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Inhibition of respiration extends *C. elegans*' lifespan via reactive oxygen species that increase HIF-1 activity

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Summary

A mild inhibition of mitochondrial respiration extends the lifespan of many organisms, including yeast, worms, flies and mice [1–10], but the underlying mechanism is unknown. One environmental condition that reduces rates of respiration is hypoxia (low oxygen). Thus it is possible that mechanisms that sense oxygen play a role in the longevity response to reduced respiration. The hypoxia-inducible factor HIF-1 is a highly-conserved transcription factor that activates genes that promote survival during hypoxia [11–12]. In this study, we show that inhibiting respiration in *C. elegans* can promote longevity by activating HIF-1. Through genome-wide screening, we found that RNAi knockdown of many genes encoding respiratory-chain components induced *hif-1*-dependent transcription. Moreover, HIF-1 was required for the extended lifespans of *clk-1* and *isp-1* mutants, which have reduced rates of respiration [1,4,13]. Inhibiting respiration appears to activate HIF-1 by elevating the level of reactive oxygen species (ROS). We found that ROS is increased in respiration mutants, and that mild increases in ROS can stimulate HIF-1 to activate gene expression and promote longevity. In this way, HIF-1 appears to link respiratory stress in the mitochondria to a nuclear transcriptional response that promotes longevity.

Results and Discussion

To identify genes that affect HIF-1 activity in *C. elegans*, we performed a genome-wide screen for RNAi clones that induced a HIF-1-responsive GFP reporter, *Pnhr-57::GFP* [14–15]. We found 248 RNAi clones targeting 245 genes that reproducibly increased the level of *Pnhr-57::GFP* under normoxic conditions (Table S1A). The screen was predicted to identify genes already known to regulate HIF-1. Under normal oxygen conditions, *C. elegans* HIF-1 is hydroxylated by the oxygen-dependent prolyl hydroxylase EGL-9, and this hydroxylation leads to HIF-1's degradation in a process that involves the E3 ubiquitin ligase VHL-1 [12,14,16]. Under hypoxic conditions, this hydroxylation does not occur and the stabilized HIF-1 activates downstream target genes required for adaptive responses to

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hypoxia [12,14]. We recovered RNAi clones targeting *egl-9*, *vhl-1* and *rhy-1* (which encodes a HIF-1-inhibitory transmembrane protein) [15], validating our screen (Table S1A).

Remarkably, a highly significant fraction of the genes we identified encoded mitochondrial proteins (26 out of 113 gene-ontology (GO)-annotated cellular components, $P<10^{-14}$, Table S1B), including components of the electron transport chain and ATP synthase such as *cyc-1* [cytochrome c1], *cco-1* [cytochrome c oxidase], *nuo-1* [NADH:ubiquinone oxidoreductase], *atp-3* [ATP synthase subunit δ], and *atp-5* [ATP synthase subunit d]; Fig. 1A–F and J). These findings implied that impaired respiration increases HIF-1 activity under normoxic conditions. Consistent with this idea, we found that *Pnhr-57::GFP* was up-regulated in two mutants that are defective in respiration, *clk-1(qm30)* (which encodes a mitochondrial hydroxylase required for ubiquinone production) and *isp-1(qm150)* (which encodes an iron-sulfur protein in complex III) (Fig. 1G–I and K).

The finding that inhibiting respiration activates *hif-1*-dependent gene expression suggested that HIF-1 might be part of the pathway by which inhibition of respiration extends lifespan. We found that the long lifespans of *clk-1* and *isp-1* mutants were significantly suppressed by *hif-1* loss-of-function mutations or *hif-1* RNAi (Fig. 2A, B and Fig. S2A, B). *hif-1* RNAi decreased the lifespans of these respiration mutants even if it was initiated during adulthood (Fig. S2C, D). This was noteworthy, as respiratory-chain RNAi only extends *C. elegans'* lifespan when initiated during larval development [5, 17]. Perhaps HIF-1 acts in the adult to maintain the operation of a regulatory state initiated by respiration inhibition during development. We also found that RNAi knockdown of *aha-1*, which encodes the HIF1β subunit [18], shortened the lifespans of *clk-1* and *isp-1* mutants. (Fig. 2C, D). HIF-1 inhibition did not shorten lifespan indiscriminately, as it did not affect the lifespan of wild type (Fig. 2A–D and Fig. S2A–D) [19].

