HHS Public Access

Author manuscript

Free Radic Biol Med. Author manuscript; available in PMC 2018 July 01.

Published in final edited form as:

Free Radic Biol Med. 2017 July ; 108: 362–373. doi:10.1016/j.freeradbiomed.2017.04.004.

Uncoupling of Oxidative Stress Resistance and Lifespan in Long-lived *isp-1* Mitochondrial Mutants in *Caenorhabditis elegans*

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Abstract

Mutations affecting components of the mitochondrial electron transport chain have been shown to increase lifespan in multiple species including the worm *Caenorhabditis elegans*. While it was originally proposed that decreased generation of reactive oxygen species (ROS) resulting from lower rates of electron transport could account for the observed increase in lifespan, recent evidence indicates that ROS levels are increased in at least some of these long-lived mitochondrial mutants. Here, we show that the long-lived mitochondrial mutant *isp-1* worms have increased resistance to oxidative stress. Our results suggest that elevated ROS levels in *isp-1* worms cause the activation of multiple stress-response pathways including the mitochondrial unfolded protein response, the SKN-1-mediated stress response, and the hypoxia response. In addition, these worms have increased expression of specific antioxidant enzymes, including a marked upregulation of the inducible superoxide dismutase genes *sod-3* and *sod-5.* Examining the contribution of *sod-3* and *sod-5* to the oxidative stress resistance in *isp-1* worms revealed that loss of either of these genes increased resistance to oxidative stress, but not other forms of stress. Deletion of *sod-3* or *sod-5* decreased the lifespan of *isp-1* worms and further exacerbated their slow physiologic rates. Thus, while deletion of *sod-3* and *sod-5* genes has little impact on stress resistance, physiologic rates or lifespan in wild-type worms, these genes are required for the longevity of *isp-1* worms. Overall, this work shows that the increased resistance to oxidative stress in *isp-1* worms does not account

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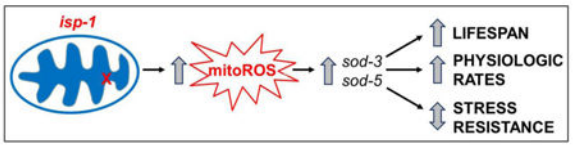
for their longevity, and that resistance to oxidative stress can be experimentally dissociated from lifespan.

Graphical Abstract



Keywords

Aging; oxidative stress; *Caenorhabditis elegans*; superoxide dismutase; *isp-1*; mitochondria; reactive oxygen species; genetics; lifespan

Introduction

Reactive oxygen species (ROS) are generated as a bi-product of normal metabolism as electrons being passed down the electron transport chain (ETC) are leaked directly to oxygen to form superoxide. These ROS have been shown to cause oxidative damage to functional units of the cell, such as DNA, protein and lipids. Since excessive oxidative damage can impair function, and oxidative damage has been shown to increase with age [1], it has been proposed that reactive oxygen species are one of the primary causes of aging [2]. As mitochondria are thought to be the primary site of ROS generation in a cell, the Mitochondrial Free Radical Theory of Aging further proposes that ROS-induced damage to the mitochondria is the main contributor to aging [3]. Based on this theory, decreasing the rate of electrons flowing through the ETC could be predicted to increase longevity by decreasing the production of ROS (assuming equivalent efficiency). Consistent with this idea, a number of mutations that affect proteins in the ETC have been shown to increase lifespan in the worm *C. elegans* [4-8], as well as in other species, including flies [9] and mice [10, 11].

isp-1 encodes the Rieske iron sulfur protein of complex III in the electron transport chain [6]. While the complete absence of ISP-1 protein results in lethality, a point mutation (*qm150*) in the exon 6 of 8 exons in the *isp-1* gene has been shown to markedly increase lifespan [6]. These mutants have been shown to have a decreased rate of oxidative phosphorylation [6, 12, 13] (note that while oxidative phosphorylation is measured by oxygen consumption, which takes place at complex V, ROS are produced at earlier points in the ETC), an increase in the levels of reduced CoenzymeQ9 resulting from decreased oxidation by complex III [14], and decreased levels of complex I [13]. Based on these observations, it was originally proposed that *isp-1* worms live long because of decreased production of ROS (see Figure 5 [6]). However, more recent findings cast doubt on this conclusion, suggesting that ROS levels are actually increased in *isp-1* worms despite their decreased rate of oxidative phosphorylation. Whole *isp-1* worms exhibit increased staining with two ROS-sensitive fluorescent dyes: DCF (dichlorofluorescein) and DHE (dihydroethidium)[15]. Further, it was also shown that mitochondrial ROS are elevated in

isp-1 worms as MitoSox staining is increased in mitochondria isolated from *isp-1* worms compared to WT [16]. While measurements of oxidative damage in whole *isp-1* worms showed no difference in the level of protein carbonyls [17], the levels of 4-HNE modified proteins were found to be increased in mitochondria isolated from *isp-1* worms compared to WT [18]. Combined these data indicate that ROS levels and oxidative damage are not decreased in *isp-1* worms.

While unchecked ROS can cause damage to various components of a cell, there are a number of antioxidant enzymes that are present to detoxify ROS [19]. Superoxide dismutase (*sod* genes) converts superoxide, the primary form of ROS generated in the mitochondria, into hydrogen peroxide, which can then be converted to water by catalase (*ctl* genes), glutathione peroxidase (*gpx* genes) or peroxiredoxin (*prdx* genes). Glutaredoxins (*glrx*) and thioredoxin (*trx*) can reduce peroxiredoxins and other targets to restore their function after oxidation. The activity of thioredoxin can then be restored by thioredoxin reductase (*trxr* genes). Glutathione-S-transferase (*gst* genes) can act to detoxify products of oxidative stress.

In *C. elegans* there are five *sod* genes: *sod-1, sod-2* and *sod-4* are the primary cytoplasmic, mitochondrial, and extracellular *sod* genes respectively, and correspond to *SOD1, SOD2* and *SOD3* in humans. These three *sod* genes account for 99% of the *sod* mRNA present in the worm [20]. *C. elegans* also express two additional *sod* genes that are not found in most organisms. *sod-3* and *sod-5* are inducible *sod* genes that are normally expressed at low levels (less than 1% of total *sod* mRNA) in the mitochondria and cytoplasm, respectively. While it is known that the expression of these *sod* genes is induced under stress [21], the function of these genes is poorly understood. Deletion of either *sod-3* or *sod-5* has no effect on lifespan, sensitivity to juglone-induced oxidative stress, development time, fertility, defecation cycle length or rate of movement in WT worms [22, 23].

In this paper we study the relationship between oxidative stress resistance and longevity in the long-lived *isp-1* mitochondrial mutant. We find that although these worms have increased ROS, they have increased resistance to oxidative stress, which is associated with an upregulation of antioxidant defense genes, and the activation of various stress response pathways. Examining the role of the inducible *sod* genes in the stress resistance and longevity of *isp-1* worms, reveals that deletion of these genes increases resistance to acute oxidative stress but decreases lifespan in *isp-1* worms. This indicates that having increased levels of ROS does not necessarily result in increased sensitivity to oxidative stress, and that increasing resistance to oxidative stress does not necessarily increase lifespan.

Materials and Methods

Strains

C. elegans strains were cultured on nematode growth medium (NGM) agar plates, which were seeded with OP50, a slow-growing mutant of *Escherichia coli*. All strains were maintained at 20°C. Wild-type animals were N2 Bristol strain. The following strains were used in these experiments:

Strain	Description
WT(N2)	Wild-type control strain
isp-1(qm150)	Long-lived mitochondrial mutant with point mutation in exon 6 of 8 of gene encoding Rieske iron sulfur protein of complex III
sod-3(tm760)	Inducible mitochondrial superoxide dismutase mutant with deletion removing exons 2 and 3 of 5 exons. These exons contain part of the active site of the enzyme
sod-5(tm1146)	Inducible cytoplasmic superoxide dismutase mutant with deletion affecting exons 2 and 3 of 5 exons. These exons contain part of the active site of the enzyme
MQ1378 *qmEx413[Psod-3::sod-3:GFP]*	Transgenic worms expressing SOD-3:GFP for visualization of SOD-3 expression levels
CL2166 *dvIs19[Pgst-4::GFP]*	Transgenic worms expressing GFP under the promoter of the SKN-1 target gene *gst-4* for monitoring activation of SKN-1 oxidative stress response
SJ4100 *zcIs13[Phsp-6::GFP]*	Transgenic worms expressing GFP under the *hsp-6* promoter for monitoring activation of the mitochondrial unfolded protein response

We also generated the following double mutant strains: *isp-1(qm150);sod-3(tm760), isp-1(qm150);sod-5(tm1146), isp-1(qm150);Psod-3::sod-3:GFP, isp-1(qm150);Pgst-4::GFP*, and *isp-1(qm150);Phsp-6::GFP*.

mRNA expression levels

Isolation of mRNA—One to two plates of well fed, synchronized, pre-fertile young adult worms were collected in M9 buffer [Na2HPO4 (42.3 uM), KH2PO4 (22uM), NaCl (85 uM), MgSO4 (1mM)]. Worms were washed three times in M9 buffer and flash frozen in Trizol (ThermoFisher Scientific). Once samples for all of the independent replicates had been collected, RNA from all of the samples was extracted at the same time. RNA isolation was done as previously described [24]. Note that the RNA samples used for RNA sequencing were completely independent of those used for quantitative real-time RT-PCR.

