Manrique and coworkers did not censor bagged animals in the survival analysis, the mean lifespan of control animals in the other study was 20 days at 20 °C on HT115 bacteria (as food source), which is slightly longer than average for a wild-type strain under normal laboratory conditions. Here we tested different constructs and generated a specific anti FRH-1 antibody, to specifically correlate level of frataxin protein expression, lifespan and other animal metabolic parameters and clearly showed that none of tested constructs actually

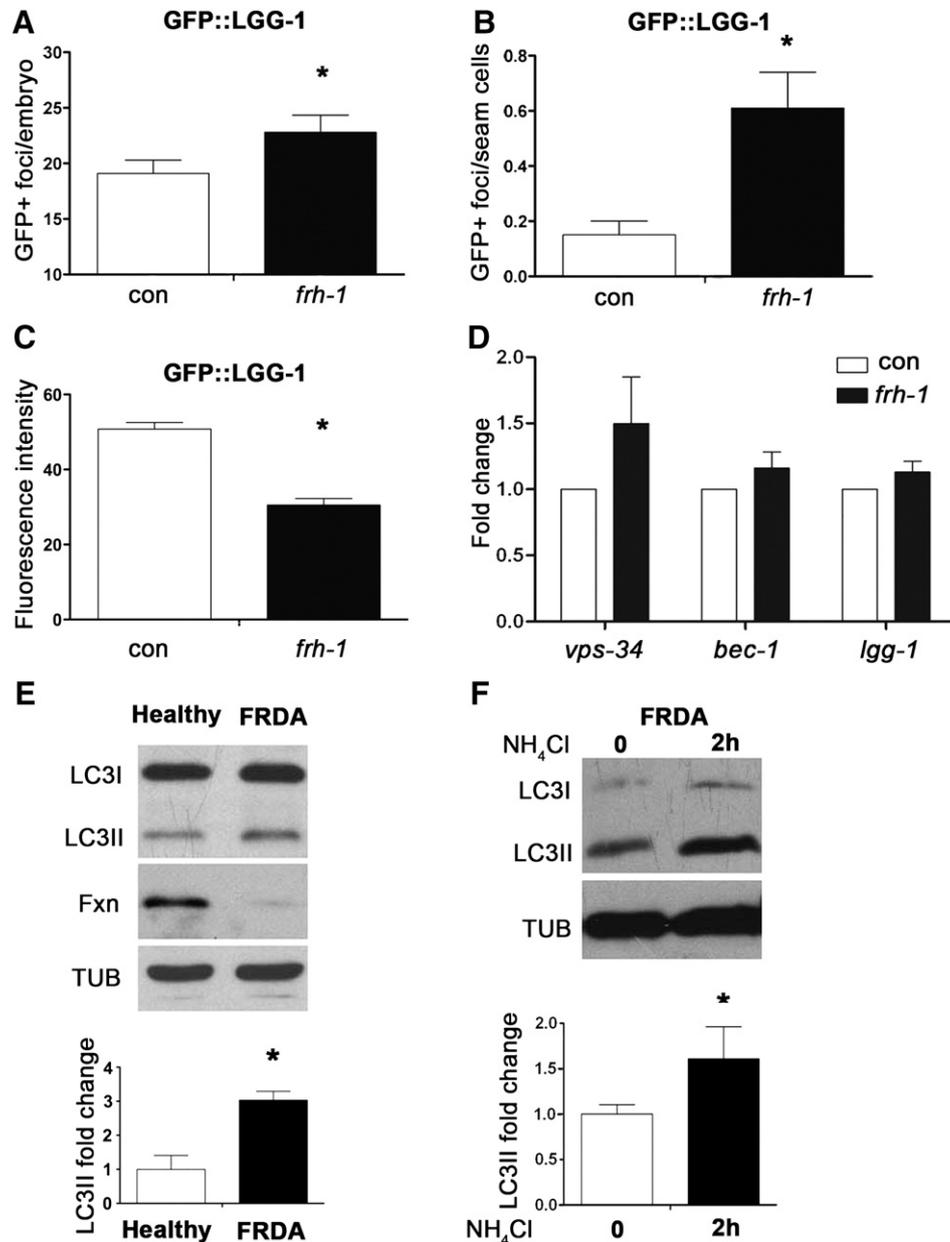


Fig. 5. Frataxin suppression induces autophagy. A–B) Number of autophagosomal GFP⁺ foci in embryos (A) and seam cells of L3 larvae (B) of *C. elegans* GFP::LGG-1 transgenic animals fed bacteria expressing empty-vector (con) or *frh-1* dsRNA (*frh-1*). C) Total amount of GFP::LGG-1 protein expression in embryos of animals treated as in A. D) Transcriptional changes of autophagy modulator genes in WT animals treated as in A. E) Autophagosome accumulation assessed by increased formation of LC3II in Friedreich's Ataxia patient derived lymphoblast (FRDA) compared to control lymphoblast derived from a healthy brother (Healthy). During the autophagic process the LC3/LGG-1 protein undergoes a posttranslational modification that accelerates its migration on acrylamide gel (from LC3I to LC3II). Fxn, human frataxin. F) Autophagosome accumulation in FRDA lymphoblasts left untreated or treated for two hours with the lysosome inhibitor NH₄Cl. In each panel, bars and errors indicate mean values \pm SEM after densitometric analysis from three independent replicates. * $p < 0.05$ vs control, *t*-test.

