EMBO reports

Cest-2.2 overexpression alters lipid metabolism and extends longevity of mitochondrial mutants

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Dear Dr. Bano,

Thank you for the submission of your research manuscript to our journal, which was now seen by three referees, whose reports are copied below.

We concur with the referees that the proposed role of CEST-2.2 in modulating the lifespan of short-lived and long-lived mutants in principle very interesting. However, referees also raise significant and largely overlapping concerns that need to be addressed to consider publication here. In particular,

- The conclusion that CEST-2.2 reverses lifespan phenotypes by modulating lipid catabolism is not sufficiently supported by the data.
- Whether CEST-2.2 depletion affects specifically mitochondrial (or complex I) mutants, or other long/shortlived mutants as well needs to be teased apart.
- Does CEST-2.2 modulate mitochondrial function or mtUPR?
- The proposed role of OAA in CEST-2.2 mediated lifespan extension requires further support.

Should you be able to address all criticisms in full, we could consider a revised manuscript. I do realize that addressing all the referees' criticisms will require a lot of additional time and effort and be technically challenging. I would therefore understand if you wish to publish the manuscript rapidly and without any significant changes elsewhere, in which case please let us know so we can withdraw it from our system.

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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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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,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

In this manuscript, Piazzesi and colleagues investigate whether enhanced metabolic flexibility is sufficient to modulate ageing in nematodes exhibiting bioenergetic abnormalities. More specifically, they assessed the epigenetic landscapes of two models of mitochondrial complex I deficiencies that differently affected C. elegans lifespan. To this end, they examined whether histone H3.3 variant, which was previously shown to impair the establishment of various lifespan extending programs, is differentially deposited into chromatin of long-lived and short-lived mitochondrial mutants. Indeed, by narrowing down the analysis of data collected from chromatin immunoprecipitation (ChIP) followed by deep sequencing and RNA seq analysis, the authors found that H3.3 was differentially loaded on the promoters of genes in long-lived and short-lived mitochondrial mutants. Indeed, H3.3 was enriched at the promoters of 179 genes in nuo-6(qm200) long-lived mutants. Analysis of RNA seq datasheets from these mutants identified 10 out of 179 genes that are consistently upregulated in nuo-6(qm200) long-lived animals while downregulated in gas-1(fc21) short-lived mutants. Further analysis was focused on the phylogenetically conserved cest-2.2 that encodes a carboxylesterase. Knockdown or knockout of cest-2.2 abrogates the extended lifespan of animals subjected to cco-1 RNAi. By contrast, cest-2.2 overexpression extends the lifespan of gas-1(fc21) mutants, improves their healthspan by rescuing locomotory defects and ameliorates mitochondrial fragmentation. Next generation RNA seq analysis in intestines isolated from gas-1(fc21) and gas-1(fc21); cest-2.2 O/E animals identified 354 dysregulated lipid metabolism genes. In particular, genes involved in fatty acid oxidation were downregulated in gas-1(fc21) animals compared to gas-1(fc21); cest-2.2 O/E animals. The longevity of the latter strain was partially reduced by knocking down the yolk protein gene vit-1, which is considered to have lipid transporter activity. Moreover, the composition of free fatty acids (FFA) was different between gas-1(fc21) animals and gas-1(fc21); cest-2.2 O/E animals, although their total lipid content was comparable. It was also shown that depletion of nuclear hormone receptors NHR-49 or NHR-80 shortens the lifespan of gas-1(fc21); cest-2.2 O/E animals. Downregulation of genes involved in lipid peroxidation in mitochondria such as acs-2 and ech-1 or in peroxisomes such as acox-1 resulted in partial and significant reduction, respectively of lifespan in gas-1(fc21); cest-2.2 O/E. On the other hand, knockdown of pck-1 did not affect the lifespan of these animals whereas mdh-2 deficiency reduced the lifespan of gas-1(fc21); cest-2.2 O/E while it enhanced the longevity of gas-1(fc21) mutants. Altogether, these findings suggest that fatty acid catabolism mediate the beneficial effects of cest-2.2 overexpression on lifespan of certain mitochondrial mutants.

