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Two modes of mitochondrial dysfunction lead independently to lifespan extension in *Caenorhabditis elegans*

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Summary

In *Caenorhabditis elegans*, longevity is increased by a partial loss-of-function mutation in the mitochondrial complex III subunit gene *isp-1*. Longevity is also increased by RNAi against the expression of a variety of mitochondrial respiratory chain genes, including *isp-1*, but it is unknown whether the *isp-1(qm150)* mutation and the RNAi treatments trigger the same underlying mechanisms of longevity. We have identified *nuo-6(qm200)*, a mutation in a conserved subunit of mitochondrial complex I (NUDFB4). The mutation reduces the function of complex I and, like *isp-1(qm150)*, results in low oxygen consumption, slow growth, slow behavior, and increased lifespan. We have compared the phenotypes of *nuo-6(qm200)* to those of *nuo-6(RNAi)* and found them to be distinct in crucial ways, including patterns of growth and fertility, behavioral rates, oxygen consumption, ATP levels, autophagy, and resistance to paraquat, as well as expression of superoxide dismutases, mitochondrial heat-shock proteins, and other gene expression markers. RNAi treatments appear to generate a stress and autophagy response, while the genomic mutation alters electron transport and reactive oxygen species metabolism. For many phenotypes, we also compared *isp-1(qm150)* to *isp-1(RNAi)* and found the same pattern of differences. Most importantly, we found that, while the lifespan of *nuo-6, isp-1* double mutants is not greater than that of the single mutants, the lifespan increase induced by *nuo-6(RNAi)* is fully additive to that induced by *isp-1(qm150)*, and the increase induced by *isp-1(RNAi)* is fully additive to that induced by *nuo-6(qm200)*. Our results demonstrate that distinct and separable aspects of mitochondrial biology affect lifespan independently.

Key words: aging; *C. elegans;* electron transport; *isp-1*; mitochondria; *nuo-6*.

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Introduction

The nematode *Caenorhabditis elegans* has been extensively used to identify molecular mechanisms that determine the lifespan of animals (Antebi, 2007). In particular, it was found that altering mitochondrial function could substantially increase lifespan (Ewbank *et al.*, 1997; Felkai *et al.*, 1999; Feng *et al.*, 2001; Dillin *et al.*, 2002; Lee *et al.*, 2003). This effect of altered mitochondrial function was initially identified through point mutations that altered the function of mitochondrial proteins, such as CLK-1 (Ewbank *et al.*, 1997; Felkai *et al.*, 1999), which is involved in ubiquinone biosynthesis, and ISP-1, which is a catalytic subunit of mitochondrial complex III (Feng *et al.*, 2001). The effect of disrupting mitochondrial function has also been extensively studied by RNAi knockdown of genes that encode mitochondrial proteins (Dillin *et al.*, 2002; Lee *et al.*, 2003; Hamilton *et al.*, 2005). By this method, many different loci coding for mitochondrial proteins were found to be able to positively affect lifespan (Hansen *et al.*, 2005; Curran & Ruvkun, 2007; Pan *et al.*, 2007). In particular, it was found that RNAi knockdown of subunits of mitochondrial complexes I, III, IV, and V, but not II, could result in lifespan extension. However, null mutations or severe knockdown by RNAi of many of these same genes leads to lethality (Tsang *et al.*, 2001; Lee *et al.*, 2003; Sonnichsen *et al.*, 2005), or to severe phenotypes that are not accompanied by increased longevity (Ishii *et al.*, 1998; Kayser *et al.*, 2001). Overall, a picture has emerged, which has also been directly tested (Rea *et al.*, 2007), that suggests that, with the exception of complex II, the more severely the function of a mitochondrial complex is disrupted by RNAi the greater the resulting lifespan increase until a threshold is reached, where deleterious effects prevent further lifespan increase, or shorten lifespan, or are lethal.

isp-1(qm150) is the only known mutation in a mitochondrial complex subunit that increases adult lifespan (Feng *et al.*, 2001). Other known mutations, such as *mev-1* in a subunit of complex II (Senoo-Matsuda *et al.*, 2001), or *gas-1* in a subunit of complex I (Kayser *et al.*, 2001), shorten lifespan. *isp-1(qm150)* has thus been often used to compare the lifespan effects of mitochondrial dysfunction with other mechanisms of longevity and to explore possible interactions between different mechanisms (Hsu *et al.*, 2003; Hansen *et al.*, 2005; Falk *et al.*, 2006; Pinkston *et al.*, 2006; Wolff *et al.*, 2006; Brys *et al.*, 2007; Ichishita *et al.*, 2008; Morcos *et al.*, 2008; Carrano *et al.*, 2009; Cristina *et al.*, 2009). Recently, gene array technology was used to examine gene expression changes resulting from *isp-1(qm150)* and RNAi against *cyc-1*, which codes for complex III

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cytochrome *c1* (Cristina *et al.*, 2009). Interestingly, the pattern of changes in gene expression induced by the two conditions was found to be substantially different, with the expression of more than 40% of all genes tested altered by knockdown of *cyc-1* (∼20% of the genes up-regulated and ∼20% of the genes down-regulated) but with the expression of only 4% of tested genes changed in *isp-1* mutant (3.5% of genes up-regulated and 0.5% of genes down-regulated), although CYC-1 and ISP-1 function in the same mitochondrial complex. This suggests that silencing by RNAi causes more profound changes than genomic point mutations.

Here, we describe the identification and characterization of a mutation in a subunit of mitochondrial complex I that increases lifespan. We take advantage of the availability of two mutations in subunits of two different respiratory complexes to show that they act on lifespan by the same mechanism, as their effects are not additive, but that RNAi against these respiratory chain subunits acts by a different mechanism that is additive to that of the mutations. In fact, the two mechanisms appear to be radically different and should be studied separately. These results underscore the importance of mitochondrial function as a key determinant of lifespan by demonstrating that distinct aspects of mitochondrial biology affect lifespan independently.

Results

Identification and characterization of *nuo-6(qm200)*

We carried out a genetic screen to identify mutants with phenotypes similar to those of the *isp-1(qm150)* mutants, which grow, reproduce, and age more slowly than the wild type, and have behavioral rhythms, including defecation, pumping and swimming, that are slower than the wild type, yet look healthy as adults, appearing of wild-type size and coloration. After chemical mutagenesis with ethyl methane sulfonate (EMS), hatching of F2 progeny was synchronized and animals with slow growth were isolated and scored for their rate of defecation. Animals that had wild-type appearance, slow growth and a slow defecation rate were studied further. One of the mutants identified in this way (*qm200)* was outcrossed 6 times, characterized further, and found to be indeed phenotypically very similar to *isp-1(qm150)*(Table 1) (Feng *et al*., 2001). Like *isp-1(qm150)* mutants, *qm200* mutants show slow embryonic and postembryonic development, slow swimming, slow defecation, slow pumping, a mild increase in embryonic and larval lethality, and a substantial increase in lifespan (see Table 1 and detailed descriptions in the next paragraph). Molecular characterization of *qm200* (see also Experimental Procedures) indicated that it is a G to A transition producing a G to E change in residue 116 of the predicted protein W01A8.4, which is homologous to a subunit of mitochondrial complex I of vertebrates (NDUFB4/B15) (Fig. 1A). Injection of *qm200* mutants with PCR-amplified W01A8.4 yielded transgenic lines producing animals whose appearance was indistinguishable from the wild type, and whose rates of pumping, swimming, defecation, and postembryonic development were partially or fully rescued (Table S1). The mutated residue is not conserved between phyla but is conserved among nematode species, including the distantly related *Brugia malayi* (not shown). Following current practice, we have renamed W01A8.4 *nuo-6* (for NADH ubiquinone oxidoreductase). NUO-6 is conserved in a wide diversity of animal species (Fig. 1A), and corresponds to a recognized protein domain (PFAM 07225). NUDFB4/B15 was first identified in bovine heart (Walker *et al.*, 1992), where it was found to be a prime target of peroxynitrite damage to mitochondria (Murray *et al.*, 2003), and its expression in people was found to be positively correlated with high maximum oxygen uptake capacity in muscle (Parikh *et al.*, 2008).

