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

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1st Editorial Decision 7th Apr 2021

Dear Prof. Van Raamsdonk

Thank you for the submission of your research manuscript to EMBO reports. It has been reviewed by the journal-independent platform Review Commons and you have submitted a point-by-point response and outlined a revision plan.

We agree that your study is potentially a nice contribution to EMBO reports and we therefore invite you to revise your manuscript along the lines outlined in your revision plan. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or reject ion of the manuscript will therefore depend on the completeness of your responses included in the next , final version of the manuscript .

We invite you to submit your manuscript within three months of a request for revision. This would be July 7th in your case. However, we are aware of the fact that many laboratories are not fully functional due to COVID-19 related shutdowns and we have therefore extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further.

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Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
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- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see <
https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below (see also <
https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843
(https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
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*** Note - All links should resolve to a page where the data can be accessed. ***

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instruction on how to label the files are available .

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases, such as the ChIP-seq data you analysed. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

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- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Martina Rembold, PhD
Senior Editor
EMBO reports

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Revision 0

Review #1

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The manuscript by Campos et al. describe the association between long-lived mitochondrial mutants and increased resistance to pathogen infection. The authors discover that mitochondrial electron transport chain mutants (nuo-6 and isp-1) display increased expression of many genes involved in innate immunity that are regulated by the p38 signaling pathway. Consistent with this finding, mito mutants displayed increased survival during infection. p38 signaling was found to be required for these innate immune gene inductions during mitochondrial stress and for their increased survival during infection. P38 signaling was also found to be required for the increased lifespan of isp-1 and nuo-6 mutant animals. Intriguingly, p38 signaling does not appear to be affected in these mitochondrial mutants, despite being required for the increase in immunity/host resistance. The authors discover that mitochondrial stress animals exhibit reduced feeding which they argue may suppress any activation of the p38 pathway caused by ROS. The mitochondrial UPR was also found to be required for the increase in innate immune gene expression in isp-1 and nuo-6 mutant animals, as well as their extended survival. The authors conclude that ATFS-1 can act in parallel to p38 signaling by directly binding to common innate immune target genes. In support of this, ATFS-1 and ATF-7 appear to bind to shared target genes but likely at independent sites due to their different consensus sequences.

1. One general consideration is that some of the key concepts outlined in this manuscript have already been described previously and are therefore not entirely novel conceptually. For example, one key citation missing from the current manuscript is from Hwang et al. 2014 (PMID 25288734). This study has already described that the isp-1 mutant strain survives longer during P. aeruginosa infection. This citation also describes that the gene expression profile of isp-1 mutants animals includes a considerable number of pathogen-responsive genes that are similarly induced during infection. While the current manuscript does go into the mechanism of this resistance with more detail, they should amend the language to more appropriately reflect previous work, notably the above reference.
2. The authors suggest that ROS activation of the p38 MAPK pathway is likely not the mechanism that explains the resistance of long-lived mitochondrial mutant animals due to their reduced food intake. However, is ROS production nonetheless involved? Does antioxidant treatment suppress the increased resistance during infection of isp-1 and/or nuo-6 mutant animals?
3. (line 278-282): the authors should elaborate on how the p38 MAPK pathway plays a permissive role. It is intriguing that ATFS-1 and ATF-7 are both bZIP transcription factors that could theoretically

heterodimerize and that they share common immune gene targets. The authors do indicate that the binding sites for ATFS-1 and ATF-7 are very different and are likely acting distinctly but some speculation would nonetheless strengthen this statement.
4. The authors suggest that reduced food consumption of nuo-6 and isp-1 animals may suppress ROS-induced activation of the p38 innate immune pathway. It is intriguing that dietary restriction was previously shown to increase resistance to infection, presumably through p38-independent mechanisms (PMID 30905669). It would be interesting to measure host survival of nuo-6 and isp-1 mutant animals that are dietary-restricted to see if the enhanced survival rates conferred by mitochondrial stress and DR are additive or not.
5. Figure 2: It is intriguing that loss of p38 signaling appears to have different effects in nuo-6 versus isp-1 animals. Specifically, loss of p38 signaling in isp-1 mutants renders them more sensitive to infection than wild-type, whereas it generally suppresses survival rates back to wild-type levels in the nuo-6 mutant background. Even within the nuo-6 mutant group, loss of SEK-1 has more dramatic effects on nuo-6 mutant animals than does loss of NSY-1, PMK-1 or ATF-7(gf). This is despite the fact that the nsy-1, sek-1, and pmk-1 alleles that are used in this study are all reported to be null. Can the authors speculate on these differences?
6. One of the main conclusions from this study is that ATFS-1 likely binds directly to innate immune genes that are in common with ATF-7. Since this is such a pivotal finding, the authors should validate some candidate genes from the referenced ChIP seq datasets using ChIP qPCR. Also, are there predicted ATFS-1 binding sites (PMID 25773600) in these promoters?

3. Significance:

Significance (Required)

As mentioned in my comments, some of the findings of the current manuscript have been shown before. Nonetheless, the authors do describe new insights into the mechanism of how mitochondrial stress signaling promotes host resistance to infection, which is noteworthy.

This manuscript would be of value to researchers in the fields of mitochondrial biology, mitochondrial stress signaling (including the UPRmt field), host-pathogen interactions, and longevity determination.

My expertise is in stress signaling in the context of longevity and host-pathogen interactions.

Review #2

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

Campos et al. show that mild mitochondrial impairment promotes C. elegans resistance against the bacterial pathogen Pseudomonas aeruginosa PA14, which is associated with increased expression of a subset of innate immunity genes in the animal. Interestingly, upregulation of the innate immunity genes in the mitochondrial electron transport chain mutants, nuo-6 (complex I) and isp-1 (complex III), does not appear to involve enhanced activation of the p38 MAPK PMK-1, which has been previously implicated in anti-bacterial immunity (Jeong et al, EMBO J 2017, 36, 1046). Because the authors also show that this increased pathogen resistance and expression of innate immunity genes in at least one of the mitochondrial mutants (nuo-6) only partly depend on the p38 PMK-1 pathway, this would argue for the involvement of another pathway. The authors show that this other pathway involves the mitochondrial unfolded protein response (mitoUPR) through activation of the transcription factor atfs-1, which not only upregulates a subset of innate immunity genes, but also presumably decreases pathogen intake. Together their data suggest that the p38 PMK-1 pathway and mitoUPR act in parallel to promote the enhanced pathogen resistance of mitochondrial mutants.

