Supplemental Information

The Mitochondria-Regulated Immune Pathway Activated in the C. elegans Intestine Is Neuroprotective

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Supplemental Figures and Figure Legends

Figure S1

A

L1s on 3-5μM Rotenone

Day 1 Adult
Screen for neuronal loss

Rotenone

neuronal degeneration (%)

control ROT control ROT control ROT

mtl-1 spp-1 thn-1

**

B

DAF-16 target

Relative mRNA levels

countor ROT control ROT control ROT

gst-4

C

SKN-1 target

Relative mRNA levels

countor ROT

gst-4

D

ZIP-2 target

Relative mRNA levels

countor ROT control ROT

irg-1 irg-2

E

L1s on 3-5μM Rotenone

Day 1 Adult
smFISH for p38MAPK/ATF-7 pathway specific target gene C17H12.8

F

L1s on 3-5μM Rotenone

Day 1 Adult
smFISH for p38MAPK/ATF-7 pathway specific target gene C17H12.8
Figure S1. Related to Figure 1. Rotenone treatment upregulates multiple stress response pathways in *C. elegans*

(A) Percent animals that lost dopaminergic neurons visualized with *Pdat-1::mCherry* compared to thermosensory neurons visualized by *Pgcy-8::GFP* when exposed to 3-5 µM rotenone. n=150 animals.

(B) mRNA levels determined by qRT-PCR of DAF-16-dependent genes *mtl-1, spp-1* and *thn-1* in wild-type animals exposed to 3-5 µM rotenone relative to controls. n ≥3 repeats of 20 animals for each treatment.

(C) mRNA levels determined by qRT-PCR of the SKN-1-dependent gene *gst-4* in wild-type animals exposed to 3-5 µM rotenone relative to controls. n ≥3 repeats of 20 animals for each treatment.

(D) mRNA levels determined by qRT-PCR of ZIP-2-dependent innate immune genes *irg-1* and *irg-2* in wild-type animals exposed to 3-5 µM rotenone relative to controls. n ≥3 repeats of 20 animals for each treatment.

(A-D) Bar Graphs: Mean± SEM. *p<0.05, **p<0.01 (Student’s *t* test). Animals: day 1-2 adults.

(B-D) Values normalized to wild-type animals on control RNAi.

(E) Micrographs showing single-molecule RNA *in situ* hybridization (smFISH) for the ATF-7 target gene *C17H12.8* transcript following the systemic exposure of wild-type animals to rotenone. Images are representative maximum projection confocal images of the head (upper panel) and tail (lower panel) of Day 1 adults. Note, mRNA is detected only in intestinal nuclei (lower panel), and not in neuronal, muscle or hypodermal nuclei in the head (upper panel), or in neuronal, hypodermal and germ cell nuclei in the tail (lower panel). Bar, 10 µm. n=3 animals.
Micrographs showing single-molecule RNA in situ hybridization (smFISH) for the ATF-7 target gene C17H12.8 transcripts following the systemic rotenone-exposure of wild-type animals on control RNAi and animals on atf-7 RNAi. Images are representative maximum projection confocal images of the anterior and posterior regions of Day 1 adults: note, mRNA is detected only in intestinal nuclei. Bar, 10 μm. n=3 animals each treatment. Inset: single intestinal nucleus. Animals: day 1-2 adults.
Figure S2

A

Relative mRNA levels

C17H12.8  K08D8.5  C32H11.4  F55G11.8

B

Relative mRNA levels

C32H11.4  F55G11.8

C

Immunoblotting with anti-Phospho-p38MAPK antibody

p-p38MAPK::GFP  p-p38MAPK::FLAG  p-p38MAPK

Tubulin

D

p38MAPK protein levels

Normalized muscle o/e

muscle  Intestine

E

Relative mRNA levels

F55G11.8  M02F4.7  F55D6.2  F35E12.6  F08G5.6  T24B8.5

F

p38MAPK o/e in body wall muscle cells

G

Pdat-1::mCHERRY worms with DA neuronal degeneration (%)

H

Relative atf-7 mRNA levels

Rotenone
**Figure S2. Related to Figure 2.** Rotenone-induced dopaminergic neuron degeneration is suppressed by p38MAPK/ATF-7 mediated innate immune activation.

(A) mRNA levels of p38MAPK/ATF-7 innate immune response genes (X-axis) in rotenone-treated animals subject to *vhp-l* RNAi measured by qRT-PCR. mRNA levels of the transcripts (X-axis) are normalized to wild-type control levels. Actin is the internal control. n≥ 3 repeats of 20 animals for each treatment.

(B) mRNA levels of p38MAPK/ATF-7 innate immune response genes (X-axis) in control and rotenone-exposed wild-type animals raised on OP50 and *Pseudomonas aeruginosa* (PA14). mRNA levels of the transcripts (X-axis) are normalized to wild-type control levels. Actin is the internal control. n= 2 with 20 animals for each treatment.

(C) Phosphorylated p38MAPK levels detected using a phospho-p38MAPK antibody, in wild-type animals, animals overexpressing p38MAPK under a muscle-specific promoter (*unc-54*), animals overexpressing p38MAPK under an intestine-specific promoter (*vha-6*), and animals overexpressing a kinase-dead p38MAPK under an intestine-specific promoter (*vha-6*).

(D) Relative expression levels of muscle- and intestine-specific phospho-p38MAPK relative to alpha-tubulin levels, normalized to phospho-p38MAPK in muscle. n=2 repeats of 20 animals each.

(E) mRNA levels of rotenone-induced innate immune response genes (X-axis) in animals overexpressing p38MAPK in intestinal cells relative to control animals as measured by qRT-PCR. mRNA levels of the transcripts (X-axis) are normalized to wild-type control levels. Actin is the internal control. n≥ 3 repeats of 20 animals for each treatment.