Table 1 Phenotypic characterization of *nuo-6* mutants and *nuo-6;isp-1* double mutants

	Wild type (N2)	***nuo-6 (qm200)***	***nuo-6(qm200);isp-1 (qm150)***
Embryonic development (h)	13.9 ± 1.0 (n = 46)	21.9 ± 1.8 P < 0.0001 (n = 58)	27.8 ± 3.1 P < 0.0001 (n = 35)
Post embryonic development (h)	46.5 ± 3.0 (n = 20)	108.0 ± 1.3 P < 0.0001 (n = 21)	105.3 ± 2.6 P < 0.0001 (n = 100)
Defecation cycle length (s)	52.3 ± 0.9 (n = 25)	100.5 ± 13.7 P < 0.0001 (n = 25)	101.6 ± 12.3 P = 0.77 (n = 25)
Brood size (eggs)	326.7 ± 37.5 (n = 30)	69.1 ± 13.9 P < 0.0001 (n = 90)	52.2 ± 12.4 P = 0.003 (n = 100)
Embryonic lethality (fraction dead)	1.0% (n = 400)	5.3% (n = 200)	18% (n = 100)
Larval lethality (fraction dead)	0% (n = 200)	5.3% (n = 400)	6% (n = 50)
Lifespan (days)	18.6 ± 6.8 (n = 150)	32.2 ± 12.5 P < 0.0001 (n = 120)	30.3 ± 7.3 P = 0.0012 (n = 100)

Means ± SD are given. The P values were determined by the Student's t-test. The P values in the ***nuo-6*** column compare ***nuo-6*** mutants to the wild type, and the p values for the double mutant column compare them to ***nuo-6.***

First, we examined the cellular and intracellular distribution of a NUO-6::GFP. As expected given its homology to a mitochondrial complex I subunit, we found that the fusion protein shows the subcellular distribution typical of mitochondrial proteins (Fig. 1B). However, we have observed expression only in body wall muscles, in the gut, in the somatic gonad, in the germ line, and, more faintly, in the hypodermis, including in the seam cells. Thus, it is possible that NUO-6 is not required to the same extent in every tissue, and might not be required at all in the nervous system. However, expression from a transgene can never guarantee a complete representation of the pattern and level of expression of the chromosomal gene, which might be more uniformly expressed.

We next characterized the primary biochemical and physiological consequence of the absence of NUO-6 by measuring mitochondrial function. We found that the activity of mitochondrial complex I is severely reduced in the *qm200* mutants

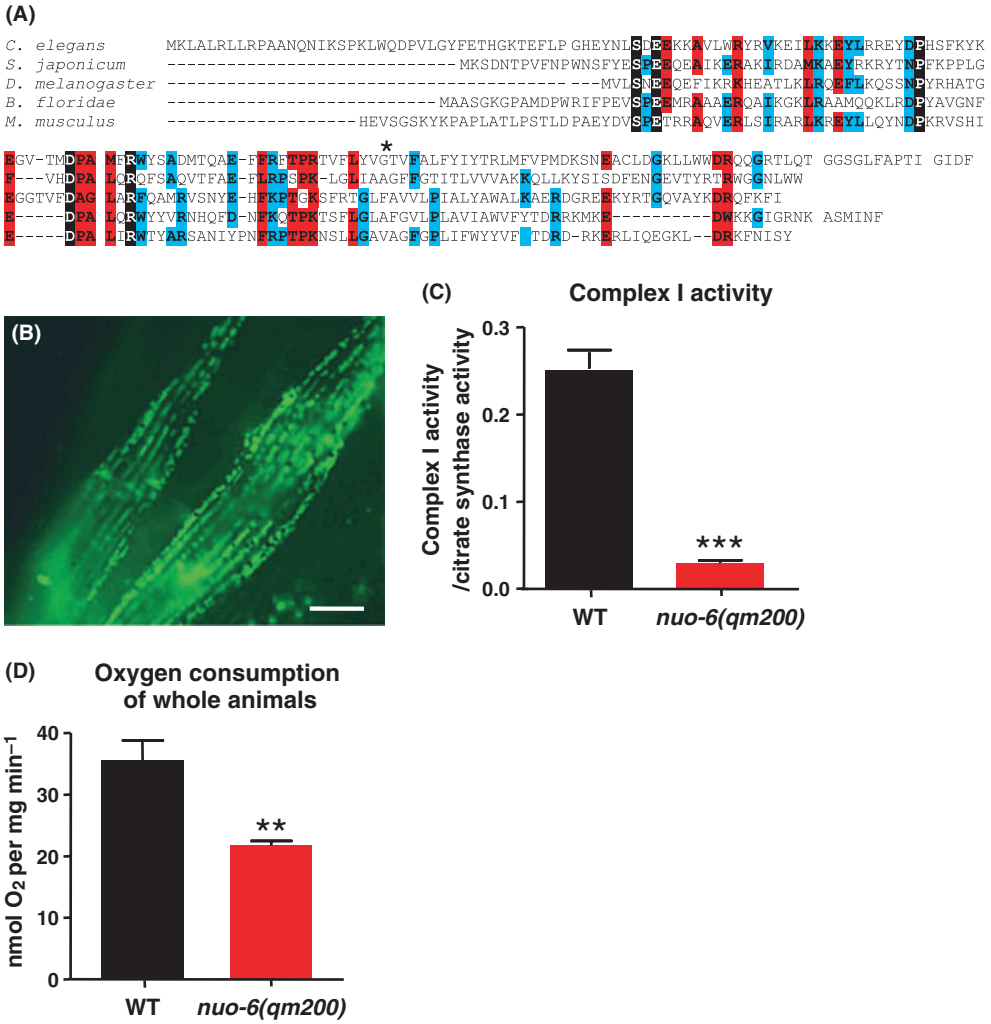

Fig. 1 Molecular and phenotypic characterization of *nuo-6(qm200)*. (A) *nuo-6* (W01A8.4) encodes a homolog of subunit B15/NUDFB4 of vertebrate mitochondrial complex I. An asterisk indicates the site of the *qm200* mutation (a G to A missense mutation). (B) NUO-6::GFP fusion protein expressed in body wall muscle cells. The distribution shows the typical mitochondrial pattern. The scale bar is 10 μm. (C) *nuo-6(qm200)* severely reduces mitochondrial Complex I activity *in vitro*. Complex I activity was normalized to citrate synthase activity; young adults were used; the wild type (WT) is N2; means ± SE are shown; $n = 4$ independent experiments; *** stands for $P < 0.001$ obtained by the Student's *t*-test. (D) The oxygen consumption of young adult wild-type N2 worms (WT) and *nuo-6(qm200)* mutants, normalized to total protein; means ± SE are shown; $n = 10$ independent experiments; ** stands for $P < 0.01$. The consumption of a large number of worms was measured simultaneously in a glass chamber with a Clark oxygen electrode (see also Fig. S2).

(Fig. 1C), similar to the reduction of complex III activity observed in *isp-1* mutants (Fig. S1). In addition, oxygen consumption, a measure of the rate of electron transport and of energy generation, is reduced in intact young adult mutants in both *nuo-6* (Fig. 1D) and *isp-1* (Fig. S2), as measured by two different methods (Fig. S2).

nuo-6(qm200) and *nuo-6(RNAi)* induce different growth and behavioral phenotypes

RNAi knockdown of *nuo-6* has repeatedly been found to be very deleterious in systematic RNAi screens (Fraser *et al.*, 2000; Rual *et al.*, 2004; Sonnichsen *et al.*, 2005). We found RNAi knockdown of *nuo-6* to be lethal in our assays as well. For this, we constructed a feeding RNAi clone with no overlap with any other gene (see Appendix S1), in particular with no overlap with W01A8.3 (*cutl-6*), a gene whose 3′ end might overlap the 3′ end of *nuo-6*. Furthermore, we have tested the level of expression of *cutl-6* after *nuo-6(RNAi)* and found it to be unaffected (not shown).

nuo-6(RNAi) resulted in a mixture of dead eggs and animals that hatched but subsequently failed to develop (not shown). However, by diluting the RNAi-inducing bacteria with bacteria carrying only an empty vector, we could decrease the severity of the effect of the RNAi. Similar techniques have been used by others to reduce the severity of the phenotypes induced by RNAi against subunits of mitochondrial complexes (Rea *et al.*, 2007). We found that providing a higher proportion of RNAi-inducing bacteria induces a proportionally more severe knockdown of mRNA levels (Fig. 2A). We also found that we could induce

