Appendix for:

Mild mitochondrial impairment enhances innate immunity and longevity through ATFS-1 and p38 signaling

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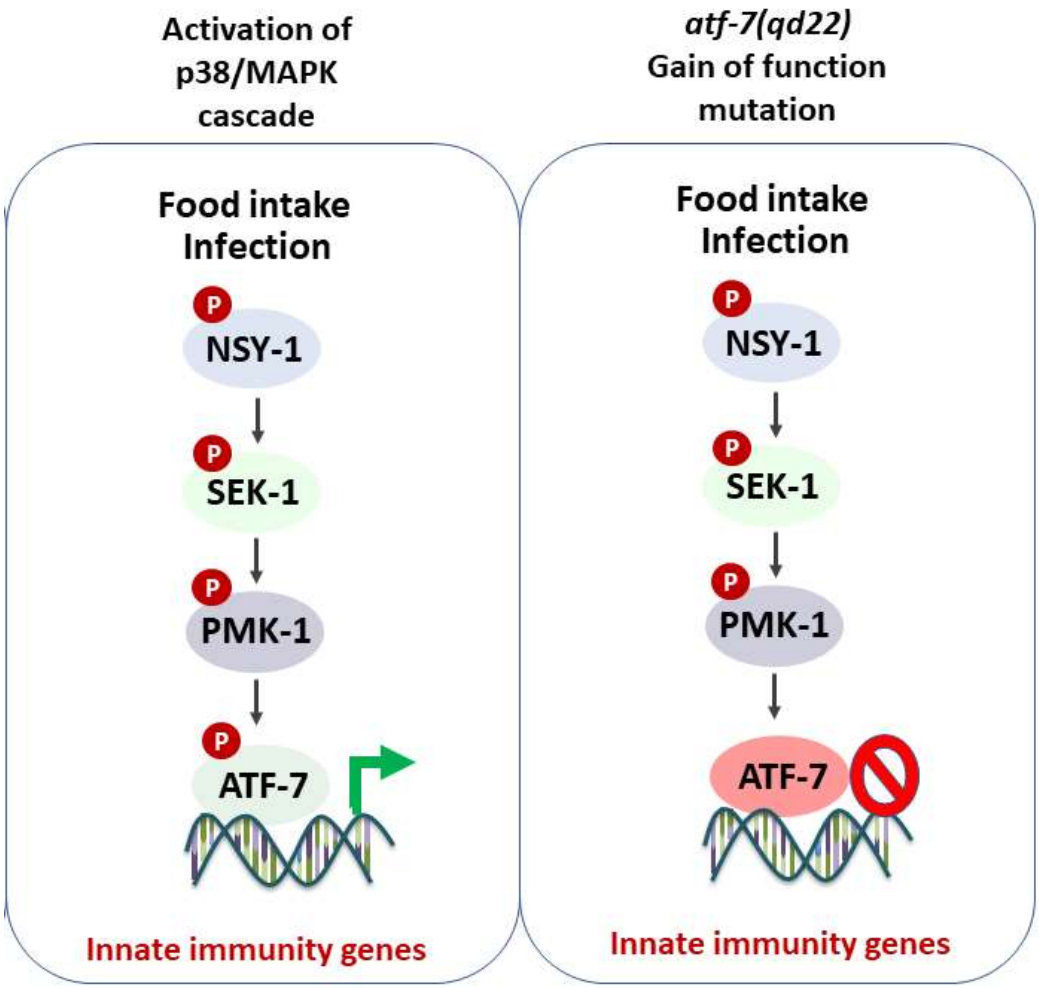
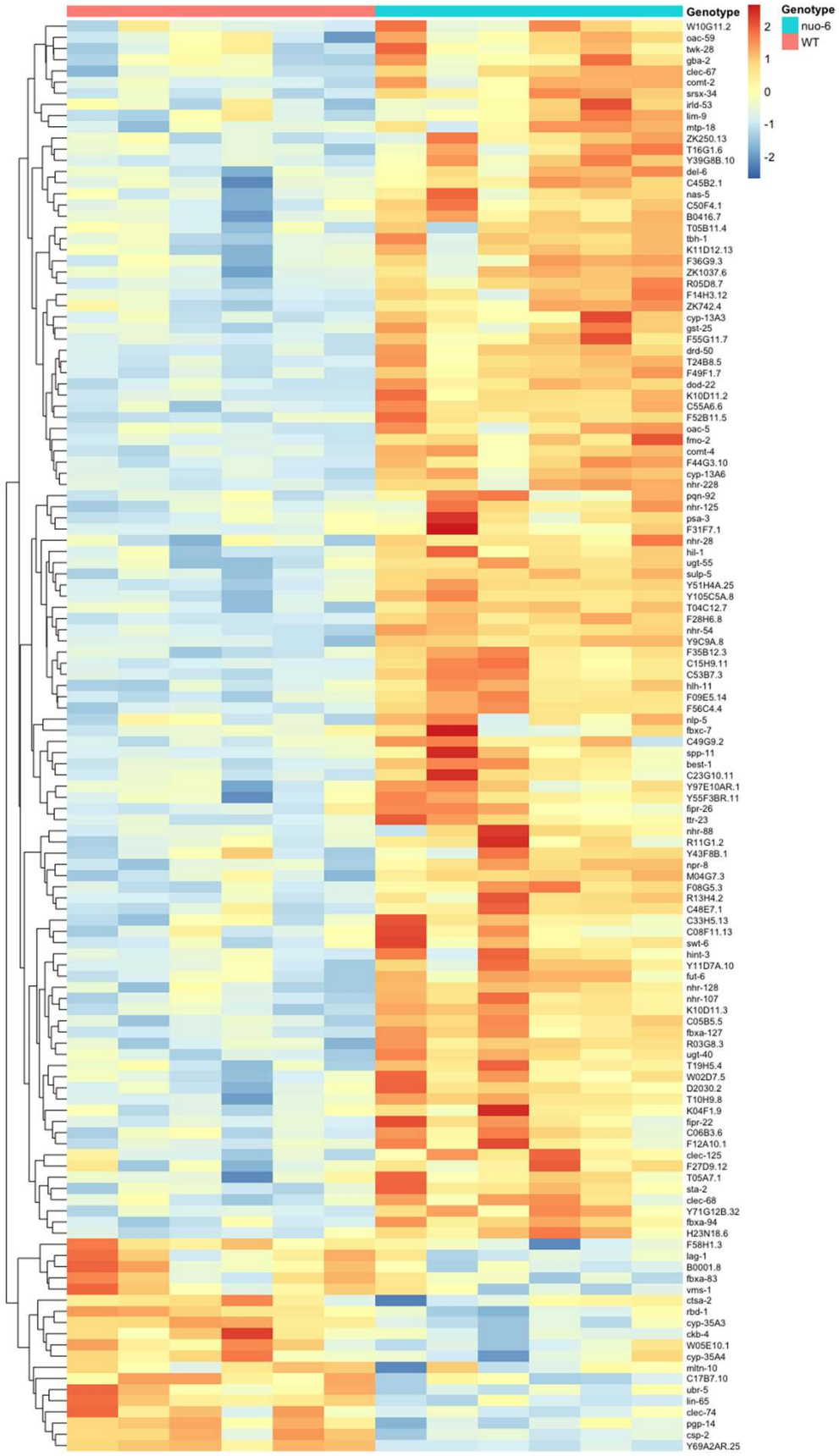
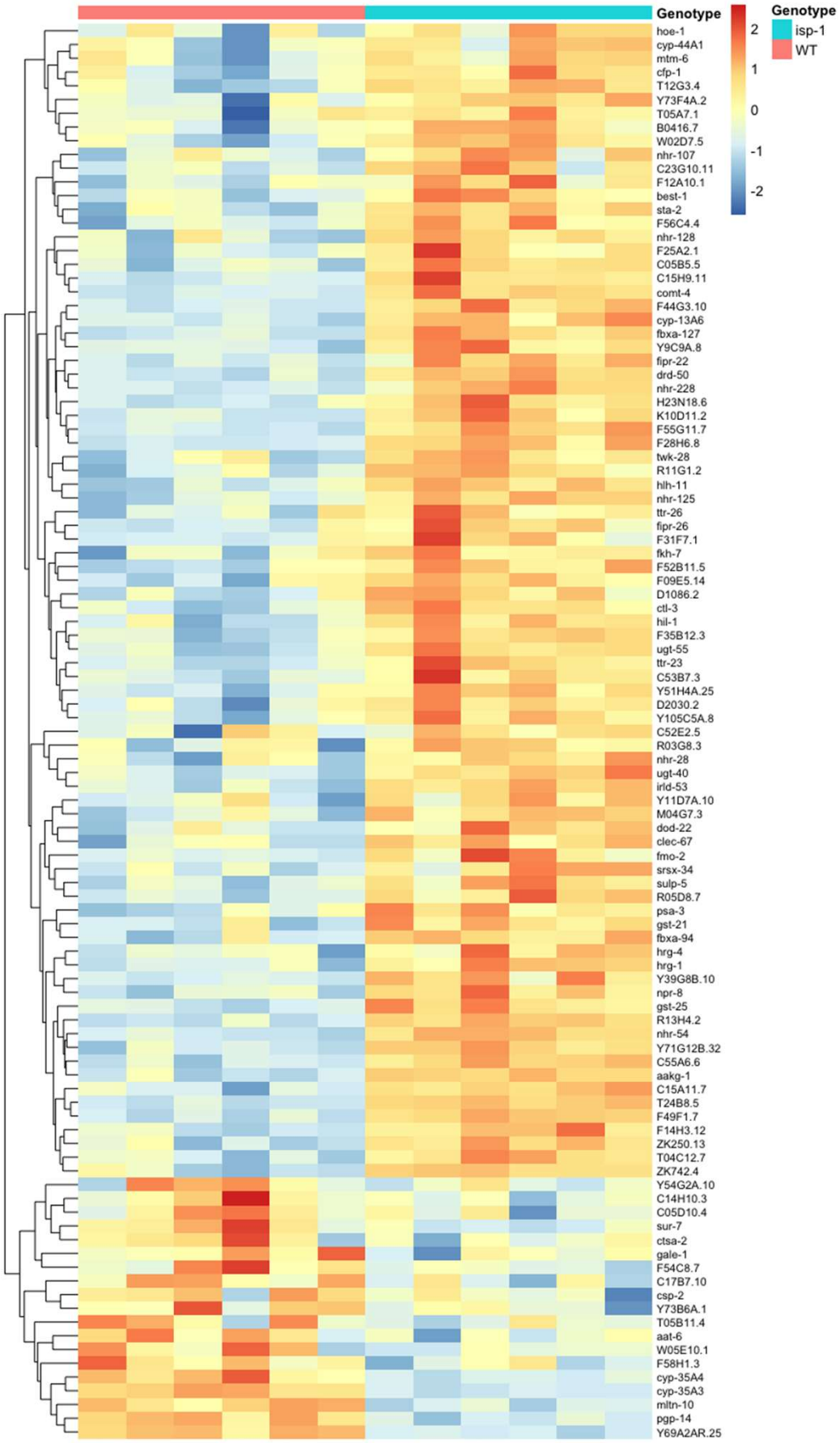