RNA sequencing and analysis

For the RNA sequencing (RNA-seq) experiments, we collected six independent samples for WT and *isp-1* worms. RNA was isolated independently for all twelve samples. Subsequently, the *isp-1* samples were pooled prior to library preparation. Sequencing libraries were prepared using the Kapa Biosystems stranded mRNA-Seq kit for the Illumina platform. Libraries were sequenced using 1×75 bp sequencing on the Illumina NextSeq 500 platform at the Van Andel Research Institute to a depth of ~30M reads per sample. Read quality was assessed using FASTQC v. 0.11.3 (www.bioinformatics.babraham.ac.uk/projects/fastqc/) and aligned first to the E. coli K-12 genome [25] to assess contamination, then aligned to the WBcel235 *C. elegans* genome assembly [26] using Subread v. 1.5.0 [27] with default parameters. Transcript abundances were quantified using featureCounts v. 1.4.6 [28] with strand specific read counting. Differential gene expression analysis was performed using the edgeR package v. 3.14.0 [29] in R v. 3.3.0.

To compare gene expression in WT, *isp-1, isp-1;sod-3* and *isp-1;sod-5* worms, we collected 9 biological replicates per strain and isolated mRNA separately. Sets of three mRNA isolates were pooled at equal concentrations to generate three samples for library preparation and sequencing. Sequencing was performed as described above at a depth of ~10M reads per

sample. RNAseq data is available at NCBI Gene Expression Omnibus (GEO): GSE95240. Heatmaps were generated using the pheatmap package v. 1.0.8 in R v. 3.3.0 (https://CRAN.R-project.org/package=pheatmap). Pathway and gene ontology enrichment analyses were performed using the commonly differentially expressed genes ($q < 0.05$) between *isp-1*, *isp-1;sod-3*, and *isp-1;sod-5* relative to WT unless stated otherwise. KEGG and Reactome pathway enrichment analysis was performed using the BACA package v. 1.3 [30] in R v. 3.3.0. Enriched pathways were required to have a minimum of five genes and $q < 0.05$. Gene ontology enrichment was performed with the GOseq package [31] v. 1.26.0 in R v. 3.3.0 and geneontology.org. Plots were generated using ggplot2 v. 2.2.1 (http://www.springer.com/us/book/9780387981413) and cowplot v. 0.7.0 in R v. 3.3.0 (https://CRAN.R-project.org/package=cowplot).

Quantitative real-time RT-PCR—Quantitative Real-Time RT-PCR (qPCR) was performed in two steps as previously described [24]. mRNA was converted to cDNA using a High-Capacity cDNA Reverse Transcription kit (Life Technologies/Invitrogen). qPCR was performed using a FastStart Universal SYBR Green kit (Roche) in an AP Biosystems RT-PCR machine.

Fluorescent reporter strains

Fluorescent reporter intensity quantification in adult worms was performed by mounting randomly selected adult worms onto an unseeded NGM plate (nematode growth medium) and imaging using a fluorescence dissecting microscope (Nikon SMZ1500) as reported previously [21]. Fluorescent reporter intensity quantification in larval stage worms was performed using a Cellomics Arrayscan high content imager as previously described [32].

Oxidative stress assays

Sensitivity to oxidative stress was assessed using multiple paradigms involving two superoxide-generating compounds: paraquat (PQ) and juglone. PQ produces superoxide through redox cycling: it receives an electron from a donor such as NADPH oxidase, then passes the electron on to dioxygen to generate superoxide, thereby regenerating PQ ion in the process[33]. PQ is thought to increase superoxide levels primarily in the mitochondria [34, 35]. Juglone (5-hydroxy-1,4-napthalenedione) is a natural extract from black walnut trees. It is also believed to generate superoxide through redox cycling [36].

Paraquat development assay—To test oxidative stress during development, a minimum of 40 eggs were transferred to plates containing low concentrations of paraquat ranging from 0.2 mM to 0.35 mM. The developing worms were then monitored for the latest developmental stage attained. The stage to which the worms developed appeared to be sensitive to the exact concentration of paraquat as some variation was observed with different batches of paraquat or different ages of plates. To eliminate this variability, all of the strains were always tested at the same time on the same batch of plates.

Acute oxidative stress during development—Worms that were hatched on NGM plates were transferred to plates containing 200 mM paraquat at either the L2 or L4 stage of development. The survival of these worms was then checked hourly for the 12 hour duration

of the assay. At each time point, dead worms were counted and removed. 25 worms per strain per trial were tested.

Acute oxidative stress during adulthood—Sensitivity to oxidative stress was tested acutely during adulthood by placing day 1 or day 8 adult worms on plates containing various concentrations of juglone ranging from 180-300 μM. Survival was monitored at 1, 2, 3 and 4 hours. For this assay, plates were made fresh on the day of the assay as the activity of juglone in plates declines over time. Each trial utilized 25 worms per strain for each concentration of juglone.

Chronic oxidative stress during adulthood—To test sensitivity to oxidative stress throughout adulthood, day 1 adult worms were transferred to plates containing 4 mM paraquat. 100 μM FUdR was also added to these plates to prevent internal hatching of progeny (bagging). Survival was monitored daily until all of the worms had died. The results represent 115 death events for both WT and *isp-1* worms.

Heat stress assay

Worms grown on NGM plates at 20°C were transferred to a 37°C incubator on day 1 of adulthood. Survival was monitored hourly until all of the worms had died. Each replicate used 20 worms per strain.

Osmotic stress assay

Worms grown on regular NGM plates (50 mM NaCl) were transferred to plates containing 500 mM NaCl on day 1 of adulthood. Survival was checked at 24 and 48 hours. Each replicate used 20 worms per strain.

Bacterial pathogen stress assay

Worms were exposed to *Pseudomonas aeruginosa* to induce bacterial pathogen stress following the slow kill assay outlined by Kirienko et. al. [37]. Each replicate used 20 worms per strain.

Lifespan

Lifespan studies were completed at 20°C on plates containing 100 μM FUdR (fluorodeoxyuridine; Sigma). Although this concentration of FUdR can increase the lifespan of certain strains [38], we have found that *isp-1* worms have increased lifespan with or without FUdR and that the lifespan of *sod-3* and *sod-5* mutants is equivalent to WT with or without FUdR. Worms that died from internal hatching of progeny, expulsion of internal contents or desiccation on the side of the dish were removed from the study. Four independent biological replicates were performed with more than 240 death events recorded for each strain.

Post-embryonic development

Eggs were collected and allowed to hatch over a period of 3 hours. After 3 hours, 25 L1 worms per strain per trial were transferred to a new plate. Beginning at 35 hours worms were checked every 4 hours. Worms that had reached adulthood were counted and removed.

Self-brood size

To determine the average number of progeny produced by each strain, L4 worms were placed on individual NGM plates. Worms were transferred daily until egg laying ceased and the total number of live progeny produced was counted. Each replicate consisted of 5 worms. Progeny were allowed to grow to the pre-fertile young adult stage to facilitate counting and were immobilized in a 4°C cold room prior to counting.

Defecation cycle length

Defecation cycle length in young adult worms was measured as the average time between consecutive pBoc contractions. Results represent 10 worms per replicate.

Thrashing rate

Thrashing rate was measured manually. Twenty day 1 adult worms were transferred to an unseeded NGM plate and 1 ml of M9 buffer was added. The total number of body bends was counted for 30 seconds for a randomly selected 10 worms.