Unexpectedly, loss of *hif-1* only partially reduced the lifespan extension caused by *cyc-1* or *cco-1* RNAi (Fig. S2E, F). This finding suggests that the mechanisms by which respiratory-chain RNAi and the *clk-1/isp-1* respiration mutations lengthen lifespan may be at least somewhat distinct from one another.

Does inhibition of respiration activate the entire *hif-1*-dependent hypoxia response? Using quantitative RT-PCR, we found that four out of five *C. elegans hif-1*-dependent hypoxia-inducible genes we tested [14] (*nhr-57* and *F22B5.4*, *fmo-2*, *egl-9* and *phy-2*) were significantly up-regulated in *clk-1* and *isp-1* mutants (Fig. 1L, M,Fig. S1A-C). The expression of two of these, *nhr-57* and *F22B5.4* (Fig. 2E, F) was partially dependent on *hif-1*, but the other two up-regulated genes, *fmo-2* and *egl-9*, were expressed independently of *hif-1* (Fig. S2G, H). Thus hypoxia and respiration inhibition activate distinct patterns of *hif-1*-dependent gene expression. Mild hypoxia has been shown to extend *C. elegans* lifespan [20], so it will be interesting to learn what, if any, role *hif-1* may have in that longevity response.

Defects in respiration in *C. elegans* not only extend lifespan, they also slow the rates of growth and behaviour [1,4–6,21], reduce brood size and delay reproduction [4,21]. We found that inhibition of *hif-1* or *aha-1* had little or no effect on these phenotypes (Table S2). Thus, HIF-1 appears to influence only one aspect of the animal's response to respiration inhibition, longevity. Interestingly, in flies and mice, respiration-inhibiting conditions that extend lifespan do not affect growth or behaviour. In *C. elegans*, respiratory-chain RNAi dose-response experiments indicate that lifespan and behaviours are affected co-ordinately [17]. These observations suggest the possibility that the growth, reproductive and behavioural responses to respiration inhibition evolved separately from the longevity response and are subject to a different mode of regulation. Consistent with this, the worm-

specific growth and behavioural phenotypes can be suppressed significantly by inhibition of the *C. elegans*-specific genes *fstr-1/2*, which appears to have a smaller effect on lifespan [22].

Is *hif-1* required for other *C. elegans* longevity pathways [10]? *hif-1* RNAi did not shorten the long lifespan of *daf-2*/insulin/IGF-1-receptor mutants or dietary-restricted *eat-2* mutants (Fig. S2I, J), consistent with recent, independent reports [19]; nor did it shorten the long lifespans of chemosensory *osm-5* or germline-defective *glp-1* mutants (Fig. S2K, L). Thus HIF-1 specifically affects lifespan in response to the inhibition of respiration.

We also asked whether elevating HIF-1 in animals with wild-type respiration genes would be sufficient to extend lifespan. As shown independently by Mehta et al. [19], we found that conditions that stabilize HIF-1; that is, inhibition of *vhl-1* or *egl-9*, significantly increased lifespan (Fig. 3A–D and Fig. S3A–D; see also Fig. S2M, N legend, and supplemental materials for discussion about recent studies of HIF-1 longevity). Importantly, *clk-1* and *isp-1* mutations did not further extend the long lifespans of *vhl-1* or *egl-9* mutants, arguing that *vhl-1*, *egl-9* and respiration mutations all promote longevity by activating HIF-1 (Fig. 3A–D and Fig. S3A–D). Likewise, *clk-1* or *isp-1* mutations did not further increase *nhr-57* and *F22B5.4* mRNA levels in *vhl-1* or *egl-9* mutants (Fig. 3E–J).

How might mutations in these respiration genes activate HIF-1? In cultured cells, hypoxia can increase the level of reactive oxygen species (ROS), which in turn activates HIF-1 by a mechanism that is not yet understood [23]. Because ROS are produced during electron transport, and because ROS increases when electron transport is reduced in isolated mitochondria [23–26], we hypothesized that ROS levels rise in respiration mutants, and that this rise in ROS, in turn, activates HIF-1.