Appendix Figure S1. Overview of p38-mediated innate immune signaling pathway.
The p38-mediated innate immune signaling pathway is a MAPK signaling pathway. Exposure to bacterial pathogens or increased food intake results in the activation of this pathway through the phosphorylation of NSY-1/ASK1 (MAPK kinase kinase). NSY-1 then phosphorylates SEK-1/MKK3/MKK6 (MAPK kinase), which phosphorylates PMK-1/p38 (MAPK), which phosphorylates the transcription factor ATF-7/ATF2/ATF7/CREB5. Under normal conditions, ATF-7 acts as a repressor inhibiting the expression of innate immunity genes. When ATF-7 is phosphorylated by PMK-1, it becomes an activator promoting the expression of innate immunity genes. The *qd22* mutation prevents phosphorylation of ATF-7 by PMK-1. As a result, ATF-7 with the *qd22* mutation acts as a constitutive repressor even in the presence of bacterial pathogens.



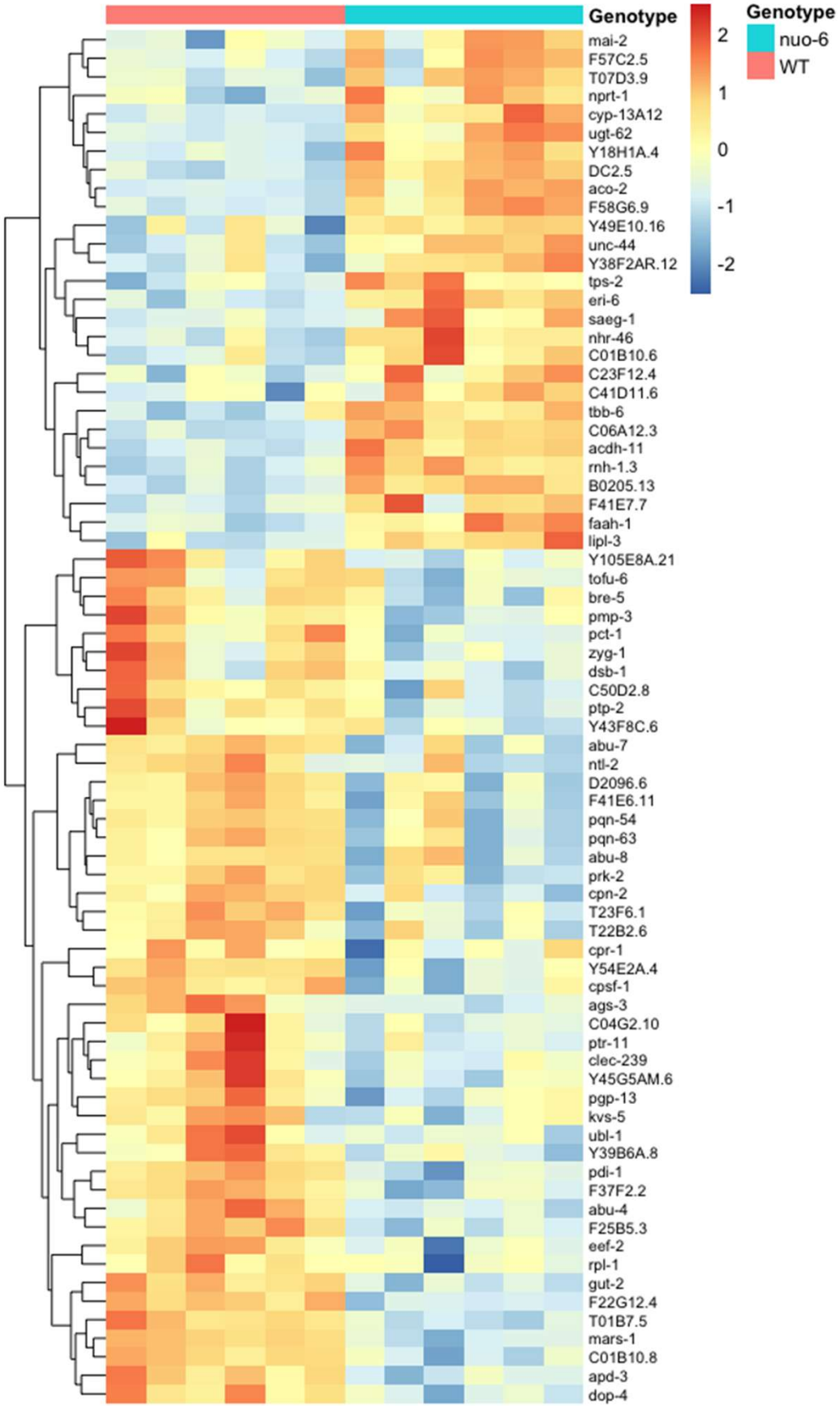
Appendix Figure S2. Genes that are upregulated by activation of the p38-mediated innate immune pathway are primarily upregulated in the long-lived mitochondrial mutant *nuo-6.*

This heatmap includes genes that are upregulated by exposure to the bacteria pathogen *P. aeruginosa* PA14 in a PMK-1- and ATF-7-dependent manner and for which the expression is significantly changed in *nuo-6* mutants.



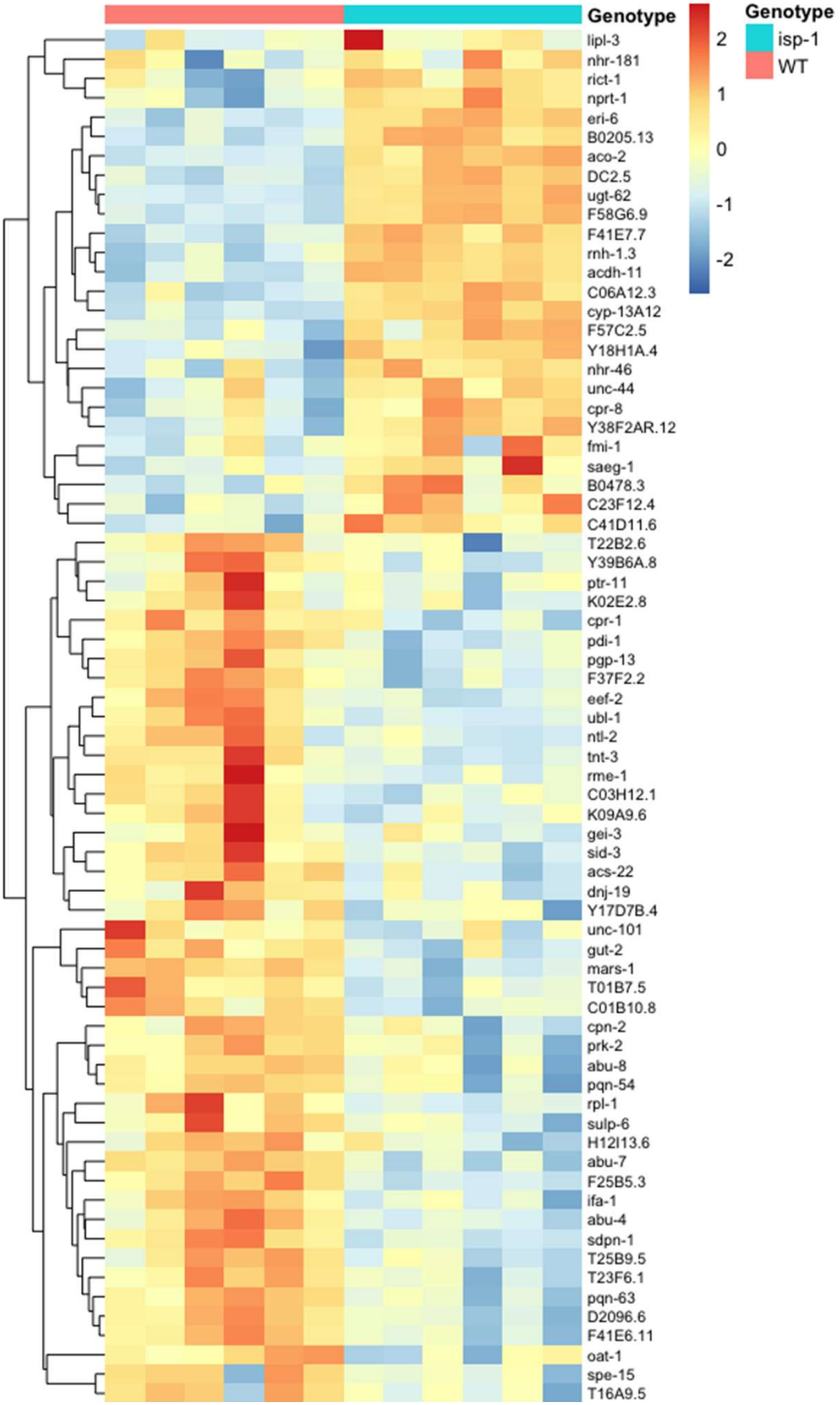
Appendix Figure S3. Genes that are upregulated by activation of the p38-mediated innate immune pathway are primarily upregulated in the long-lived mitochondrial mutant *isp-1.*

