Inhibition of elongin C promotes longevity and protein homeostasis via HIF-1 in C. elegans

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Summary

The transcription factor hypoxia-inducible factor 1 (HIF-1) is crucial for responses to low oxygen and promotes longevity in Caenorhabditis elegans. We previously performed a genome-wide RNA interference screen and identified many genes that act as potential negative regulators of HIF-1. Here, we functionally characterized these genes and found several novel genes that affected lifespan. The worm ortholog of elongin C, elc-1, encodes a subunit of E3 ligase and transcription elongation factor. We found that knockdown of elc-1 prolonged lifespan and delayed paralysis caused by impaired protein homeostasis. We further showed that elc-1 RNA interference increased lifespan and protein homeostasis by upregulating HIF-1. The roles of elongin C and HIF-1 are well conserved in eukaryotes. Thus, our study may provide insights into the aging regulatory pathway consisting of elongin C and HIF-1 in complex metazoans.

Key words: aging; C. elegans; elc-1; hypoxia-inducible factor 1; protein homeostasis.

Introduction

Proper levels of oxygen are essential for the survival of aerobic organisms. Hypoxia-inducible factor 1 (HIF-1) is a key transcription factor that governs cellular responses to low oxygen (reviewed in Semenza, 2012). In normal oxygen conditions, HIF-1 is hydroxylated by the proline hydroxylase EGL-9. This leads to the ubiquitination and degradation of HIF-1 by an E3 ligase containing the von Hippel–Lindau-1 (VHL-1) tumor suppressor. VHL-1 determines the substrate specificity of the E3 ligase. In contrast, hypoxia or inhibition of EGL-9 or VHL-1 promotes the stabilization of HIF-1. Stabilized HIF-1 translocates to the nucleus and regulates the transcription of genes that control hypoxic responses. The crucial functions of human HIF-1 are highlighted by the findings that HIF-1 is associated with various diseases and pathological conditions, including cancer, arterial diseases, and organ transplant rejection (reviewed in Semenza, 2012).

C. elegans HIF-1 also plays key roles in various physiologic processes, including stress and pathogen responses, axon guidance, iron and protein homeostasis, and reproduction (Pocock & Hobert, 2008; Beller et al., 2009; Mehta et al., 2009; Zhang et al., 2009; Powell-Coffman, 2010; Romney et al., 2011; Ackerman & Gems, 2012; Fawcett et al., 2015). Recent studies demonstrate that HIF-1 activation promotes longevity in C. elegans (Mehta et al., 2009; Muller et al., 2009, Zhang et al., 2009; Lee et al., 2010, Leiser et al., 2011, Hwang et al., 2014). However, the components that mediate longevity in response to HIF-1 activation remain unclear.

In our previous report, we performed a genomewide RNA interference (RNAi) screen using an HIF-1 reporter, nhr-57p::gfp transgenic C. elegans. We found 245 putative HIF-1 regulators (Lee et al., 2010, Fig. S1, Supporting information). Here, we characterized the functions of these potential HIF-1 regulatory genes in lifespan regulation. We found six genes whose knockdown increased the lifespan of worms. Among those, knockdown of elc-1, which encodes a worm homolog of elongin C, lengthened lifespan by stabilizing HIF-1. In addition, genetic inhibition of ELC-1 increased protein homeostasis in a HIF-1-dependent manner. Elongin C is evolutionarily conserved and therefore may affect aging in complex animals, such as mammals as well as C. elegans.

Results

Our previous genomewide screen was performed in a liquid culture system (Lee et al., 2010); however, conventional lifespan assays are performed in solid culture systems. Therefore, we re-examined 53 RNAi clones that were strong nhr-57p::gfp inducers and found 16 RNAi clones that robustly increased nhr-57p::gfp levels in the solid culture system [Fig. S1, Table S1, Supporting information, and Fig. 1A. Note: Commercially available RNAi clones that were designed to target elc-1 and Y82E9BR.16 have another common target, Y82E9BR.3. We therefore designated these two RNAi clones as elc-1/Y82E9BR.3 RNAi and Y82E9BR.16/Y82E9BR.3 RNAi (Fig. S2, Supporting information)]. Surprisingly, we found that 12 of the 16 RNAi clones induced nhr-57 independently of HIF-1 (Fig. 1A). These data suggest that factors other than HIF-1 also regulate the induction of nhr-57.