Moreover, while they show that the FOXO transcription factor daf-16 is also required for the enhanced pathogen resistance of mitochondrial mutants (i.e,, isp-1), they rule out daf-16 involvement in the activation of innate immunity genes. Instead, daf-16 decreases pathogen intake and upregulates other stress-response genes. Thus, this study highlights the requirement for multiple pathways to promote pathogen resistance through multiple mechanisms.

Major comments:

(1) The authors state that the p38 MAPK PMK-1 is not activated in the long-lived mitochondrial mutants. However, it might be better to state that there is "no enhanced activation" of PMK-1, since they clearly show in nuo-6 and isp-1 mutants the presence of phosphorylated PMK-1 (Fig. 4A), which would indicate an activated form of PMK-1 in these mutants.

(2) Are the food-intake behaviors of all mutants in liquid culture (Fig. 4B-F) the same as their food-intake behaviors on solid agar media, the environment where pathogen resistance was measured?

(3) Does the p38 pathway single mutant nsy-1 or sek-1 live shorter than wild type on dead E. coli OP50 (Fig. S9) than they do on live OP50 (Fig. 3)? If so, what might that mean? These mutants are also living shorter than wild type on PA14 (Fig. 2), but live as long as wild type on OP50 (Fig. 3). What is in the live OP50 that allows these mutants to live like wild type?

At the same time, wouldn't it be simpler to call the multiple antibiotic-treated OP50 as "dead bacteria", instead of "non-proliferating bacteria"? Some of the antibiotics used to treat OP50 are bactericidal and not bacteriostatic.

(4) Since nuo-6 and isp-1 do not always behave exactly the same in their dependence on certain genes (e.g., Fig. 2C vs Fig 2D), what happens in isp-1; atfs-1 double mutants? Do these mutants behave in the same manner as nuo-6; atfs-1?

Regarding nuo-6; atfs-1, why does the double mutant live shorter on PA14 than either single mutant (Fig. 6A)? Is this because atfs-1 is needed to activate the p38 MAPK-dependent and -independent pathways? In Fig. 7B, the atfs-1(gof) appears to have slightly more phosphorylated p38 compared to wild type, although it is not statistically significant?

In Fig. 6B, the atfs-1 loss-of-function single mutant also increases the expression of Y9C9A.8, but suppresses it in a nuo-6 mutant background? What might that mean?

Some of my comments can be easily addressed with written comments. Others might require generation of a strain, like the isp-1; atfs-1 double mutant, prior to any assays.

3. Significance:

Significance (Required)

Please see the above summary for the significance of this manuscript to the field. Importantly, this study highlights the requirement for multiple pathways to promote pathogen resistance through multiple mechanisms. Readers interested in aging, mitochondrial function, innate immunity and stress responses should find this study thought-provoking. I include myself in this group of readers, since I study the genetics of C. elegans aging and stress responses.

Review #3

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Campos et al provide evidence that mild mitochondrial dysfunction in C. elegans induces genes involved in innate immunity and promotes bacterial pathogen resistance and longevity, while inhibits food intake through an ATFS-1-mediated mechanism. The manuscript is well-written and the experiments are well-performed and reported. However, there are several points that need to be addressed before the manuscript can be published.

Major concerns

1) Some studies propose that OP50 offers some toxicity to worms which is not observed in other bacterial strains like HT115. The authors should test the role of the p38-innate immune signaling pathway in nuo-6 and isp-1 lifespan using other non-pathogenic E. coli strains.

2) The authors should measure food intake in worms exposed to pathogenic bacteria, given that reduced

bacterial intake may be related to reduced mortality.

3) The authors should check if ROS is required for the activation of the p38-mediated innate immune signaling pathway and reduction in food intake.

4) Since ATFS-1 and the p38 pathway control food intake, how related to dietary restriction the phenotypes the authors are studying are?

5) Somewhat related to the previous points, I am not so sure whether the changes in food intake are cause or consequence of the alterations in the innate immunity-related genes. Reduced food intake is depicted in Fig. 8 as the cause of the activation of the p38 pathway, but there is not enough evidence to unequivocally prove that. In fact, food intake might be controlled by the p38 or ATFS-1 pathway or by a common regulator such as ROS.

6) I am not so convinced of the role of DAF-16. In fact, in Fig. 5A daf-16 mutation reduces pathogen resistance and that could represent a toxic effect of the mutation. Furthermore, the results in Fig. 4D do not exclude the possibility that daf-16 and isp-1 act in parallel.

7) Loss of innate immunity related genes may result in toxicity and sensitize worms to pathogenic bacteria. This is further supported by an even lower resistance to pathogens in the double mutants mainly in Fig. 2D.

8) The blots are saturated, particularly in Fig. 4A, and this can be masking the differences in p38 phosphorylation. In fact, the fact that p38 phosphorylation is not changed is contradictory to the other results. How is p38 regulated by mitochondrial mutations then? I am concerned that p38 is actually not altered and the changes in gene expression are exclusively due to ATFS-1. The interaction with the p38 pathway demonstrated genetically could be due to the toxicity elicited by the loss of function mutations in this pathway.

Minor concerns

1) Lines 167 and 174: What are these p values referred to?

2) Line 258: I partially agree with the conclusions, since the functions may not necessarily be associated with innate immune signaling but rather other functions of p38.

3) Why in figures 4D and E different mutants were used?

4) Line 498: revise writing.

5) Show blots in Fig. 7B.

6) It would be interesting to know where the activation of the immune-related genes by the mitochondrial mutations is happening, whether this is a cell autonomous or cell non-autonomous mechanism.

3. Significance:

Significance (Required)

This study provides significant advance in mechanistic aspects of lifespan regulation in worms, linking mitochondrial metabolism, food intake, innate immunity, resistance to pathogen infections and longevity. The work presents novel mechanistic insights that could be applied to understand how mild mitochondrial dysfunction leads to increased lifespan. Overall, the audience interested in this study are expected to be

aging biologists and possibly immunologists with particular interest in mechanistic aspects of longevity and innate immunity, as well as C. elegans as a model organism. I am part of this group of scientists with particular interest in studying the interplay between metabolism and aging.