Detecting ROS using dihydroethidium (DHE)

ROS levels were measured using DHE (ThermoFisher Scientific, D1168) as previously described [39] with the following modifications. 30mM DHE stock in DMSO was aliquoted and stored at -80°C. 5 µl of stock DHE was diluted in 5 mL of PBS (30µM concentration). Age matched day 1 adult worms (approximately 100 individuals) were picked into a 1.5 tube and washed 3 times in 1 mL PBS. On the final wash, all but 100 µl PBS was removed, and 100 ul of 30µM DHE was added to a final concentration of 15uM DHE in 200 µl. Worms were incubated for 1 hour on a shaker at room temperature, then washed 3 times in PBS and mounted on a 1.5% agarose pad and immobilized with 5mM levamisole. Worms were imaged at 40× using an upright Leica compound microscope (DM5500B). 30-40 worms were imaged for 3 biological replicates. Fluorescence intensity of ethidium labeled ROS was quantified in the anterior pharynx using a ROI (region of interest) method and ImageJ.

Measuring oxidative damage

Carbonyl groups were detected using the Protein Oxidation Detection Kit (Millipore, S7150). Briefly, pellets of day 1 adult worms (1-2 60 mm plates per sample) were collected and flash frozen in lysis Buffer (150 mM KCl, 1mM EDTA, 0.25% SDS, 1% NP-40, 50 mM Tris/HCL pH 7.4), with DTT added to a final concentration of 50mM to prevent oxidation occurring after cell lysis. Samples were freeze-thawed on ice, and sonication was performed using a Bioruptor (Diagenode). Protein concentration of diluted samples (5mM DTT) was determined using the reducing agent compatible BCA kit (Pierce/Thermoscientfic 23250). 5 µg total protein was put into each DNPH and control reaction, and separated on a 10% gel, transferred, and developed according to standard methods. Quantification was performed

using ImageJ by collecting total pixel intensity of equally thresholded ROI, with the negative control background subtracted from each sample. Intensity was normalized to WT, and 3 biological replicates were averaged.

Statistical analysis

For all assays, a minimum of three independent biological replicates were performed. Statistical significance of differences was determined using a one-way ANOVA, two-way ANOVA, or repeated measures ANOVA, and a Bonferroni posttest. For lifespan and chronic paraquat data, differences between the survival curves were assessed using a log-rank test. Error bars indicate standard error of the mean.

Results

Increased resistance to oxidative stress in *isp-1* worms

During the original characterization of *isp-1* worms, it was reported that *isp-1* worms fail to develop on plates containing the superoxide-generating compound paraquat at concentrations that did not prevent WT worms from reaching adulthood [6]. We confirmed this result by showing that WT worms can develop to adulthood at concentrations up to at least 0.35 mM paraquat, while *isp-1* worms fail to develop to adulthood even at 0.2 mM paraquat (Fig. 1A). While this result is consistent with *isp-1* worms having increased sensitivity to oxidative stress, it is also possible that the difference results from the slower development time of *isp-1* worms, thereby giving them a more prolonged exposure to paraquat in order to reach adulthood.

To determine whether the long development time of *isp-1* worms was responsible for their increased sensitivity to oxidative stress in the paraquat development assay, we performed an acute oxidative stress assay on developing *isp-1* worms at the L2 and L4 stages of development. In this assay, we exposed *isp-1* and WT worms to 200 mM paraquat and monitored survival. In contrast to the paraquat development assay, we found that *isp-1* worms exhibited increased survival compared to WT worms at both the L2 and L4 stage of development (Fig. 1 B,C). This suggests either that *isp-1* worms have a differential ability to handle acute high doses of oxidative stress, compared to chronic low doses, or that the increased sensitivity to oxidative stress in the paraquat development assay results from the long development time of *isp-1* worms.

To gain more insight into oxidative stress sensitivity in *isp-1* worms, we performed both acute and chronic assays of oxidative stress in adult *isp-1* worms. In an acute assay of oxidative stress resistance in which worms are exposed to another superoxide-generating compound juglone, *isp-1* worms were found to be more resistant to oxidative stress than WT worms at day 1 and day 8 of adulthood (Fig. 1 D,E). Finally, we tested resistance to oxidative stress during adulthood using a chronic exposure to 4 mM paraquat beginning at day 1 of adulthood. Again, we found that *isp-1* worms have markedly increased survival compared to WT worms (Fig. 1F).

Since *isp-1* worms have been reported to have elevated levels of ROS [16, 39], it is counterintuitive that these worms would have increased resistance to oxidative stress. As

such, we sought to confirm that *isp-1* worms have increased ROS. We stained *isp-1* and WT worms with dihydroethidium (DHE), a compound that emits red fluorescence when oxidized and has been used as an *in vivo* sensor of ROS [39, 40]. We found that *isp-1* worms exhibited increased DHE fluorescence compared to WT worms, thereby confirming that these worms have elevated ROS (Fig. 1G). Similarly, we measured oxidative damage to proteins by measuring protein carbonylation and found an increase in *isp-1* worms compared to WT worms (Fig. 1H). Combined our results suggest that *isp-1* worms have increased resistance to oxidative stress throughout development and adulthood despite having elevated levels of ROS.

Upregulation of inducible *sod* genes in *isp-1* worms

Based on our observation that *isp-1* worms exhibit increased resistance to oxidative stress, we hypothesized that increased ROS production in *isp-1* worms might trigger the upregulation of antioxidant defence genes, and activation of stress response pathways leading to oxidative stress resistance. To determine whether antioxidant defenses and stress response pathways are activated in *isp-1* worms, we initially used RNAseq to obtain a global view of the transcriptional changes present in *isp-1* worms. We focussed specifically on genes encoding antioxidant defense enzymes and those involved in known stress response pathways including the cytoplasmic unfolded protein response, the mitochondrial unfolded protein response, the endoplasmic reticulum unfolded protein response, the hypoxia response, and the SKN-1-mediated oxidative stress response.

Among the superoxide dismutase genes (SOD), we found that only *sod-3* and *sod-5* were upregulated (Supplementary Fig. S1A). Among the catalase genes, we observed upregulation of *ctl-3* (Supplementary Fig. S1B), while there was no upregulation of any of the peroxiredoxin (*prdx*), glutathione peroxidase (*gpx*) or glutaredoxin (*glrx*) genes (Supplementary Fig. S1C-E). We found that one thioredoxin gene (*trx-2*) was upregulated, as was the thioredoxin reductase *trxr-2* (Supplementary Fig. 1F). Finally, we observed upregulation of multiple glutathione-S-transferase genes (*gst-3, gst-4, gst-8, gst-12, gst-13, gst-14, gst-15, gst-16, gst-19, gst-20, gst-21, gst-24, gst-25, gst-29, gst-31, gst-33, gst-34, gst-37, gst-41, gst-44, gsto-1*, and *gsto-2*) (Supplementary Fig. 1G).

To confirm the results of the RNAseq experiment, we performed quantitative real-time RT-PCR for selected antioxidant genes to ensure that the results are reproducible and no significant differences were missed. As with the RNAseq data, we found that *sod-3* and *sod-5* are significantly upregulated in *isp-1* worms, while the expression of other *sod* genes are unchanged (Fig. 2A). Similarly, we found that the expression of *ctl-3* is increased in *isp-1* worms, while *ctl-1, ctl-2*, and all three *prdx* genes are equivalent to wild-type (Fig. 2B,C). Finally, we observed a significant upregulation of specific glutathione-S-transferase genes (Fig. 2D).

Activation of stress response pathways in *isp-1* worms

Next, we sought to determine the extent to which stress response pathways are activated in *isp-1* worms. The RNAseq data indicated no activation of the cytosolic unfolded protein response (*hsp-16.11, hsp-16.2*) or the ER unfolded protein response (*hsp-4*) (Supplementary

Fig. S2A). With respect to the mitochondrial unfolded protein response, we observed a 50% increase in *hsp-6* levels, but no difference in the expression of *hsp-60* (Supplementary Fig. S2A). Examining the expression of the top genes shown to be induced during short term hypoxia [41] revealed that almost all of these genes are also upregulated in *isp-1* worms, suggesting that the hypoxia response is activated (Supplementary Fig. S2B). Finally, we found that the expression of SKN-1 target genes [42] were not consistently upregulated in *isp-1* worms but we did see a marked increase in *gst-4* (Supplementary Fig. S2C).

In order to confirm the results from the RNAseq data, we used fluorescent reporter strains or quantitative real-time RT-PCR. We found that reporter strains for the SKN-1-mediated oxidative stress response (*Pgst-4::GFP*) and the mitochondrial unfolded protein response (*Phsp-6::GFP*) were both upregulated in *isp-1* worms (Fig. 3A). Similarly, we found that target genes for the hypoxia response (*nhr-57, F22B5.4*) were upregulated in *isp-1* worms by quantitative real-time RT-PCR (Fig. 3B).