To measure ROS, we used a 2′,7′-dichlorofluorescein diacetate (DCF-DA) fluorescence assay that we found to reliably report ROS levels in *C. elegans* (Fig. 4A–C, Fig. S4A). We found that ROS levels were significantly increased in whole body lysates of *clk-1* and *isp-1* mutants (Fig. 4A). We observed a similar increase in fluorescence *in vivo*, using another fluorescent ROS-sensor, dyhydroxy ethidium (DHE) (Fig. S4B–F). These data indicate that the defective mitochondria in the *clk-1* and *isp-1* mutants generate elevated levels of ROS.

Consistent with our results, Yang et al. showed that isolated submitochondrial particles from *clk-1(qm30)* mutants produce more H_2O_2 than do those from wild type [27]. We note that recently, Dingley et al. reported that mitochondrial superoxide level measured by using the fluorescent MitoSOX dye was slightly decreased in *isp-1(qm150)* mutants [28]. However, they also showed that mitochondria of respiration mutants, including *isp-1* mutants, have prominent defects in the uptake of fluorescent dyes. Therefore, as they themselves speculated, it is possible that mitochondrial ROS levels in the *isp-1* mutants, as measured by MitoSOX in their study, were underestimated.

To test whether ROS can extend *C. elegans* lifespan, we measured the lifespan of animals treated chronically with various concentrations of paraquat, which generates superoxide in mitochondria [29]. Low paraquat levels (0.125 mM, 0.25 mM, 0.5 mM and 1 mM) increased lifespan significantly, whereas, as expected, higher concentrations of paraquat (4, 16, and 64 mM) decreased lifespan in a dose-dependent manner (Fig. 4D and Table S4). Using the two ROS indicators, we confirmed that high concentrations of paraquat increased ROS (Fig. 4C and Fig. S4H). Unexpectedly, whereas one of the dyes showed a significant increase in ROS levels in animals treated with low levels of paraquat, the other dye reported reduced ROS levels at low paraquat concentrations (Fig. 4B and Fig. S4G). One possible explanation is that the two dyes sense different types of ROS, and that the animal's protective response to paraquat can actually decrease the levels of certain ROS species relative to untreated

controls. Although these findings raise new questions, overall the data suggest that moderate levels of ROS can extend lifespan. Consistent with these findings, low levels of juglone, another ROS-generating chemical, also extends *C. elegans* lifespan [30].

Next, we asked whether paraquat increases lifespan by activating HIF-1. We found that animals chronically treated with low levels of paraquat (0.25 mM) displayed an increase in *nhr-57* expression (Fig. 4E, F, I and J) that was largely *hif-1* dependent (Fig. 4G–J). Moreover, *hif-1* was partially required for paraquat to increase lifespan (Fig. 4K). Together these data suggest that ROS generated by defects in respiration activate HIF-1, which in turn can promote longevity.

Historically, it has been believed that ROS generated in mitochondria are one of the main determinants of aging [31]. However, several recent studies suggest that modest increases in ROS levels can have beneficial effects on lifespan by triggering the expression of cell-protective pathways [25,30,32–33]. For example, 2-deoxyglucose activates AMP kinase and extends lifespan in *C. elegans* in a ROS-dependent fashion [32]. In addition, we showed previously that antimycin A, a respiration inhibitor that increases ROS [23], extends *C. elegans'* lifespan [5]. Here we showed that ROS generated when respiration rates are reduced in *C. elegans* increase HIF-1 transcriptional activity, which in turn is sufficient to lengthen lifespan.

These findings reinforce the emerging idea that a little ROS may be beneficial [25,33]. However, the finding that low and high levels of ROS have opposite effects on lifespan makes the interpretation of experiments in which antioxidant proteins are eliminated a bit complicated. Specifically, unless loss of an antioxidant produces high ROS levels, one might expect to find an increase rather than decrease in lifespan, even if high levels of ROS do accelerate the normal aging process.

It was noteworthy that *clk-1* and *isp-1* mutants exhibited comparably-elevated ROS levels, as the overall respiration defect of *isp-1* (complex III—defective) mutants appears to be greater than that of *clk-1* (ubiquinone-defective) mutants [2,4,13]. This would be consistent with our interpretation that reactive oxygen species, rather than, say altered oxygen consumption rate, which is reduced in *isp-1* but not *clk-1* mutants [2,4,13], trigger longevity.