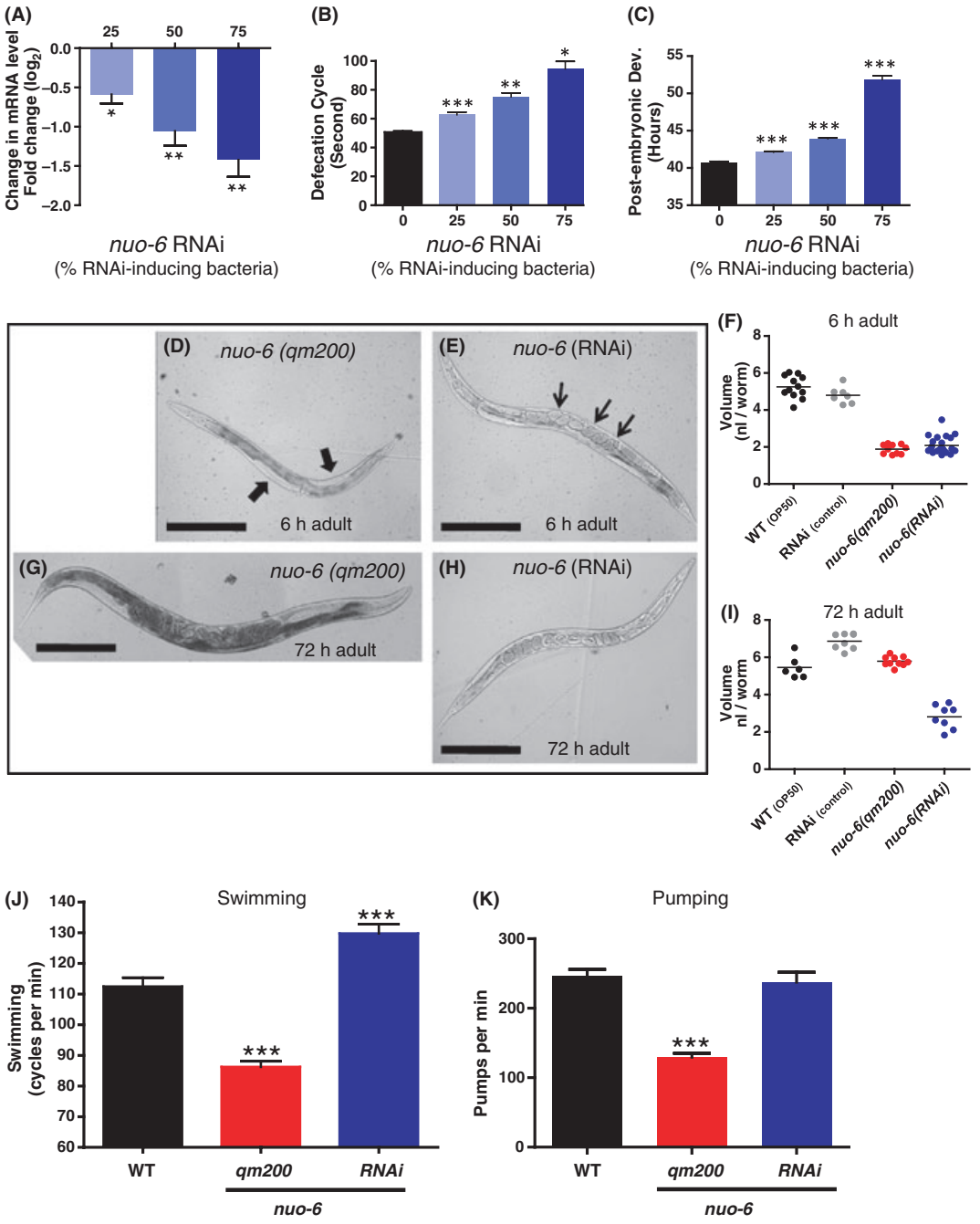

Fig. 2 *nuo-6(qm200)* mutants and *nuo-6* RNAi-treated animals display different growth and behavioral phenotypes. (A) *nuo-6(RNAi)* reduces the level of *nuo-6* mRNA in comparison with the wild-type in direct relation to the concentration of RNAi-inducing bacteria; the RNAi-inducing bacteria are diluted with bacteria carrying only an empty vector; means ± S.E. are shown; *, ** and *** stand for $P < 0.05$, 0.01, 0.001, respectively, and correspond to *t*-tests comparing the values at the particular concentration to those at the immediately lower concentration. (B) The rate of defecation at three dilutions of RNAi-inducing bacteria; $n \geq 10$ at each concentration. (C) The same analysis as in B but for the duration of postembryonic development; $n \geq 60$ at each concentration. (D–F) 6 h after the adult molt *nuo-6(qm200)* (D), and *nuo-6(RNAi)*(at 50% RNA-inducing bacteria) (E), are of similar volume (F) but *nuo-6(qm200)* mutants have not yet produced any eggs. The arrows in D point to the empty gonads and point to eggs in E. (G–I) Seven-two hours after the adult molt *nuo-6(qm200)* mutants (G) have reached a wild-type size and now produce abundant eggs, while *nuo-6(RNAi)* animals (H) have not increased in size or volume (I). The scale bar is 200 μm in all panels. (J) The swimming rate (frequency of thrashing) of *nuo-6(qm200)* mutants is slower than that of the wild type, while *nuo-6(RNAi)* animals swim faster than the wild type; $n = 20$; ***stands for $P < 0.001$ by the Student's *t*-test. (K) The pumping rate (number of pumps per minute) of *nuo-6(qm200)* mutants is lower than that of the wild type, while that of *nuo-6(RNAi)* animals is identical; $n = 10$; *** as in I. 50% RNAi is used in panels E, H, F, I and J.

graded phenotypic changes in this way, as observed with the length of the defecation cycle, which was slowed down as in the *qm200* mutants (Fig. 2B), postembryonic development, which was lengthened as in the *qm200* mutants (Fig. 2C), and brood size, which was reduced as in the *qm200* mutants (Fig. S3). However, RNAi treatment (at 50% RNA-inducing bacteria) had

a very different effect than the *qm200* mutation on gross anatomy and germline development. Six hours after having reached adulthood (assessed by appearance of the vulva) *nuo-6(qm200)* mutants (Fig. 2D) and *nuo-6* RNAi-treated animals (Fig. 2E) had a similar body size, which was significantly smaller than wild type (Fig. 2F), and both were relatively transparent. However, while *qm200* mutants had not yet formed a single egg (Fig. 2D), RNAi-treated worms had already produced and sometimes laid numerous eggs (Fig. 2E). Furthermore, after 72 hours *qm200* mutants had become as large as the wild type (Fig. 2G,I), had a dark coloration, and produced eggs (Fig. 2G). RNAi-treated worms, however, remained as small and transparent as after 6 hours (Fig. 2H,I). RNAi treatment and the *qm200* mutation also had very different effects on swimming rates (Fig. 2J) (young adult *qm200* mutants swam more slowly than the wild type, while RNAi-treated animals swam more quickly), and on pumping (Fig. 2K)(*qm200* mutants pumped slowly but RNAi treatment had no effect on pumping). For swimming (Fig. S4A), size (Fig. S4B) and brood size (Fig. S3), the severity of the phenotype produced by RNAi depended on the proportion of RNAi-inducing bacteria as for defecation and the rate of development (Fig. 2B,C). For size and swimming (Fig. S4), we also studied the differences between *isp-1(qm150)* mutants and animals treated with *isp-1* RNAi at 25% RNAi-inducing bacteria, which induced a > 2-fold decrease in mRNA (not shown), and observed the same differences as with *nuo-6* (Fig. S4). *isp-1* RNAi with more than 25% RNAi-inducing bacteria was lethal (not shown).

isp-1(qm150) and *nuo-6(qm200),* but not *isp-1(RNAi)* or *nuo-6(RNAi),* induce similar changes in gene expression.

isp-1(qm150) leads to up-regulation of ~3.5% of the genes of the genome as assessed by gene arrays (Cristina *et al.*, 2009). From these genes, we chose the 14 genes (see Appendix S1) whose expression was found to be the most reliably elevated (lowest q values), and that had homologs in non-nematode species, and tested their expression by qPCR in *isp-1(qm150)* mutants. The expression of eight of these was significantly elevated as well when evaluated by this different technique (Fig. 3A). The expression levels of the other six genes (T05G5.6; T04C4.1; B0286.3; Y41D4B.5; T12D8.8; T23G11.2) were either not elevated or not significantly elevated under our conditions (not shown), and were not considered further. The expression levels of the eight genes with elevated expression in *isp-1(qm150)* mutants were also assessed in *nuo-6(qm200)* mutants, as well as in *isp-1* RNAi animals with 25% RNAi-inducing bacteria and in *nuo-6* RNAi animals with 50% RNAi-inducing bacteria (Fig. 3A). In the figure, the statistical significance of the differences of level of expression in comparison to the wild type or to wild type treated only with bacteria containing the empty vector (L4440) is indicated by asterisks associated with each of the bars representing the change of level of expression. In addition, the bars are color-coded in the following way: blue represents a change that is significant, and in the same direction as that observed in *isp-1(qm150)* mutants, while red represents a change that is either in a different direction or is not significant. In *nuo-6(qm200)* mutants the expression levels of 7/8 of the genes were significantly elevated, and the eighth (C27D8.4) showed a trend in the same direction, indicating a very similar reaction of the organism to the two mutations (Fig. 3A). However, in *isp-1(RNAi)* animals only one gene (R02E12.6) was significantly elevated and only 3/8 genes (F39H2.5, C24F3.4, H04M03.3) were significantly elevated in *nuo-6(RNAi)* animals. Furthermore, for three of the genes (R02E12.6, M18.5, F41E6.5) the expression in *nuo-6(RNAi)* was in fact significantly down-regulated (Fig. 3A). Although gene expression in *nuo-6(qm200)* or *nuo-6(RNAi)* has not yet been studied in a genome-wide manner, the knockdown of *cyc-1*, which results in a very similar overall phenotype as that of *nuo-6* and *isp-1*, results in ~ 20% of all genes being overexpressed (Cristina *et al.*, 2009). Thus, if *nuo-6(RNAi)* and *isp-1(RNAi)* are at all similar to *cyc-1(RNAi)* in this respect, one would expect to see by chance alone some overlap with any other condition (here *nuo-6(qm200)* and *isp-1(qm150)*) that elevates the expression of some genes. In conclusion, the pattern of changes in gene expression induced by each of the two point mutations is very similar, which is consistent with the other similarities in their phenotypes, yet is remarkable as the two mutant proteins function in different mitochondrial complexes. The patterns of gene expression induced by *nuo-6(RNAi)* and *isp-1(RNAi),* however, appear to be quite different.