Having shown that antioxidant genes and stress response pathways are activated in *isp-1* worms at day 1 of adulthood, we sought to determine if these pathways are also activated during development and whether their activation persists throughout adulthood. We found that at 1 day post-hatching fluorescent reporter strains for *sod-3* (antioxidant gene expression), *gst-4* (SKN-1-mediated oxidative stress response), *hsp-6* (mitochondrial unfolded protein response) and *nhr-57* (hypoxia response) were already activated in *isp-1* worms (Fig. 3C). This result is consistent with the increased resistance to oxidative stress observed in developing *isp-1* worms. The upregulation of *sod-3* persisted until at least day 14 of adulthood (Fig. 3D), while the increased expression of *gst-4* and *hsp-6* diminished with age (Fig. 3E,F). Note that the ability to activate the *gst-4* and *hsp-6* reporters is lost with age [21].

Paradoxical increase in resistance to oxidative stress with deletion of inducible *sod* genes

Based on the marked upregulation of *sod-3* and *sod-5* in *isp-1* worms, we sought to determine whether the increased expression of these genes contributed to the increased resistance to oxidative stress of *isp-1* worms. Since SOD acts to detoxify ROS, deletion of *sod* genes should result in a decreased ability to detoxify ROS resulting in increased sensitivity to oxidative stress. However, both *sod-3* and *sod-5* are normally expressed at very low levels each accounting for less than 1% of the total *sod* mRNA [20]. According to our RNAseq data, *sod-3* makes up 1.6% of the total *sod* mRNA in WT worms, while *sod-5* account for just 0.1% (Supplementary Fig. S3). In *isp-1* worms, *sod-3* mRNA accounts for 16.6% of total *sod* mRNA and *sod-5* accounts for 2.1% (Supplementary Fig. S3). Nonetheless, the expression of *sod-3* and *sod-5* in *isp-1* worms is still less than *sod-2* and *sod-1*, respectively. To define the contribution of *sod-3* and *sod-5* to oxidative stress resistance in *isp-1* worms, we generated *isp-1;sod-3* and *isp-1;sod-5* double mutants and compared their stress resistance to *isp-1* worms.

Surprisingly, we found that the deletion of *sod-3* or *sod-5* resulted in increased resistance to oxidative stress of *isp-1* worms in the paraquat development assay. As with WT worms, *isp-1;sod-3* and *isp-1;sod-5* worms were able to develop to adulthood at concentrations up to at least 0.35 mM paraquat, a concentration at which *isp-1* worms fail to develop past the L1

larval stage (Fig. 4A). Similarly, we found that deletion of *sod-3* or *sod-5* increased resistance to oxidative stress in *isp-1* worms in an acute juglone oxidative stress assay on day 1 of adulthood (Fig. 4B). In contrast, *isp-1;sod-3* and *isp-1;sod-5* worms were found to have decreased survival in a chronic oxidative stress survival assay compared to *isp-1* worms (Fig. 4C).

To determine if the deletion of *sod-3* or *sod-5* resulted in increased resistance to other stresses, we compared *isp-1* worms to *isp-1;sod-3* and *isp-1;sod-5* worms in a heat stress assay (exposure to 37°C heat)(Fig. 4D), an osmotic stress assay (exposure to 500 mM NaCl) (Fig. 4E), and a bacterial pathogen stress assay (exposure to *Pseudomonas aeruginosa*) (Fig. 4F). In each case, we found that *isp-1* worms are more resistant to stress than WT worms, but that deletion of *sod-3* or *sod-5* did not further increase resistance to stress. Combined, this indicates that the loss of either of the inducible *sod* genes specifically affects sensitivity to oxidative stress.

The inducible *sod* genes are required for *isp-1* longevity and mitigating the slowing of physiologic rates

To determine whether the increased expression of *sod-3* and *sod-5* in *isp-1* worms contributes to their long lifespan, we examined the lifespan of *isp-1;sod-3* and *isp-1;sod-5* double mutants. While deletion of *sod-3* or *sod-5* has no impact on lifespan in wild-type worms [22], deletion of either of the inducible *sod* genes in *isp-1* worms resulted in a significant decrease in lifespan (Fig. 5A, Supplementary Table S1).

Since the presence of the inducible *sod* genes contributes to the increased longevity of *isp-1* worms, we sought to determine whether these genes impacted other physiologic rates, which have been shown to be altered in *isp-1* worms [6, 17]. Unlike lifespan where the deletion of the inducible *sod* genes resulted in a reversion towards WT, we found that deletion of *sod-3* or *sod-5* resulted in exacerbation of the slow development, decreased brood size, slow defecation, and slow thrashing phenotypes of *isp-1* worms (Fig. 5B-E). It is important to note that the development time of *isp-1;sod-3* and *isp-1;sod-5* worms is significantly longer than *isp-1* and thus cannot account for their increased ability to develop to adulthood in the paraquat development assay. Overall, while deletion of *sod-3* or *sod-5* has little impact on the physiology, stress resistance and lifespan of wild-type worms, these genes are required for *isp-1* longevity, and their absence significantly worsens the slow physiology of *isp-1* worms.

Deletion of inducible *sod* genes does not affect ROS levels or oxidative damage in *isp-1* worms

To gain further insight into the effect of deleting inducible *sod* genes on lifespan and resistance to oxidative stress in *isp-1* worms, we compared the levels of ROS and oxidative damage in *isp-1;sod-3* and *isp-1;sod-5* worms to *isp-1* worms. Using DHE fluorescence to measure ROS levels, we found that while ROS levels are increased in *isp-1;sod-3* and *isp-1;sod-5* worms compared to WT, there was no difference from *isp-1* worms (Supplementary Figure 4A). Similarly, measuring oxidative damage, we found no difference in the levels of protein carbonylation between *isp-1* worms and *isp-1;sod-3* or *isp-1;sod-5*

worms (Supplementary Figure 4B,C). Thus, the differences in oxidative stress resistance and lifespan caused by deletion of the inducible *sod* genes cannot be attributed to changes in ROS levels or oxidative damage. This result is consistent with the fact that *sod-3* and *sod-5* are not the primary *sod* gene in the mitochondria or cytoplasm respectively.

Deletion of inducible *sod* genes does not increase expression of antioxidant or stress response genes in *isp-1* worms

Based on our observation of increased resistance to oxidative stress in *isp-1;sod-3* and *isp-1;sod-5* worms, we used quantitative real-time RT-PCR to determine how the deletion of the inducible *sod* genes affected antioxidant genes expression and the expression of genes involved in specific stress response pathways. Among the antioxidant genes tested (*sod-1, sod-2, sod-4, ctl-1, ctl-2, ctl-3, prdx-2, prdx-3, prdx-6, gst-8*), we did not observe any differences between *isp-1* worms and *isp-1;sod-3* or *isp-1;sod-5* worms (Supplementary Fig. S5). Similarly, most of the stress response genes we tested (*nhr-57, F22B5.3, sodh-1, gst-4, hsp-16.2, hsp*-6) showed similar expression between *isp-1* and *isp-1;sod-3* or *isp-1;sod-5* worms (Supplementary Fig. S5). We did find that two HIF-dependent hypoxia response genes, *mtl-1* and *comt-4*, exhibit decreased expression in the *isp-1;sod* double mutant strains compared to *isp-1* worms (Supplementary Fig. S5).

Deletion of inducible *sod* genes alters gene expression in *isp-1* worms

To gain a more comprehensive view of the changes in gene expression induced by deletion of *sod-3* or *sod-5* in *isp-1* worms, we performed RNAseq. Overall, we found that *isp-1;sod-3* and *isp-1;sod-5* worms exhibit similar changes in gene expression to *isp-1* worms (Supplementary Fig. S6, S7). Of the genes that are upregulated in *isp-1* worms, 39.9% and 40.7% are upregulated in *isp-1;sod-3* and *isp-1;sod-5* worms, respectively (Supplementary Fig. S8). Of genes that are downregulated in *isp-1* worms, 29.6% and 45.7% are also downregulated in *isp-1;sod-3* and *isp-1;sod-5* worms respectively (Supplementary Fig. S8). The top GO terms for the genes that are modulated in all three mutants are indicated in Supplementary Fig. S9.