This heatmap includes genes that are upregulated by exposure to the bacteria pathogen *P. aeruginosa* PA14 in a PMK-1- and ATF-7-dependent manner and for which the expression is significantly changed in *isp-1* mutants.



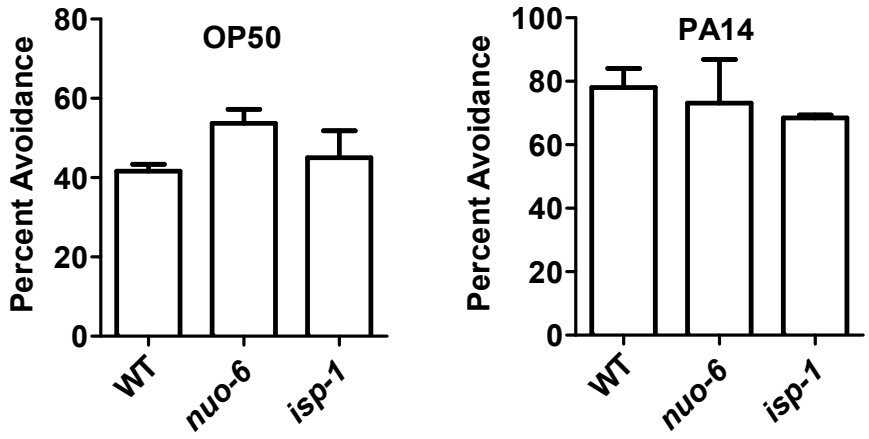
Appendix Figure S4. Expression of genes that are downregulated by activation of the p38-mediated innate immune pathway is altered in the long-lived mitochondrial mutant *nuo-6.*

This heatmap includes genes that are downregulated by exposure to the bacteria pathogen *P. aeruginosa* PA14 in a PMK-1- and ATF-7--dependent manner and for which the expression is significantly changed in *nuo-6* mutants.



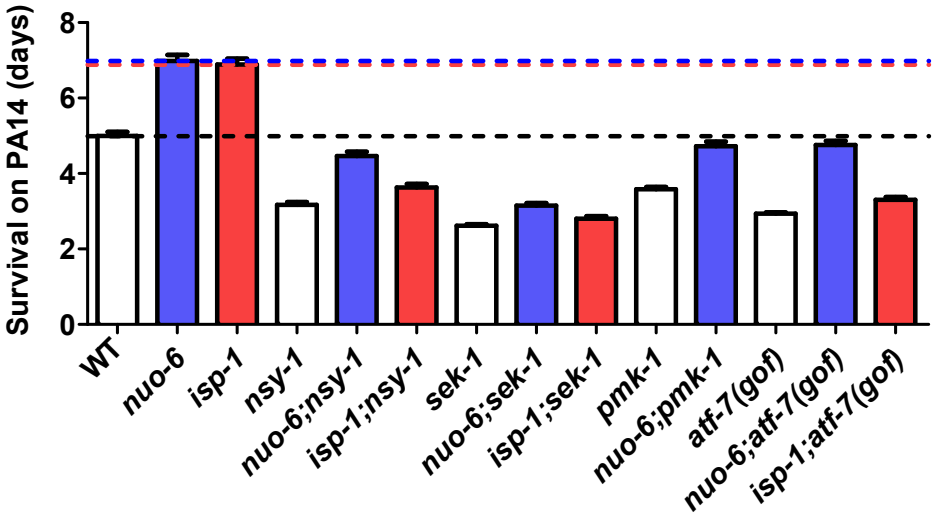
Appendix Figure S5. Expression of genes that are downregulated by activation of the p38-mediated innate immune pathway is altered in the long-lived mitochondrial mutant *isp-1.*

This heatmap includes genes that are downregulated by exposure to the bacteria pathogen *P. aeruginosa* PA14 in a PMK-1- and ATF-7--dependent manner and for which the expression is significantly changed in *isp-1* mutants.



Appendix Figure S6. Increased resistance to bacterial pathogens does not result from bacterial avoidance.
A,B. To explore the mechanisms underlying the increased resistance to bacterial pathogens in *nuo-6* and *isp-1* mutants, bacterial avoidance and food consumption were examined. There was no significant difference in bacterial avoidance between wild-type worms and *nuo-6* or *isp-1* mutants on OP50 bacteria (**A**) or *P. aeruginosa* (**B**). Three biological replicates per strain were quantified.