Reviewer #1

1. One key citation missing from the current manuscript is from Hwang et al. 2014 (PMID 25288734). This study has already described that the isp-1 mutant strain survives longer during P. aeruginosa infection. This citation also describes that the gene expression profile of isp-1 mutants animals includes a considerable number of pathogen-responsive genes that are similarly induced during infection. While the current manuscript does go into the mechanism of this resistance with more detail, they should amend the language to more appropriately reflect previous work, notably the above reference.

We apologize for the oversight and have added the suggested citation. Hwang et al. show that *isp-1* worms have increased resistance to bacterial pathogens that is dependent on HIF-1/HIF1 and AAK-2/AMPK. In future work, it will be interesting to examine whether HIF-1 and AAK-2 act in concert with, or independently of, ATFS-1 and the p38-mediated innate immune signaling pathway to mediate pathogen resistance and longevity in *isp-1* worms. We have added these points to our discussion **(see lines 577-582).**

2. The authors suggest that ROS activation of the p38 MAPK pathway is likely not the mechanism that explains the resistance of long-lived mitochondrial mutant animals due to their reduced food intake. However, is ROS production nonetheless involved? Does antioxidant treatment suppress the increased resistance during infection of isp-1 and/or nuo-6 mutant animals?

To address this question, we treated wild-type, *isp-1* and *nuo-6* worms with antioxidant and then measured resistance to bacterial pathogens using the *P. aeruginosa* strain PA14 slow kill assay. For the antioxidant treatment, we used 10 mM Vitamin C as we have previously shown that this concentration is effective at reducing ROS in mitochondrial mutant worms to decrease *isp-1* and *clk-1* lifespan (Van Raamsdonk and Hekimi 2012, *PNAS;* Schaar et al., 2015, *PLoS Genetics*) but did not impact lifespan in wild-type worms. We found that treatment with Vitamin C did not suppress the increased resistance to bacterial pathogens in *nuo-6* or *isp-1* worms. We have included this data in **Appendix Figure S11,** and added this point to our manuscript **(see lines 376-388)**.

3. (line 278-282): the authors should elaborate on how the p38 MAPK pathway plays a permissive role. It is intriguing that ATFS-1 and ATF-7 are both bZIP transcription factors that could theoretically heterodimerize and that they share common immune gene targets. The authors do indicate that the binding sites for ATFS-1 and ATF-7 are very different and are likely acting distinctly but some speculation would nonetheless strengthen this statement.

While ATFS-1 and ATF-7 were shown to bind to the promoter regions of the same innate immunity genes, the apparent consensus binding sites are different suggesting that they may bind to different regions of the promoter. One way in which the p38 MAPK pathway could be playing a permissive role is that ATF-7 binding and relief from its repressor activity is required for any transcription of p38-mediated innate immunity target genes to occur. This is consistent with our data showing that disruption of *nsy-1, sek-1, pmk-1* or *atf-7* decreases the expression of innate immunity genes in wild-type worms. In contrast, it may be that the role of ATFS-1 is for enhanced expression of innate immunity genes such that when ATFS-1 is bound to the promoter region, or perhaps enhancer elements, the baseline expression of innate immunity genes that results from the binding of ATF-7 is increased. This idea is

supported by our data showing that disruption of *atfs-1* does not affect the expression of innate immunity genes in wild-type worms but prevents *nuo-6* mutants from having increased expression. We have updated our manuscript to include these points **(see lines 530-546)**.

4. The authors suggest that reduced food consumption of nuo-6 and isp-1 animals may suppress ROS-induced activation of the p38 innate immune pathway. It is intriguing that dietary restriction was previously shown to increase resistance to infection, presumably through p38-independent mechanisms (PMID 30905669). It would be interesting to measure host survival of nuo-6 and isp-1 mutant animals that are dietary-restricted to see if the enhanced survival rates conferred by mitochondrial stress and DR are additive or not.

According to this suggestion, we compared the bacterial pathogen resistance of wild-type, *isp-1* and *nuo-6* worms that have undergone dietary restriction to the same strains under ad libitum conditions. We found that while dietary restriction increased bacterial pathogen resistance in wild-type worms, it did not further increase the enhanced pathogen resistance in *nuo-6* or *isp-1* worms. Thus, the enhanced survival rates resulting from dietary restriction and mild impairment of mitochondrial function are not additive. We have included this data in **Appendix Figure S9,** and added this point to our manuscript **(see lines 358-364)**.

5. Figure 2: It is intriguing that loss of p38 signaling appears to have different effects in nuo-6 versus isp-1 animals. Specifically, loss of p38 signaling in isp-1 mutants renders them more sensitive to infection than wild-type, whereas it generally suppresses survival rates back to wild-type levels in the nuo-6 mutant background. Even within the nuo-6 mutant group, loss of SEK-1 has more dramatic effects on nuo-6 mutant animals than does loss of NSY-1, PMK-1 or ATF-7(gf). This is despite the fact that the nsy-1, sek-1, and pmk-1 alleles that are used in this study are all reported to be null. Can the authors speculate on these differences?

While the *isp-1* and *nuo-6* mutations both alter mitochondrial function, they affect different components of the electron transport chain. *isp-1* mutations affect Complex III (Feng et al. 2001, *Dev. Cell*), while *nuo-6* mutations affect Complex I (Yang and Hekimi 2010, *Aging Cell*). Although these mutants both have increased lifespan and a similar slowing of physiologic rates, it is not uncommon to observe differences between these mutants. For example, while treatment with the antioxidant NAC completely reverts *nuo-6* lifespan to wild-type, it only partially reduces *isp-1* lifespan (Yang and Hekimi 2010, *PLoS Biology*), suggesting that *nuo-6* lifespan may be more dependent on ROS than *isp-1.* We have recently shown that deletion of *atfs-1* reduces *nuo-6* lifespan, but completely prevents *isp-1* worms from developing to adulthood (Wu et al. 2018, *BMC Biology*), suggesting that *isp-1* worms are more dependent on ATFS-1 than *nuo-6* worms. The fact that loss of p38 signaling decreases the survival of *isp-1* worms to a greater extent than *nuo-6* worms when exposed to bacterial pathogens suggests that *isp-1* worms are more dependent on this pathway for bacterial pathogen resistance, while *nuo-6* worms might rely on other pathways more. Disruption of *sek-1* has a greater impact on pathogen resistance than *nsy-1* and *pmk-1* because SEK-1 is absolutely required for innate immune signaling, while some partial redundancy exists for NSY-1 and PMK-1. We have added these points to our manuscript **(see lines 222-227)**.