In summary, in this study we showed that respiration mutations in *clk-1* and *isp-1* extend lifespan by increasing *hif-1*-dependent gene expression, and that increased *hif-1* activity is sufficient for longevity. HIF-1 is a transcription factor, implying that HIF-1 extends longevity via changes in downstream gene expression. How ROS in the mitochondria impact HIF-1 and cause it to influence nuclear gene expression is not clear, nor is it clear which genes HIF-1 activates to extend lifespan. Several lines of evidence suggest that increased expression of *nhr-57* is not sufficient to increase lifespan. First, 4 mM paraquat treatment shortened lifespan although this condition increased *Pnhr-57::GFP*. In addition, we found that *nhr-57* level was higher in *egl-9* and *vhl-1* mutant animals than *clk-1* and *isp-1* mutant animals in spite of the fact that they all have similar longevity phenotypes. Presumably, the activity of a group of HIF-1 regulated genes, which may or may not include *nhr-57*, is responsible for increasing lifespan in *clk-1* and *isp-1* mutants. Inhibition of respiration is known to trigger a conserved gene expression response called the retrograde response that activates alternative energy pathways and cell protective mechanisms [3,34]. It will be interesting to learn to what extent HIF-1 influences the expression of these genes.

It is interesting to speculate that the longevity response to reduced respiration played a role in the evolution of mammalian lifespan, as larger species of mammals tend to have lower rates of respiration and to live longer than smaller mammals [10]. A *hif-1*-dependent response to the inhibition of a fundamental oxygen-dependent process like respiration might

well have arisen early during evolution. Like the *C. elegans* respiration mutants described here, long-lived *mClk-1*$^{+/-}$ mice, which lack one copy of the mouse ortholog of *C. elegans clk-1*, have increased levels of mitochondrial ROS [35]. While this manuscript was in preparation, the Hekimi lab reported that the elevated ROS in *mClk-1*$^{+/-}$ mice activate HIF-1 to affect the immune response [36]. This is intriguing, as it suggests a possible conservation of mechanism from worms to mammals. It would be interesting to test whether increased HIF-1 activity contributes to the extended lifespan of these mice as well.

Highlights

- Reduced mitochondrial respiration extends lifespan in many species.
- Inhibition of mitochondrial respiration increases HIF-1 activity in *C. elegans*.
- HIF-1 is required for the longevity caused by mutations that inhibit respiration.
- HIF-1 is likely activated by reactive oxygen species that are generated when respiration is inhibited.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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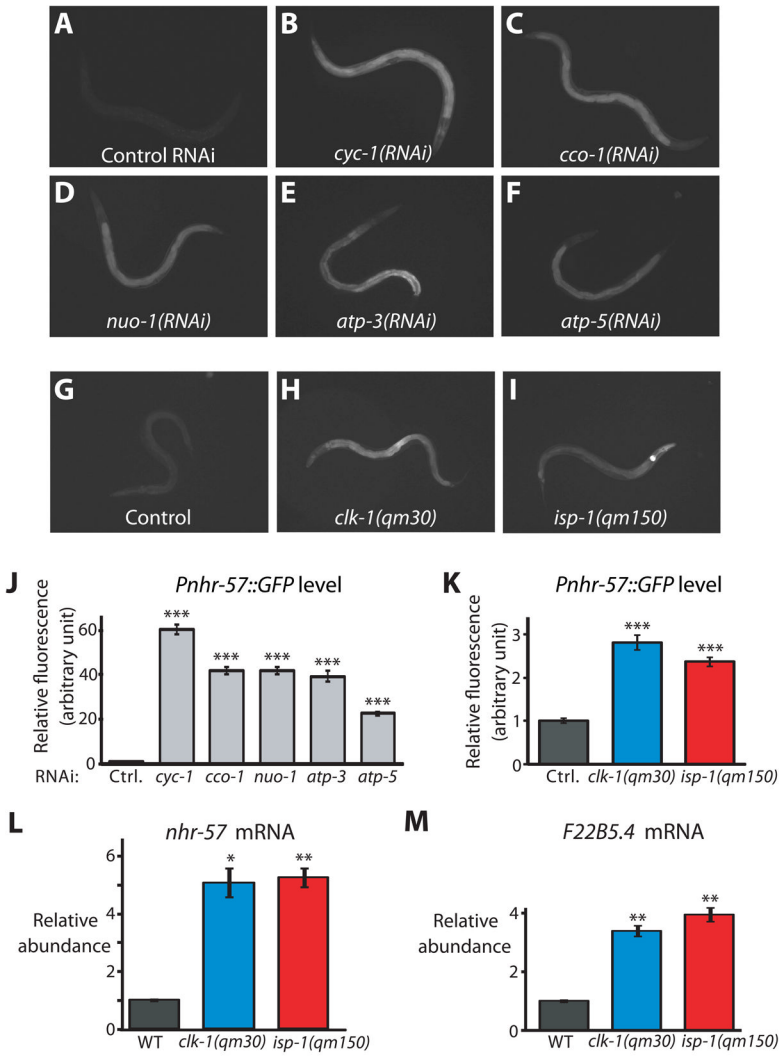