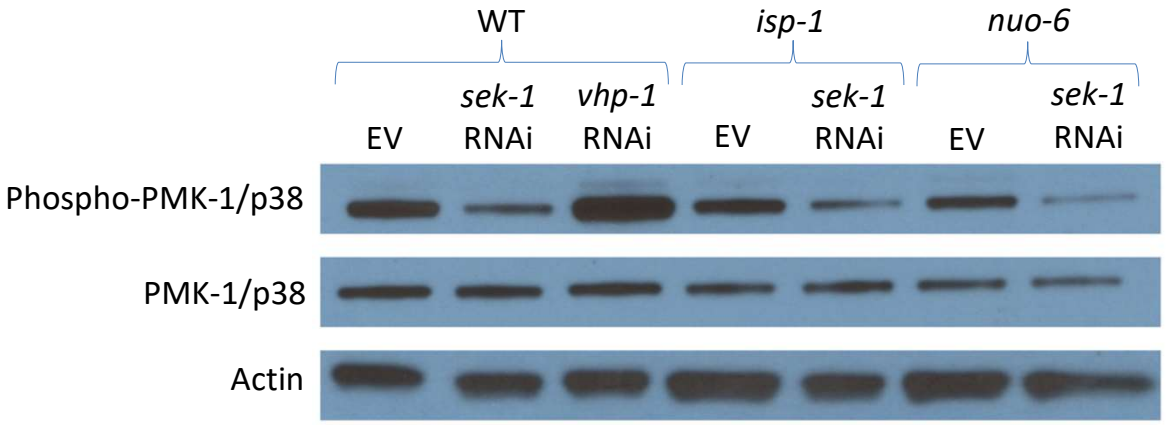
Data information: Error bars indicate SEM. Statistical significance was assessed using a one-way ANOVA with Dunnett's multiple comparison test.



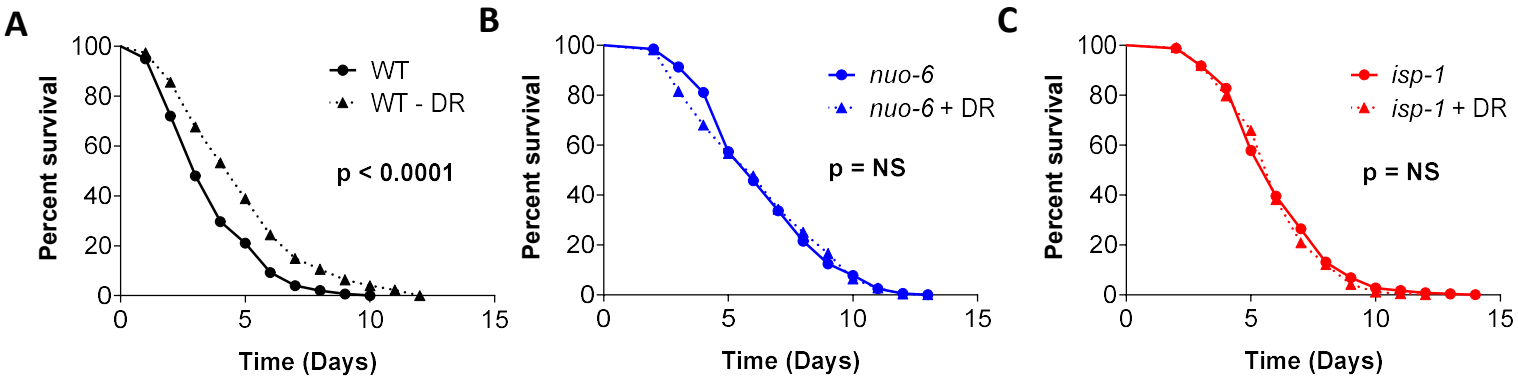
Appendix Figure S7. Disruption of p38-mediated innate immune signaling pathway decreases resistance to bacterial pathogens.

Resistance to bacterial pathogens was tested by exposing worms to *Pseudomonas aeruginosa* strain PA14 in a slow kill assay. Long-lived mitochondrial mutants, *nuo-6* and *isp-1,* have increased survival on pathogenic PA14 bacteria. Mutations affecting genes involved in the p38-mediated innate immune signaling pathway including *nsy-1, sek-1, pmk-1* and *atf-7(gof)* cause decreased resistance to bacterial pathogens in wild-type (white bars), *nuo-6* (blue bars) and *isp-1* (red bars) worms. Black dotted line indicates wild-type survival. Blue dotted line indicates *nuo-6* survival. Red dotted line indicates *isp-1* survival. Survival is measured as the number of days from exposure to PA14 (day 3 of adulthood) until death. Two biological replicates per strain were completed.

Data information: Error bars indicate SEM of survival of individual animals. This bar graph is a summary of data shown in **Figure 2** to facilitate comparison across all strains.



Appendix Figure S8. Proportion of activated PMK-1/p38 is not increased in *nuo-6* and *isp-1* worms.
Activation of PMK-1/p38 was measured by levels of phosphorylated p38/PMK-1 compared to total levels of PMK-1 by western blotting. As a positive and negative controls, we examined the effect of *vhp-1* RNAi and *sek-1* RNAi. RNAi against *sek-1* resulted in decreased phosphorylation of PMK-1/p38 without affecting total PMK-1 levels. RNAi against *vhp-1* increased phospho-p38/PMK-1 levels without affecting total PMK-1 levels. RNAi treatment was begun at the L3 developmental stage and worms were collected 2 days later.