6. One of the main conclusions from this study is that ATFS-1 likely binds directly to innate immune genes that are in common with ATF-7. Since this is such a pivotal finding, the authors should validate some candidate genes from the referenced ChIP seq datasets using ChIP qPCR. Also, are there predicted ATFS-1 binding sites (PMID 25773600) in these promoters?

Our data shows that activation of ATFS-1 increases the expression of innate immunity genes without increasing activation of p38. The simplest explanation for this observation is that ATFS-1 can upregulate the same innate immunity genes as ATF-7. Accordingly, we hypothesized that ATFS-1 can bind to the same genes as ATF-7, either in the promoter or enhancer regions, to modulate gene expression. Fortunately, two previous ChIP-Seq studies, from well-established laboratories who have extensive experience studying ATFS-1 and ATF-7, had already determined which genes are bound by these two transcription factors (Nargund et al. 2015, *Molecular Cell*; Fletcher et al. 2019, *PLoS Genetics*). Comparing the results of these two published studies confirmed our hypothesis by demonstrating that the same innate immunity genes are bound by both ATF-7 and ATFS-1 *in vivo*. In order to provide additional support for the conclusion that ATFS-1 and ATF-7 can bind to the same genes, we examined the genetic sequence of innate immunity genes that were shown to be bound by both ATFS-1 and ATF-7 in the published ChIP-seq studies to identify predicted binding sites for ATFS-1 and ATF-7. Of the 24 genes that were in common between the two ChIP-seq studies, there were 13 genes where the binding peaks for ATFS-1 and ATF-7 show overlap, and for 10 of those genes the overlap occurs in the promoter region. Based on this analysis, we have added **Figures EV5** and **Figure S15** which illustrate both the region of each innate immunity gene that was identified in the ChIP-seq studies as well as the location of the consensus binding sites within this region for the 10 genes in which ATFS-1 and ATF-7 bind in the promoter region in close proximity. We have added these additional points to our manuscript **(see lines 485-495)**.

Reviewer #2:

(1) The authors state that the p38 MAPK PMK-1 is not activated in the long-lived mitochondrial mutants. However, it might be better to state that there is "no enhanced activation" of PMK-1, since they clearly show in nuo-6 and isp-1 mutants the presence of phosphorylated PMK-1 (Fig. 4A), which would indicate an activated form of PMK-1 in these mutants.

According to this suggestion, we have changed the text to indicate that there is no enhanced activation of p38/PMK-1 in *nuo-6* and *isp-1* worms **(see lines 290-291, 309-310)**.

(2) Are the food-intake behaviors of all mutants in liquid culture (Fig. 4B-F) the same as their food-intake behaviors on solid agar media, the environment where pathogen resistance was measured?

For our previous study on the role of innate immune signaling in dietary restriction and reduced insulin/IGF-1 signaling (Wu et al. 2019, *Cell Metabolism*), we compared assays measuring food intake on solid agar media versus the liquid culture approach used in the current study to determine which method is the most robust. While both assays produced generally similar results, performing the food intake assay on solid agar plates was highly variable as it is challenging to scrape off all of the uneaten bacteria from solid plates in order to measure it. Since the approach of measuring food intake in liquid media produces far more consistent and reliable results, we chose to use this assay for the current study. We have updated our manuscript to include this justification **(see lines 730-736)**.

(**3) Does the p38 pathway single mutant nsy-1 or sek-1 live shorter than wild type on dead E. coli OP50 (Fig. S9) than they do on live OP50 (Fig. 3)? If so, what might that mean? These mutants are also living shorter than wild type on PA14 (Fig. 2), but live as long as wild type on OP50 (Fig. 3). What is in the live OP50 that allows these mutants to live like wild type?**

In a previous publication, we found that *sek-1* mutants live shorter than wild-type worms, and *nsy-1* live slightly shorter than wild-type worms in a lifespan assay performed in liquid medium with non-proliferating OP50 bacteria (Wu et al. 2019, *Cell Metabolism*). In the current study, we performed lifespan assays on solid NGM plates with live OP50 bacteria and observed a wild-type lifespan in *sek-1* and *nsy-1* worms. Since there are multiple experimental variables that are different between the previous and current study, most notably liquid versus solid media, the lifespan results cannot be directly compared. In the case of measuring survival of these strains on PA14, the simplest explanation is that they are dying sooner because their innate immune signaling pathway is disrupted, and so they are less able to mount an immune response against the pathogenic bacteria. The fact that genes involved in the p38-mediated innate immune signaling pathway are not required to achieve a normal lifespan on OP50 bacteria, suggests that OP50 bacteria is not pathogenic enough to require innate immune activation for normal lifespan. We have updated our manuscript to include these points **(see lines 248-253)**.

At the same time, wouldn't it be simpler to call the multiple antibiotic-treated OP50 as "dead bacteria", instead of "non-proliferating bacteria"? Some of the antibiotics used to treat OP50 are bactericidal and not bacteriostatic.

We previously monitored the OD600 of the antibiotic-treated, cold-treated OP50 that we used in our experiment, and found that there is only a very small decrease in OD600 after 10 days (Moroz et al. 2014, *Aging Cell*). Since dead bacteria are rapidly broken down leading to a decrease in OD600, this result is consistent with the bacteria being alive but not proliferating. We have added this point to our manuscript **(see lines 705-708)**.

(4) Since nuo-6 and isp-1 do not always behave exactly the same in their dependence on certain genes (e.g., Fig. 2C vs Fig 2D), what happens in isp-1; atfs-1 double mutants? Do these mutants behave in the same manner as nuo-6; atfs-1?