The expression of the genes for mitochondrial heat-shock proteins and the heat-shock factor HSF-1 are upregulated in *nuo-6(RNAi)* and *isp-1(RNAi)* but not in *nuo-6(qm200)* or *isp-1(qm150).*

RNAi treatment against a subunit of a multi-protein complex such as the mitochondrial respiratory complexes is expected to result in difficulties in assembling functional complexes and might therefore lead to the accumulation of improperly assembled complexes, or to an excess of unassembled and unfolded subunits, all of which is likely to trigger a stress response leading to an increase in the expression of the global regulator heat-shock factor 1 (HSF-1) and of specific heat-shock proteins. Such an effect is not necessarily expected from the *nuo-6(qm200)* or *isp-1(qm150)* mutations that are predicted to encode proteins with single amino acid changes that might be normally incorporated into their respective complexes. We tested this by using qPCR to determine the expression of *hsf-1* as well as of *hsp-6* and *hsp-60*, two previously studied mitochondrial heat-shock proteins (Hsu *et al.*, 2003; Yoneda *et al.*, 2004; Hamilton *et al.*, 2005; Kimura *et al.*, 2007), including a finding that *hsp-6* was upregulated after RNAi against subunits of mitochondrial complexes (Hamilton *et al.*, 2005). We found that all three genes were strongly upregulated in *nuo-6(RNAi),* and two of them (*hsf-1* and *hsp-6*) in *isp-1(RNAi),* but not in *nuo-6(qm200)* nor in *isp-1(qm150)* mutants (Fig. 3B). In fact, *hsf-1* is even significantly down-regulated in *isp-1(qm150)* mutants. Thus,

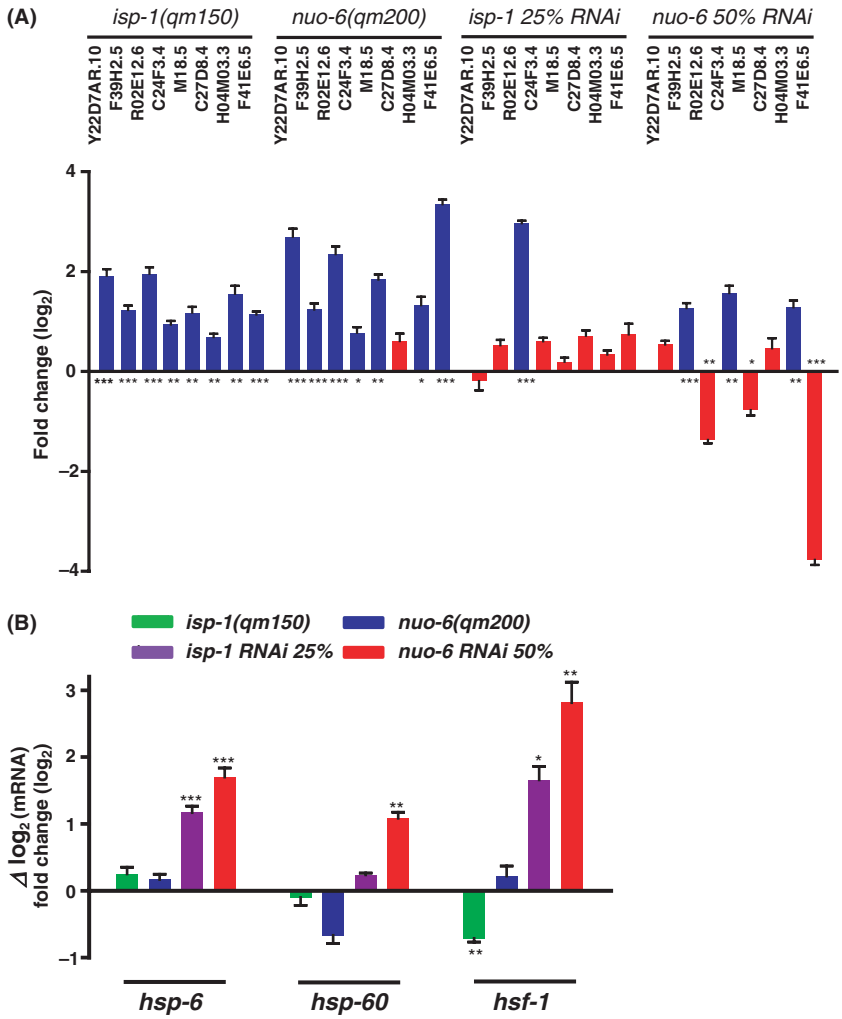

Fig. 3 *isp-1(qm150)* and *nuo-6(qm200),* but not *isp-1(RNAi)* or *nuo-6(RNAi),* induce similar changes in gene expression as measured by qRT-PCR. Fold changes on a $\log_2$ scale are shown. (A) Eight genes that were significantly up-regulated in *isp-1(qm150)* mutants are also elevated in *nuo-6(qm200)* mutants, of which seven significantly, but the expression of only one and three of these genes is upregulated in *isp-1(RNAi)* and *nuo-6(RNAi),* respectively. Moreover, three of the genes are significantly down-regulated in *nuo-6(RNAi).* In the figure, the statistical significance of the difference of expression in comparison to the wild type is indicated by asterisks associated with each of the bars that represent the magnitude of the change of expression. In addition, the bars are color-coded in the following way: blue represents a change that is significant, and in the same direction as that observed in *isp-1(qm150)* mutants, while red represents a change that is either in a different direction or is not significant. Mean ± SE are given; $n = 10$. (B) The expression of mitochondrial heat-shock protein genes (*hsp-6* and *hsp-60*) and the heat-shock factor gene (*hsf-1*) were measured. The expression of these genes tended to be elevated in *isp-1(RNAi)* and *nuo-6(RNAi)*, but not in the mutants, with *hsf-1* even down-regulated in *isp-1(qm150).* Mean ± SE are given; $n = 6$.

alterations in cellular physiology that result from an activated heat-shock response are likely to participate in producing the phenotypic differences between the point mutants and the RNAi treatment. Differences between the ways the assembly of complexes I and III is being monitored *in vivo*, or differences between the actual degree of knockdown achieved by the treatments at the protein level, could explain the difference of expression of *hsp-60* following the two different RNAi treatments.

nuo-6(qm200) and *nuo-6(RNAi)* induce different metabolic phenotypes

NUO-6 is a subunit of mitochondrial complex I, and we have found that the *qm200* mutation indeed affects mitochondrial function (Fig. 1C,D). We therefore also compared the effect of the *nuo-6* mutation and RNAi treatment on oxygen consumption (Fig. 4A), ATP levels (Fig. 4B) resistance to oxidative stress induced by treatment with the pro-oxidant paraquat (Fig. 4C), and levels of the superoxide dismutases SOD-1 (Fig. 4D) and SOD-2 (Fig. 4E). Sensitivity to paraquat is an indication of the balance between endogenous reactive oxygen species (ROS) generation and detoxification. Increased levels of SODs is generally indicative of increased intracellular ROS levels, and is also often associated with abnormal function of the electron transport chain (ETC) (Yang *et al.*, 2007). In our initial characterization of *nuo-6(qm200)* (Fig. 1D), we measured oxygen consumption from a large number of animals (200-1000) at once in a 1.5-mL chamber with a Clark oxygen electrode.