Next, we performed lifespan assays with the 16 strong nhr-57 inducer RNAi clones and found that six RNAi clones significantly increased lifespan (Fig. 1B–H, Fig. S3, and Table S2, Supporting information). RNAi targeting elc-1, a worm homolog of mammalian elongin C, and Y82E9BR.3, a worm homolog of ATP synthase subunit C, significantly promoted longevity (Fig. 1B,H). Likewise, RNAi targeting Y82E9BR.16, a worm homolog of solute carrier family 22 member 21, and Y82E9BR.3 promoted longevity (Fig. 1C,H). In addition, knockdown of the nema-tode-specific gene nrl-2 increased lifespan (Fig. 1D,H); this result is consistent with those presented in a previous report (Hansen et al., 2005). We also found that knockdown of the mitochondrial genes F29C4.2 (a worm homolog of cytochrome C oxidase subunit 6C), C16A3.5 (a worm homolog of NADH dehydrogenase [ubiquione] 1 subcomplex subunit 9), and C34C12.8 (a worm homolog of mitochondrial GrpE) extended lifespan (Fig. 1E–H). These results are consistent with many reports showing that mild inhibition of mitochondrial
components confers longevity (reviewed in Van Raamsdonk & Hekimi, 2010; Hwang et al., 2012). We then examined whether the longevity caused by these six RNAi clones was dependent on HIF-1. The RNAi clone that targeted elc-1/Y82E9BR.3 was the only one that increased lifespan in a slightly hif-1-dependent manner (Figs 1H and S4, Supporting information). Together, these data indicate that RNAi clones that increase lifespan and induce nhr-57 expression levels do not necessarily act through HIF-1.

Because RNAi clones against elc-1/Y82E9BR.3 and Y82E9BR.16/Y82E9BR.3 had a common target gene, we generated RNAi clones

Fig. 1 Semiquantification of nhr-57p::gfp levels and the effects of nhr-57 inducer RNAi clones on lifespan. (A) Among 53 candidate RNAi clones selected from our previous screen in a liquid culture system (Lee et al., 2010), 16 RNAi clones consistently increased the level of nhr-57p::gfp in a solid culture system, mostly in a hif-1-independent manner. The arbitrary cutoff value was 0.5 as indicated by a dotted line. egl-9 RNAi was used as a positive control. Error bars indicate standard error of the mean (SEM) (n > 12). (B–G) Lifespan curves of wild-type (WT) animals treated with commercially available RNAi clones targeting elc-1/Y82E9BR.3 (B), Y82E9BR.16/Y82E9BR.3 (C), nil-2 (D), F29C4.2 (E), C16A3.5 (F), or C34C12.8 (G). Lifespan assays were performed at least twice independently. See Fig. S3 for the results of lifespan assays upon treating with other nhr-57p::gfp inducer RNAi clones that did not increase lifespan. (H) Percent changes in the lifespan of WT and hif-1 mutant worms after treatment with RNAi clones shown in Figs 1B–G and S4. The mean lifespan was compared with those of control RNAi-treated worms in at least two trials, and error bars indicate SEM. See Table S2 for additional trials and statistical analysis for lifespan data shown in this figure.
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We found that elc-1-specific knockdown increased lifespan in a largely hif-1-dependent manner (in five of nine trials) (Fig. 2A,B Table 1 and Table S2). Based on these results, we focused on the regulation of HIF-1 by ELC-1.

ELC-1 is a worm homolog of mammalian elongin C. The amino acid sequences and structures of these proteins are well conserved from yeast to humans (Fig. 3A–C). Elongin C has two distinct roles. First, elongin C acts as a component of a transcription elongation factor in association with elongin A and elongin B (Bradsher et al., 1993a,b; Shilatifard et al., 2003). Second, elongin C functions as a component of an E3 ubiquitin ligase by binding to other components, including elongin B and pVHL; this complex determines the specificity for HIF-1α degradation (Duan et al., 1995; Kim & Kaelin, 2003). Although mammalian elongin C has been functionally characterized, it is unknown whether C. elegans elc-1 regulates HIF-1 and modulates HIF-1-dependent phenotypes.

We generated GFP-fused elc-1-expressing transgenic animals to determine the expression patterns of elc-1. We detected bright expression of ELC-1::GFP in the vulval muscle and dim expression in the pharynx, hypodermis, and intestine (Fig. 3D–F). We found that ELC-1 localized to both the cytoplasm and the nucleus (Fig. 3F). This result is consistent with the dual roles of mammalian elongin C as a transcription elongation factor and component of an E3 ligase.