This is an interesting question. Unfortunately, *isp-1;atfs-1* mutants arrest during development (Wu et al. 2018, *BMC Biology*), which is why we only examined the effect of *atfs-1* deletion in *nuo-6* mutants. We have updated the manuscript to note this point **(see lines 430-431)**.

Regarding nuo-6; atfs-1, why does the double mutant live shorter on PA14 than either single mutant (Fig. 6A)? Is this because atfs-1 is needed to activate the p38 MAPK-dependent and -independent pathways?

It is possible that the *nuo-6* mutation makes worms more sensitive to bacterial pathogens, perhaps due to decreased energy production, and that activation of ATFS-1 is required not only to enhance their resistance to pathogens but also to increase their resistance back to wild-type levels. In a previous study, we showed that loss of ATFS-1 slows down the rate of nuclear localization of DAF-16. Thus, loss of *atfs-1*

may also be decreasing resistance to bacterial pathogens by diminishing the general stress resistance imparted by the DAF-16-mediated stress response pathway. We have updated the manuscript to include these points **(see lines 435-439)**.

In Fig. 7B, the atfs-1(gof) appears to have slightly more phosphorylated p38 compared to wild type, although it is not statistically significant?

While there was a trend towards a very modest increase in phosphorylated p38 in the constitutively-active *atfs-1* mutant compared to wild-type, quantification of four biological replicates indicated that the difference is not significant. To ensure that the lack of statistical significance was not due to an insufficient number of replicates or sub-optimal exposure times for the Western blots, we repeated this Western blot with four additional replicates. With all eight replicates, there is still a trend towards increase in the *atfs-1(gof)* mutant but this difference is not significant. This result is consistent with the fact that the levels of phosphorylated p38 are not significantly increased in *nuo-6* or *isp-1* mutants, both of which show activation of ATSF-1. We updated **Figure 7** to include the additional replicates and have provided raw images of all of these Western blots in the **Appendix**. We have added these points to the manuscript **(see lines 465-471)**.

In Fig. 6B, the atfs-1 loss-of-function single mutant also increases the expression of Y9C9A.8, but suppresses it in a nuo-6 mutant background? What might that mean?

It is possible that in wild-type animals disruption of *atfs-1* causes a compensatory upregulation of specific stress response genes. We have previously shown that deletion of *atfs-1* results in upregulation of chaperone genes involved in the cytoplasmic unfolded protein response (*hsp-16.11, hsp-16.2*; Wu et al. 2018; *BMC Biology*). Perhaps Y9C9A.8 is acting in a similar way. In *nuo-6,* the upregulation of Y9C9A.8 is driven by activation of ATFS-1, and thus is prevented by *atfs-1* deletion. We have added these points to the manuscript **(see lines 442-446)**.

Reviewer #3:

1) Some studies propose that OP50 offers some toxicity to worms which is not observed in other bacterial strains like HT115. The authors should test the role of the p38-innate immune signaling pathway in nuo-6 and isp-1 lifespan using other non-pathogenic E. coli strains.

To determine if the effect of disrupting the p38-mediated innate immune signaling pathway on the lifespan of *isp-1* and *nuo-6* mutants was simply the result of losing protection against OP50 bacteria, we examined the effect of *nsy-1, sek-1* and *atf-7(gof)* mutations on *isp-1* and *nuo-6* lifespan using non-proliferating bacteria (**Figure EV2**). We found that even when no proliferating bacteria are present, disruption of the p38-mediated innate immune signaling pathway markedly decreases *isp-1* and *nuo-6* lifespan. This suggests that the p38-mediated innate immune signaling pathway is required for their long lifespan independently of its ability to protect against bacterial infection. Similarly, we have previously shown that lifespan extension resulting from dietary restriction is dependent on the p38-mediated innate immune signaling pathway even when non-proliferating bacteria are used (Wu et al. 2019, *Cell* Metabolism). We have clarified this important point in the manuscript **(see lines 272-280)**.

2) The authors should measure food intake in worms exposed to pathogenic bacteria, given that reduced bacterial intake may be related to reduced mortality.

Unfortunately, it is not feasible to perform the food intake assay using the pathogenic bacteria because the bacteria cause death thereby complicating the calculation of food consumed per worm (which requires at least 3 days to assess). As an alternative to measuring food intake, we attempted to measure intestinal accumulation of *P. aeruginosa* using a *P. aeruginosa* strain that expresses GFP. We examined time points up to 24 hours, a time point when the worms start to die from the pathogenic bacteria. Unfortunately, at 24 hours the fluorescent signal is still very weak in the pharynx and not detectable in the intestine. Because the signal was so weak, we concluded that quantification of fluorescence at his time point would not be reliable.

3) The authors should check if ROS is required for the activation of the p38-mediated innate immune signaling pathway and reduction in food intake.

To determine if the elevated levels of ROS that are present in *isp-1* and *nuo-6* worms affects activation of the p38-mediated innate immune signaling pathway, we treated wild-type, *isp-1* and *nuo-6* worms with Vitamin C and measured the ratio of phosphorylated p38 to total p38 by Western blotting. Similarly, to examine the effect of ROS on food intake, we treated wild-type, *isp-1* and *nuo-6* worms with Vitamin C and then quantify its effect on food intake. For these experiments, we used 10 mM Vitamin C as we have previously shown that this concentration is effective at reducing ROS in mitochondrial mutant worms to decrease *isp-1* and *clk-1* lifespan (Van Raamsdonk and Hekimi 2012, *PNAS;* Schaar et al., 2015, *PLoS Genetics*) but did not impact lifespan in wild-type worms. We found that treatment with Vitamin C did not affect activation of p38 or food intake in *nuo-6* or *isp-1* worms. We have included these results in **Appendix Figure S12** and **Figure S13**, and added these points to our manuscript **(see lines 376-388)**.

4) Since ATFS-1 and the p38 pathway control food intake, how related to dietary restriction the phenotypes the authors are studying are?