shortened lifespan compared to control animals (which have a mean lifespan of almost 18 days, Table 1). Of note, also *frh-1* knock-out animals, although arrested as L2/L3, live longer than wild-type (Ventura and Rea, 2007).

Neurons and myocytes are the cells mostly affected in FRDA patients. *C. elegans* neurons are relatively resistant to the effect of feeding RNAi, the technique routinely used for gene knock-down. Although this might lead to underestimation of specific neuronal effects, it notably allows one to studying the function of genes whose deficiency in the nervous system would be lethal. Nonetheless, *frh-1*(RNAi) animals displayed a mild but selective deficit in specific sensory neurons. An interesting possibility is that lifespan extension in response to *frh-1* deficiency is achieved via disruption of specific subtypes of neurons, such as the AWA pair of neurons, which is

known to extend lifespan in *C. elegans* (Apfeld and Kenyon, 1999). *frh-1*(RNAi) animals also have decreased pumping, a feature that might reflect altered functionality of the pharynx, interestingly the organ that more closely resembles mammalian heart's organogenesis (Haun et al., 1998). Here, we find that stimulation of autophagy is an evolutionarily conserved adaptive response to severe frataxin suppression. Autophagy induction in non-renewable cells (such as neurons and myocytes) might therefore represent an attempt to preserve cell viability upon frataxin deficiency in the human pathology. Importantly, although accumulation of autophagosomal foci was described in neurons of conditional *frataxin* KO mice (Simon et al., 2004), this could be ascribed to increase autophagy flux or block in autophagosomes degradation. Our results are indicative of an intact, and therefore increased autophagic flux, an observation with