Next, we determined whether ELC-1 regulated HIF-1 levels in C. elegans. Knockdown of elc-1 increased HIF-1::MYC protein levels (Fig. 4A,B). However, elc-1 RNAi did not affect mRNA levels of hif-1 in quantitative RT–PCR (qRT–PCR) results (Fig. 4C). Thus, ELC-1 affected HIF-1 at the posttranscriptional level. We measured the expression levels of three hif-1 target genes, nhr-57, fmo-2, and phy-2 (Shen et al., 2005). We found that elc-1 RNAi upregulated mRNA levels of these genes in a hif-1-dependent manner (Fig. 4D–F). The effects of elc-1 RNAi were comparable to those of vhl-1 RNAi, which was used as a positive control (Fig. 4D–F). Thus, we concluded that C. elegans ELCL-1 negatively regulates HIF-1 at the protein level, and this consistent with its function as an E3 ligase component in mammals.

We examined whether knockdown of elc-1 affected other phenotypes, including impaired reproduction and improved protein homeostasis, which are caused by upregulation of HIF-1 (Mehta et al., 2009). We found that elc-1 RNAi conferred a severe sterile phenotype, which was mostly independent of hif-1 (Fig. 5A). Age-dependent paralysis in a transgenic worm model of Huntington’s disease caused by expression of aggregation-prone Q35 was reduced by elc-1 knockdown (Fig. 5B). The effect of elc-1 RNAi was similar to that of vhl-1 RNAi (Fig. 5C and Mehta et al., 2009). Knockdown of elc-1 or vhl-1 did not improve the motility of Q35 transgenic animals in a hif-1-mutant background (Fig. 5B,C). In addition, similar to vhl-1 RNAi (Mehta et al., 2009), elc-1 RNAi also delayed the paralysis caused by overexpression of aggregation-prone Aβ, a worm model of Alzheimer’s disease (Fig. 5D; two of three trials). Together, these data suggest that inhibition of ELC-1 reduces proteotoxicity via HIF-1 but affects reproduction independently of HIF-1.

**Discussion**

In this study, we analyzed the lifespan-regulatory roles of putative HIF-1 regulators in C. elegans. We showed that the inhibition of C. elegans elongin C promoted longevity by upregulating HIF-1. Inhibition of elongin C also delayed paralysis in a C. elegans model of Huntington’s disease that expresses a polyglutamine protein, in a HIF-1-dependent manner. In contrast, elongin C affected reproduction independently of HIF-1. Thus, we propose that ELC-1 regulates different aspects of animal physiology through both HIF-1-dependent and HIF-1-independent mechanisms.

Longevity caused by knockdown of the putative HIF-1 regulators in this study was independent of hif-1 with the exception of elc-1 knockdown. In addition, a majority of the strong nhr-57 inducer RNAI clones increased the nhr-57p::gfp levels in a largely hif-1-independent fashion. These findings are consistent with those of our previous report;

### Table 1 Summary of lifespan results

| Strain                          | Mean lifespan ± SEM (days) | % change | Total number of worms |
|---------------------------------|---------------------------|----------|-----------------------|
| WT control                      | 20.7 ± 0.5               | +12%     | 1960                  |
| elc-1(RNAi)                     | 23.1 ± 0.6               | +12%     | 1186                  |
| elc-1(Y82E9BR.3(RNAi)           | 33.6 ± 2.8               | +63%     | 425                   |
| YB2EQ9BR.16(RNAi)               | 21.3 ± 2.5               | +3       | 275                   |
| YB2EQ9BR.16(YB2EQ9BR.3(RNAi)    | 33.4 ± 0.8               | +62%     | 250                   |
| YB2EQ9BR.3(RNAi); hif-1(ia4)    | 28.0 ± 0.2               | +35%     | 192                   |
| elc-1(RNAi); hif-1(ia4)         | 21.1 ± 0.7               | +4%      | 1181                  |
| elc-1(Y82E9BR.3(RNAi); hif-1(ia4)| 31.7 ± 1.4              | +56%     | 425                   |
| YB2EQ9BR.3(RNAi); hif-1(ia4)    | 27.5 ± 0.4               | +35%     | 176                   |

This table contains summary of the lifespan results in this study except lifespan screen data. Percent changes in mean lifespan of RNAI-treated wild-type and mutant animals were calculated against control RNAI-treated wild-type and mutant worms, respectively. See Table S2 for the results of each trial and statistical analysis for lifespan data.