Careful analysis of the gene expression heat map (Supplementary Fig. S6) revealed specific genes that show opposite patterns of gene expression in *isp-1* worms from *isp-1;sod-3* and *isp-1;sod-5* worms (Supplementary Fig. S10). This includes genes that are significantly upregulated in *isp-1* worms but significantly downregulated in *isp-1;sod-3* and *isp-1;sod-5* mutants (*dod-24, T22B7.7, B1086.3*) and genes that are significantly downregulated in *isp-1* worms but significantly upregulated in *isp-1;sod-3* and *isp-1;sod-5* worms (*clec-43, Y38F1A.6, C09B8.4, B0511.11*). Taken together, the inverted expression profiles for these genes in *isp-1* and *isp-1;sod-3* and *isp-1;sod-5* double mutants demonstrates that changes in transcriptional and metabolic profiles exist, which may begin to explain the differences we observed in lifespan, stress resistance and physiologic rates.

To further explore what genes or groups of genes might account for the phenotypic differences between *isp-1* worms and *isp-1;sod-3* and *isp-1;sod-5* worms, we performed KEGG enrichment analysis and Reactome enrichment analysis on the RNAseq gene expression results to identify functional groups of changes that show a different pattern of

gene expression in *isp-1* worms compared to *isp-1;sod-3* and *isp-1;sod-5* worms. In the KEGG analysis, we found that genes in the "ribosome" category are upregulated in *isp-1* worms but not in the double mutants (Supplementary Fig. S11). Interestingly, we found that genes involved in "oxidative phosphorylation" and the "citrate cycle" are downregulated in *isp-1;sod-3* and *isp-1;sod-5* worms but not in *isp-1* worms (Supplementary Fig. S11). This result is consistent with our observation that *isp-1;sod-3* and *isp-1;sod-5* worms have slower physiologic rates than *isp-1* worms. In the reactome analysis, we found that there are seven functional categories of genes that are upregulated in *isp-1* worms but not in *isp-1;sod-3* or *isp-1;sod-5* worms (Supplementary Fig. S12). Future studies will be required to determine the extent to which the changes in gene expression between *isp-1* and the *isp-1;sod* double mutants that we have identified contribute to the phenotypic differences between these strains.

Discussion

Lack of correlation between levels of ROS and resistance to oxidative stress

In this work, we show that *isp-1* worms exhibit increased sensitivity to oxidative stress in a paraquat development assay, but increased resistance to oxidative stress in an acute juglone assay at the L2 and L4 stages of development. These results are consistent with previous studies showing that fewer *isp-1* worms develop to adulthood on plates containing 0.2-0.8 mM paraquat compared to WT worms [6], but that *isp-1* worms at the L4 developmental stage have increased survival on 16 mM paraquat plates compared to WT worms [43]. We further go on to show using two different assays that *isp-1* worms are also resistant to oxidative stress during adulthood. Combined, our data supports the interpretation that the increased sensitivity to oxidative stress in *isp-1* worms in the paraquat development assay is an artifact of their extended development time (100 hours for *isp-1* versus 45 hours for WT, [6]) and that *isp-1* worms have increased resistance to oxidative stress throughout development and adulthood. This demonstrates that when performing assays involving externally-generated oxidative stress (e.g. exposure to ROS-generating compounds such as paraquat) it is important to consider the duration of exposure and uptake of the compound in the interpretation of the results, as these factors can also influence survival.

We also observed differing results in chronic versus acute assays of oxidative stress sensitivity during adulthood. We found that, in comparison to *isp-1* worms, *isp-1;sod-3* and *isp-1;sod-5* worms show increased resistance to acute oxidative stress during adulthood, but increased sensitivity to chronic oxidative stress. The fact that *isp-1;sod-3* and *isp-1;sod-5* worms have decreased lifespan compared to *isp-1* worms likely contributes to the fact that these worms also have decreased survival in the chronic paraquat assay. Combined, these results suggest that it is important to perform multiple different assays of sensitivity to oxidative stress to obtain a more complete understanding of stress resistance in a given strain.

Previous studies have demonstrated that *isp-1* worms have increased levels of ROS as measured by staining with three different ROS-sensitive dyes: DCF, DHE and MitoSox [16, 39] and either normal or increased oxidative damage [17, 18]. Because *isp-1* worms demonstrated increased resistance to oxidative stress, we sought to confirm the elevation of

ROS levels and oxidative damage in *isp-1* worms by staining with a ROS-sensitive fluorescent dye, DHE, and measuring protein carbonylation, respectively. Our results showing increased ROS in *isp-1* worms clearly indicate that it is possible to have increased resistance to oxidative stress and elevated levels of ROS. Furthermore, this result suggests that measuring sensitivity to oxidative stress cannot be used to infer ROS levels.

Upregulation of stress response pathways in *isp-1* mutants

To explain the mechanism by which *isp-1* worms have increased resistance to oxidative stress despite elevated ROS, we hypothesized that these worms activate antioxidant defenses and stress response pathways to compensate for the increased levels of ROS. This hypothesis is supported by our data that demonstrates increased expression of multiple antioxidant genes and genes associated with stress response pathways. These findings are supported by previous studies showing increased expression of *sod-3* [6, 18], and the HIF-1-mediated hypoxia response genes *nhr-57* and *F22B5.4* [39] in *isp-1* worms, as well as the activation of SKN-1-mediated oxidative stress reporter *Pgst-4::GFP* [44], and mitoUPR reporter *Phsp-6::GFP* [45-47].

Of the stress pathways examined, we found that the majority of the top genes that are activated by short term hypoxia are upregulated in *isp-1* worms. Previous work has demonstrated that the hypoxia-induced HIF-1 target genes *nhr-57* and *F22B5.4* are upregulated in *isp-1* worms [15]. The fact that these genes are activated by treatment with paraquat, and that HIF-1 is required for both the upregulation of those genes and for the long lifespan on *isp-1* worms suggests that a ROS-activated, HIF-dependent hypoxia response contributes to the longevity of *isp-1* worms. Since it has been shown that hypoxia can cause increased generation of mitochondrial ROS [48, 49], it is likely that the overlap in gene expression changes between short term hypoxia and *isp-1* mutation results from the fact that both interventions increase mitochondrial ROS.

Two previous studies have examined gene expression changes in *isp-1* worms using microarray technologies [50, 51]. The study by Cristina *et al.* used an Illumina microarray, while the study by Yee *et al.* used an Affymetrix microarray. We compared the results of the two previous experiments with our RNAseq data using genes in our dataset that exhibited a two-fold change. We found that there were only 39 genes that were found to be upregulated in all three studies (Supplementary Fig. S13, Supplementary Table S2). This represents a small fraction of the 1428, 703 and 1797 genes that were found to be upregulated in our study, the study by Cristina *et al.*, and the study by Yee *et al.* Similarly, there were only 3 genes out of 376, 105 or 1765 downregulated genes that were found to be in common between all three studies (Supplementary Fig. S13, Supplementary Table S2). While different technologies were utilized in each study, it is still uncertain why the degree of overlap between the three studies represents such a small percentage of the total genes identified in each experiment. Of the genes that did show overlap between all three studies were *sod-3*, the focus of our current study, and *cdr-2* the focus of the study by Cristina *et al.* [50].

Role of inducible superoxide dismutase genes in longevity, stress resistance and physiologic rates

While humans and many other organisms have three *SOD* genes – one for each compartment of the cell (cytoplasm, mitochondria and extra-cellular) – *C. elegans* have five *sod* genes. In addition to the primary cytoplasmic (*sod-1*), mitochondrial (*sod-2*), and extracellular (*sod-4*) *sod* genes, the additional *sod* genes include *sod-3*, which is localized to the mitochondrial matrix [52, 53], and *sod-5* in the cytoplasm [54]. The function of these two additional *sod* genes is not known. Moreover, our previous work suggested that these *sod* genes may be expendable as the deletion of *sod-3* or *sod-5* had no effect on lifespan, development, fertility or movement [22, 23]. While we did observe a small decrease in survival in *sod-3* worms exposed to 4 mM paraquat, there was no difference when exposed to 240 μM juglone, and *sod-5* mutants exhibited normal sensitivity to oxidative stress in both assays [22].

The fact that we observed little or no phenotypic abnormalities in *sod-3* and *sod-5* deletion mutants is consistent with the fact that these genes are normally expressed at very low levels. Previous results relying on quantitative real-time RT-PCR indicated that *sod-3* and *sod-5* account for less than 1% of the total *sod* mRNA in a worm [20]. Since this result could have been influenced by the efficiency of the primers used for the qPCR reaction, we looked at the distribution of *sod* mRNA in our RNAseq data. We obtained similar results with *sod-3* accounting for 1.6% of the total *sod* mRNA and *sod-5* accounting for just 0.1% of the total *sod* mRNA, compared to 34.5% and 57.9% for *sod-2* and *sod-1* respectively.