(F) Percent rotenone-treated animals overexpressing p38MAPK in muscle cells that lost dopaminergic neurons on control and *atf-7* RNAi, compared to wild-type. n=160-181.
(G) Percent wild-type animals that lost dopaminergic neurons when exposed to 3-5 µM rotenone on control RNAi, and percent that lost dopaminergic neurons in p38MAPK overexpressing animals on control, *skn-1* and *daf-16* RNAi. *n*=150 animals.

(H) mRNA levels of *atf-7* measured in animals following RNAi-induced knockdown of *atf-7*. Samples are from wild-type and *rrf-3(mg373)* animals. mRNA levels are normalized to wild-type levels on control RNAi. Actin is the internal control. *n*=2 repeats of 20 animals each. Graph represents average mRNA levels from two experiments.

(A, B, E-G) Graphs: Mean± SEM. * *p*<0.05, *** *p*<0.001 (Student’s *t* test). Data from paired experiments and controls are compared. (A-H) Animals: day 1-2 adults.
Figure S3

A

L1s on 3-5μM Rotenone

Day 1 Adult
Active p38MAPK
Western blot

p-p38MAPK

Tubulin

p-p38MAPK protein levels normalized to control

Wild-type
Rotenone
Control

0.00
0.50
1.00
1.50

B

Relative mRNA levels

C17H12.8
F56D6.2
M02F4.7
F49F1.6

Wild-type
p38MAPK Δ
km25

Wild-type
p38MAPK Δ
km25

Wild-type
p38MAPK Δ
km25

Wild-type
p38MAPK Δ
km25
(A) Levels of phosphorylated p38MAPK following rotenone treatment of wild-type animals relative to control untreated animals. Levels of phospho-p38MAPK (top lanes) were determined by western blot and quantified relative to alpha-tubulin (bottom lanes) and normalized to control, non-rotenone treated animals. n≥3 repeats of 15-20 animals per treatment. Bar Graph: Mean±SEM. Data from paired experiments and controls are compared.

(B) mRNA levels of ATF-7 dependent innate immune genes in wild-type animals and pmk-1(km25) animals on control RNAi and Complex I RNAi, determined by qRT-PCR. mRNA levels of the transcripts following RNAi treatment (RNAi treatments are in the data legend) were normalized to wild-type control levels. Actin is the internal control. n≥3 repeats of 20-25 animals per treatment. Bar Graphs show Mean; data labels attached. Data from paired experiments and controls are compared. ** p<0.01, *** p<0.001 (Student’s t test). Animals: day 1-2 adults.
Figure S4. Related to Figure 4. Neuroprotection conferred by p38MAPK/ATF-7 mediated innate immune activation does not occur through changes in ATP levels, mitochondrial biogenesis or UPR\textsuperscript{mito}.

(A) ATP levels in control and rotenone treated animals: animals were on control RNAi or \textit{atf-7} RNAi or were overexpressing p38MAPK and on OP50. All data are normalized to ATP levels in wild-type animals on control RNAi, without rotenone exposure.

(B) Quantitative RT-PCR measurements of mRNA levels of mitochondrial ETC genes (genes are on X-axis) under control conditions without exposure to rotenone, in wild-type animals and p38MAPK overexpressing animals on control RNAi and \textit{atf-7} RNAi. mRNA levels of the transcripts (X-axis) were normalized to levels in wild-type animals. Actin is the internal control. \(n \geq 3\) repeats of 20-25 animals per treatment.

(C) Quantitation of ATPB protein levels by western blot analysis in wild-type animals on control and \textit{atf-7} RNAi, and p38MAPK overexpressing animals under control conditions and upon rotenone treatment. Levels of ATPB were quantified relative to alpha-tubulin and normalized to control animals not exposed to rotenone. \(n \geq 3\) repeats of 20-25 animals per treatment.

(D) Quantitative RT-PCR measurements of mRNA levels of \textit{gst-4} and \textit{sod-2} in control conditions and upon rotenone exposure in wild-type animals and p38MAPK overexpressing animals. \(n \geq 3\) repeats of 20-25 animals per treatment.

(E) Representative micrographs of rotenone-exposed animals expressing the \textit{hsp-6pr::GFP UPR}\textsuperscript{mito} reporter construct on control RNAi and \textit{atf-7} RNAi. Bar, 0.1 mm.

(F) mRNA levels of \textit{hsp-6} and \textit{hsp-60} upon rotenone treatment in wild-type animals on control RNAi, animals subject to \textit{atf-7} RNAi, \textit{pmk-1(km25)} animals and animals overexpressing p38MAPK. mRNA levels of the transcripts (X-axis) were normalized to levels in wild-type
animals on rotenone. Actin is the internal control. n≥3 repeats of 20-25 animals per treatment.

(G) Quantitation of HSP-60 protein levels by western blot analysis in wild-type animals on control RNAi and atf-7 RNAi and pmk-1(km25) animals all exposed to rotenone. Levels of HSP-60 were quantified relative to alpha-tubulin and normalized to control animals on rotenone. n≥3 repeats of 20-25 animals per treatment.

(H) Percent wild-type animals and pmk-1(km25) animals on control and atfs-1 RNAi that lost dopaminergic neurons on rotenone following RNAi treatment. n=2; 200-300 animals per treatment.