One of the key objectives of this study is to show the importance of the carboxylesterase CEST-2.2 in determining the survival

threshold of complex I deficient nematodes through epigenetic changes that confer metabolic flexibility. However, despite the authors claim, CEST-2.2 depletion appears to adversely affect the lifespan of wild-type animals as well as long-lived mitochondrial mutants. Indeed, cest-2.2 knockdown significantly shortens the lifespan of wild type worms (Fig. 1J and Table S1,), while only slightly influences the lifespan of nuo-6(qm200) mutants (Fig. 1I and Table S1). The same is true for the cest-2.2 null mutants which showed significantly reduced lifespan (P=0.0007) compared to wild type animals. Together, these findings suggest that CEST-2.2 depletion is detrimental in both wild type and mutant backgrounds. The authors should examine whether their findings are relevant to other mitochondrial mutants or are mostly specific to complex I deficient mutants.

The authors state that CEST-2.2 O/E transgene ameliorates the aberrant mitochondrial fragmentation in gas-1 mutant animals and provide some images to support this statement (Fig. 2I). To strengthen their findings, they could provide a quantification of fragmented versus healthy tubular mitochondria as well as measurements of ATP production and mitochondrial membrane potential as evidenced either by direct measurements of this parameter of indirectly by TMRE staining.

The authors state that cest-2.2 null mutation inhibited the lifespan extension due to cco-1 RNAi. First, were CRISPR-Cas9-generated mutant worms outcrossed for several generations to eliminate off target effects before being used to experiments? Second, Fig. 1 O shows that cest-2.2 RNAi shortens the increased lifespan of wild type animals subjected to cco-1 RNAi, suggesting that CEST-2.2 is required for the lifespan extension conferred by cco-1 knockdown in wild type background. On the other hand, cco-1 RNAi significantly increases the lifespan of cest-2.2 mutants (Fig. 1P and Table S1). These results are somehow confusing and need further investigation. Please, explain which strains /treatments were compared in Fig. 1P. In other words, the different P values (asterisks) to which comparisons correspond. This information should be included in Figure legend.

While it is apparent that cest-2.2 predominantly affects the lifespan of mitochondrial mutants, it is not obvious whether/how cest-2.2 itself affects mitochondrial homeostasis or function per se. Towards this direction, the authors could assess OCR, mitochondrial morphology and other functional mitochondrial parameters in wild-type, cest-2.2 knockout and cest-2.2 o/e animals. Additionally, activation of mitochondrial unfolded protein response (UPRmt) is often indispensable for the extended lifespan of mitochondrial mutants (Durieux and Dillin, Cell, 2011). Does cest-2.2 overexpression employ UPRmt to extend lifespan?

In Fig. 3A, an adult animal expressing a cest-2.2p::GFP extrachromosomal array is shown. The age of the animal should be defined. Also, a non-transgenic age-matched control should be included in the Figure in order to confirm that the green signal is not due to autofluorescence. Based on the finding that vit-1 knockdown partially reduced the lifespan of gas-1(fc21); cest-2.2 O/E animals, the authors propose that these animals have less lipid droplets because of enhanced lipid catabolism rather than as a consequence of a preferential nutrient transferring to the developing progeny. It is not clear how this conclusion is drawn from the above mentioned data.

The authors show that cest-2.2 overexpression is sufficient to extend lifespan of gas-1 mitochondrial mutants, which are defective for the ETC complex I. Can cest-2.2 overexpression extend the lifespan of mev-1 mutants, which are also inherently short-lived due to ETC complex II deficiency? Moreover, how does cest-2.2 silencing affects the lifespan of long-lived isp-1 mutants?

The finding that cest-2.2 overexpression extends the lifespan of short-lived gas-1 mutants, while it does not affect the lifespan of wild-type animals should be adequately explained. Is lipid catabolism induced in wild-type animals upon cest-2.2 o/e? Are free fatty acid pools exhausted, similarly to gas-1 mutants? If cest-2.2 overexpression is accompanied by similar metabolic changes, why don't wild-type animals live longer?

Figures denoted in the legend of Fig. S2, are missing. More specifically, lifespan curves of gas-1(fc21) and gas-1(fc21); cest-2.2 O/E animals are not shown although mentioned in the legend. Regarding this point, it would be helpful to include survival curves of wild type animals upon acox-1 RNAi or mdh-2 RNAi in the curves of mutant worms presented in Fig. 5H and J, respectively for better comparison.

The authors claim that CEST-2.2 enhances longevity in gas-1(fc21) cest-2.2 O/E animals by refilling intermediates through oxaloacetate production, ultimately buffering the deleterious loss of complex 1 activity. To support this statement, they can test whether exogenous supplementation of oxaloacetate increases the lifespan of gas-1(fc21) mutants.