**Fig. 2** The effect of RNAI targeting elc-1 on lifespan. (A and B) Lifespan curves of wild-type (A) and hif-1(ia4) mutants (B) that were treated with an in-house RNAI clone specifically targeting elc-1. See Table 1 and Table S2 for additional information for lifespan data shown in this figure. The lifespan-extending effect of elc-1 RNAI was modest (+12% on average) compared to that of vhl-1 mutation (+12% to +62%, Mehta et al., 2009). However, the lifespan-extending effect of elc-1 RNAI is actually comparable to that of vhl-1 RNAI on wild-type (+11%, Mehta et al., 2009). Please note that we were unable to determine the lifespan of elc-1 deletion mutants because they display a lethal phenotype.

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RNAi of several mitochondrial ETC components increases the level of nhr-57p:gfp and lifespan, but longevity is only marginally suppressed by hif-1 mutations (Lee et al., 2010). Thus, factors other than HIF-1 appear to contribute to the regulation of nhr-57 expression and longevity. These factors may include transcription factors, DVE-1, CEH-23, and CEP-1, which regulate longevity in response to inhibition of mitochondrial...
ELC-1 modulates HIF-1 protein levels. (A) Western blot analysis of HIF-1::MYC protein levels in elc-1 RNAi-treated C. elegans. Knockdown of elc-1 increased HIF-1::MYC protein levels. (B) Quantification of band intensity for data in panel A (n = 3). (C) Knockdown of elc-1 did not affect hif-1 mRNA levels in wild-type or in hif-1 mutants (n = 4). (D–F) The effects of elc-1 RNAi on mRNA levels of the known HIF-1 target genes, nhr-57 (D), fmo-2 (E), and phy-2 (F). elc-1 RNAi increased the mRNA levels of these genes in a hif-1-dependent manner (n = 4). An empty vector (L4440) was used as a negative control (Control RNAi). vhl-1 RNAi upregulates HIF-1 and was used as a positive control. Error bars represent SEM (*P < 0.05, **P < 0.01, ***P < 0.001, two-tailed Student’s t-test).

The effects of elc-1 RNAi on reproduction and protein homeostasis. (A) elc-1 RNAI caused severe sterility, which was not affected by hif-1(ia4) mutations. (B, C) RNAI knockdown of elc-1 (four of five trials) (B) or vhl-1 (C) delayed the paralysis of transgenic worms expressing Q35::YFP (Q35). Delayed paralysis by elc-1 RNAI or vhl-1 RNAI was suppressed by hif-1 mutations. (D) elc-1 RNAI delayed the age-dependent paralysis of Aβ (AP)-expressing worms (two of three trials), similar to vhl-1 RNAI. See Table S3 for statistical analysis. Error bars represent SEM (n.s.: not significant, *P < 0.05, ***P < 0.001, two-tailed Student’s t-test).

C. elegans has served as an excellent animal model for studying HIF-1 biology, in particular due to a variety of available genetic tools, including viable hif-1 and its regulator mutants (Reviewed in Powell-Coffman, 2010; Hwang & Lee, 2011). In addition, many studies have employed biochemical methods to measure the protein levels of HIF-1 (Powell-Coffman, 2010; Hwang & Lee, 2011; Romney et al., 2011; Ackerman & Gems, 2012; Hwang et al., 2014; this study). To our knowledge, however, no report has shown the ubiquitination patterns of HIF-1 using components (Durieux et al., 2011; Walter et al., 2011; Baruah et al., 2014), because nhr-57 is induced by impaired mitochondrial functions (Lee et al., 2010). In addition, transcription factors, such as ELT-3, EOR-1, BLMP-1, ALR-1, PHA-4, PQM-1, SKN-1, MDL-1, and PES-1, bind to the promoter region of nhr-57, based on modENCODE data analysis (Gerstein et al., 2010; Van Nostrand & Kim, 2013). It would be interesting to determine whether these transcription factors cooperate with HIF-1 to regulate nhr-57 induction and longevity.
C. elegans. Thus, it will be crucial for future research to determine the ubiquitination patterns of HIF-1 to mechanistically dissect the E3 ligase functions of ELC-1 and VHL-1.