(A-D, F,G) Bar Graphs show Mean± SEM. Data from paired experiments and controls are compared. * p<0.05, ** p<0.01 (Student’s t test).
Figure S5

**A**

RNAi: Intestine only

Pdat-1::mCherry worms with DA neuronal degeneration (%)

|          | ctrl RNAi | mboa-1 RNAi |
|----------|-----------|-------------|
| rotenone |           |             |

**B**

RNAi: Neurons only

Pdat-1::GFP worms with DA neuronal degeneration (%)

|          | ctrl RNAi | mboa-1 RNAi |
|----------|-----------|-------------|
| rotenone |           | **          |
Figure S5. Related to Figure 5. Knockdown of the autophagy gene mboa-1 in neurons affected neurodegeneration on rotenone.

(A) Percent of rotenone-induced neurodegeneration upon intestine-specific RNAi: rde-1(ne219) mutants expressing RDE-1 under the intestinal nhx-2 promoter were exposed to control and mboa-1 RNAi.

(B) Percent of rotenone-induced neurodegeneration upon neuron-specific RNAi: sid-1(pk3321) expressing SID-1 under the neuronal unc-119 promoter were exposed to control and mboa-1 RNAi

(A-B) Graphs show Mean± SEM. ** p<0.01 (Student’s t test). Animals: day1-2 adults. n>3 experiments of 100-125 animals each.
Figure S6. Related to Figure 6. Effect of p38MAPK/ATF-7 pathway on autophagy dependent gene products

(A-C) Western blot analysis of GFP levels in GFP::LGG-1 expressing animals under control conditions and upon rotenone treatment:

(A) wild-type, and p38MAPK o/e animals that have been crossed to the GFP::LGG-1 strain, on control and rotenone,

(B) animals on control RNAi and atf-7 RNAi, on control and rotenone

(C) wild-type and pmk-1(km25) animals on control and rotenone.

Levels of GFP (top lanes) were quantified relative to alpha-tubulin (bottom lanes). Bar Graphs show Mean± SEM. * p<0.05, *** p<0.001 (Student’s t test).

(D) Micrograph of head of wild-type animal: ADE neurons were identified by crossing the dat-l المتوسط::mCHERRY animals with GFP::LGG-1 animals. Bar, 25µm.

(E) Quantitation of GFP::LGG-1 puncta in the ADE neurons under control conditions and upon rotenone treatment in wild-type animals on control RNAi, wild-type animals on atf-7 RNAi, pmk-1(km25) animals, wild-type animals on vhp-1 RNAi, and animals overexpressing p38MAPK.

(F) Quantitation of PINK-1::GFP expression levels in transgenic worms expressing PINK-1::GFP driven by its endogenous promoter, in wild-type animals exposed to control RNAi, animals exposed to atf-7 RNAi and animals overexpressing p38MAPK. Measurements were under control, non-rotenone conditions. n>200 animals. * p<0.05, ** p<0.01, *** p<0.001 (Student’s t test).
Supplemental Experimental Procedures

C. elegans strains

pdat1::mCHERRY was a generous gift from Dr. Anton Gartner (University of Dundee, UK). The following stains were obtained from Caenorhabditis Genetics Center (CGC) at University of Minnesota, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440):

N2 Bristol strain,

MAH236- sqIs13 [Plgg-1::GFP::lgg-1 + Podr-1::RFP]

AY102 - acEx102 [Pvha-6::pmk-1::GFP + rol-6(su1006)]

SJ4100 - zcIs13 [hsp-6::GFP] V

KU25 - pmk-1(km25) IV

RB2547 - pink-1(ok3538) II,

rrf-3(mg373),

rrf-3(mg373); acEx102 [Pvha-6::pmk-1::GFP + rol-6(su1006)]

VP303-(rde-1(ne219); [nhx-2:rde-1, rol-6]),

sid-1(pk3321) V; uIs69 [pCFJ90 (myo-2p::mCherry) + unc-119p::sid-1]

SJ4143-Is[pges-1::GFPmt],
BR4006- *pink-1(tm1779)* II *Ex655 [pink-1::pink-1::GFP + myo-2p::mCherry + herring sperm DNA]*

SJ4100-zcIs13[hsp-6p::GFP]

MAH236-sqIs13 [lgg-1p::GFP::lgg-1 + odr-1p::RFP]

The *pdat-1::mCHERRY* marker, or a *pdat-1::GFP* marker was crossed in all the strains, when needed to assess dopaminergic neuron loss.

**Constructs and Generation of transgenic Lines**

We generated the transgenic worms using the standard microinjection method as described previously\(^1\). The plasmid carrying the gene of interest (15-20 ng/μl) with or without the reporter plasmid (15-20 ng/μl) was used for these microinjections. All constructs were sequenced prior to injection.

1. **Generation of *atf-7::GFP* overexpression strain**

Animals expressing *Pdat1::RFP* were microinjected with yeast DNA carrying 22794 bp *C. elegans* genomic DNA covering the *atf-7* gene tagged with GFP, a kind gift from Dr. Dennis Kim (Massachusetts Institute of Technology, Cambridge, MA)\(^2\), to obtain transgenic worms.

2. **Generation of *Punc-54::pmk-1::FLAG* strain**

Construction of *Punc54::mCHERRY* was amplified using the following primers from pCFJ104 plasmid (kind gift from Dr. Bryan Philips, University of Iowa) and introduced under *unc-54* promoter in pPD30.38 vector.
pmk-1 cDNA was obtained from Open Biosystems ORFeome (GE Dharmacon, Fisher Scientific, Pittsburgh, PA). pmk-1 cDNA along with FLAG coding region was amplified and inserted into pPD30.38 under the unc-54 promoter. The following primers were used:

Pmk1FlagSalIFor(CGACGTCGACAAAAATGGATTACAAGGATGATGACGATAAGTTTC CACAGACAAACAATGG)
Pmk1NcoIRev(GACCCATGGCTACGATTCCATTTTCTCCTC)

Punc-54::pmk-1::FLAG was co-injected with Punc54::mCHERRY to obtain this transgenic strain.