In *isp-1* worms, we found that the large increase in *sod-3* and *sod-5* expression resulted in these genes making up a much larger proportion of total *sod* mRNA (16.6% and 2.1% respectively). Because of the marked upregulation of the inducible *sod* genes in *isp-1* worms, we examined their contribution to stress resistance, lifespan and physiologic rates in *isp-1* worms by deleting *sod-3* or *sod-5* in an *isp-1* mutant background. Since SOD acts to detoxify superoxide, it would be predicted that deletion of *sod* genes would lead to increased levels of ROS, due to less ROS detoxification. Thus, it is a surprising result that deletion of either *sod-3* or *sod-5* resulted in increased resistance to oxidative stress in *isp-1* worms in both the paraquat development assay and the acute juglone assay on day 1 of adulthood. It is also surprising that deletion of either of the inducible *sod* genes has the opposite effect on survival in the chronic paraquat assay. This indicates that the outcome of oxidative stress assays needs to be carefully interpreted, and highlights the importance of using multiple paradigms to assess sensitivity to oxidative stress.

Despite the fact that deletion of the inducible *sod* genes increased resistance to oxidative stress (except in the chronic paraquat assay), both *isp-1;sod-3* and *isp-1;sod-5* double mutants had decreased lifespan compared to *isp-1* worms. This indicates that resistance to oxidative stress can be experimentally dissociated from longevity. Furthermore, since deletion of *sod-3* or *sod-5* decreased *isp-1* lifespan without affecting resistance to heat stress, osmotic stress or bacterial pathogen stress, this indicates that the increased resistance to stress in *isp-1* worms does not account for their longevity.

Conclusions

In this work we show that *isp-1* worms have increased resistance to oxidative stress despite having elevated levels of ROS, and that resistance to oxidative stress can be further increased through the deletion of either of the inducible superoxide dismutase genes. The fact that deletion of *sod-3* or *sod-5* increases resistance to oxidative stress but decreases lifespan demonstrates that resistance to oxidative stress can be experimentally dissociated from longevity. Importantly, while the function of the inducible *sod* genes in WT worms is unknown, we show that these genes impact stress resistance, lifespan and physiologic rates in the *isp-1* mutant background (Supplementary Table S3).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institute of General Medical Sciences (NIGMS) by grant number RO1 GM121756 (PI: Jeremy Van Raamsdonk) and the Van Andel Research Institute (VARI). Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). We would like to thank Eric Collins, Julie Koeman, Mary Rhodes and Marie Adams from the VARI Genomics Core Facility for library preparation and RNA sequencing. We would also like to acknowledge the *C. elegans* knockout consortium and the National Bioresource Project of Japan for providing strains used in this research.

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Highlights

- *isp-1* worms have increased resistance to oxidative stress despite having elevated levels of ROS
- Deletion of inducible superoxide dismutase genes paradoxically increases resistance to oxidative stress
- Inducible superoxide dismutase genes can impact longevity, stress resistance and physiologic rates
- Resistance to oxidative stress can be experimentally dissociated from longevity

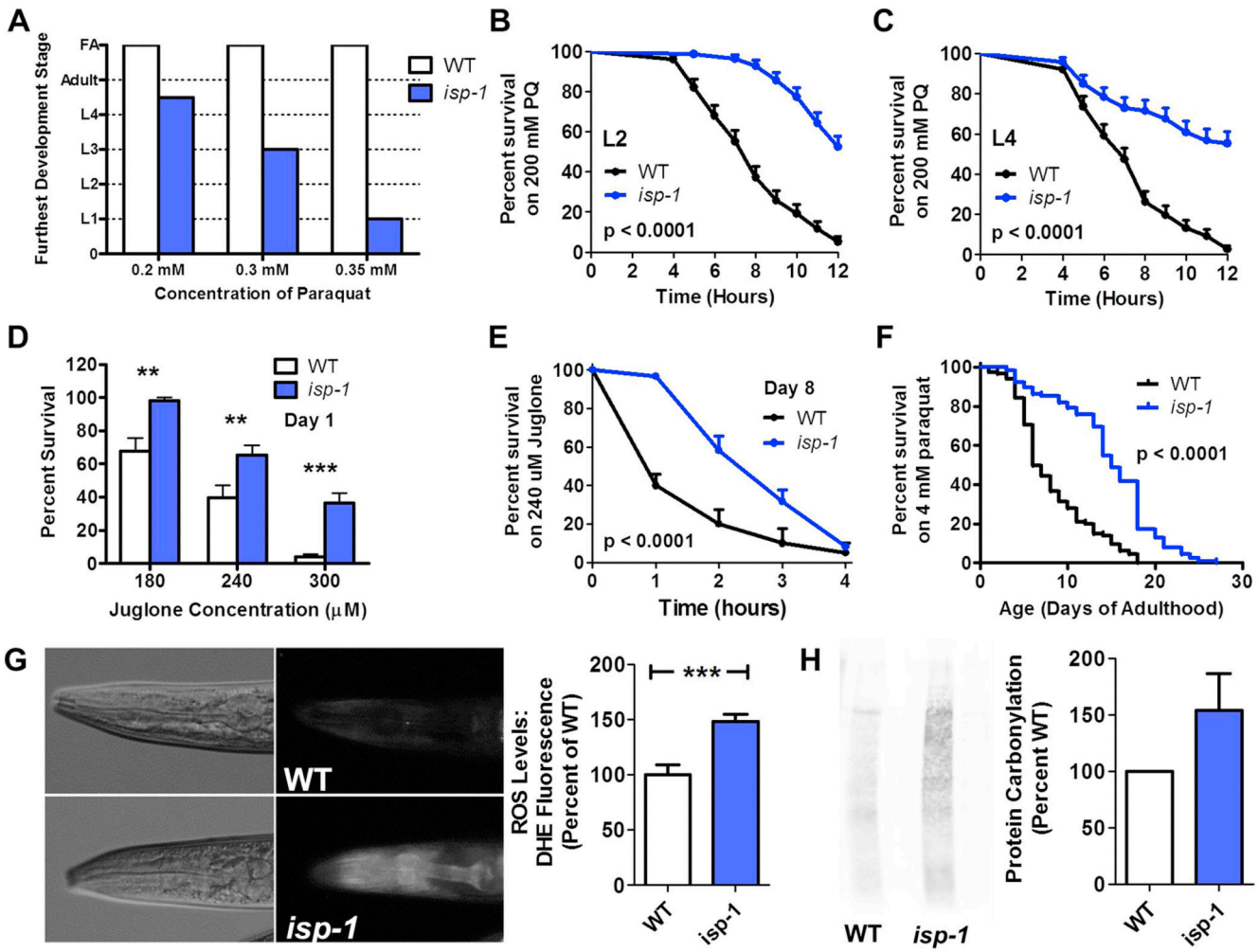

Fig. 1. *isp-1* worms have increased resistance to oxidative stress

(**A**) *isp-1* eggs exposed to paraquat fail to develop to fertile adults at concentrations (0.2 mM – 0.35 mM) that do not prevent wild-type N2 worms from reaching adulthood. However, in an acute oxidative stress assay where worms are exposed to 200 mM paraquat, *isp-1* worms show increased survival compared to wild-type worms at the L2 (**B**) and L4 stages (**C**) of development. Increased resistance to oxidative stress is also observed during adulthood in *isp-1* worms as day 1 adult *isp-1* worms (**D**) and day 8 adult *isp-1* worms (**E**) both survive juglone-induced oxidative stress (180-300 μM) better than wild-type worms. Similarly, in a chronic oxidative stress assay where worms are exposed to a low concentration of paraquat (4 mM) beginning on day 1 of adulthood, *isp-1* worms survive significantly longer than wild-type worms. This indicates that *isp-1* worms have increased resistance to oxidative stress that begins before the L2 stage of development. Error bars indicate SEM. FA = fertile adult. **p<0.01, ***p<0.001.