Genetic inhibition of VHL-1 and ELC-1 exerted similar and distinct effects on C. elegans physiology. Both elc-1 RNAi and vhl-1 mutation reduced fertility (shown in this study and in Mehta et al., 2009). However, elc-1 RNAi compromised reproduction independently of HIF-1 (this study), whereas vhl-1 mutations do so in a HIF-1-dependent manner (Mehta et al., 2009). These differences may be due to the different roles of ELC-1 and VHL-1 in the E3 ligase complex. ELC-1 is a core factor of E3 ligase and binds to multiple substrate-specific subunits, including VHL-1, which has limited specificity for the degradation of substrate proteins. Thus, it seems highly likely that ELC-1 affects the stability of a broader range of substrates than VHL-1 does. Indeed, an ELC-1-containing E3 ligase that contains ZIF-1 as a substrate-specific subunit affects reproduction by destabilizing PIE-1, an essential factor for germ line development (DeRenz et al., 2003). Thus, the reduced fertility caused by ELC-1 may act through both the VHL-1/HIF-1 and ZIF-1/PIE-1 axes.

Studies have shown that elongin C has dual functions as an E3 ubiquitin ligase component and a transcription elongation factor (reviewed in Kim & Kaelin, 2003; Shilatifard et al., 2003). Nevertheless, the postembryonic functions of metazoan elongin C remain unclear. This is mainly due to the lethal phenotype of Drosophila (Mummery-Widmer et al., 2009) and C. elegans (http://www.wormbase.org/species/c_elegans/gene/elc-1) mutants and the lack of a knockout mouse model. Here, we showed that elongin C modulated several physiologic aspects, including aging and reproduction. Thus, our work will serve as a guide for future research by improving our understanding of how elongin C regulates specific physiologic processes in vivo.

Experimental procedures

Strains

The following strains, including strains provided by Caenorhabditis Genetics Center, were funded by the NIH National Center for Research Resources, were examined in this study: N2 wild-type, ZG120iais7[nhr-57p::gfp; unc-119(+)] a gift from Powell-Coffman laboratory, U3 hif-1(ia4) V;iais7[nhr-57p::gfp; unc-119(+)]; U6 hif-1 (ia4) V obtained by outcrossing ZG31 3 times to Lee laboratory N2, U159iais28[hif-1::hif-1::myc; unc-119(+)] obtained by outcrossing ZG580 4 times to Lee laboratory N2, US77 yehX136(elc-1:: elc-1p::elc-1::gfp; odr-1::::fp]; AM140 rms132[unc-54p::Q35::yfp]; CL2006 dvls2[unc-54p::Al1-42; rol-60], and U674 hif-1(ia4) V; rms132[unc-54p::Q35::yfp].

Examination of nhr-57p::gfp expression upon RNAi treatment

RNAi bacteria were seeded onto the wells of 24-well NGM plates in triplicate, and dsRNA was induced with 1 μM isopropyl-β-D-thiogalactopyranoside (IPTG, Gold biotechnology, St. Louis, MO, USA) at room temperature for 24 h. nhr-57p::gfp and hif-1(ia4); nhr-57p::gfp transgenic worms were synchronized on the RNAi bacteria lawns, and GFP expression was scored by three researchers in three independent experimental sets. GFP expression was scored as zero to three based on the intensity of GFP fluorescence. Zero indicated no induction, while three indicated the highest induction of GFP upon RNAi treatment. We set the criteria for the GFP scores using control RNAi (score: zero)- and egl-9 RNAi (score: three)-treated nhr-57p::gfp. Among 53 strong candidates, RNAi targeting 16 genes increased the nhr-57p::gfp levels (arbitrary cutoff value = 0.5) in the solid culture system (Fig. 1A).

Lifespan assays

Lifespan assays were performed as described previously with some modifications (Seo et al., 2013). Briefly, synchronized young adult worms were transferred onto 5 μL 5-fluoro-2′-deoxyuridine (FUDR, Sigma, St. Louis, MO, USA)-treated NGM plates with E. coli food. In the case of lifespan assays without FUDR treatment, worms were transferred to a new plate every one or 2 days until they stopped laying eggs. Approximately 100 worms for each condition were examined for death every 2 or 3 days until all the animals were dead. Animals that ruptured, bagged, burrowed, or crawled off the plates were censored but used as censored subjects for the statistical analysis. Lifespan assays were performed at 20°C. OASIS (http://ski.postech.ac.kr/oasis) was used for statistical analysis (Yang et al., 2011).