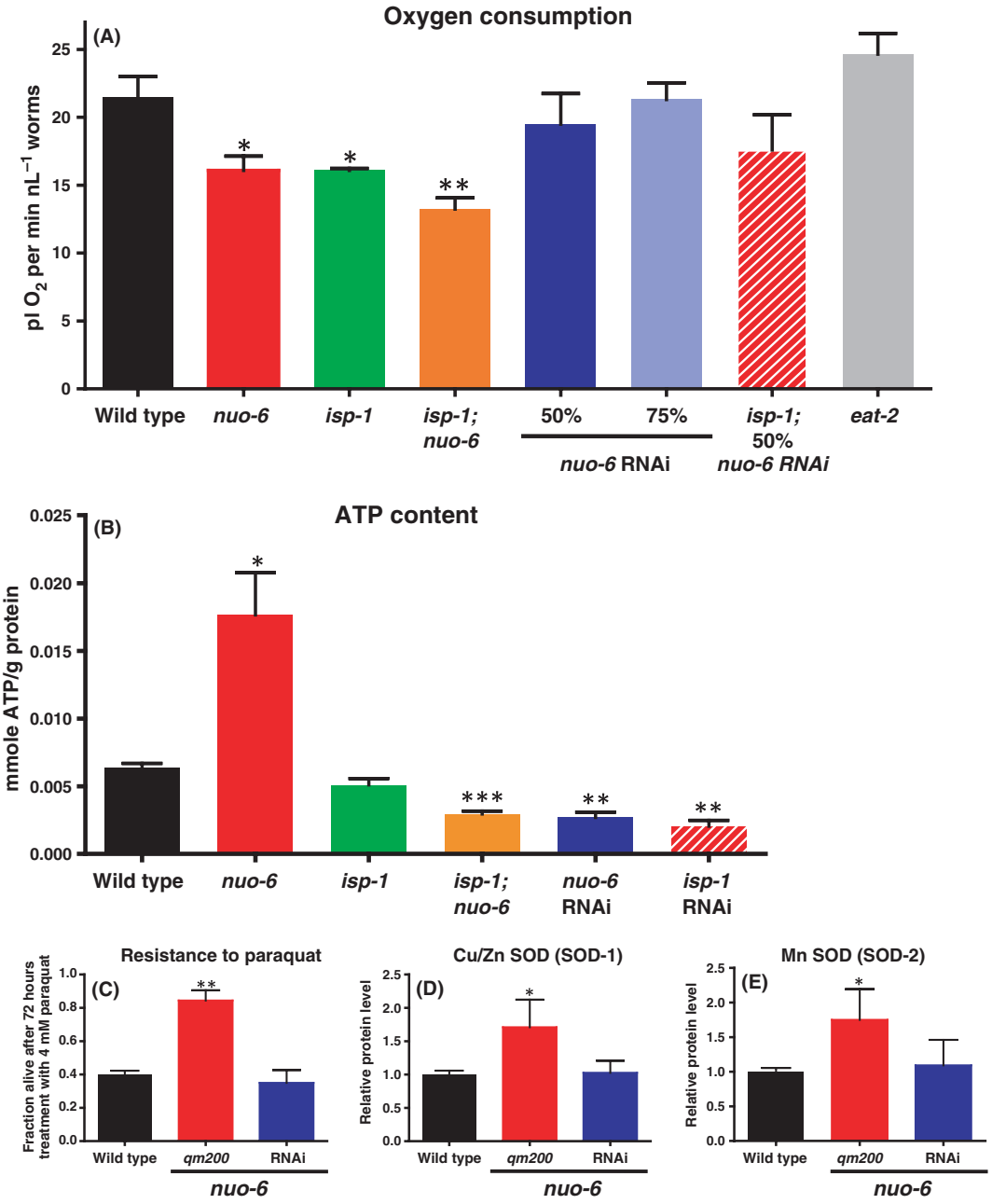

Fig. 4 *nuo-6(qm200)* and *nuo-6(RNAi)* induce different changes in oxygen consumption and ROS metabolism. (A) Oxygen consumption of various strains were measured with an optical oxygen sensor in a 0.5-μL chamber and normalized to the worms' body volume. Only *isp-1(qm150), nuo-6(qm200),* and *isp-1;nuo-6* double mutants have low oxygen consumption. Mean ± SE are given; $n = 15$ (three experiments with five worms each). (B) ATP contents was measured (see Experimental Procedures) and normalized to the total amount of soluble protein. RNAi against *isp-1* and *nuo-6*, and *isp-1;nuo-6* double mutants have significantly lower ATP content, while *nuo-6(qm200)* mutants have higher ATP content, than the wild type. Mean ± SE are given; $n = 3$ repeats of the experiment. (C) L4 larvae were transferred to 4 mM paraquat plates and surviving worms were counted after 3 days. *nuo-6(qm200)* mutants are significantly more resistant than *nuo-6(RNAi)* worms, which were no different from the wild type. Mean ± SE are given; $n = 60$. (D and E) The levels of SOD-1 and SOD-2 were measured from 100 worms-samples by Western blotting. *C. elegans* superoxide dismutases-specific antibodies were used to identify SOD-1 (cytoplasmic Cu/Zn SOD) (D) and SOD-2 (mitochondrial Mn SOD) (E). Western blots were quantified; band densities were normalized to α-tubulin and to the ratio observed in the wild type (N2) (see Experimental Procedures). *qm200* mutant significantly increases both SODs protein levels while knocking down *nuo-6* does not have such effects. Means and ± SE are shown; $n = 6$; * and ** correspond to $P < 0.05$ and $P < 0.01$, respectively, by the Student's *t*-test.

However, for the comparison between mutant and RNAi, we used a different method (Suda *et al.*, 2005) with which the oxygen consumption of five worms at a time is measured in a micro-chamber using a coated optic fiber. The two methods yield similar results (Fig. S2), but the latter method is more practical when it is difficult to obtain many developmentally synchronized

animals, as can be the case with RNAi. Like *isp-1* mutants, *nuo-6(qm200)* mutants have lower oxygen consumption. However, the mutants were different regarding ATP levels, which were wild type in *isp-1(qm150)* mutants but elevated in *nuo-6(qm200)* mutants. The elevated ATP levels suggest that in *nuo-6* mutants energy-consuming processes were slowed more severely than is the generation of ATP by the mitochondria, or that a feedback control on ATP generation by the rate of consumption has been lost. However, more experiments measuring the ADP/ATP and AMP/ATP ratios will be needed to firmly establish how the mutants differ energetically from the wild type and from each other.

Metabolically, *nuo-6(qm200)* and *nuo-6(RNAi)* were very different in every respect. *nuo-6(RNAi)* animals do not show reduced oxygen consumption, but show reduced ATP levels (as has been found previously for RNAi knockdown of other respiratory chain subunits (Dillin *et al.*, 2002)), and, in contrast to *nuo-6(qm200),* were not paraquat resistant (Fig. 4C) nor showed elevated levels of SOD-1 and SOD-2 (Fig. 4D,E). It was of interest to observe the increased expression of SOD-1 and SOD-2 in *nuo-6* mutants as increased superoxide detoxification could contribute to longevity by reducing oxidative stress. However, as previously observed with *isp-1(qm150)* mutants, which also show increased SOD-1 and SOD-2 levels (Yang *et al.*, 2007), RNAi knockdown of *sod-1* or *sod-2* had no effect on the lifespan of *nuo-6(qm200)*(not shown), although the increased expression of the SODs does likely contribute to the increased resistance of the mutants to paraquat (Van Raamsdonk & Hekimi, 2009).

Mitochondrial mutations and the RNAi treatments induce additive mechanisms of longevity

The phenotype of *nuo-6(qm200)* is very similar to that of *isp-1(qm150)*, although the two mutations affect different mitochondrial complexes. We therefore wondered whether they affected lifespan by the same mechanism. To test this, we first constructed *isp-1(qm150); nuo-6(qm200)* double mutants and determined their lifespan (Fig. 5A, Tables 1 and S2). We found that the longevity of the double mutants was very similar, although slightly shorter, than that of each of the constituent mutants. The double mutants are also very similar to *nuo-6(qm200)* for growth, fertility and behavior (Table 1). However, oxygen consumption and ATP levels were more severely affected in the double mutants (Fig. 4A,B). This suggests that the two mutations affect lifespan by the same mechanism, and that the maximum longevity increase that can be triggered by this mechanism is reached in each of the mutants. In fact, the slightly shorter lifespan of the double mutants (2 days on average) suggests that the more severe reductions in oxygen consumption and ATP levels are somewhat deleterious. Our observations on ATP levels also suggest that neither the high level of ATP in *nuo-6(qm200)* nor the normal levels in *isp-1(qm150)* are necessary for their enhanced longevity.