Fig. 1. Inhibiting respiration increases HIF-1 activity

(**A**) Animals expressing the HIF-1-regulated *Pnhr-57::GFP* transgene displayed low levels of GFP when grown on control bacteria carrying the empty RNAi vector. (**B**–**F**) In contrast, RNAi of respiratory-chain or ATP synthase genes *cyc-1* (**B**), *cco-1* (**C**), *nuo-1* (**D**), *atp-3* (**E**), or *atp-5* (**F**) induced the expression of *Pnhr-57::GFP*. RNAi treatment of *cyc-1* or *cco-1* only during adulthood did not increase the level of *Pnhr-57::GFP* (Fig. S1D-H). (**G**–**I**) Mutations in *clk-1* (**H**) and *isp-1* (**I**), which reduce respiration, elevated *Pnhr-57::GFP* expression (**G**). (**J**) Quantification of fluorescence in **A** to **F** (n>30); and (**K**), in **G** to **I** (n>42). mRNA levels of *nhr-57* (**L**) and *F22B5.4* (**M**), another HIF-1-regulated gene, were significantly increased in *clk-1(qm30)* and *isp-1(qm150)* mutants [Please see Fig. S1A-C for qRT-PCR data of other hypoxia-responsive genes]. Data were obtained from 3 independent quantitative RT-PCR analyses and error bars represent s.e.m (**P*<0.01, ***P*<0.001, ****P*<0.0001, two-tailed Student's *t*-test compared to wild type).