3. Generation of Pvha-6:pmk-1AGF::FLAG strain

First Pvha-6:pmk-1::FLAG construct was made by replacing the unc-54 promoter in Punc54::pmk-1::FLAG with the vha-6 promoter. The following primers were used to amplify the vha-6 promoter:

Pvha-6HindIIIFor (GACAAGCTTAAGTATACTATTTATTACTCG)
Pvha-6SalIRev (GACGTCGACTTTTTATGGGTTTTGGTAGGTTTTTAG)

Using a Site Directed Mutagenesis (SDM) Kit (QuickChange XL site-directed mutagenesis kit ; Catalog # 200517-5, Agilent Technologies, Inc., Santa Clara, CA 95051, United States) PMK-1 kinase active site TGY was modified into inactive AGF dead-kinase. The following primers were used for SDM protocol:

P38AGFFor (CTGATTCTGAAATGGCTGGATTCGTGGCAACAAGATGG)
P38AGFRev (CCATCTTGTTGCCACGAATCCAGCCATTTTCAGAATTCAG)
Pvha-6:pmk-1AGF-FLAG was co-injected with ttx3::GFP (a kind gift from Dr. Bryan Philips, University of Iowa) into N2 strains to obtain transgenic lines.

**Crosses used**

*acEx102 [Pvha-6::pmk-1::GFP + rol-6(su1006)]; Pdat1::mCHERRY*: The AY102 strain carrying the *acEx102[pDB09.2(pvha-6::pmk-1::gfp); pRF4(rol-6(su1006))]* transgene originally harbored a deletion in the *pmk-1* gene. We back-crossed these animals with animals expressing the dopaminergic neuron reporter *Pdat1::mCHERRY*, four times to both restore the wild-type *pmk-1* background and to introduce the dopaminergic neuron reporter into the p38MAPK overexpression background. We confirmed that these animals harbored a wild-type *pmk-1* background by PCR genotyping.

*RB2547 pink-1(ok3538) II strains were crossed with animals expressing the *Pdat-1::mCHERRY* reporter, and F2 animals homozygous for the *pink-1(ok3538)* deletion were genotyped using PCR.*

*KU25 - pmk-1(km25) IV strains were crossed with animals expressing the *Pdat-1::mCHERRY* reporter, and F2 animals homozygous for the *pmk-1(km25)* deletion were identified using PCR genotyping.*

Similarly, the *Pvha-6:pmk-1AGF-FLAG* and the *Punc-54::pmk-1::FLAG* strains were also crossed to animals expressing the *Pdat-1::mCHERRY* reporter, and F2 animals homozygous for both transgenes were selected for our experiments.

**Growth conditions**

All strains were grown and maintained at low densities at 20°C under standard conditions. Ambient temperature was maintained at 20-22°C. All animals included in the experiments, unless
stated otherwise, were one or two day old hermaphrodites that were age-matched by bleaching or picking as L4 juveniles. Unless otherwise stated, animals were fed with OP50 obtained from the Caenorhabditis Genetics Center (CGC, Twin Cities, MN). Stock strains were maintained by passaging 10 larval stage 4 animals (L4s) onto NGM plates, and 4 days later picking L4s onto fresh plates for experiments. The NGM plates were standardized by pouring 8.9 ml of liquid NGM/plate that yielded plates with an average weight of 13.5 ± 0.2 g. For RNAi experiments, RNAi bacteria (HT115) with empty or RNAi constructs were grown overnight in LB liquid culture until OD595 was approximately 2.0, and induced with IPTG (1 μM) for 1 hour before seeding the bacteria on NGM plates. Rotenone NGM plates were made as following: 10mM Rotenone (Catalog # 45656, Sigma-Aldrich, St. Louis, MO 63178 USA) stocks were made in DMSO and stored at -20°C. NGM agar was allowed to cool to approximately 40-50°C following autoclaving, and rotenone was added to make its final concentration 3-5 μM. NGM plates were then allowed to dry for 48 hours and bacteria (OP50 or HT115 with empty plasmid or RNAi plasmid) were seeded. For all the rotenone experiments, worms were grown on plates from larval stage 1 (L1s). To obtain L1s, gravid adults from a whole plate were bleached and eggs were spread on empty NGM plates for around 18 hours, and allowed to hatch.

**RNAi Experiments and Constructs**

All RNAi clones obtained from the Arhinger library were sequenced. *atf-7* RNAi was obtained from Open Biosystems (GE Dharmacon, Fisher Scientific, Pittsburgh, PA). All the results with *atf-7* RNAi were also repeated using a different *atf-7* RNAi plasmid that was used in Shivers et al.², a kind gift from Dr. Dennis Kim (MIT). For all RNAi experiments (control and rotenone), NGM plates were supplemented with tetracycline and ampicillin. Since rotenone inhibits bacterial
growth, to optimize RNAi mediated knockdown using feeding vectors we grow RNAi cultures to an OD of 2.0, and induce them in culture prior to seeding the bacteria on the plate. We have confirmed using RT-PCR that this leads to a robust knockdown of mRNA levels of the target genes.