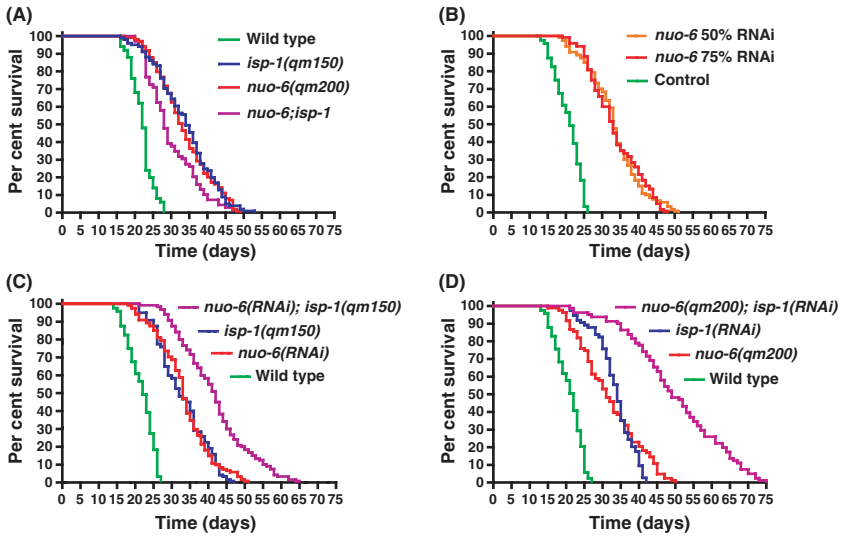

Fig. 5 The longevity-increasing effects of genetic mutations in subunits of mitochondrial respiratory complexes are additive to the longevity-increasing effects of knocking down the expression of the same genes by RNAi. (A) *isp-1(qm150)* (median lifespan 34 days) and *nuo-6(qm200)* (median lifespan 33 days) mutants increase lifespan to a similar degree (wild-type (N2) median lifespan 22 days; $P < 0.001$ for the difference with the lifespans of both *isp-1* and *nuo-6*), and *isp-1(qm150); nuo-6(qm200)* double mutants fail to further increase lifespan (median lifespan 28 days) (n = 70–200). (B) Knocking down *nuo-6* by RNAi with 50% of RNAi-inducing bacteria (median lifespan 33 days, while control median lifespan 21 days, $P < 0.0001$) increases lifespan, but a more severe knockdown with 75% of RNAi-inducing bacteria failed to prolong lifespan further (median lifespan 33 days, $P = 0.9038$) (n = 120). (C) The longevity-increasing effect of *nuo-6(RNAi)* at 50% RNAi-inducing bacteria (median lifespan 42 days) is fully additive to that of the *isp-1(qm150)* mutation (median lifespan 32 days, $P < 0.0001$) (n = 80). (D) The longevity-increasing effect of *isp-1(RNAi)* (median lifespan 49 days) is fully additive to that of the *nuo-6(qm200)* mutations (median lifespan 31 days, $P < 0.0001$) (n = 80). See Table S2 for all details of lifespan experiments. All statistic analyses were carried out by curve comparisons using Graphpad 4.0.

To test the significance of the overall difference in phenotype between *nuo-6(qm200)* and *nuo-6(RNAi)* in terms of mechanisms of longevity, we first tested the effect on lifespan of *nuo-6(RNAi)* with two different concentrations of RNAi-inducing bacteria (Fig. 5B). RNAi at both concentrations substantially increases lifespan but 75% RNAi-inducing bacteria did not induce a more severe phenotype (longer life) than 50% RNAi, in contrast to what is observed for defecation (Fig. 2A), postembryonic development (Fig. 2B), brood size (Fig. S3), swimming (Fig. S4A) and size (Fig. S4B). Thus, at 50% RNAi, the effect on lifespan appears to be already maximal. Not surprisingly, given that severe knockdown of *nuo-6* is lethal, the effect of *nuo-6(RNAi)* on *nuo-6(qm200)* mutants is lethal even at 50% RNAi; that is, such animals died during embryogenesis, most never hatched, and none reached adulthood (not shown). We therefore treated *isp-1(qm150)* with *nuo-6(RNAi)* at 50% RNAi. The treatment did not affect oxygen consumption of the mutants (Fig. 4A), consistent with the lack of effect on oxygen consumption of *nuo-6(RNAi)* by itself (Fig. 4A). For lifespan, however, we observed strong additivity (Fig. 5C). We also conducted the converse experiment of treating *nuo-6(qm200)* mutants with RNAi against *isp-1* at 25% RNAi and again observed dramatic additivity (Fig. 5D). This additivity suggests that the mutations in respiratory chain subunits and the knockdown of the expression of such subunits prolong lifespan by different mechanisms.

The lifespan of *nuo-6(qm200)* and *isp-1(qm150)* mutants is independent of the induction of autophagy

One of the simplest and most striking difference between the phenotypes of mutants and RNAi-treated animals is their overall anatomy and behavior: while the mutants are large, dark and slow, the RNAi-treated animals are small, transparent, relatively infertile, and very active. In fact in several respects, the phenotype of RNAi-treated worms appears similar to that of calorie-restricted animals such as the slowly pumping *eat-2* mutants (Lakowski & Hekimi, 1998). In addition, we found, like others (Houthoofd *et al.*, 2002), that *eat-2* mutants, like RNAi-treated animals, show a normal level of oxygen consumption (Fig. 4A). The cellular and molecular mechanisms behind the lifespan extension of *eat-2* mutants have recently been explored by several groups (Jia & Levine, 2007; Panowski *et al.*, 2007; Hansen *et al.*, 2008; Carrano *et al.*, 2009; Greer & Brunet, 2009). In particular, it was found that the increased lifespan of *eat-2* mutants might result in part from a high level of autophagy (Hansen *et al.*, 2008). Thus, we used the *gfp::lgg-1* autophagy reporter (Melendez *et al.*, 2003) to test the level of autophagy in *isp-1(qm150), nuo-6(qm200)* and RNAi-treated animals (Fig. 6A). LGG-1 is predicted to be required for the degradation of cellular components by autophagy. GFP::LGG-1 shows diffuse cytoplasmic localization, but can show a marked increase in punctate staining in hypodermal seam cells that is believed to reflect an increase in the number of autophagosomes. By this assay, we found that the mutants had normal, but the RNAi-treated animals have high levels of autophagy, consistent with the overall phenotypic similarity of RNAi-treated animals with *eat-2* mutants. By the same assay, previous workers have found some increase in autophagy in *isp-1* mutants but to a much lesser degree than in *eat-2* mutants (Hansen *et al.*, 2008). We further scored the lifespan of *nuo-6; eat-2* double mutants and of *eat-2* mutants treated with 50% RNAi against *nuo-6* (Fig. 6B). We found that, although *nuo-6(qm200)* and *nuo-6(RNAi)* have identical lifespans, the combination of *nuo-6(qm200)* and *eat-2(ad1116)* is fully additive, but *nuo-6(RNAi)* is only partially additive to *eat-2(ad1116)* (Fig. 6B). The lifespan of *eat-2* on RNAi control bacteria with empty vector is 1.8 days shorter than on

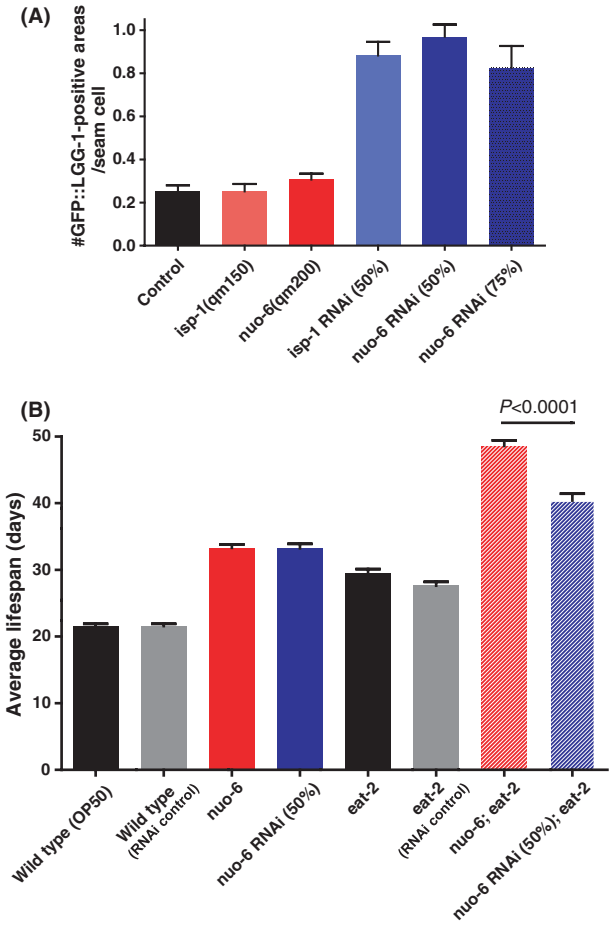

Fig. 6 The longevity-increasing effect of *nuo-6(qm200)* is not linked to the induction of autophagy. (A) The *gfp::lgg-1* reporter gene for autophagy was expressed in all animals tested and the number of GFP::LGG-1 positive puncta in seam cells were counted in L3 larvae. *isp-1(RNAi)* and *nuo-6(RNAi)* increased the number of positive *gfp::lgg1* puncta but not the *isp-1(qm150)* and *nuo-6(qm200)* mutations (n = 100-400). (B) The longevity-increasing effect of *eat-2(ad1116),* which induces calorie restriction, is fully additive to that of *nuo-6(qm200)* but not to that of *nuo-6(RNAi).* Even though the lifespans of *nuo-6* mutants and *nuo-6* RNAi are similar, the *nuo-6(qm200); eat-2(ad1116)* double mutants live significantly longer than *nuo-6(RNAi); eat-2(ad1116)* mutants (n = 80-200). All mutant genotypes lived significantly longer than the wild type. Independent experiments for each genotype or condition (Table S2) were pooled and mean lifespans and ±SE were calculated for the entire pool and are shown; the *P*-value was obtained by the Student's *t*-test. See Table S2 for all details of lifespan experiments.

OP50. However, the lifespan of *eat-2; nuo-6(qm200)* is 8.2 days longer than that of *eat-2; nuo-6(RNAi)*. Thus, these findings are consistent with the notion that the increased lifespan of *nuo-6(RNAi)* is partially the result of high level of autophagy as is the increased longevity of *eat-2(ad1116),* but that the increased longevity of *nuo-6(qm200)* is independent of this mechanism, which allows it to be almost fully additive to that of *eat-2(ad1116)*.