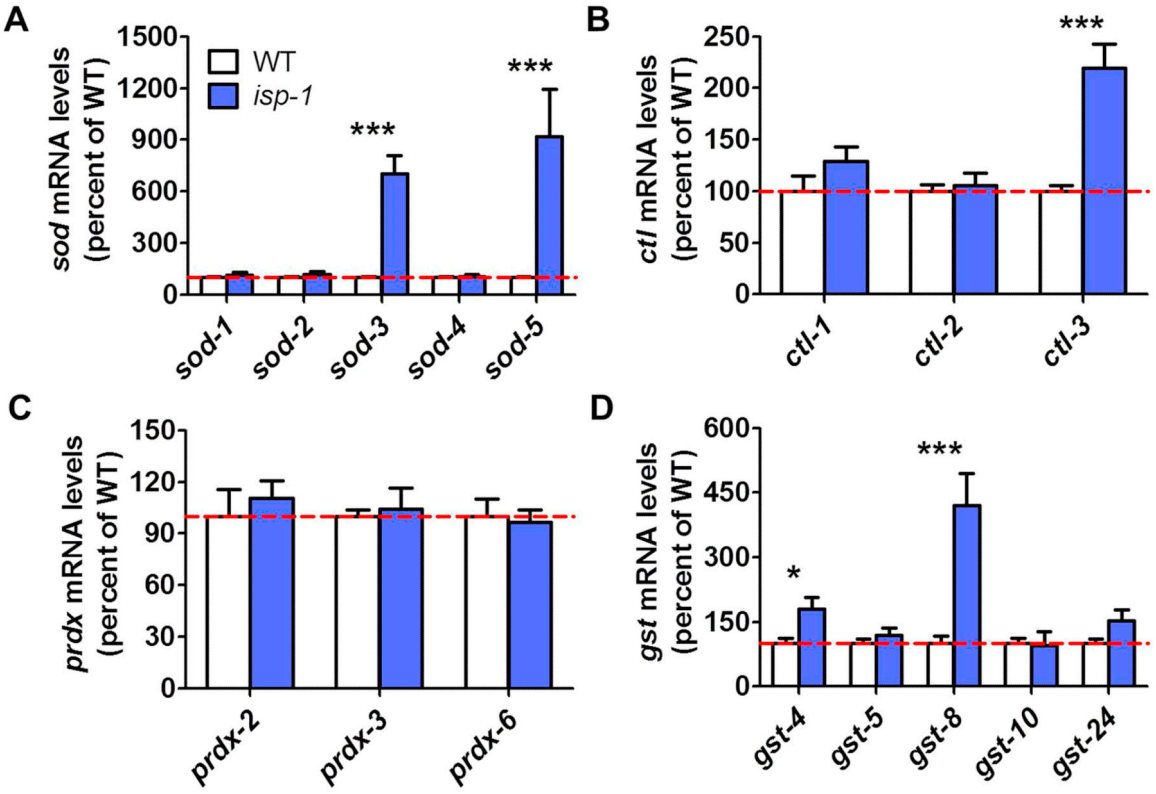

Fig. 2. Inducible *sod* genes are upregulated in *isp-1* worms

The expression of antioxidant enzymes was assessed using quantitative real-time RT-PCR **A.** *sod-3* and *sod-5* were markedly upregulated in *isp-1* worms (**A**). Among the catalase genes *ctl-3* expression was increased in *isp-1* worms (**B**), but none of the peroxiredoxins (**C**) showed increased expression. Specific glutathione-S-transferase genes (*gst-4, gst-8*) were also significantly upregulated in *isp-1* worms. Error bars indicate SEM. *p<0.05, ***p<0.001.

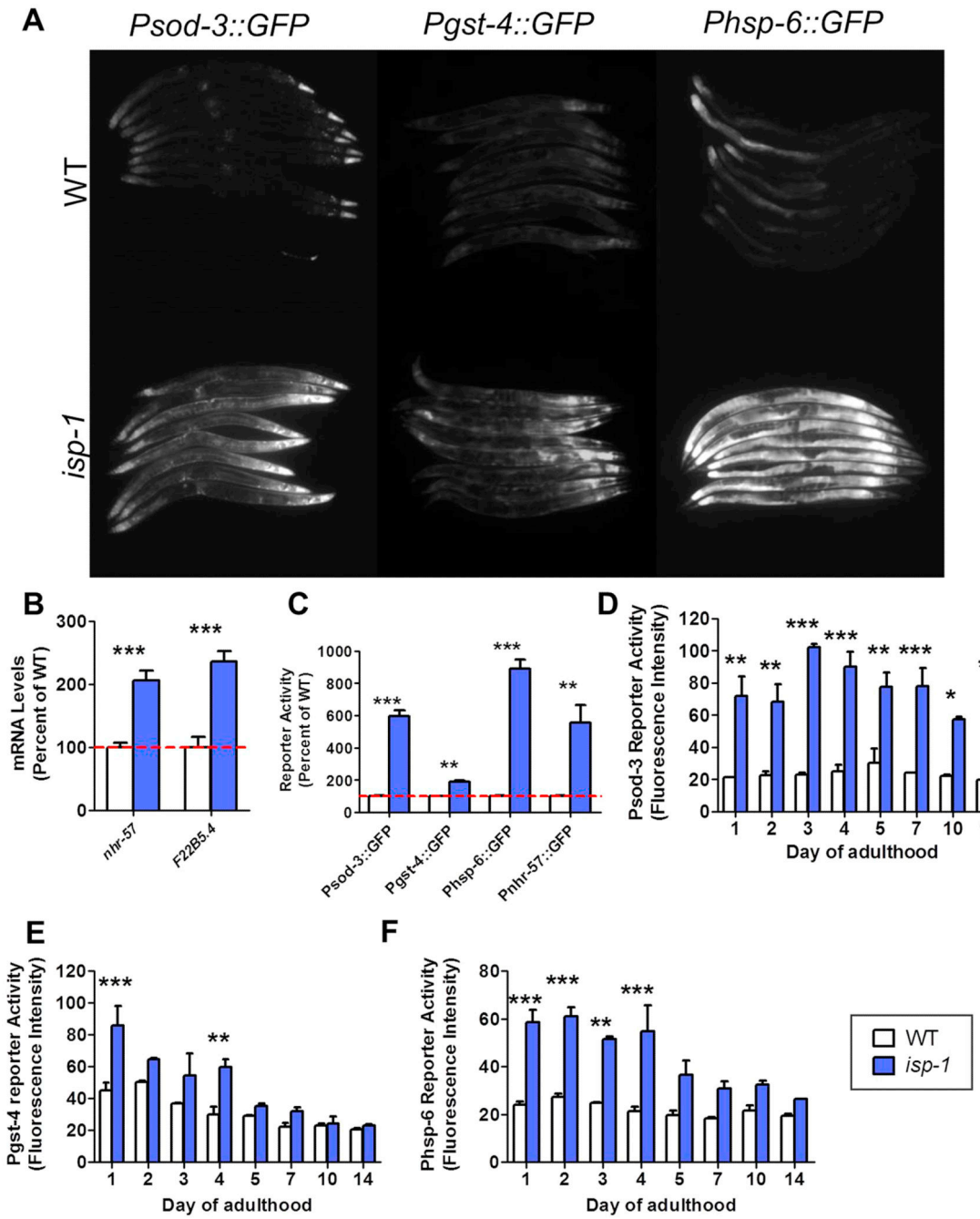

Fig. 3. Multiple stress response pathways are activated in *isp-1* worms

Fluorescent reporter strains and quantitative real-time RT-PCR were used to monitor the activation of stress response pathways in *isp-1* worms. **A.** Fluorescent reporter strains for an antioxidant stress response (*Psod-3::sod-3:GFP)*, the SKN-1-mediated oxidative stress response (*Pgst-4::GFP)*, and the mitochondrial unfolded protein response (*Phsp-6::GFP*) were all activated in *isp-1* worms. **B.** Two representative targets of the HIF-1-mediated hypoxia response (*nhr-57, F22B5.4*) were upregulated in *isp-1* worms. **C.** Examining the time course of stress response activation revealed that all four stress response reporters

(*Psod-3::GFP, Pgst-4::GFP, Phsp-6::GFP*, and *Pnhr-57::GFP)* already showed increased activation compared to WT worms in developing worms that were 1 day post-hatching. **D.** The *Psod-3::sod-3:GFP* reporter shows increased activation throughout adulthood in *isp-1* worms. **E.** The increased activity of the *Pgst-4::GFP* reporter in *isp-1* worms decreases with age, but this stress response pathway has been shown to lose function with age. **F.** The mitochondrial unfolded protein response exhibits increased activation in *isp-1* worms throughout adulthood but the magnitude of increase decreases with age (the ability of this pathway to respond to stress is known to decline with age. Error bars indicate SEM. *p<0.05,**p<0.01, ***p<0.001.