While the lifespan extension that results from mild impairment of mitochondrial function and the lifespan extension resulting from dietary restriction are both dependent on the p38-mediated innate immune signaling pathway, these interventions modulate innate immunity gene expression in opposite directions. We previously reported that dietary restriction primarily downregulates innate immunity genes (Wu et al. 2019 *Cell Metabolism*). Here, we show that mutations in *isp-1* or *nuo-6* primarily result in upregulation of innate immunity genes. To more globally examine gene expression changes between dietary restriction and mild impairment of mitochondrial function, we compared differentially expressed genes. We found that there was very little overlap of either upregulated or downregulated genes between dietary restriction and *isp-1/nuo-6* mutants. In fact, we observed a similar degree of overlap comparing upregulated genes to upregulated genes as comparing upregulated genes to downregulated genes. We have included this data in **Appendix Figure S10**, and added these points to our manuscript **(see lines 366-374)**.

5) Somewhat related to the previous points, I am not so sure whether the changes in food intake are cause or consequence of the alterations in the innate immunity-related genes. Reduced food intake is depicted in Fig. 8 as the cause of the activation of the p38 pathway, but there is not enough evidence to unequivocally prove that. In fact, food intake might be controlled by the p38 or ATFS-1 pathway or by a common regulator such as ROS.

We apologize that we didn't make this clearer. In our previous work, we showed that dietary restriction results in decreased activation of the p38 pathway (Wu et al. 2019, *Cell Metabolism*). We also showed that disabling the p38-regulated immunity pathway did not affect food intake (*sek-1* and *atf-7(gof)* mutants). Here, we show that activation of ATFS-1 results in decreased food intake. Based on our previous study, this decrease in food intake should similarly decrease p38 pathway activation. In Figure 8, we have depicted ATFS-1 inhibiting food intake, and food intake activating the p38-mediated innate immune signaling pathway. Combined, our model suggests that activation of ATFS-1 should act to decrease p38-mediated innate immune signaling. We have clarified this in the **Figure 8 legend**.

6) I am not so convinced of the role of DAF-16. In fact, in Fig. 5A daf-16 mutation reduces pathogen resistance and that could represent a toxic effect of the mutation. Furthermore, the results in Fig. 4D do not exclude the possibility that daf-16 and isp-1 act in parallel.

We agree that the role of DAF-16 could be non-specific. While we show that disruption of *daf-16* leads to decreased bacterial pathogen survival in *isp-1* worms, our results also indicate that DAF-16 is not involved in the upregulation of p38/ATF-7-regulated innate immunity genes in *isp-1* worms. Moreover, disruption of *daf-16* also decreases bacterial pathogen survival in wild-type worms. Thus, DAF-16 may be enhancing resistance to pathogens through activation of different immunity genes or through a general increase in resistance to stress and or survival. We have emphasized these points in our manuscript **(see lines 431-434).**

7) Loss of innate immunity related genes may result in toxicity and sensitize worms to pathogenic bacteria. This is further supported by an even lower resistance to pathogens in the double mutants mainly in Fig. 2D.

We agree. Our data confirms that disruption of the p38-mediated innate immune signaling pathway makes worms more susceptible to bacterial pathogens. We have emphasized this point **(see lines 416-417 and 575-577)**.

8) The blots are saturated, particularly in Fig. 4A, and this can be masking the differences in p38 phosphorylation. In fact, the fact that p38 phosphorylation is not changed is contradictory to the other results. How is p38 regulated by mitochondrial mutations then? I am concerned that p38 is actually not altered and the changes in gene expression are exclusively due to ATFS-1. The interaction with the p38 pathway demonstrated genetically could be due to the toxicity elicited by the loss of function mutations in this pathway.

To address this concern, we have repeated the Western blotting experiment to compare the ratio of phosphorylated p38 to total p38 between wild-type, *isp-1* and *nuo-6* worms, and have taken multiple exposures to ensure that the blots are not over-saturated. Having now completed eight replicates, we

believe that there is not a major change in p38 activation in *nuo-6* and *isp-1* worms. Our data suggests that the p38-mediated innate immunity pathway is playing a permissive role such that it is required for baseline expression of innate immunity genes, but that activation of ATFS-1 is driving the enhanced expression of innate immunity genes that we observe in the long-lived mitochondrial mutants and constitutively active *atfs-1* mutants. We have updated our manuscript to clarify this **(see lines 465-471 and 530-546)**. We have updated **Figure 4** to include the quantification of all replicates and have included all of the raw Western blot images in the **Appendix**.

****Minor concerns****

1) Lines 167 and 174: What are these p values referred to?

The p-values indicate the significance of the difference between the observed number of overlapping genes between the two gene sets, and the expected number of overlapping genes if the genes were picked at random **(see lines 169-171, Figure 1 legend, and Figure EV4 legend).**

2) Line 258: I partially agree with the conclusions, since the functions may not necessarily be associated with innate immune signaling but rather other functions of p38.

Since *isp-1* and *nuo-6* worms have extended longevity even when grown on non-proliferating bacteria this indicates that their long life is not dependent on their enhanced resistance to bacterial pathogens. Similarly, since disruption of genes in the p38-mediated innate immune signaling pathway decrease *isp-1* and *nuo-6* lifespan even when the worms are grown on non-proliferating bacteria, this suggests that this pathway enhances longevity independently of its ability to increase innate immunity.

3) Why in figures 4D and E different mutants were used?

We only used *isp-1* mutants to examine the effect of *daf-16* because we were unable to generate *nuo-6 daf-16* mutants due to close proximity of the two genes on the same chromosome. We only used *nuo-6* mutants to examine the effect of *atfs-1* because *isp-1;atfs-1* worms arrest during development. We have included this explanation in our manuscript **(see lines 400-402 and 430-431)**.

4) Line 498: revise writing.

We have rewritten this sentence to improve clarity **(see lines 623-627)**.

5) Show blots in Fig. 7B.

We have added an image of a representative Western blot in **Figure 7**, and have provided the raw images for all of Western blots in the **Appendix**.

6) It would be interesting to know where the activation of the immune-related genes by the mitochondrial mutations is happening, whether this is a cell autonomous or cell non-autonomous mechanism.