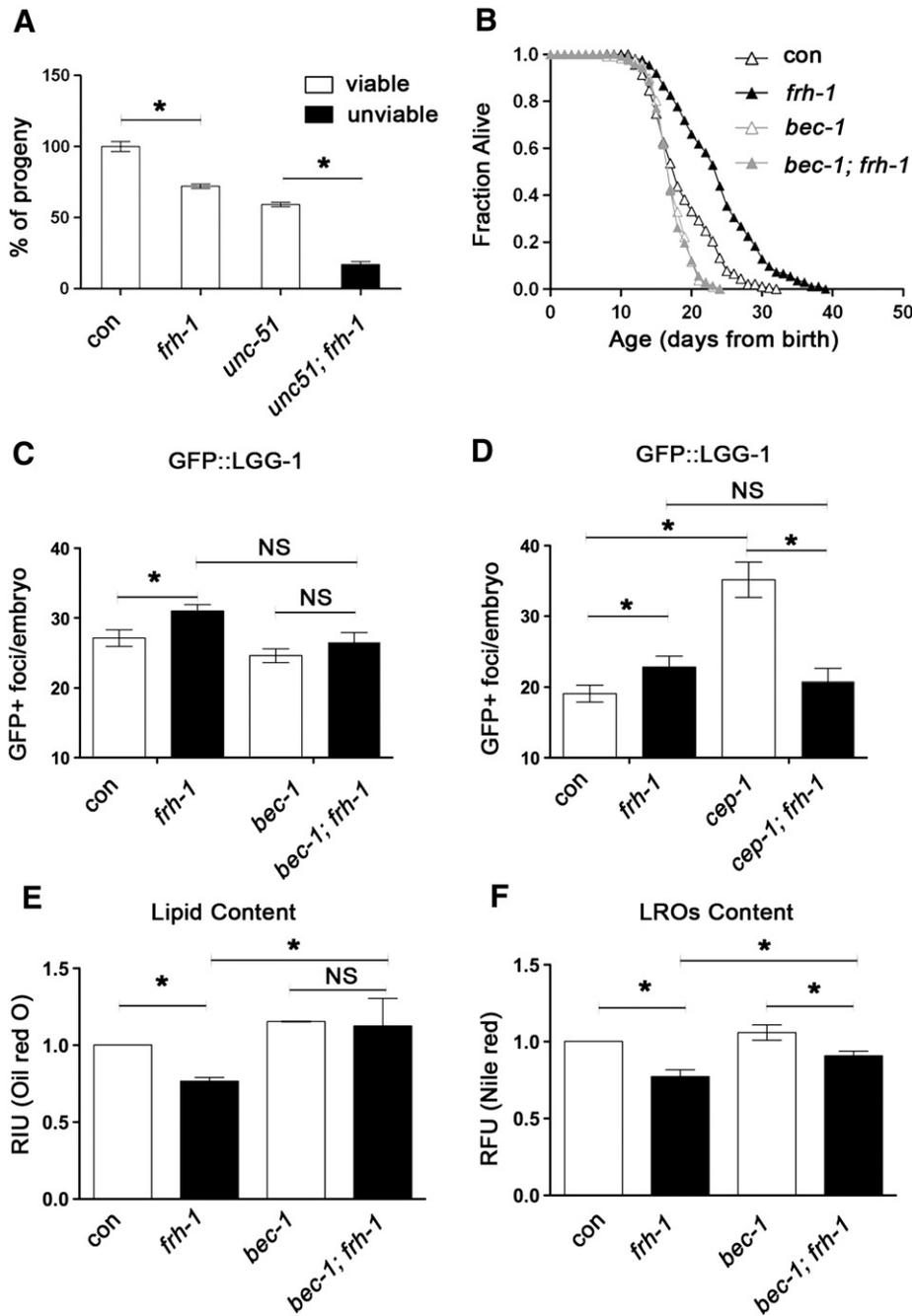


Fig. 6. Autophagy is required to extend lifespan and reduce lipid accumulation in *frh-1*(RNAi) animals. A) Fertility and viability of WT and *unc-51* mutants fed bacteria expressing either empty-vector (con) or *frh-1* dsRNAi (*frh-1*). B) Survival curves of WT animals fed bacteria expressing empty-vector (con), an equal mix of empty-vector and *frh-1* dsRNA (*frh-1*), an equal mix of empty-vector and *bec-1* dsRNA (*bec-1*), or an equal mix of both dsRNA (*bec-1; frh-1*). C) Number of autophagosomal GFP⁺ foci in embryos of GFP::LGG-1 animals fed as in B. D) Number of autophagosomal GFP⁺ foci in embryos of GFP::LGG-1 and of GFP::LGG-1;*cep-1*(lg12501) strains fed bacteria expressing either empty-vector (con) or *frh-1* dsRNA (*frh-1*). E–F) Content of lipid and of lysosomes-related organelles (LROs) were respectively measured by fixing animals and staining them with Oil-Red-O (E, RIU=relative intensity unit), and by feeding animals with the vital dye Nile Red (F, RFU=relative fluorescence unit) in WT animals treated as in B. Bars and errors indicate mean values \pm SEM from at least three independent replicates. * $p < 0.05$ vs control, *t*-test.

significant biological implications for Friedreich's ataxia disease pathogenesis and new potential therapeutic approaches.

Consistent with silencing of other MRC regulatory proteins (Dillin et al., 2002; Rea et al., 2007), we found that ATP or ROS levels in *frh-1*(RNAi) worms do not linearly correlate with lifespan. The induction of protective stress responses, such as autophagy, only upon severe frataxin suppression could account for these findings and explain the inability of the non-extending *frh-1III* dsRNA construct to decrease ROS and to maintain ATP levels. A threshold effect could also explain why contrary to the original observation (Simon et al., 2004), Palomo and coworkers did not observe any significant

autophagy induction in neuronal-like cells treated with frataxin shRNA (Palomo et al., 2011).