Analysis of the functional protein association networks

Functional protein association networks of genes identified from the genomewide RNAi screen (Lee et al., 2010) were analyzed using STRING (http://string-db.org/) (Franceschini et al., 2013). Networks were modified and visualized using Cytoscape (http://www.cytoscape.org/) (Smoot et al., 2011).

Modeling and alignment of protein structures

Modeling of C. elegans ELC-1 and human TCEB1, a homolog of elongin C, was performed by using SWISS-MODEL (http://swissmodel.expasy.org/) (Biasini et al., 2014), based on the crystal structures of mammalian VHL–elongin C–elongin B complex and SOCS3–elongin B–elongin C complex (Stebbins et al., 1999; Babon et al., 2008). Structures were visualized and aligned using PyMOL (http://www.pymol.org/) (Schrodinger, 2010).

Generation of plasmids for RNA interference and transgenesis

Genomic regions of elc-1, Y82E9BR.3, and Y82E9BR.16 were cloned into pPD129.36 (Timmons et al., 2001) using In-Fusion HD Cloning Kits (Clontech Laboratories, Inc., Mountain View, CA, USA), by following the manufacturer’s instruction. Constructs were then transformed into HT115 competent cells. Genomic regions (approximately 3.5 kb, Fig. S2) of elc-1::elc-1 were cloned into pPD95.75 (Timmons et al., 2001) using HindIII and Acc65I restriction enzymes. Approximately 2 kb upstream sequences of elc-1 coding region were used as a promoter of elc-1. Sequences of oligonucleotides were used for generating the RNAi clones and the elc-1::elc-1::gfp plasmid are as follows.

- elc-1-F-TCCACCGCGTCCATCATGGCAAAATGTTGGGAAAATCCCGT
elc-1-R-GGCGAAAGTGATTGGCTACATCTAAATCTGCTTGGTCTGAT
Y82E9BR.3-F-GAATTCCAAGCTATTTAAGTTGAAGACCTTTTC
Y82E9BR.3-R-CTTAGGAGCCATTTAAGCTACATCTAAATCTG

CAG
Y82E9BR.16-F-GTGGATTTCCCCGGGAGATTTACAGTTTTCC
Y82E9BR.16-R-CTTAGGAGCCATTTAAGCTACATCTAAATCT
TTTGCAGAAGGCTTC

elc-1p::elc-1-F-CCGGGCAAGCTTTGAGGATTTAAGCTACATCTAAATC
elc-1p::elc-1-R-CAGTCGCTACCCCAATC

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Generation of elc-1p::elc-1::gfp transgenic animals

elc-1p::elc-1::gfp transgenic animals were generated as described previously with some modifications (Gaglia et al., 2012). The elc-1p::elc-1::gfp construct (25 ng μL⁻¹) was injected into the gonad of day 1 adult hermaphrodites with a co-injection marker, odr-1p::rfp (75 ng μL⁻¹).

Quantification of paralysis

The quantification of paralyzed worms was performed as described previously (Mehta et al., 2009) with some modifications. Briefly, the paralysis of Q35 (Q35::YFP)- and ABi-expressing animals was determined by visual analysis. Worms were classified as paralyzed if they did not show any forward movement in response to tapping. Approximately 100 worms for each condition were examined for paralysis every 3 or 4 days until day 14 to day 16. Animals that died, ruptured, bagged, burrowed, or crawled off were censored but used for statistical analysis as censored subjects. All the paralysis assays were performed at 20°C. OASIS (http://sbi.postech.ac.kr/oasis) was used for statistical analysis (Yang et al., 2011). The format of Table S3 (Supporting information) that shows the statistical analysis of the paralysis experiments was based on a previous report (Zhang et al., 2013).

Measurement of brood size

RNAi-treated single L4 stage worm was placed on each RNAi bacteria-seeded plate. Worms were transferred to new plates every day until they stopped laying eggs. The number of hatched progeny was counted. Six to nine P0 hermaphrodites were used for measuring average brood sizes.