**Dopaminergic (DA) neuronal loss assay**

NGM plates with 3-5 μM rotenone were used for this assay. Rotenone was dissolved in DMSO, and control plates had vehicle only. Control and rotenone plates were poured at the same time. For RNAi experiments on animals exposed to rotenone, RNAi bacteria were grown overnight in LB liquid culture and induced with IPTG (1 μM) for 1 hour before seeding the bacteria on rotenone plates. The *Pdat1::mCHERRY* adult worms were bleached and eggs were spread on an empty NGM plate for around 18 hours. Approximately 100-150 hatched L1s were placed on rotenone plates and scored for the DA neuron loss phenotype in one day old adults. The DA neuron loss was assessed under a Leica MZ10F stereo microscope (Leica, Solms, Germany) at 8X magnification, and subsequently randomly verified under a compound microscope (Zeiss, Oberkochen, Germany). Animals that had lost at least one or more of their six anterior dopaminergic neurons (2 pairs of CEPs and 1 pair of ADEs) were counted. The representative images for the DA neuron loss were taken using Leica TCS SP5 confocal microscope (Leica, Solms, Germany).

**Pseudomonas treatment:**

A single colony of *P. aeruginosa* PA14 was used to inoculate 3 ml LB liquid media, and incubated overnight at 37°C. 200 μl of this culture was seeded on 60 mm NGM plates (with or without
rotenone). Freshly poured NGM plates were allowed to dry overnight at room temperature and subsequently the bacteria were grown in a 37°C incubator for 24 hours before the plates were cooled down to 20°C, and plated with L1s. *E.coli* OP50 plates were used as a control and were made in the same way as PA14 plates. The elicitation of innate immune response by PA14 treatment was confirmed by measuring levels of classic PA14 induced genes by qRT-PCR (see results section).

**Single molecule fluorescent in situ hybridization (smFISH)**

smFISH probes were designed against C17H12.8 by utilizing the Stellaris FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA) available online at www.biosearchtech.com/stellarisdesigner. The fixed worms were hybridized with the C17H12.8 Stellaris FISH Probe set labeled with Cy5 dye (Biosearch Technologies, Inc., Petaluma, CA), following the manufacturer’s instructions available online at www.biosearchtech.com/stellarisprotocols. Briefly, at least 10-20 one day old adult worms per condition (rotenone treatment, non-rotenone plates, control RNAi, and *atf-7* RNAi) were harvested, fixed using 4% paraformaldehyde and subsequently resuspended in 70% ethanol at 4°C for approximately 24 hours to permeabilize the animals. Samples were washed using Stellaris Wash Buffer A (Catalog # SMF-WA1-60, Biosearch Technologies, Inc., Petaluma, CA) and then the hybridization solution (Catalog # SMF-HB1-10, Biosearch Technologies, Inc., Petaluma, CA) containing the probes was added. The samples were kept in hybridization solution at 37 °C for 16 hours, after which they were washed three times with Wash Buffer A and incubated for 30 minutes in Wash Buffer A with DAPI. Following DAPI staining, worms were washed with Wash Buffer B (Catalog # SMF-WB1-20, Biosearch Technologies, Inc., Petaluma, CA) and finally the samples
were resuspended in approximately 12 μl of Vectashield hard set (Catalog # H-1000; Vector Laboratories, Burlingame, CA), placed on slides and imaged on the Leica TCS SP5 Confocal Microscope (Leica, Solms, Germany) using a 63x oil objective. LAS AF software (Leica, Solms, Germany) was used to obtain maximum projections of Z stacks. ImageJ software was used to overlay DAPI maximum projection with smFISH maximum projection.

Animals were separated into pools that retained all their dopaminergic neurons, and those that lost one or more dopaminergic neurons prior to processing for smFISH. The number of C17H12.8 puncta in intestinal cells was scored blind.

**Estimation of ATP concentrations:**

To estimate ATP concentrations, we followed previously used protocol\(^5\). Briefly, ~200 synchronized one day old adult hermaphrodites were collected in M9 buffer and washed three times. Worm pellets were freeze/thawed three times and boiled for 15 minutes to release ATP and destroy ATPase activity. Then worms were pelleted at 11,000 x g for 10 minutes at 4°C. ATP contents were measured with ATP detection kit (Catalog # A22066, Life Technologies – Molecular Probes, Carlsbad, CA) according to the manufacturer’s instructions. This bioluminescence assay quantitatively determines the ATP levels with recombinant firefly luciferase and its substrate D-luciferin. This assay measures the bioluminescence emitted by the ATP-dependent oxidation of D-luciferin catalyzed by luciferase. Known dilutions of ATP (Catalog # A22066, Life Technologies – Molecular Probes, Carlsbad, CA) were used to generate standard curves. A Luminometer 20/20 (Turner BioSystems, Promega, Madison WI) was used to measure bioluminescence levels. A
Pierce BCA Protein Assay Kit (Catalog # 23227, Thermo Fisher Scientific, Grand Island, NY) was used to measure protein concentrations using an Eppendorf BioPhotometer Plus spectrophotometer (Eppendorf North America, Hauppauge, NY). The concentrations of ATP were normalized to absolute protein concentrations to get the final values.