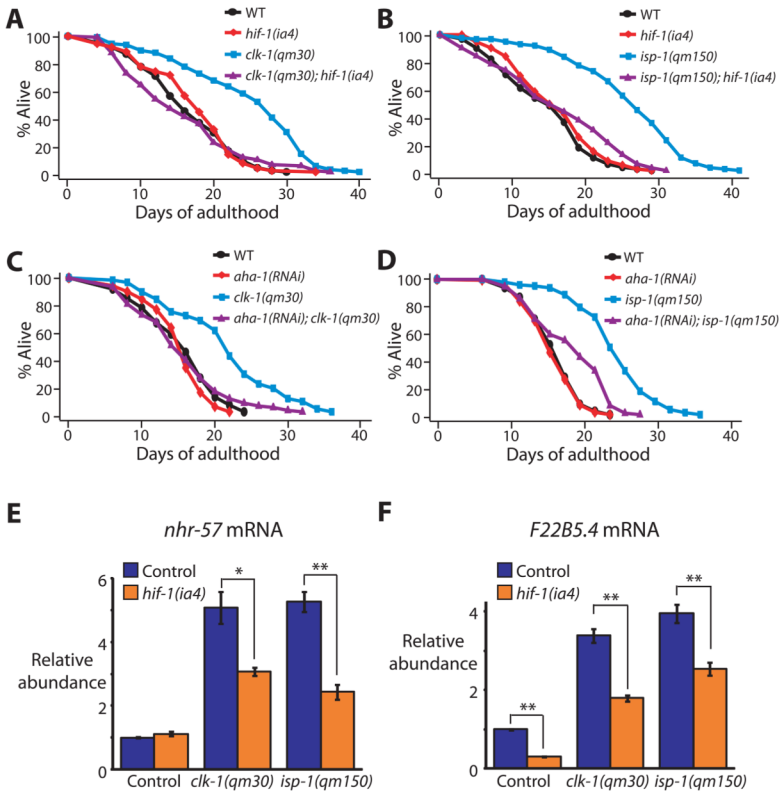

Fig. 2. The lifespan extension conferred by respiration mutants requires *hif-1*

(**A**, **B**) *hif-1(ia4)* loss-of-function mutations decreased the longevity of *clk-1(qm30)* (**A**) and *isp-1(qm150)* (**B**) mutants significantly (in three out of four trials and four out of four trials, respectively; See Supplemental Table S2). (**C**, **D**) The long lifespan of *clk-1(qm30)* (**C**) and *isp-1(qm150)* (**D**) mutants was significantly shortened by *aha-1* [HIF1β] RNAi. Neither the *hif-1(ia4)* mutation nor *aha-1* RNAi affected the lifespan of wild type (WT). (See Supplemental Table S2 for statistical analysis.) [We note that whereas Mehta et al. and we both found that *hif-1* mutations do not affect the lifespans of wild type [19], Chen et al. and Zhang et al. reported that *hif-1* mutants live longer than wild type [37–38]. We carried out additional experiments to resolve this discrepancy, which are described in supplemental material (Fig. S2M, N)] (**E**, **F**) The increased mRNA levels of the HIF-1-dependent genes *nhr-57* (**E**) and *F22B5.4* (**F**) in *clk-1(qm30)* and *isp-1(qm150)* mutants were significantly decreased by *hif-1(ia4)* mutation. Error bars represent s.e.m (n=3, **P*<0.05, ***P*<0.01, two-tailed Student's *t*-test). See Fig. S2G, H for quantitative RT-PCR data of other *hif-1*-dependent hypoxia-inducible genes.

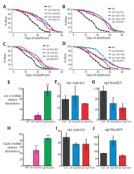

Fig. 3. Activation of HIF-1 by *vhl-1* or *egl-9* mutations does not further lengthen the lifespan of respiration mutants

(**A**, **B**) Mutations in *vhl-1* increased the lifespan of wild type but did not further extend the lifespan of *clk-1(qm30)* (**A**) or *isp-1(qm150)* (**B**) mutants. (**C**, **D**) The *egl-9(sa307)* mutation did not further increase the lifespans of *clk-1(qm30)* (**C**) or *isp-1(qm150)* (**D**) mutants. (**E**) Consistent with previous reports [14,39], mRNA levels of *nhr-57* were significantly increased by *vhl-1(ok161)* and *egl-9(sa307)* mutations. (**F**, **G**) The increased mRNA levels of *nhr-57* in *vhl-1(ok161)* (**F**) or *egl-9(sa307)* (**G**) mutants were not further augmented by *clk-1(qm30)* and *isp-1(qm150)* mutations (control, Ctrl.). (See Supplemental Table S3 for statistical analysis.) (**H**) Expression of *F22B5.4* was highly induced in *vhl-1(ok161)* and *egl-9(sa307)* mutants as reported previously [14,39]. (**I**, **J**) this induction was not significantly further increased by *clk-1(qm30)* and *isp-1(qm150)* mutations. Note that the increased *F22B5.4* mRNA levels in *egl-9(sa307)* mutants by *clk-1(qm30)* mutation was marginally not significant (P=0.06). Error bars represent s.e.m (n=3, *P<0.01, **P<0.001, two-tailed Student's *t*-test).