While it would be interesting to explore whether specific tissues are important in sensing mitochondrial impairment in order to upregulate genes involved in innate immunity, it is beyond the scope of this manuscript. Previous work has shown that knocking down the expression of the cytochrome c oxidase gene *cco-1* in neurons can activate the ATFS-1 target gene *hsp-6* in the intestine (Durieux et al., 2011). Based on this, one could hypothesize that a similar cell non-autonomous mechanism might be involved. We have noted this possible future direction in our discussion **(see lines 635-638).**

1st Revision - Editorial Decision 9th Sep 2021

Dear Prof. Van Raamsdonk

Thank you for the submission of your revised manuscript to EMBO reports. I apologize for the delay in handling your manuscript, but we have only now received the last referee report. Please find the full set of referee reports copied below.

As you will see, all referees are very positive about the study and request only minor changes to the text and to discuss alternative scenarios regarding the role of ROS.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

1) Appendix:
- Please add page numbers to the table of content
- Please remove the reference to Tables EV1 to EV3
- Please change the figure labels and callouts in the text to "Appendix Figure Sx".
- Source data: Please specify which of the source data in the Appendix are for Appendix Figure S12 and which blots are related to Fig. 4 and Fig. 7

2) EV tables: Please add a legend in a separate tab of each .xls file.

3) Please update the reference to the related paper from Soo SK et al, if possible.

4) The lifespan experiments in Figure 2 and Fig. EV2 are based on data from 2 biological replicates (n = 2). Our editorial policies require that the individual data points instead of mean or median are shown in such cases and a statistical analysis should not be performed.

5) Please add space between the individual images in Fig 1C.

6) I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

7) Please complete the information on funding in our online submission system. Currently, the information in the system does not match the funding information in the manuscript. Many grant reference numbers appear to be missing.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

****

Referee #1:

The authors have satisfactorily addressed all my concerns, but the there is a minor point brought up by the new data that should be considered before publication. Based on the new data in figures S11-S13, the authors state the following: "We found that treatment with Vitamin C had no effect on bacterial pathogen resistance (Appendix Fig S11), PMK-1/p38 activation (Appendix Fig S12), or food intake (Appendix Fig S13) in nuo-6 or isp-1 worms. This suggests that the elevated levels of ROS in the long-lived mitochondrial mutants is not driving these phenotypes,..." I partially agree with this conclusion given that in WT, vitamin C appears to confer the same level of survival against pathogenic bacteria than that conferred by nuo-6 and isp-1 mutations (Fig. S11). Likewise, vitamin C, nuo-6 and isp-1 mutations all reduce food intake (Fig. S13) and these effects are not additive. It is not possible to draw similar conclusions regarding p38 phosphorylation since it appears each condition is normalized by its respective vehicle control (that should be revised). Overall, one may conclude that nuo-6 and isp-1 mutations are promoting an antioxidant response similar to the exogenous supplementation with vitamin C and this is contributing to protection against pathogenic bacteria and reduced food intake. Perhaps after exposure with pathogenic bacteria the baseline of pro-oxidant species is high enough to allow antioxidants to be beneficial. In fact, mitochondrial dysfunction has been shown to promote longevity by acting during development in a ROS-dependent manner and creating an epigenetic memory that is sustained throughout adulthood (Andy Dillin's work). Considering how the authors designed their experiments, by the time nuo-6 and isp-1 mutants are exposed to PA or vitamin C, elevated ROS may have already created this memory that sustains an efficient antioxidant response that appears to be protective against pathogenic bacteria and is mimicked by exogenous supplementation with antioxidants in WT worms. Looking through this perspective, ROS may still be playing an important role in these phenotypes.

Referee #2:

The authors have addressed all my concerns. I am satisfied with the current state of the manuscript.

Referee #3:

The authors have addressed all reviewers' comments. I recommend publication. At the copy-edit stage, I only suggest a few grammatical edits.
(1) On page 8, the sentence in lines 177-178 reads awkward.
(2) On page 16, line 413, please introduce a space between "mutants" and "(Troemel...)".
(3) The genotypes of double mutants, for example, isp-1; atfs-1 on page 17, line 431, should have a space after the semicolon.
(4) On page 21, the sentence from lines 536-539 reads awkward. The next sentence also reads like a run-on sentence.
(5) On page 21, line 552, please introduce a space between "pathway" and "(Pellegrino...)".
(6) In the figure 1 legend, line 1072, I think it is better to write "significant", instead of "significantly".

Editorial modifications

1) Appendix:
- Please add page numbers to the table of content
- Please remove the reference to Tables EV1 to EV3
- Please change the figure labels and callouts in the text to "Appendix Figure Sx".
- Source data: Please specify which of the source data in the Appendix are for Appendix Figure S12 and which blots are related to Fig. 4 and Fig. 7

All modifications were made as suggested.

2) EV tables: Please add a legend in a separate tab of each .xls file.

A legend was added to Tables EV1 and EV3 (now called Dataset EV1 and Dataset EV2).

3) Please update the reference to the related paper from Soo SK et al, if possible.

The Soo et al paper has been accepted at *Life Science Alliance.* The reference has been updated to indicate the journal name and "Accepted", which can eventually be replaced by the volume and page numbers when that information is available.

4) The lifespan experiments in Figure 2 and Fig. EV2 are based on data from 2 biological replicates (n = 2). Our editorial policies require that the individual data points instead of mean or median are shown in such cases and a statistical analysis should not be performed.

The bacterial pathogen stress and lifespan experiments in Figure 2 and Figure EV2 show the data with a Kaplan-Meier survival plot. This plot does not show a mean/median but rather incorporates all of the data to generate the survival curve. The N for Figure 2 is a minimum of 175 animals per strain and for Figure EV2 is a minimum of 274 animals per strain (the N for each strain and the individual survival of all of the animals is provided in Table EV2). The two biological replicates indicates that these numbers of animals were not all tested in the same trial but were tested in two completely separate trials. Because the N is so high for these experiments, it is not possible to visualize the data for each individual animal if plotted on a graph. We have updated the Figure legends to indicate the number of animals. Please advise if there are other changes that could help to clarify this.

5) Please add space between the individual images in Fig 1C.

Space was added between the images.

6) I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

We have addressed the comments using tracked changes.

7) Please complete the information on funding in our online submission system. Currently, the information in the system does not match the funding information in the manuscript. Many grant reference numbers appear to be missing.

We have updated the funding information on the submission system to match the manuscript text and included the grant reference numbers.