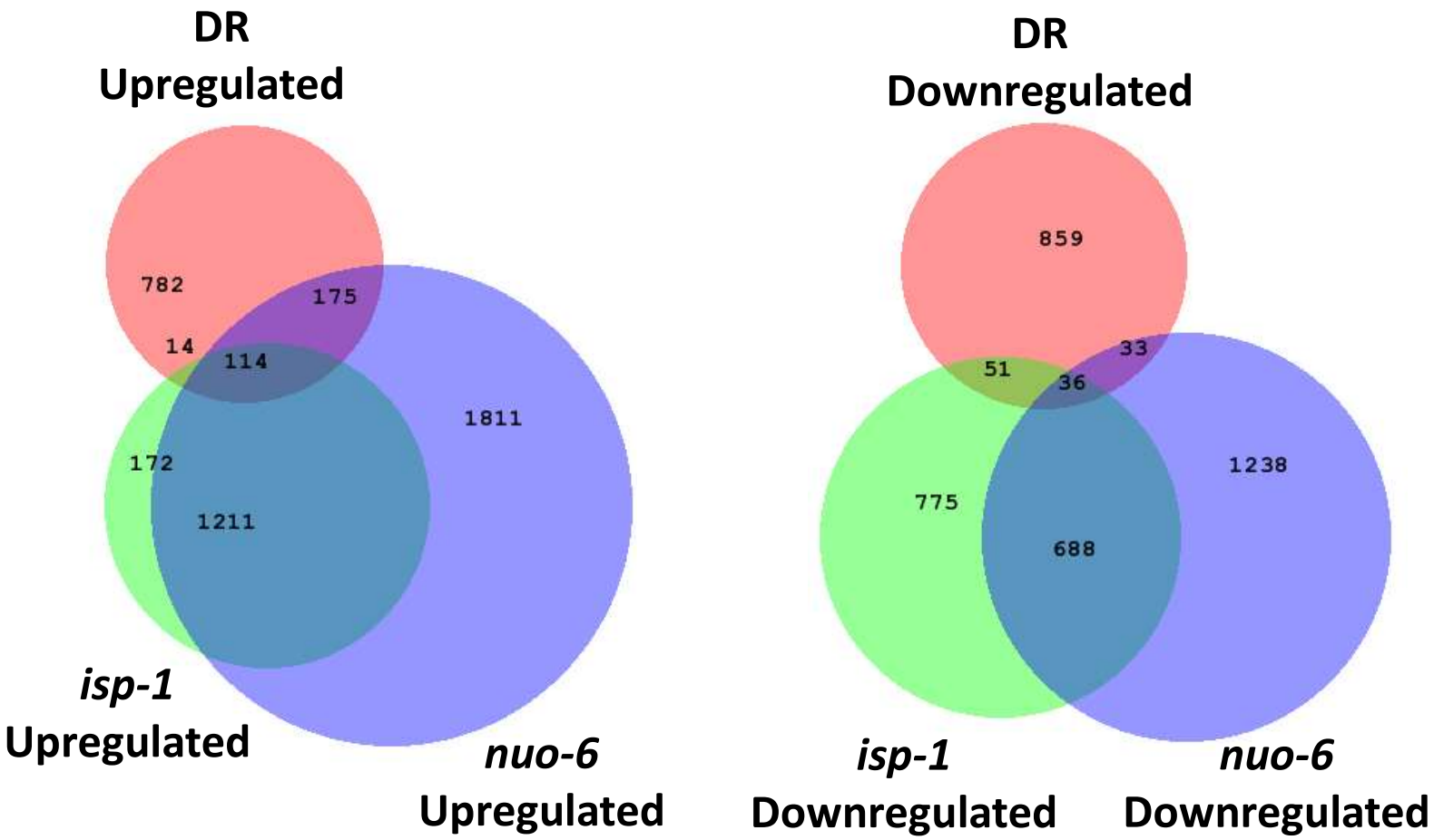


Appendix Figure S9. Dietary restriction does not further increase resistance to bacterial pathogens in long-lived mitochondrial mutants.

A-C. Dietary restriction (DR) increases resistance to *P. aeruginosa* strain PA14 in wild-type worms (**A**) but does not further increase bacterial pathogen resistance in *nuo-6* (**B**) or *isp-1* (**C**) mutants. DR was performed in liquid from day 3 to day 6 of adulthood and then worms were exposed to *P. aeruginosa* strain PA14. Ad libitum (AL) food concentration is OD600 at 3 and DR is OD600 at 0.1 of non-proliferating bacterial food. Two biological replicates per strain per condition were performed.

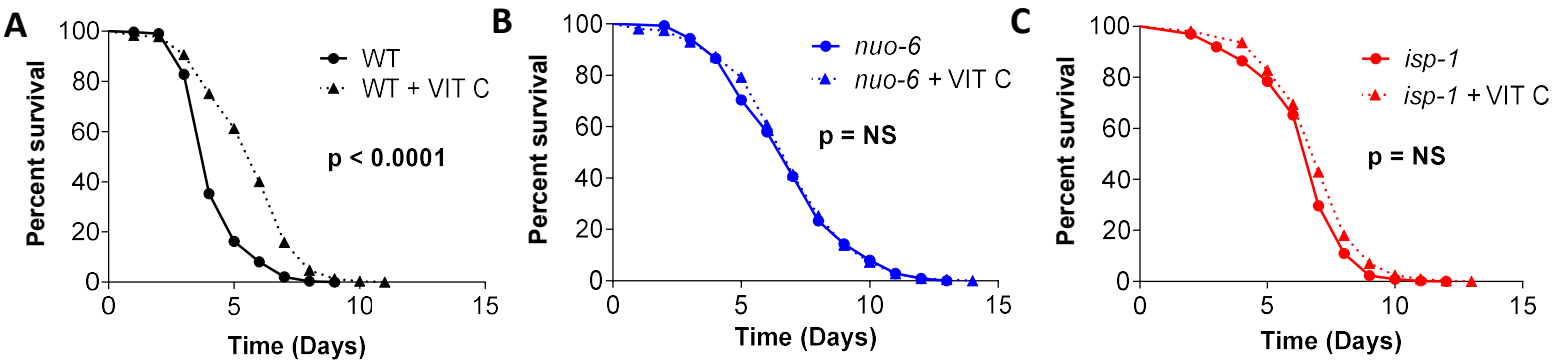
Data information: Statistical significance was assessed using the log-rank test.

Source data are available online for this figure.



Gene set 1	Gene set 2	Number of Overlapping Genes	Percent Overlap
nuo-6 upregulated (3311)	DR upregulated (1085)	289	27%
	DR downregulated (979)	327	33%
isp-1 upregulated (1511)	DR upregulated (1085)	128	12%
	DR downregulated (979)	120	12%
nuo-6 downregulated (1995)	DR upregulated (1085)	68	6%
	DR downregulated (979)	69	7%
isp-1 downregulated (1550)	DR upregulated (1085)	52	5%
	DR downregulated (979)	87	9%

Appendix Figure S10. Long-lived mitochondrial mutants exhibit limited overlap in differentially expressed genes with worms exposed to dietary restriction.
Venn diagrams show the overlap between genes that are significantly upregulated (**Left**) or significantly downregulated (**Right**) in *nuo-6* mutants, *isp-1* mutants or worms undergoing dietary restriction (DR). Gene expression data for mitochondrial mutants is from Senchuk et al. 2018, *PLoS Genetics.* Gene expression data for dietary restriction worms is from Wu et al. 2019, *Cell Metabolism.* For comparison, we examined the overlap between genes upregulated in the long-lived mitochondrial mutants and downregulated by dietary restriction, and vice versa. The percent overlap between upregulated-upregulated genes and upregulated-downregulated genes was similar. Overall, the gene expression changes resulting from dietary restriction have little similarity with gene expression changes caused by mild impairment of mitochondrial function.