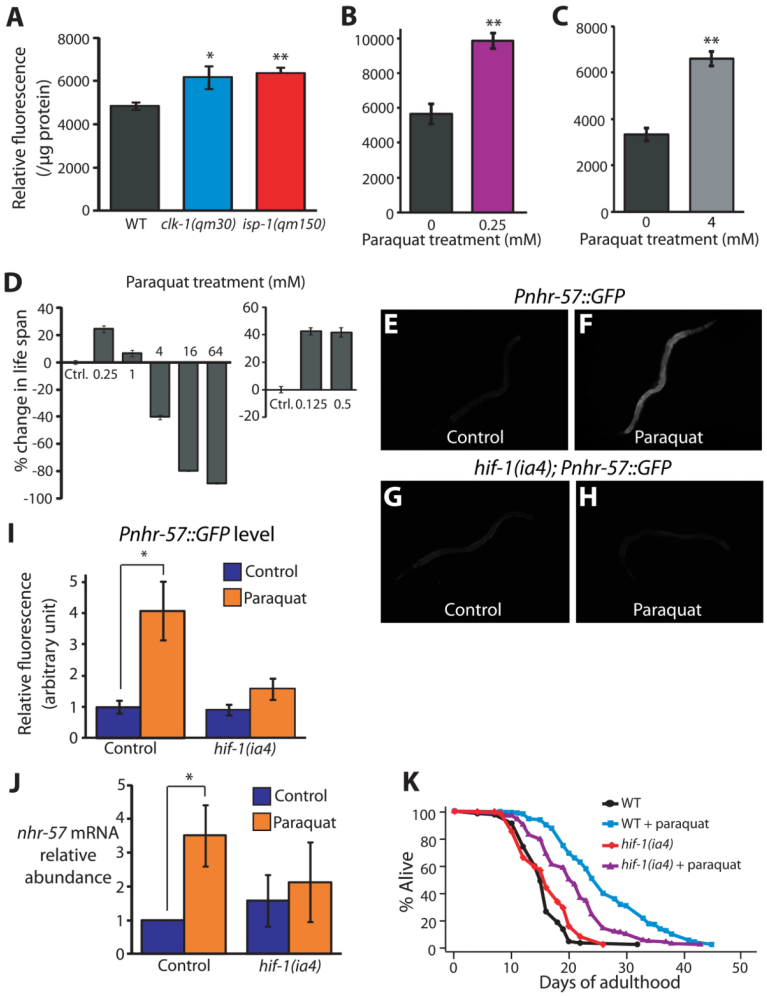

Fig. 4. Increased ROS causes HIF-1 to promote longevity in respiration-defective mutants

(**A**) ROS levels, measured using a 2′,7′-dichlorofluorescein diacetate (DCF-DA) fluorescence assay, were significantly increased in *clk-1(qm30)* and *isp-1(qm150)* mutants (n=5). See also Fig. S4A for data using *mev-1(kn1)* mutant animals, which were previously shown to have increased ROS levels [40]. (**B**–**C**) A low dose (0.25 mM) (**B**) and high dose (4 mM) (**C**) of paraquat significantly increased the level of DCF-DA fluorescence. 4 mM paraquat was introduced from L4 to day 3 of adulthood because worms arrest as larvae if treated with 4 mM paraquat from hatching (n=3). Error bars represent s.e.m (**P*<0.05, ***P*<0.01, two-tailed Student's *t*-test). (**D**) Low doses of paraquat (0.125 mM, 0.25 mM, 0.5 mM and 1 mM) lengthened lifespan, whereas higher concentrations (4, 16, and 64 mM) shortened lifespan. Paraquat was introduced during adulthood. The lifespan measurements for 0.125 mM and 0.5 mM paraquat treatment were performed separately and therefore shown with different controls. See also Table S4. (**E**, **F**) Compared to untreated control *Pnhr-57::GFP* animals (**E**), animals treated with low levels of paraquat (0.25 mM) displayed increased GFP levels (**F**). Paraquat treatment further increased *Pnhr-57::GFP* levels in *clk-1(qm30)* and *isp-1(qm150)* mutant animals (Fig. S4J, K), suggesting that the induction was not saturated by either of the mutations or by the paraquat treatment. (**G**, **H**) The induction of *Pnhr-57::GFP* by paraquat treatment was significantly diminished in the *hif-1(ia4)* mutant. (**I**) Quantification of fluorescence in **E** to **H** (n >15). (**J**) The increased *nhr-57* mRNA abundance caused by paraquat treatment, assayed using quantitative RT-

PCR, was significantly decreased by *hif-1(ia4)* mutation. Data analysis was done from 10 independent quantitative RT-PCR experiments. [In two of our data sets, the levels of *nhr-57* mRNA in paraquat-treated wild-type animals were increased by a very-large 681 and 755 fold compared to those in control animals. We excluded these two datasets from our analysis by using rejection analysis of QP-test for outliers (confidence level: 0.99) [41]]. (**K**) Mutations in *hif-1* significantly decreased the longevity caused by paraquat treatment. Some long-lived mutants require the *daf-16*/FOXO transcription factor gene for their longevity, but respiration mutants do not [1,4,10]. We found that 0.25 mM paraquat treatment increased the lifespan of *daf-16(mu86)* null mutants (Fig. S4L). See Supplemental Table S4 for statistical analysis. Error bars represent s.e.m (**P*<0.05, ***P*<0.01, two-tailed Student's *t*-test). Previously, Rea et al. observed an increased trend in protein carbonylation levels at doses of respiratory-chain RNAi that increased lifespan, and also at higher RNAi doses, which did not extend lifespan [17]. On this basis, they concluded that ROS did not play a role in this longevity. One way to reconcile their findings with ours is to suggest that a sharp reduction in respiration prevents lifespan extension in spite of elevated ROS because it compromises the animals' health.