Discussion

Two different types of mitochondrial dysfunction

isp-1(qm150) mutants provide a particularly clear-cut demonstration of the involvement of mitochondrial electron transport in lifespan determination. Indeed, the *isp-1(qm150)* mutation severely reduces electron transport without affecting ATP levels (Fig. 4B) and results in a large increase in lifespan but spares many other aspects of physiology as evidenced by the overall wild-type appearance of the mutants. Furthermore, the phenotype results from a single amino acid change that induces a partial loss-of-function in a very well studied and highly conserved catalytic subunit of a mitochondrial complex (Feng *et al.*, 2001). In addition, several of the phenotypes that accompany the increased longevity, including slow development and behavior, and reduced fertility, but not lifespan or electron transport themselves, can be completely or partially suppressed by a compensatory mutation in *ctb-1* (coding for the cytochrome *b1* of complex III), which suggests that increased longevity is directly caused by slow electron transport, and is not the indirect consequence of one of the other phenotypes. The *nuo-6(qm200)* point mutant that we describe here is phenotypically very similar to *isp-1(qm150)*, although it affects a subunit of a different mitochondrial complex. It is thus a conservative hypothesis to suggest that both mutations result is a slowing down of the rate at which electrons are transferred from electron donors to acceptors in their respective complexes, while leaving the electron transport chain and mitochondrial structure intact. This might also explain why the effects of these mutations are not additive for lifespan. In fact, oxygen consumption is only mildly more depressed in the double mutants than in the singles.

RNAi treatments against subunits of respiratory complexes also increase lifespan (Dillin *et al.*, 2002; Lee *et al.*, 2003; Hamilton *et al.*, 2005; Hansen *et al.*, 2005; Curran & Ruvkun, 2007; Pan *et al.*, 2007), and it has generally been assumed that the mutants and RNAi treatments prolong lifespan by the same mechanism. Yet the consequences on the assembly of mitochondrial complexes of preventing the production of a subunit are likely to be very different from those of incorporating mutant subunits with altered function. One would expect depressed production of one subunit to lead to the assembly of fewer functional mitochondrial complexes but the assembled complexes should have wild-type function. One would also expect such mitochondria to contain unfolded subunits (because these subunits could not be incorporated into a complex), as well as misassembled complexes (Yoneda *et al.*, 2004). Animals treated by RNAi against *nuo-6* show the overall phenotype that is typical of other RNAi treatments against subunits of mitochondrial complexes, being smaller, more transparent, and less fertile than the wild type. In fact, we found that virtually every aspect of the phenotype of *nuo-6(RNAi)* animals was distinct from that of *nuo-6(qm200)* mutants. Furthermore, we found that in *nuo-6(RNAi)* animals but not in the mutants, the heat-shock response was activated and autophagy was enhanced, which is consistent with the hypothesis that normal complex assembly is disrupted after RNAi, but that *isp-1* or *nuo-6* mutant subunits can be more or less normally incorporated into their respective complexes.

Interestingly, the oxygen consumption of RNAi-treated animals is not really affected when normalized to body size (Fig. 4A), as was observed previously for RNAi knockdown of other subunits (Lee *et al.*, 2003). This suggests that there is a developmental adjustment to reduced mitochondrial function. The fact that this adjustment does not occur in *isp-1* and *nuo-6* mutants in spite of a very severe loss of complex III (Fig. S1) and complex I activity (Fig. 1C), respectively, is one more indication that the cellular consequences of the two modes of dysfunction are quite different.

Different mechanisms of longevity are triggered by different modes of mitochondrial dysfunction

We have found that the two modes of mitochondrial dysfunction, i.e. inactivation by RNAi or by genomic mutation, trigger mechanisms of lifespan prolongation that are additive to each other. Additivity of lifespan increases, especially when the mutants or treatments that are additive induce very distinct overall phenotypes, is generally considered as a strong criterion for the identification of distinct mechanisms of aging, (Dorman *et al.*, 1995; Lakowski & Hekimi, 1996, 1998; Hekimi *et al.*, 2001; Crawford *et al.*, 2007; Chen *et al.*, 2009; Mehta *et al.*, 2009). Another possibility is that each of two additive interventions triggers only partially a single mechanism, such that both interventions are required for the greatest possible effect. However, in addition to the very distinct phenotypes of mitochondrial RNAi and mitochondrial mutants, there are several other reasons why this is likely not the case here: (i) The most severe level of knockdown of *nuo-6* is lethal and 75% RNAi is substantially more severe than 50% RNAi for defecation, postembryonic development, brood size, swimming, and size (Figs 2AB, S3, S4A,B), yet the lifespan increase produced by 75% RNAi is no greater than that produced by 50% RNAi (Fig. 5B). Thus, the effect of *nuo-6(RNAi)* on lifespan is saturated at 50% RNAi. (ii) The *isp-1*, *nuo-6* double mutants have lower oxygen consumption than either of the single mutants yet do not live longer. As reduced oxygen consumption through reduced electron transport is likely the primary defect

in these mutants this suggests again that a more severe defect of one type only cannot improve lifespan beyond what is observed in single mutants. (iii) The phenotype of mutants treated with RNAi against the other gene (*nuo-6(qm200); isp-1(RNAi)* or *isp-1(qm150); nuo-6 (RNAi)*) appears simply additive, not more severe for any given phenotype observed in mutants or in RNAi-treated worms. For example, these animals are not more sluggish than the mutants without treatment, or smaller than when wild-type worms are treated with *nuo-6* or *isp-1* RNAi, and they are still fertile (not shown). We conclude that the two modes of mitochondrial dysfunction prolong lifespan by distinct mechanisms, not each by triggering the same mechanism in a different way.

Retrograde signaling

Mitochondrial dysfunction triggers the retrograde response in yeast (Epstein *et al.*, 2001), in which the expression of nuclear genes is altered in a stereotypical way in response to reduced mitochondrial function to minimize the impact of the dysfunction on the organism's performance (Liu & Butow, 1999). The authors of a recent publication (Cristina *et al.*, 2009) suggest that the patterns of changes in gene expression produced by *clk-1(qm30)* [a mutation in an enzyme necessary for ubiquinone biosynthesis (Ewbank *et al.*, 1997)], *isp-1(qm150)* and RNAi against *cyc-1* (coding for cytochrome *c1*) might be analogous to those observed in the retrograde response. In yeast, the retrograde response has been linked to lifespan (Kirchman *et al.*, 1999). However, it is controversial whether the relationship between mitochondrial dysfunction and increased lifespan in fact involves the retrograde response (Woo & Poyton, 2009). Our findings here suggest that the gene expression changes that were found to be common between mutants and RNAi treatments (Cristina *et al.*, 2009) are not responsible *per se* for the lifespan increase of either type as we have shown that these have different underlying mechanisms. However, this does not necessarily imply that these changes in gene expression are not part of the changes that are necessary in one of the conditions (point mutation or RNAi), or even in both, but rather that they are not sufficient.

Autophagy, the heat-shock response, and protein degradation

There is much agreement that, in one way or another, the aged phenotype involves the accumulation of damage to macromolecules. It was therefore illuminating that links were found recently between autophagy, a mechanism that helps to eliminate damaged cellular components, and lifespan extension in worms (Melendez *et al.*, 2003; Hars *et al.*, 2007; Jia & Levine, 2007; Hansen *et al.*, 2008) and aging in mice (Zhang & Cuervo, 2008). In particular, it was proposed that at least part of the lifespan extension produced by calorie restriction in worms required intact mechanisms of autophagy. On the other hand, here we found that an increase in autophagy is readily detected and substantial in RNAi-treated worms but not in the mutants. This is consistent with our findings that the heat-shock response is activated after RNAi treatment but not in the mutants. This suggests that the damage (i.e. unfolded proteins and partially assembled complexes) induced by RNAi is sensed and an appropriate response is mounted. We also found that the lifespan of *nuo-6* is additive to that of *eat-2* but that *nuo-6(RNAi)* is only partially additive to *eat-2* at best. This is consistent with recent findings that a pathway of ubiquitin-dependent proteolysis underlies the lifespan extension of *eat-2* mutants but not that of *isp-1(qm150)* (Carrano *et al.*, 2009). Furthermore, the lifespan of *cyc-1(RNAi)*, in contrast to *isp-1(qm150),* was at least partially dependent on the activity of this pathway (Carrano *et al.*, 2009). Similarly, it was observed that RNAi during adulthood against *bec-1*, a gene necessary for autophagy, did not suppress the longevity of *isp-1(qm150)* (Hansen *et al.*, 2008), and *atp-3(RNAi)* could not extend the lifespan of short-lived *bec-1* mutants (Toth *et al.*, 2008). Taken together, these findings suggest that at least part of the lifespan increase associated with RNAi against respiratory chain complex subunits might result from an increase in turnover and repair mechanisms. However, the lifespan of the mutants appears independent of autophagy or proteolysis, which also suggests that calorie restriction and mitochondrial mutations affect longevity by distinct mechanisms.