**RNA extraction and quantitative RT-PCR**

For RNA extraction, 15-20 one day old adult animals were picked into 10 μl of RNAlater (Catalog # 76104; Qiagen, Venlo, Limburg) and snap-frozen immediately in liquid nitrogen for at least 20 minutes. The following steps were either carried out immediately after snap-freezing, or samples were stored at -80°C. Samples were thawed on ice and 250 μl of Trizol (Catalog # 400753; Life Technologies, Carlsbad, CA) was added, followed by brief vortexing at room temperature. Samples were then vortexed at 4°C for an hour to lyse worms completely. RNA was then purified as detailed in the manufacturer’s protocol with appropriate volumes of reagents modified to 250 μl Trizol. The RNA pellet was dissolved in 17 μl RNase-free water. RNA was treated with DNase using a TURBO DNA-free kit (Catalog # AM1907; Life Technologies, Carlsbad, CA) as per manufacturer’s protocol. cDNA was generated by using the iScript™ cDNA Synthesis Kit (Catalog # 170-8891; Bio-Rad, Hercules, CA). Real-time PCR was performed using iQ™ SYBR® Green Supermix (Catalog # 170-8880; Bio-Rad, Hercules, CA), in LightCycler® 480 (Roche, Basel, Switzerland) at a 10 μl sample volume, in a 96 well white plate (Catalog # 04729692001; Roche, Basel, Switzerland). The relative amounts of mRNA were determined using the “Delta Delta CT” (ddCT) Method for quantification. Actin mRNA was used as an internal control. All relative changes of mRNA in the RNAi animals were normalized to that of RNAi-empty vector animals, except where otherwise noted. The CT values were obtained in duplicates or triplicates.
for each sample (technical replicates). Each experiment was then repeated a minimum of three times. For qPCR reactions, the amplification of a single product with no primer dimers was confirmed by melt-curve analysis performed at the end of the reaction. All qPCR reactions included minus reverse transcriptase controls to exclude any possible genomic DNA amplification. qPCR primers were designed using Roche’s Universal Probe Library Assay Design Center software and or using primer 3 software.

The sequences of different primers used for the PCR analysis are provided below (all sequences read from 5’ to 3’):

| Primer Name      | Sequence                        |
|------------------|---------------------------------|
| ATG-18FOR        | ACTTGAGAAACGGAAGGTGTT          |
| ATG-18REV        | TGATAGCATCGAACCATCCA           |
| ATG-9FOR         | GATTCACCTGGCCAGGAG             |
| ATG-9REV         | ATCGTCGAGAGTITAGCTGTGA         |
| C17H12.8FOR      | TGTCATTCAATGGAGGATATTGT        |
| C17H12.8REV      | TGATGGAGTTGGAGGATATTGA         |
| C32H11.4 F1      | TCCTTTGAAATGCGGATCT            |
| C32H11.4 R1      | GGCTTGTGAAACCACATTACC          |
| CNC2-FOR         | TGATGGGAGGTATGGAGGA            |
| CNC2-REV         | GAGCATTCCAAGGAGTCCAG           |
| F49F1.6FOR       | CCATCAAACCTACGCAAGGC           |
| F49F1.6REV       | TCCGTTGGATAGAAGGTGTT           |
| F55G11.8F1       | TCAAAACAACCCACGAAAA            |
| F55G11.8R1       | AGCAAATCTCTCGTTGGAGA           |
| F56D6.2RTFOR     | GGTGACAGTTCAAAGCCATGT          |
| F56D6.2RTREV     | TCCAAAAATGCACCAGTGA            |
| HSP60-RTFOR      | AGGCTCTTACACCTCTGTTCAAT        |
| HSP60-RTREV      | GCCTGTCTTTGCGGTATCT            |
| Gene       | Forward Primer | Reverse Primer |
|------------|----------------|----------------|
| HSP6-RTFOR | AACCGGAAAGGAAACAACAGAT | TCTCGATTTGGTCCTTGGA |
| HSP6-RTREV | TCTCGATTTGGTCCTTGGA | TCTCGATTTGGTCCTTGGA |
| IRG-1FOR   | TGAAGGAAACTAATCCATGAAGA | CATCCTTTGATTGTTTGGAGACCA |
| IRG-1REV   | CATCCTTTGATTGTTTGGAGACCA | TGAAGGAAACTAATCCATGAAGA |
| IRG-2FOR   | CGAAGGGAATCTGATATTTCG | GGAAGGGAATCTGATATTTCG |
| IRG-2REV   | GGAAGGGAATCTGATATTTCG | CGAAGGGAATCTGATATTTCG |
| K08D8.5F1  | TACATTTTCACGTCCCCACA | TACATTTTCACGTCCCCACA |
| K08D8.5R1  | TACATTTTCACGTCCCCACA | TACATTTTCACGTCCCCACA |
| LMP1-RTFOR | AATCGTTTGTCATCTTTGTTG | GTTGTTACATAGTAATGCAG |
| LMP1-RTREV | TTTGTTACATAGTAATGCAG | AATCGTTTGTCATCTTTGTTG |
| LYS7RTFOR  | TTGTGCAGTTTTCGTTGCGT | CAGGCTCTTCAACAGATCTCC |
| LYS7RTREV  | CAGGCTCTTCAACAGATCTCC | TTGTGCAGTTTTCGTTGCGT |
| M02F4.7RTFOR | GGAACACTGGGCTTCTCTCAT | CATCTTTACAGATCTCC |
| M02F4.7RTREV | GGAACACTGGGCTTCTCTCAT | CATCTTTACAGATCTCC |
| MTL-1_F    | TGGATGTAAGGAGACTGCA | TGGATGTAAGGAGACTGCA |
| MTL-1_R    | TGGATGTAAGGAGACTGCA | TGGATGTAAGGAGACTGCA |
| SPP1RTFOR  | AGGAAGAGGCTGCTGACGACGA | AGGAAGAGGCTGCTGACGACGA |
| SPP1RTREV  | AGGAAGAGGCTGCTGACGACGA | AGGAAGAGGCTGCTGACGACGA |
| SQST-1FOR  | CAGGCTCTTCAACAGATCTCC | CAGGCTCTTCAACAGATCTCC |
| SQST-1REV  | CAGGCTCTTCAACAGATCTCC | CAGGCTCTTCAACAGATCTCC |
| THN2RTFOR  | ATGGACTGGCCGGGAAGAGTT | ATGGACTGGCCGGGAAGAGTT |
| THN2RTREV  | ATGGACTGGCCGGGAAGAGTT | ATGGACTGGCCGGGAAGAGTT |
| VHA15RTFOR | TCAGCTCGAGGTCTCAGGA | TCAGCTCGAGGTCTCAGGA |
| VHA15RTREV | TCAGCTCGAGGTCTCAGGA | TCAGCTCGAGGTCTCAGGA |
| VHA16RTFOR | TTGTGCAGTCATCTCTGCTTC | TTGTGCAGTCATCTCTGCTTC |
| VHA16RTREV | TTGTGCAGTCATCTCTGCTTC | TTGTGCAGTCATCTCTGCTTC |
| VHA17RTFOR | CACGGGCCTGCGTCTGAGA | CACGGGCCTGCGTCTGAGA |
| VHA17RTREV | CACGGGCCTGCGTCTGAGA | CACGGGCCTGCGTCTGAGA |
| VPS11-RTFOR | GCAGTTCTGGAGGATCTGTGT | GCAGTTCTGGAGGATCTGTGT |
| VPS11-RTREV | GCAGTTCTGGAGGATCTGTGT | GCAGTTCTGGAGGATCTGTGT |
| VPS18-RTFOR | GGAAGCAGGCGGATTTGAT | GGAAGCAGGCGGATTTGAT |
| VPS18-RTREV | GGAAGCAGGCGGATTTGAT | GGAAGCAGGCGGATTTGAT |
| Gene     | Forward | Reverse |
|----------|---------|---------|
| Nuo-2 Forward | ATGTGCTGGGACTGGGTCTCG |          |
| Nuo-2 Reverse | GGTGATTTTGCCCGCTTCT |          |
| cyc-1 Forward  | GGGCTCTTAAGATCGTCGTGC |          |
| cyc-1 Reverse  | GTGGCTCTTCTCCCTTGACC |          |
| nuo-1 Forward  | ACACGAATCTTGCGGACAGT |          |
| nuo-1 Reverse  | TGGCGAATGAGTCCTGGAAC |          |
| Cox5b Forward  | CTCCGATACGGAGAAAGC |          |
| Cox5b reverse | CACTGTCCCTCTTGCGGAAT |          |
| atp-5 Forward  | GACGTTTCCTCGGAACTGG |          |
| atp-5 Reverse  | TCGAAGACTGCGAAGATGAC |          |
| gas-1 Forward  | CTTGTGTGTTGGAGGAAGCTG |          |
| gas-1 Reverse  | AGCTGACAGACAGTCCGATGTC |        |
| Pink-1 Forward  | GGCTGTTCTCCTGGAAAGAATGTA |        |
| Pink-1 Reverse  | GGATTTGATCGTGTTAGAAGC |          |