Western blot analysis

Synchronized young adult worms were harvested and washed using M9 buffer and then centrifuged at 2000 g for 5-10 seconds. More than 1000 worms (approximately 50 μL of worm pellets) for each condition were used for one set of sample. Worms were then immediately frozen at −80°C and mixed with 2× SDS sample buffer. The samples were boiled at 100°C for 10 min and were vortexed until the samples were broken. After 30-min centrifugation at 15 000 g, supernatant was used for the assay. The worm lysates were electrophoresed using 8% SDS-PAGE and transferred to PVDF membrane. The membrane was treated with 5% skim milk for blocking condition were used for one set of sample. Worms were then immediately frozen at −80°C and mixed with 2× SDS sample buffer. The samples were boiled at 100°C for 10 min and were vortexed until the samples were broken. After 30-min centrifugation at 15 000 g, supernatant was used for the assay. The worm lysates were electrophoresed using 8% SDS-PAGE and transferred to PVDF membrane. The membrane was treated with 5% skim milk for blocking and subsequently incubated with primary antibodies against c-Myc (Santa Cruz, Paso Robles, CA, USA; 1:1000) or α-tubulin (Santa Cruz, 1:1000). The membrane was then incubated with goat anti-mouse secondary antibody conjugated with horseradish peroxidase (Thermo, Waltham, MA, USA, 1:10 000). The PVDF membrane was then treated with the chemiluminescent horseradish peroxidase substrate (Thermo) for 1 min, and the signal was detected using X-ray film (Agfa, Mortsel, Belgium). The band intensity was quantified using ImageJ (http://imagej.nih.gov/ij/).

Fluorescence microscopy

Images of elc-1p::elc-1::gfp animals were taken using an AxioCam HRC CCD digital camera (Zeiss Corporation, Jena, Germany) with a Zeiss Axio Scope A1 compound microscope (Zeiss Corporation). Tetramisole hydrochloride (0.4 mM, Sigma) was used as an anesthetic.

Quantitative RT-PCR

Approximately 500–1000 RNAi-treated young adult worms were used for the quantitative RT-PCR analysis. Preparation of cDNA samples was performed as previously described (Lee et al., 2010). Quantitative PCR from the cDNA was executed in a StepOne Real Time PCR System (Applied Biosystems, Foster City, CA, USA) and analyzed using comparative CT method. mRNA levels of ama-1 (the large subunit of RNA polymerase II) were used for normalization.

List of oligonucleotides used for the quantitative RT-PCR

AMA-1 F: Y82E9BR.3-F-CCTCCTCGGCTCGAGAGCCCCACTC
AMA-1 R: Y82E9BR.3-R-CGGCTCCAGCTCCGATGTACTTGGC
HIF-1 F: GGAGACGACGTTGCTCTCGTTCTCG
HIF-1 R: GTCACTTGTGGAGCCTGAGATCTG
NHR-57 F: GACCTCTGTTGGAGATGGTAGAGGAG
NHR-57 R: GTGCGCTTGTTGTTGCAATTCTCAGG
Fmo-2 F: GTCACTTGTGGAGCCTGAGATCTG
Fmo-2 R: CATCAACTGACGACTCATTCGTTCGC
PHY-2 F: GTATGAGGACATGCTTCAAGGAAAG
PHY-2 R: CATCGTACTCACTCCTCTTTGTAAC
ELC-1 F: GGAGACGCGCTGCCTACCTCTGGAAC
ELC-1 R: GTAGACGAGCTTGCTTGCTTCTCG
Y82E9BR.3-F: CCGCTCCAGCTCCGATGTAATGGC
Y82E9BR.3-R: CGGCTCCAGCTCCGATGTAATGGC

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Conflict of interest

The authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

Fig. S1 Network analysis of putative negative regulators of HIF-1.

Fig. S2 Graphical information of elc-1p::elc-1 and RNAi clones against elc-1, Y82E9BR.3, and Y82E9BR.16.

Fig. S3 Lifespan results of wild-type animals treated with inducer elc-1p::elc-1 and RNAi inducer clones, which did not extend lifespan.

Fig. S4 Lifespan results of hif-1 mutants treated with nhr-57 inducer RNAi clones, which did not extend lifespan.

Fig. S5 Dissection of the effects of the nhr-57p::nhr-57p:: elc-1p::elc-1 inducible factor modulates lifespan in C. elegans. PLoS ONE 4, e6348.

Zhang P, Judy M, Lee SJ, Kenyon C (2013) Direct and indirect gene regulation by a life-extending FOXO protein in C. elegans: roles for GATA factors and lipid gene regulators. Cell Metab. 17, 85–100.

Table S1 The list of RNAi clones that highly increased the level of nhr-57p::gfp in a liquid culture system.

Table S2 Analysis of lifespan assay results.

Table S3 Analysis of paralysis assay results.