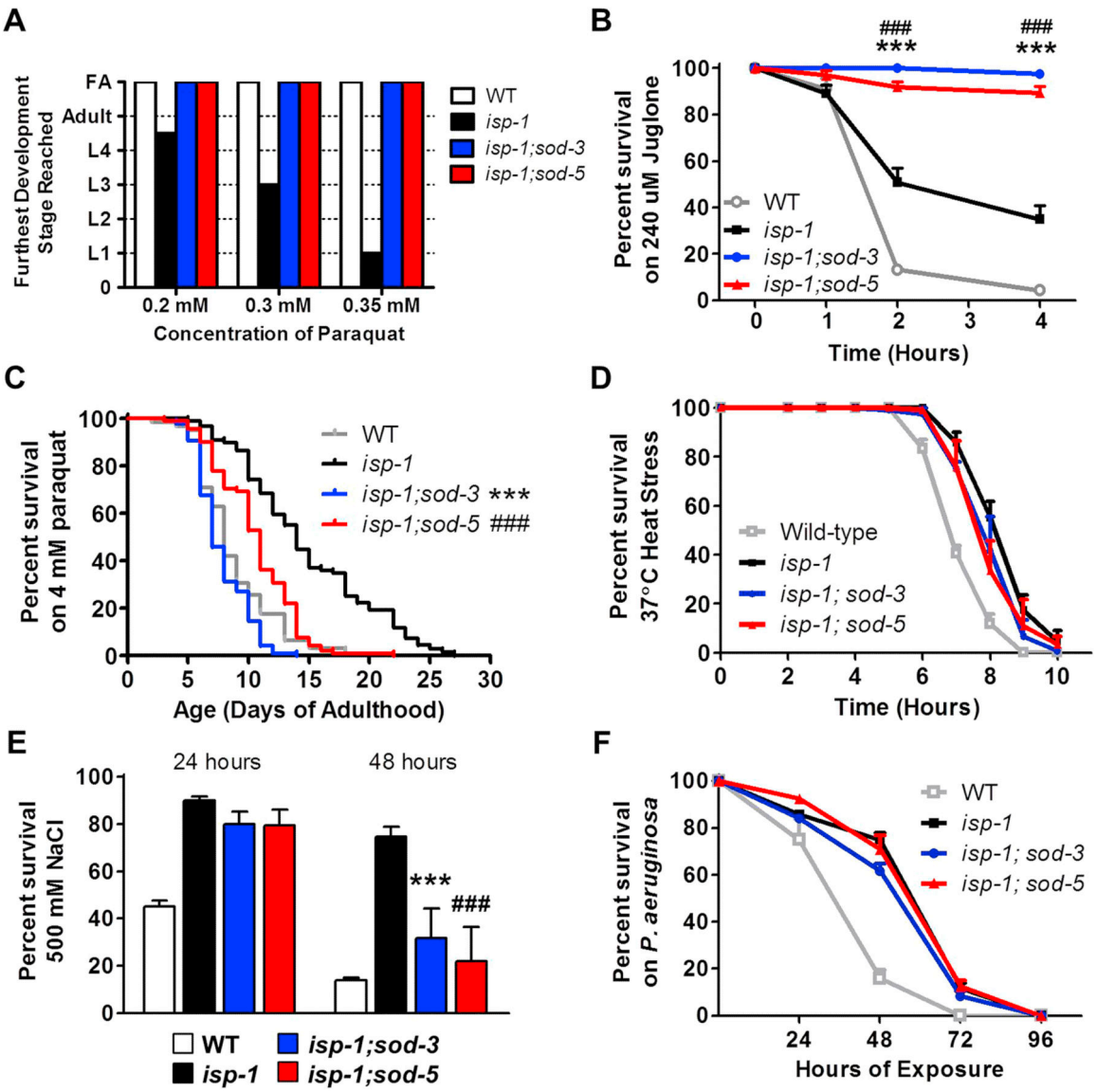

Fig. 4. Deletion of inducible *sod* genes increases resistance to oxidative stress

Deletion of either of the inducible *sod* genes, *sod-3* or *sod-5*, resulted in increased resistance to oxidative stress during development in the paraquat development assay (**A**) and on day 1 of adulthood in the juglone assay (240 μM juglone) (**B**). Conversely, *isp-1;sod-3* and *isp-1;sod-5* double mutants were more sensitive to a chronic exposure to oxidative stress (4 mM paraquat) beginning at day 1 of adulthood (**C**). The increased resistance to stress was specific to oxidative stress as *isp-1;sod-3* and *isp-1;sod-5* worms have stress sensitivity equivalent to *isp-1* worms in assays of heat stress (37°C) (**D**), osmotic stress (500 mM NaCl) (**E**), and bacterial pathogen stress *(Pseudomonas aeruginosa)* (**F**). Error bars indicate SEM. Significant differences between *isp-1* and *isp-1;sod-3* are indicated by ***, those between *isp-1* and *isp-1;sod-5* are indicated by ###.

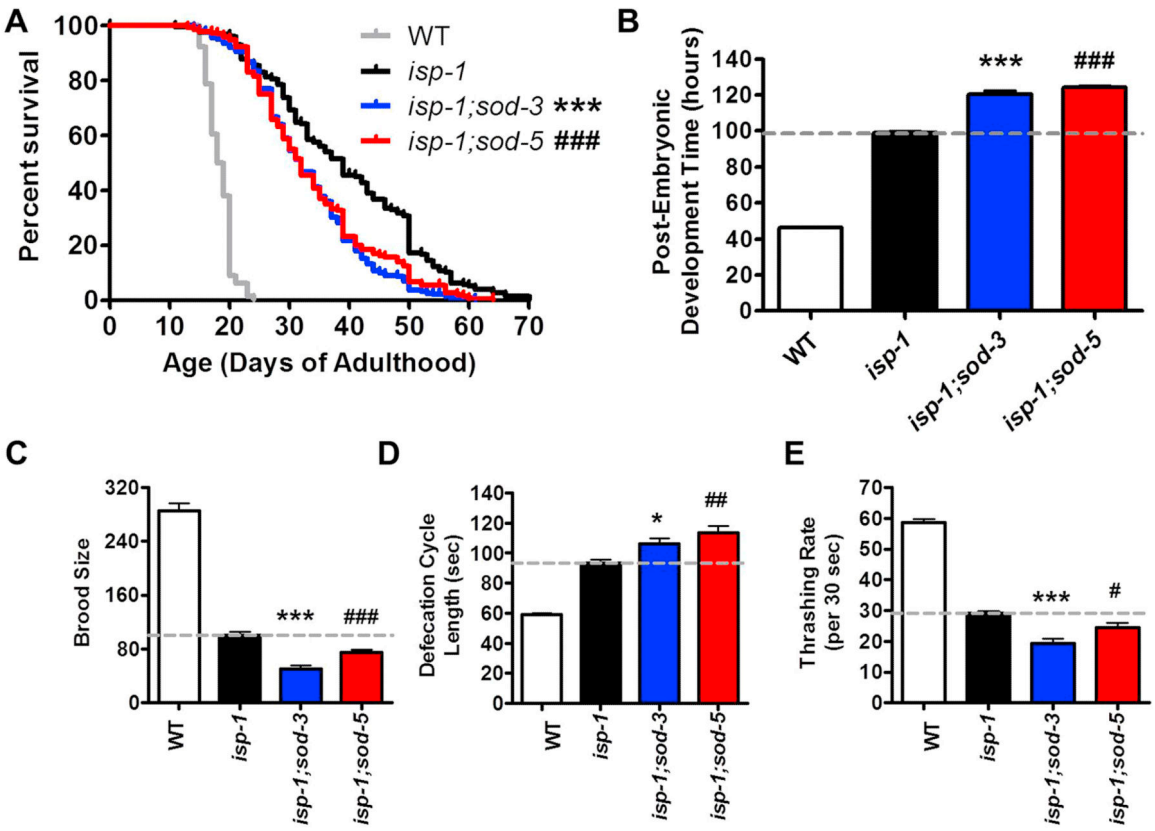

Fig 5. Deletion of inducible superoxide dismutase genes reduces lifespan and exacerbates abnormal physiological rates in *isp-1* worms

A. *isp-1* worms have increased lifespan compared to wild-type worms. Deletion of either *sod-3* or *sod-5* significantly decreases the lifespan of *isp-1* worms. **(B)** *isp-1* worms develop slower than wild-type worms. The post-embryonic development time of *isp-1* worms was further slowed by deletion of *sod-3* or *sod-5*. **(C)** *isp-1* worms have decreased brood size compared to wild-type worms. *isp-1* brood size is further decreased by deletion of *sod-3* or *sod-5*. **(D)** The defecation cycle length of *isp-1* worms is slower than wild-type worms. This phenotype is exacerbated by deletion of *sod-3* or *sod-5*. **(E)** The thrashing rate of *isp-1* worms is slower than wild-type worms and is further decreased by deletion of *sod-3* or *sod-5*. Note that the deletion of *sod-3* or *sod-5* has no effect on any of these phenotypes in wild-type worms. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001.