**Western blotting:**

For protein analysis, 15-20 worms were collected into 10 μl of water and then 2X laemmli sample buffer (Catalog # 1610737, Bio-Rad, Hercules, CA) supplemented with fresh 5% betamercaptoethanol was added to it before boiling for 30 minutes. For immunoblotting, lysates were separated on 12% SDS-PAGE gels and transferred to Nitrocellulose membrane (Catalog # 1620115, Bio-Rad, Hercules, CA) for one hour at room temperature. Immunoblots were imaged using Li-Cor® Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE). AntiGFP antibody (Catalog # 632592, Clontech Laboratories Inc., Mountain View, CA) was used to detect GFP::LGG-1, Rabbit Anti-ACTIVE p38 pAb, antibody (Catalog # V1211, Promega Corporation, Madison, WI) was used to detect active phospho-p38 MAPK, and Rabbit Anti-GFP Antibody (Catalog # 600-401-215; Rockland antibodies and Assays, Limerick, PA) was used to
detect GFP\textsuperscript{mt}. The Anti-tubulin alpha antibody (AA4.3), developed by Walsh, C was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. The following secondary antibodies were used: Sheep anti-Mouse IgG (H&L) Antibody IRDye800CW\textsuperscript{®} Conjugated (Catalog # 610-631-002, Rockland antibodies & assays, Limerick, PA), Alexa Fluor\textregistered 680 goat anti-rabbit IgG (H+L) (Catalog # A21109, Molecular Probes, Invitrogen, Carlsbad, CA). The Li-Cor Image Studio software was used to quantify the relative protein levels in different samples. Alpha-tubulin levels were used as an internal control.

**Autophagy analysis by GFP::LGG-1 Reporter:**

MAH236 line\textsuperscript{7} obtained from the CGC was used for the analysis of GFP::LGG-1 puncta in intestinal cells with slight modifications using the previously described protocol\textsuperscript{8,9}. Two day old adult worms were immobilized using 1% levamisole on 2% agarose pads and images were taken on a Leica TCS SP5 confocal microscope within 5 minutes of mounting the animal on slides (Leica, Solms, Germany). The images of proximal intestinal cells were taken with a 63X oil lens and foci were counted in the two proximal intestinal cells beyond the pharyngeal grinder. Stacks of images were taken in order to cover the complete depth of intestinal cells and images were analyzed on LAS AF software (Leica, Solms, Germany). At least 20-25 worms were imaged for each condition and the average foci number was taken for comparison.