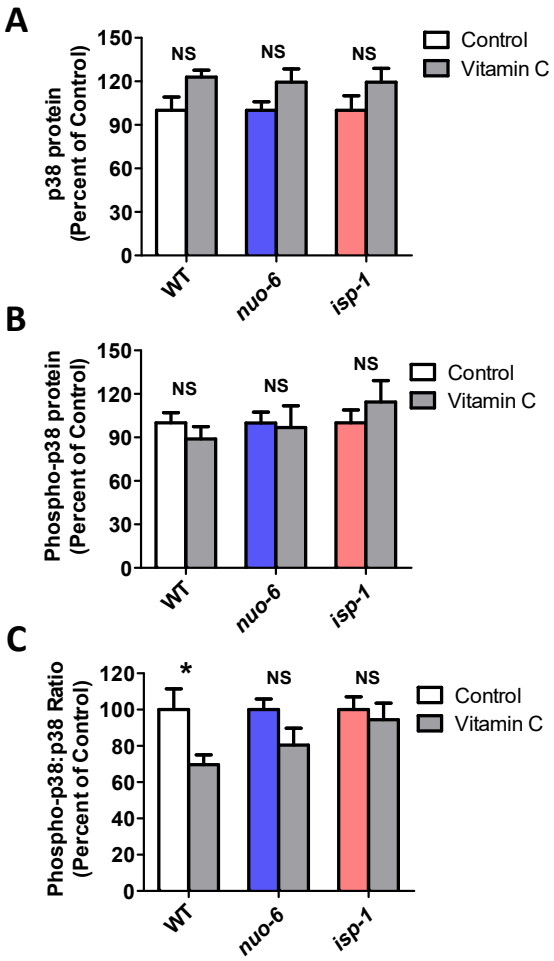


Appendix Figure S11. **Treatment with antioxidant Vitamin C does not decrease bacterial pathogen resistance in long-lived mitochondrial mutants.**

A-C. Treatment with 10 mM Vitamin C increased survival in wild-type worms exposed to *Pseudomonas aeruginosa* strain PA14 (**A**), but did not affect survival in either *nuo-6* (**B**) or *isp-1* (**C**) worms. Vitamin C treatment was performed on plates from the L4 stage of the parental generation to day 3 of adulthood of the experimental generation prior to exposure to *P. aerugonisa* on day 3 of adulthood. Two biological replicates per strain per condition were performed.

Data information: Statistical significance was assessed using the log-rank test.

Source data are available online for this figure.

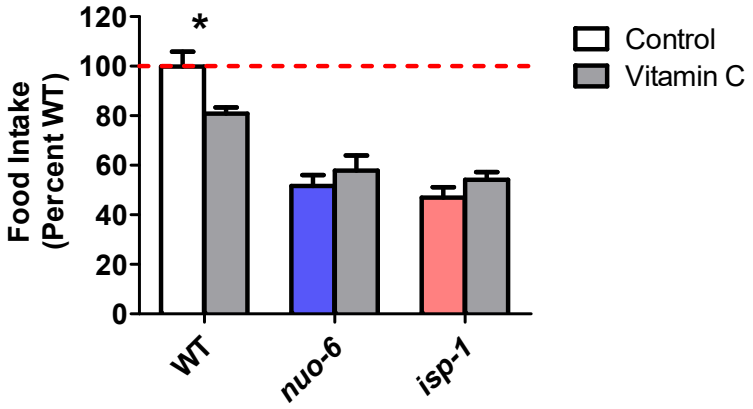


Appendix Figure S12. **Treatment with antioxidant Vitamin C does not decrease activation of the p38 in long-lived mitochondrial mutants.**

A-C. Western blotting was used to measure the activation of the p38-mediated innate immune signaling pathway by quantifying the ratio of phosphorylated p38/PMK-1 to total p38/PMK-1. Treatment with the antioxidant Vitamin C did not significantly affect the levels of total p38/PMK-1 (**A**) or phosphorylated p38/PMK-1 (**B**) in wild-type, *nuo-6* or *isp-1* worms. Treatment with Vitamin C did not affect the ratio of phosphorylated p38/PMK-1 to total p38/PMK-1 in *nuo-6* or *isp-1* worms (**C**). Vitamin C treatment (10mM) was performed on NGM plates beginning at the L4 stage of the parental generation to day 3 of adulthood of the experimental generation when the worms were collected for protein isolation. Five biological replicates per strain per condition were performed.

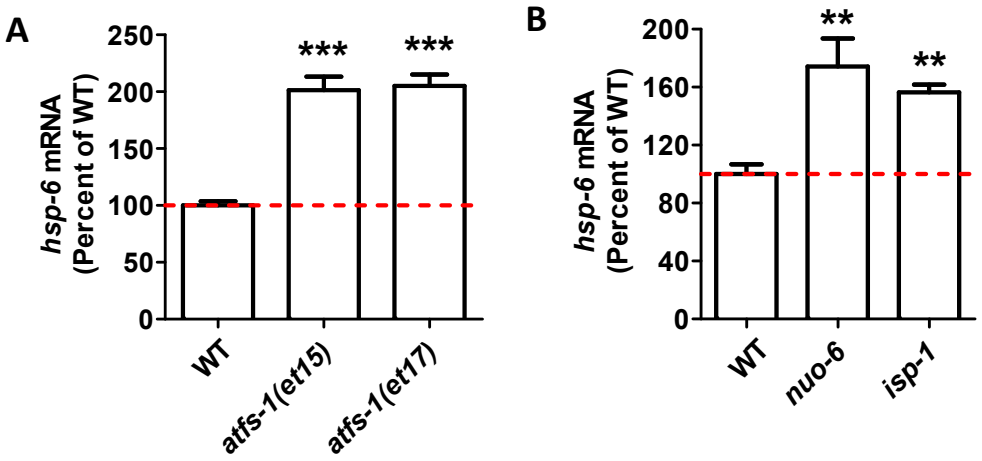
Data information: Error bars indicate SEM. *p<0.05. Statistical significance was assessed using a two-way ANOVA with Bonferroni posttest.