Our findings indicate that frataxin suppression does not generally slow down animal metabolism, but rather plays a specific role in controlling lipid remodeling through the autophagic pathway. Interestingly, lipid metabolism is finely regulated within the autophagolysosomal compartments (Kiselyov et al., 2007; Singh et al., 2009) and although the two dyes utilized to quantify lipid and LROs content stain different compartments they can colocalize under altered metabolic conditions (such as peroxisomal β -oxidation alterations Zhang et al., 2010). The amount of lipid accumulation inversely correlates

Table 3
Beclin control of *frh-1*(RNAi) animals lifespan — summary statistics.

Genotype	RNAi	Mean lifespan	±SEM	<i>p</i> vs con*	<i>p</i> vs N2*	Sample size (censored) ^a	<i>N</i> of replicates
Wild-type (N2)	<i>con</i>	18.4	0.3			212(31)	3
	<i>frh-1</i> ^b	23.1	0.5	2.4E−13		212(45)	3
	<i>bec-1</i> ^b	16.8	0.3	0.00065		140(36)	2
	<i>bec-1;frh-1</i>	16.7	0.3	0.82070 [§]	4.1E−24 ^{§§}	140(11)	2
<i>bec-1(ok691)</i>	<i>Con</i>	9.9	0.3	1.31E−54		70(0)	1
	<i>frh-1</i>	8.4	0.4	0.02004	2.88E−65	70(0)	1

* *p*-values were calculated using the log-rank test between pooled populations.

[§] *p*-value versus *bec-1*.

^{§§} *p*-value versus *frh-1*.

^a Pooled worms from *N* replicate experiments (censored individuals were included in the log rank analysis).

^b Lifespan analyses were performed on worms fed an equal mix of bacteria expressing empty-vector and *frh-1* dsRNA or *bec-1* dsRNA.

with lifespan extension but it remains to be established whether these are two independent consequences of autophagy induction or whether instead a direct causal relationship between lipid metabolism and mitochondrial control of longevity exists. We found that the expression of the *C. elegans* lipase LIPL-4, which is overexpressed in the *glp-1* germline less mutant and cooperates with the autophagic pathway to extend its lifespan (Lapierre et al., 2011; Wang et al., 2008), is dramatically reduced by *frh-1* RNAi. Overexpression of *lipl-4* in the germline deficient mutant is *daf-16* dependent while *frh-1* RNAi extends lifespan in a *daf-16* independent manner (Ventura et al., 2009); moreover the expression of different lipid-metabolizing enzymes, including *lipl-4*, is also reduced by the *frh-1III* dsRNA construct that does not extend longevity. Taken together these observations suggest that autophagy and lipid metabolism may differently coordinate longevity in a context-specific manner and that at least *lipl-4* does not play a role in longevity specification in *frh-1*(RNAi) animals. More advanced analysis of animal metabolic composition (Butler et al., 2010; Mishur and Rea, 2011) will help to elucidate whether or not lipid metabolism plays a direct role in mitochondrial control of longevity.

Lifespan extension and autophagy in response to *frh-1* RNAi required the *C. elegans* p53 orthologs, *cep-1*. Reciprocally, frataxin deficiency reduces the amount of autophagosomes in *cep-1* mutants, leading to the interesting possibility that mitochondrial functionality is required to modulate autophagy in response to specific insults. Specifically, reduced frataxin expression (and thus mitochondrial functionality) could facilitate elimination of autophagosomes in conditions of impaired autophagic flux, as possibly revealed in the *cep-1* KO mutant by the elevated total amount of GFP::LGG-1 in their embryos (our unpublished observation). In support of a functional interplay between frataxin and p53 we recently showed that frataxin participates in the hypoxia-induced response in mammalian tumors together with p53 (Guccini et al., 2011), suggesting that a similar p53-dependent control of autophagy could be orchestrated in frataxin-deficient mammalian cells.

5. Conclusions

In summary, our findings suggest that stimulation of an intact autophagic flux is an evolutionarily conserved mechanism activated in response to severe frataxin deficiency, which is required in *C. elegans* to extend lifespan and reduce lipid content in a p53-regulated manner. Since frataxin deficiency affects the expression of different MRC subunits (Rotig et al., 1997) and induces same phenotypic features observed in other RNAi-mediated Mit mutants, a similar autophagic control of lipid content and longevity is envisioned upon suppression of other mitochondrial proteins, as in part suggested by another study (Toth et al., 2008). Importantly, our results imply a role for autophagy as a protective compensatory response aimed at coping with dysfunctional mitochondria in frataxin-deficient cells, and provide a platform

for testing novel therapeutic strategies for FRDA and possibly other human mitochondrial-associated diseases.

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