**Mitochondrial DNA/Nuclear DNA Ratio:**

For relative quantification of mtDNA/nuDNA we modified a previously published protocol\textsuperscript{12-14}. Briefly, 10 age synchronized one day old adult animals were harvested in 45 μl of 1x worm lysis
buffer (A 3x worm lysis buffer was prepared in nuclease-free H₂O and stored at -20 degrees: 82.5 mM tricine (pH 8) 264 mM potassium acetate 36.2% (w/v) glycerol 7.425% (v/v) DMSO ) supplemented with proteinase K (final concentration of 1 mg/µl) in a PCR tube. The worms were then flash-frozen in liquid nitrogen and either stored at -80°C or used immediately for lysing worms with lysis protocol using a thermocycler (65°C for 1 hour followed by 95°C for 15 minutes). The lysate was then mixed thoroughly and 2 µl was used for real-time PCR for quantifying the mitochondrial genomic copies of nduo-1 (NADH-ubiquinone oxidoreductase chain 1) and cox-4 (nuclear DNA coding gene). Relative values of nduo-1 and cox-4 were compared within each sample to represent the mtDNA per nuclear genome. The following primers are used for the qPCR reaction.

nduo-1-fw: 5’-AGC GTC ATT TAT TGG GAA GAA GAC-3’,
nduo-1-rv: 5’- AAG CTT GTG CTA ATC CCA TAA ATG T -3’
Cox-4 Forward: 5’-GCC GAC TGG AAG AAC TTG TC-3’
Cox-4 Reverse: 5’-GCG GAG ATC ACC TTC CAG TA-3’

Dihydroethidium staining:

In vivo ROS levels were measured by using DHE staining as described previously15 with a few modifications. 6 mM DHE (Catalog # D11347, Life Technologies – Molecular Probes, Carlsbad, CA) stock solution was prepared in DMSO and was diluted to working stock (2X) solution of 6 µM stock in 1X PBS. Around 50 worms were harvested into 1X PBS and washed three times. Worms were collected in 100 µl PBS and 100 µl of 6 µM DHE stock was added to make the final concentration of 3 µM DHE. After 30-40 minutes of incubation in the dark, worms were washed once with 1X PBS and mounted on a 2% Agarose pad to image on the confocal microscope (Leica,
Solms, Germany). Excitation: 514 nm, Emission: 570-641 nm conditions were used for imaging. The fluorescence intensity was quantified using ImageJ software. A minimum of 20-30 samples were measured per genotype for each RNAi condition. All images were taken with the same exposure and magnification.
Supplemental References

1 Mello, C. C., Kramer, J. M., Stinchcomb, D. & Ambros, V. Efficient gene transfer in C. elegans: extrachromosomal maintenance and integration of transforming sequences. *EMBO J* **10**, 3959-3970 (1991).

2 Shivers, R. P. *et al.* Phosphorylation of the conserved transcription factor ATF-7 by PMK-1 p38 MAPK regulates innate immunity in Caenorhabditis elegans. *PLoS Genet* **6**, e1000892, doi:10.1371/journal.pgen.1000892 (2010).

3 Bolz, D. D., Tenor, J. L. & Aballay, A. A conserved PMK-1/p38 MAPK is required in Caenorhabditis elegans tissue-specific immune response to Yersinia pestis infection. *J Biol Chem* **285**, 10832-10840, doi:10.1074/jbc.M109.091629 (2010).

4 Prahlad, V., Cornelius, T. & Morimoto, R. I. Regulation of the cellular heat shock response in Caenorhabditis elegans by thermosensory neurons. *Science* **320**, 811-814, doi:10.1126/science.1156093 (2008).

5 Yang, W. & Hekimi, S. A mitochondrial superoxide signal triggers increased longevity in Caenorhabditis elegans. *PLoS Biol* **8**, e1000556, doi:10.1371/journal.pbio.1000556 (2010).

6 Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685 (1970).

7 Lapierre, L. R. *et al.* The TFEB orthologue HLH-30 regulates autophagy and modulates longevity in Caenorhabditis elegans. *Nat Commun* **4**, 2267, doi:10.1038/ncomms3267 (2013).
Lapierre, L. R., Gelino, S., Melendez, A. & Hansen, M. Autophagy and lipid metabolism coordinately modulate life span in germline-less C. elegans. *Curr Biol* **21**, 1507-1514, doi:10.1016/j.cub.2011.07.042 (2011).

Zhang, H. *et al.* Guidelines for monitoring autophagy in Caenorhabditis elegans. *Autophagy* **11**, 9-27, doi:10.1080/15548627.2014.1003478 (2015).

Dagda, R. K. *et al.* Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. *J Biol Chem* **284**, 13843-13855, doi:10.1074/jbc.M808515200 (2009).

Zhu, J., Dagda, R. K. & Chu, C. T. Monitoring mitophagy in neuronal cell cultures. *Methods Mol Biol* **793**, 325-339, doi:10.1007/978-1-61779-328-8_21 (2011).

Bratic, I., Hench, J. & Trifunovic, A. Caenorhabditis elegans as a model system for mtDNA replication defects. *Methods* **51**, 437-443, doi:10.1016/j.ymeth.2010.03.003 (2010).

Mouchiroud, L. *et al.* The NAD(+)/Sirtuin Pathway Modulates Longevity through Activation of Mitochondrial UPR and FOXO Signaling. *Cell* **154**, 430-441, doi:10.1016/j.cell.2013.06.016 (2013).

Hunter, S. E., Jung, D., Di Giulio, R. T. & Meyer, J. N. The QPCR assay for analysis of mitochondrial DNA damage, repair, and relative copy number. *Methods* **51**, 444-451, doi:10.1016/j.ymeth.2010.01.033 (2010).

Morcos, M. *et al.* Glyoxalase-1 prevents mitochondrial protein modification and enhances lifespan in Caenorhabditis elegans. *Aging Cell* **7**, 260-269, doi:10.1111/j.1474-9726.2008.00371.x (2008).

Gonzalez-Hunt, C.P., Rooney, J.P., Ryde, I.T., Anbalagan, C., Joglekar, R., Meyer, J.N. 2016. PCR-based analysis of mitochondrial DNA copy number, mitochondrial DNA
damage, and nuclear DNA damage. Curr. Protoc. Toxicol. 67:20.11.1-20.11.25. doi: 10.1002/0471140856.tx2011s67