Reduced mitochondrial function and aging

It has frequently been proposed that reduced mitochondrial energy metabolism could affect lifespan by reducing the level of reactive oxygen species (ROS) and the damage they can inflict on macromolecules. However, recent results in various organisms, in particular in worms (Yang *et al.*, 2007; Doonan *et al.*, 2008; Van Raamsdonk & Hekimi, 2009), suggest that damage from oxidative stress is not the cause of aging. Furthermore, increasing oxidative damage in *isp-1(qm150)* mutants to the wild-type level does not shorten their lifespan (Yang *et al.*, 2007). This suggests that some other consequence of reduced mitochondrial function is particularly important for aging. However, according to the results presented here, which changes are relevant to lifespan cannot be established by comparison to the changes triggered by RNAi.

Systematic RNAi surveys have found that genes expressed in the mitochondria represent the largest group of genes that can extend lifespan using this technique (Dillin *et al.*, 2002; Lee *et al.*, 2003; Hamilton *et al.*, 2005; Hansen *et al.*, 2005). We, on the other hand, have found that identifying mutations that induce hypomorphic phenotypes such as slow growth and slow behavior also leads systematically to the identification of genes whose mutant phenotype involves reduced mitochondrial function and increased lifespan, and that such genes are abundant (Hekimi *et al.*, 1995; Feng *et al.*, 2001; Van Raamsdonk *et al.*, 2010). Therefore, the results presented here that targeting mitochondria can lead to at least two different mechanisms of lifespan extension which are crucial to the analysis of the

mechanisms of action of a substantial fraction of all the genes of the genome that affect lifespan.

Experimental procedures

Screening

Wild-type animals were mutagenized with 25 mM ethyl methane sulfonate (EMS) following the standard protocol (Sulston & Hodgkin, 1988). Approximately 130 000 F1 worms (approximately 260 000 haploid genomes) were screened for progeny that displayed an *isp-1*-like phenotype. Animals from the F2 generation that grew very slowly (~ 2% of the animals) and had slow defecation were isolated on individual plates. Most arrested before adulthood, or were sterile, or displayed visible phenotypes and were discarded. Those animals that produced a full brood of slow growing progeny without obviously abnormal phenotypes were analyzed further. The selected animals were scored for both slow phenotypes at every generation until the 4th generation. Animals that could maintain these phenotypes were outcrossed to the wild type to select recessive, nonmaternal effect candidates. In the end, only five candidates were kept. *nuo-6(qm200)* was one of these candidates. Others have not yet been characterized.

Phenotype scoring

Most of the methods were as reported previously (Feng *et al.*, 2001). Lifespan scoring was as reported, including replacing worms that died prematurely with spare worms (Lakowski & Hekimi, 1998). Briefly, worms that die prematurely from internal hatching (bagging) are replaced by spare worms. For this, we keep a plate with a large number of worms that are not monitored for survival, just transferred away from their progeny. When a worm of the group that is monitored for survival dies by bagging we randomly pick a worm from the spare group to replace the worm that bagged. Nothing is known about the worm that serves as replacement, and it has been treated exactly in the same way as the worms in the group whose survival is monitored. For measuring the rate of swimming, young adult worms were suspended in M9 buffer, and the number of full thrash cycles in one minute was scored. For paraquat sensitivity which was measured by picking L4 larvae from normal NGM plates onto plates with 4 mM paraquat. After 1 h, a group of 20 young adults were transferred from the initial plate onto fresh 4 mM paraquat plates. Worms were supplied with sufficient fresh OP50, and checked daily. After 72 hr, the total number of worms that still survived was counted.

Metabolic and biochemical measurements

Oxygen consumption was measured by two methods. The first method that uses a Clark electrode and measures the consumption of many animals at once is standard and was as described previously (Feng *et al.*, 2001; Yang *et al.*, 2007). In the second method, five 1-day-old adults were placed into 0.25 μL M9 buffer in a 0.5-μL sealed chamber at 22°C. A fiber optical oxygen sensor (AL300 FOXY probe from OceanOptics, Dunedin, Florida, USA) was inserted into this chamber, and oxygen partial pressure was monitored for 15–30 min and normalized to body volume (Suda *et al.*, 2005). Briefly, for volume measurements: pictures were taken of living worms on clear agar plate with an appropriate ruler using a Leica MZFL III microscope and a Leica DC300F digital camera. The cross-section of each worm was calculated using IMAGEJ software and used for calculation the worm's volume. Mitochondria preparations and the activity of complex I were carried out by standard methods (Lapointe & Hekimi, 2008). For ATP measurements, synchronized young adult worms were collected in M9 buffer and washed three times. Worms pellets were treated with three freeze/thaw cycles and boiled for 15 min to release ATP and destroy ATPase activity, and then spun at 4°C and 11 000 *g* for 10 min. ATP contents were measured with a kit (Invitrogen, Carlsbad, California, USA; Cat: A22066). Western blots were performed with extracts of 100 young adult worms of each genotype using 12% SDS–polyacrylamide gels. The primary antibodies were used at 1:1000 (anti-SOD-1) and 1:2000 (anti-SOD-2), respectively.

Feeding RNAi

The methods were as described (Kamath *et al.*, 2001). For diluted RNAi, the overnight cultures with inserts were diluted with bacteria carrying only an empty vector at similar concentration right before plate seeding. For example, a mix that contained 0.5 mL of *nuo-6* RNAi bacteria and 1.5 mL of control bacteria represents a 25% RNAi mix. The optical densities (ODs) of the cultures were measured and uniformized before mixing. The sequences used to produce RNAi clones for *nuo-6* and *isp-1* are in the Appendix S1. For *nuo-6*, the initial amplicon was further digested by EcoR V and Hind III, and this fragment was inserted in the vector. The full sequence of the clone is given in Appendix S1.

Autophagy

The level of autophagy in various mutants was assessed using an LGG-1::GFP translational reporter previously characterized (Melendez *et al.*, 2003). Animals were raised at 20°C. GFP-positive puncta were counted in the seam cells of L3 animals. Three to ten seam cells were examined in each of 10–40 animals from at least two independent trials and averaged. Young adults were fed the RNAi bacteria, and the L3 larvae from their progeny ('F2 generation') were examined. Data were analyzed by *t*-test.

Cloning of *nuo-6*

nuo-6 was found to lie on linkage group I and mapped immediately to the right of *dpy-5(e61)* (1.60-1.92 cM from *dpy-5*).

Cosmids covering the *nuo-6* locus were microinjected into *nuo-6(qm200)* worms. The cosmid W01A8 was able to rescue the *nuo-6* phenotypes. The W01A8.4 gene was PCR-amplified from the cosmid with primers SHP2577 and SHP2578 (see Appendix S1). The 2.0-kb amplicon encompasses the entire predicted gene and was injected in worms and found to rescue the slow development and slow defecation of *nuo-6(qm200)* animals. This gene was then PCR-amplified and sequenced from *nuo-6(qm200)* worms. By comparing with published sequences, we found an A to G transition at position 781 of the predicted cDNA of the W01A8.4 gene. This mutation was not observed when sequencing the gene from our wild-type stock.

Construction of a NUO-6::GFP fusion reporter

The full-length *nuo-6* coding region including 1 kb of the upstream promoter region was PCR amplified using primer pairs SHP1281 and SHP1282 (sequences can be obtained upon request). This PCR fragments were inserted into the pPD95.77 plasmid (kind gift from the Fire lab) between the Sal I and Sph I restriction sites.

Quantitative PCR measurements

Adult worms were washed into a 1.5-mL tube with M9. After three washes with 100 μL M9, all M9 was carefully removed. Total mRNA was extracted using the TRIZOL (Invitrogen; Carlsbad, California, USA) and quantified by spectrophotometer. One microgram of total mRNA was reverse transcribed to cDNA using the OMNISCIPT RT kit (Qiagen, Mississauga, Ontario, Canada). One-fortieth of the reverse transcription product was used as template to perform real-time PCR using the Quantitect SYBR Grenn PCR kit (Qiagen) and an icycler apparatus (Bio-Rad Version 4.006). Each sample was obtained with 3 independent preparations and each preparation was measured at least twice. Data were analyzed with icycler software (version: 3.0.6070). The level of expression of each gene of interest was first normalized to the *tba-1* expression level, then normalized to an appropriate control sample: OP50-fed wild-type (N2) worms were used as control for *isp-1* and *nuo-6* mutants, and wild-type (N2) worms fed control RNAi bacteria (containing the empty vector L4440) were used for *isp-1* and *nuo-6* RNAi-treated worms.

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Author contributions

W.Y. carried out all experiments. Both authors were involved in experimental designs and interpretations. S.H. wrote the text.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 Electron transport in *isp-1(qm150).*

Fig. S2 Comparison between methods of oxygen consumption measurement.

Fig. S3 Brood size after *nuo-6* RNAi.

Fig. S4 Additional swimming and size measurements.

Table S1 Rescue of *nuo-6(qm200)* by W01A8.4

Table S2 Compilation of all individual lifespan experiments

Appendix S1 Supplemental data: Primers, sequences, strain constructions, genotyping methods

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