Source data are available online for this figure.



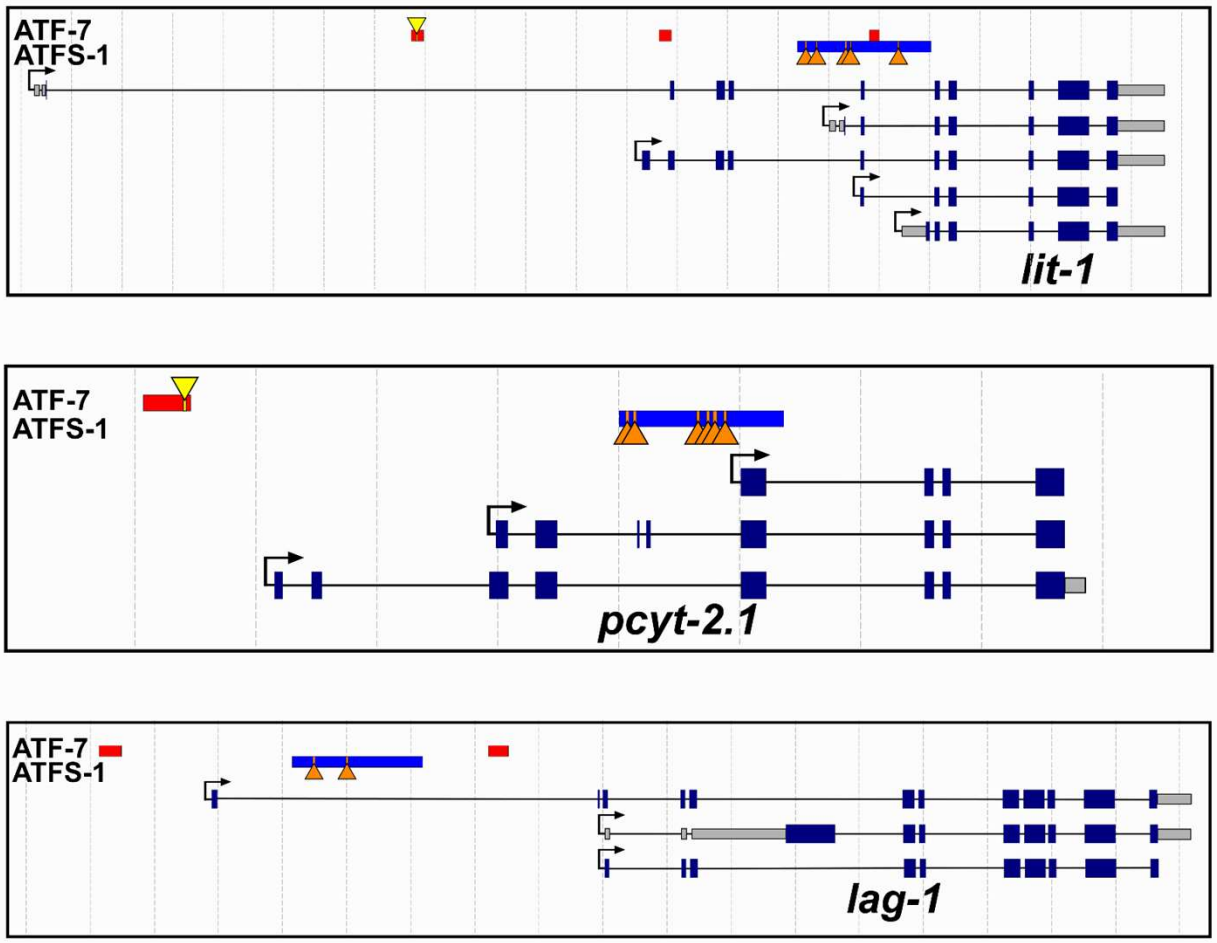
Appendix Figure S13. **Treatment with antioxidant Vitamin C does not rescue reduced feeding in long-lived mitochondrial mutants.**
Food consumption in *nuo-6* and *isp-1* mutants is not increased by treatment with the antioxidant Vitamin C. This suggests that elevated ROS is not responsible for the decrease in food consumption in these strains. Vitamin C treatment (10mM) was performed on NGM plates beginning at the L4 stage of the parental generation to day 3 of adulthood of the experimental generation. Food intake was quantified by measuring the relative amount of non-proliferating bacteria (OP50) between day 3 and day 12 of adulthood and normalized to the number of worms per well. Two biological replicates per strain per condition were performed, each including at least 7 technical replicates.

Data information: Error bars indicate SEM. Statistical significance was assessed using a two-way ANOVA with Bonferroni posttest.



Appendix Figure S14. *hsp-6* levels are increased in constitutively active *atfs-1* mutants.
A,B. Activation of ATFS-1 through the constitutively active *atfs-1* mutations *et15* or *et17* (**A**) or through mild impairment of mitochondrial function (**B**; *nuo-6, isp-1* mutants) results in upregulation of the ATFS-1 target gene *hsp-6.* Gene expression changes were determined by RNA sequencing of six biological replicates of each genotype. Results represent counts per million (CPM) expressed as a percentage of wild-type.

Data information: Error bars indicate SEM. **p<0.01, ***p<0.001. Statistical significance was assessed using a one-way ANOVA with Dunnett's multiple comparison test.



Appendix Figure S15. ATF-7 and ATFS-1 can bind to the promoter region of the same innate immunity genes. To better understand how ATF-7 and ATFS-1 act to modulate the same innate immunity genes, we mapped out the regions identified by ChIP-seq studies as well as the location of the consensus binding sites. Ten of the 24 genes that show binding of both ATF-7 and ATFS-1 demonstrated binding of ATF-7 and ATFS-1 in close proximity in the promoter region of innate immunity target genes. Diagrams of three of those genes are shown here, while diagrams of the other seven genes can be found in **Figure EV5.** The binding regions identified for ATF-7 and ATFS-1 by ChIP-seq experiments are shown by red and blue bars, respectively. The consensus binding sites for ATF-7 and ATFS-1 are indicated by yellow triangles and orange triangles, respectively. The distance between dotted lines is 1 kb. Exons are indicated by dark blue bars. Untranslated regions are indicated by grey bars and introns are indicated by black lines joining together two exons. Note that not all transcripts for each gene are illustrated, only those with differing promoter regions and or markedly different structures.