EMBO Reports Quality Check (Sept. 13)

1. The Source Data should be removed from the Appendix and uploaded as individual files, ie one file per figure.

The Raw Western blot images were removed from the Appendix and uploaded as individual files.

2. Tables EV1 & EV3 should be renamed as Datasets EV1 & EV3, and uploaded with the file type 'Data Set'.

Table EV1 was renamed Data Set EV1. Table EV2 was renamed Data Set EV2.

3. Table EV2 seems like it is source data, so should be uploaded as one file per figure. If there are two different file types for the one figure then these should be ZIPed together in a folder.

Table EV2 was separated into 7 individual Source data files. Figure legends were modified to "Source data for this figure is available online. Source data for Figure X."

4. The funding information should be included in the Acknowledgement section, not as a separate paragraph.

Funding information was moved to Acknowledgements.

5. Please add the heading 'Expanded View Figure Legends' to the manuscript.

Heading was added.

Referee #1:

The authors have satisfactorily addressed all my concerns, but the there is a minor point brought up by the new data that should be considered before publication. Based on the new data in figures S11-S13, the authors state the following: "We found that treatment with Vitamin C had no effect on bacterial pathogen resistance (Appendix Fig S11), PMK-1/p38 activation (Appendix Fig S12), or food intake (Appendix Fig S13) in nuo-6 or isp-1 worms. This suggests that the elevated levels of ROS in the long-lived mitochondrial mutants is not driving these phenotypes,..." I partially agree with this conclusion given that in WT, vitamin C appears to confer the same level of survival against pathogenic bacteria than that conferred by nuo-6 and isp-1 mutations (Fig. S11). Likewise, vitamin C, nuo-6 and isp-1 mutations all reduce food intake (Fig. S13) and these effects are not additive. It is not possible to draw similar conclusions regarding p38 phosphorylation since it appears each condition is normalized by its respective vehicle control (that should be revised). Overall, one may conclude that nuo-6 and isp-1 mutations are promoting an antioxidant response similar to the exogenous supplementation with vitamin C and this is contributing to protection against pathogenic bacteria and reduced food intake.

According to this suggestion, we have specifically noted in the text that Vitamin C treatment increased bacterial pathogen resistance and decreased food intake in wild-type worms. Since we have previously shown that antioxidant enzymes are upregulated in *nuo-6* and *isp-1* worms (Dues et al., 2017; Wu et al., 2018), we have now described the possibility that this upregulation of antioxidant genes is acting similarly to the exogenous antioxidant Vitamin C and contributing to bacterial pathogen resistance and decreased food intake (**please see lines 389-392**)

In fact, mitochondrial dysfunction has been shown to promote longevity by acting during development in a ROS-dependent manner and creating an epigenetic memory that is sustained throughout adulthood (Andy Dillin's work). Considering how the authors designed their experiments, by the time nuo-6 and isp-1 mutants are exposed to PA or vitamin C, elevated ROS may have already created this memory that sustains an efficient antioxidant response that appears to be protective against pathogenic bacteria and is mimicked by exogenous supplementation with antioxidants in WT worms. Looking through this perspective, ROS may still be playing an important role in these phenotypes.

For the experiments involving Vitamin C, we began treatment at L4 stage of parental generation and continued Vitamin C treatment throughout the development and early adulthood of the experimental worms in which we tested bacterial pathogen resistance, food consumption and p38 activation. Thus, by the time worms were exposed to PA14 to test pathogen resistance, they had already been exposed to Vitamin C for multiple days including the important developmental period identified by Andy Dillin's work. We have indicated the period of time that worms were treated with Vitamin C in the figure legends of Figure S11, S12 and S13.

Referee #3:

(1) On page 8, the sentence in lines 177-178 reads awkward.
(2) On page 16, line 413, please introduce a space between "mutants" and "(Troemel...)".
(3) The genotypes of double mutants, for example, isp-1; atfs-1 on page 17, line 431, should have a space after the semicolon.
(4) On page 21, the sentence from lines 536-539 reads awkward. The next sentence also reads like a run-on sentence.
(5) On page 21, line 552, please introduce a space between "pathway" and "(Pellegrino...)".
(6) In the figure 1 legend, line 1072, I think it is better to write "significant", instead of "significantly".

Thank you for these suggestions. We have completed all of these modifications as suggested.

2nd Revision - Editorial Decision 17th Sep 2021

Prof. Jeremy Van Raamsdonk
McGill University
1001 Decarie Boulevard
Montreal, Quebec H4A 3J1
Canada

Dear Prof. Van Raamsdonk,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Corresponding Author Names: Jeremy Van Raamsdonk and Keith Blackwell

Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2021-53116V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes were chosen based on our experience with the assays involved. Because the experiments were performed with an invertebrate model organism, we are typically able to use a much higher N than is needed to observe a particular effect size based on a power calculation.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	See reply to 1.a.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For lifespan assays animals were removed if they exhibited internal hatching of progeny, expulsion of internal organs or dessicated in the side of the dish. These criteria were pre-established.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Experiments were typically performed with the experimenter blinded to the genotype of the animals. Within each genotype worms were picked randomly.
For animal studies, include a statement about randomization even if no randomization was used.	Animals were picked randomly for inclusion in experiments.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Experiments were typically performed with the experimenter blinded to the genotype of the animals. Within each genotype worms were picked randomly.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Experiments were typically performed with the experimenter blinded to the genotype of the animals.
5. For every figure, are statistical tests justified as appropriate?	We used the standard statistical tests for each assay (e.g. one-way ANOVA, two-way ANOVA, log-rank).
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. We examined QQ plots using GraphPad Prism to confirm normality of the data.
Is there an estimate of variation within each group of data?	Error bars showing standard error of the mean are shown. Raw data for many assays is provided in a supplemental table.

Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We have previously used these antibodies for Western blotting in C. elegans: Wu et al. 2019, Cell Metabolism.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were utilized.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	These studies used hermaphrodite C. elegans. Worms were grown at 20 degrees Celsius on NGM plates with OP50 bacteria. Mutant strains utilized are outlined in the Materials and Methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not applicable.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We have consulted the ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not applicable.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Done.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	All data is provided.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.