The authors state on page 12 that "CEST-2.2 defines the survival threshold of complex I deficient nematodes, since it balances potentially deleterious mitochondrial lesions by engaging catabolic pathways that enable the usage of alternative resources to generate energy and sustain longevity". Indeed, a possible increase in b-oxidation which feeds into the TCA cycle could increase oxygen consumption rate as well as ATP levels of gas-1;cest-2.2 OE strain compared to gas-1 mutants. In figure 2J-L, maximum respiration is indeed increased in cest-2.2 OE animals. However, basal OCR is not affected, which suggests that the basal supply of ETC is not affected upon cest-2.2 OE. What are the ATP levels of control, gas-1 and gas-1;cest-2.2OE animals?

Fatty acid levels are the net result of fatty acid dietary intake, de novo fatty acid synthesis and fatty acid catabolism. The authors believe that cest-2.2 OE leads to increased FAO, based on prediction from RNA seq data. To verify whether this is indeed the case the authors could either measure FAO upon cest-2.2 OE directly (PMID: 24862277) or try increasing FAO by other means

and test whether it can still rescue gas-1 short lifespan (i.e PMID: 32597196).

Do gas-1 mutants exhibit reduced FAO, which is reversed upon CEST-2.2 OE?

Some lifespan results are puzzling. The most prominent example is the purple-colored curve in figure 2B. Survival drops steeply within a very narrow time frame (between day 28 and day 32), while no death event has been recorded till day 25. I recommend that the authors carefully consider the validity of these lifespan results, so that they are consistent and meaningful.

In the transcriptomic analysis, shown in Figure 3H wild type control would also be needed for a more direct association of the differences in the expression profile and a potential role in longevity of the presented genes.

Figure 5: to my understanding, the contribution of NHR-49 and NHR-80 on the beneficial effects of cest-2.2 OE is not easily deduced by the data, as depletion of each of those key lipid metabolism players severely shortens lifespan of wt animals. In order to conclude that a gene mediates the pro-longevity effects of cest-2.2 OE, ideally it should not affect the lifespan of wt animals. This criterion is only met for acs-2 gene, which corroborates the proposed hypothesis.

Several controls are missing throughout the manuscript. The authors compare wt, gas-1(fc21) and gas-1(fc21); cest-2.2 O/E animals, without presenting or mentioning the effects of cest-2.2 overexpression in wt animals' physiology and lipid metabolism. Does CEST-2.2 overexpression result in increased lipid catabolism in wt nematodes similar to gas-1(fc21) mutants? Do CEST-2.2 overexpressed animals display reduced lipid droplets compared to wt nematodes?

A growing body of evidence suggest the several longevity pathways, including caloric restriction, low insulin signaling and germline ablation among others, converge on lipid catabolism and metabolic rewiring. Does CEST-2.2 depletion or overexpression affect the lifespan of other long-lived mutants, such as daf-2, eat-2 and glp-1?

The authors suggest that "The differences in median lifespan between cest-2.2 KO and cest-2.2 silenced animals are probably due to the confounding effect of the two bacteria strains (i.e., OP50 and HT-115, respectively) used in the two experimental settings". To validate their hypothesis, they should use the OP50-RNAi strain (Xiao et al., Cell Rep 2015), which is available in CGC, and knockdown cest-2.2 by utilizing the same bacterial background in their assay.

In Figure 3D the authors investigate whether supplementation of ascaroside-8 extends the lifespan of gas-1 mutants. However, they did not observe any significant difference. Have they checked the effects of supplementation with other ascarosides? Is ascr#8 efficiently absorbed by animals? It has been reported in the literature that ascr#2 supplementation extends the median lifespan of wt animals. In those studies, animals were exposed to ascr#2 at the L4 developmental stage, and not from the egg stage (Ludewig, Izrayelit et al. PNAS, 2013). The authors should repeat this experiment by supplementing gas-1 mutants with ascr#8 at L4, to avoid possible developmental side-effects. They should also use wt animals as controls in Fig 3D.

Referee #2:

The manuscript submitted for review by Piazzesi et al. describes a novel approach in identifying gene products that distinguish lifespan extending vs lifespan shortening effects of mitochondrial complex I mutants using an interesting ChIP based strategy as a follow up of a previous study published in Cell Reports. One of 5 differentially targeted genes they identified was used to evaluate if its overexpression might be responsible for the different lifespan affecting outcomes of these complex I mutants.The manuscript delineates how a carboxyesterase cest-2.2 extends the lifespan of gas-1 mutants while is also required for the lifespan extension of nuo-6 and another unrelated complex IV mutant (cco-1). Although these observations are in and of themselves very interesting, the authors' attempts to understand how the overexpression this enzyme results in the observed lifespan extension take the manuscript into several metabolic analyses that yield very little concrete data that can explain how this enzyme, when overexpressed, actually extends lifespan. The genetic analysis is quite reasonable, leaving the reader with a solid foundation that suggests that vit-1, some aspects of fatty acid oxidation, and nhr-49/80 are likely required for the prolonged lifespan. How these genes function together is not clear from the work however. The authors rely on some systems biology approaches to suggest that many of the observed changes are dependent on alterations to the animals' lipid metabolism, but this is not reflected by the TLC analysis of the various lipid species that are separated using this method. There are VERY few differences, although the authors focus on the very small changes (abundance) in free fatty acids. In fact, there are a few changes probably in phospho or more polar lipids, but that are not identified in the analysis, that are equally changed, perhaps more convincingly than the free fatty acids, the molar difference of which is really minimal. The rest of the work pushes down these avenues that are weak and unsupported leaving the authors with a model to explain all the data that is jumbled, confusing and mostly unsubstantiated-barring the genetic data, which is, in my opinion convincing. I have listed below my major concerns and elaborated on some of the points that I have already brought up. In addition I have pointed out some other minor issues in the text and the figures that are more editorial in nature.

Major points of concern and comments:

If an allele is generated it is much more valuable than the RNAi analysis. This data should simply be replaced with similar curves using the CRISPR allele (give it an allele name and do not call it KO). RNAi experiments are notoriously inconsistant and difficult to control. In addition, this would also remove any inconsistencies associated with the differences in food. HT115 and OP50 may have very different lipid compositions and are likely to contribute quite differently to the TAG levels and the TAG composition. Stay consistent and use only one strain throughout the study.

What is the result of the nuo-6; cest-2.2 double mutant? Does it suppress the lifespan extension similar to the RNAi treatment?

If cest-22 RNAi can suppress the prolonged lifespan of a complex IV mitochondrial specific mutant then is it specific? How does it affect clk-1 mutants or other non-mitochondrial based long-lived mutants that have been described ie...eat-5; daf-2 or germ line ablated mutants. Does it also affect lifespan in animals treated with weak complex I inhibitors (ie..metformin) or animals treated with compounds that affect other complexes?

Does the cest-2.2 o/e extend the lifespan of nuo-6 (lf)?

The statement in the final sentence of paragraph 1-page 7 "Collectively, our findings show that CEST-2.2..." might be more accurate by simply stating that CEST-2.2 overexpression is sufficient to suppress the premature lethality and alternatively extend the lifespan of gas-1 mutants since no other complex 1 mutants that cause premature lethality were tested. With an n of 1 it is hard to make a general statement.

The mitochondrial morphological analysis needs to be quantified. Images of several mitochondria must be subjected to accepted analysis using available algorithms that assess various aspects of mitochondrial states/morphology.

The ORO staining data is strange. Why do the authors focus on a small section of the animal (hind gut) and ignore the rest of the animal? The germ line looks strange in these animals-the embryos also look very advanced. Are they long lived because of the effects of cest-2.2 on the germ line/gonad? Getting a better comparative picture of overall neutral lipid levels would be advantageous. In addition, these differences do not correlate with the TLC analysis whatsoever. The levels of all the neutral lipids were virtually indistinguishable by TLC, including the TAGs. The VERY small change in FFA was detectable but really, not convincing, and although composition may be altered the molar differences associated with these changes are unlikely to be significant. If there is a role of lipid metabolism in this phenotype it is not due to gross changes in the lipidome, but potentially some link to an important signalling lipid. It is almost certainly not due to changes in total energy (J) lost or gained due to lipid catabolism or lack thereof. This is consistent with their observations obtained by removing nhr-49.

The alternative hypothesis might include consequent generation of reducing equivalents following FA oxidation. Small changes in the NADH levels might contribute to the suppression of gas-1 and the observed extension. Can the authors extend lifespan in these mutants by blocking NADH consuming pathways and/or by manipulating an NADH generating system? How do the NAD:NADH ratios compare in these long lived mutants?

Could cest-2.2 o/e animals somehow use ROS or generate more ROS to extend lifespan? Have you measured and compared ROS levels between gas-1 and gas-1; cest-2.2 o/e lines?

C14, 15, 19 and C21 FAs are all absent or below the level of detection in the LC/MS analysis. These appear to be FAs that are normally obtained from the diet, consistent with some dysfunction in uptake. It is not clear what the role of these lipids might be but if they are somehow directly associated with, or important for, the observed lifespan extension, adding them back to the medium should revert the o/e animals to a gas-1 lifespan.

Page 8. The authors arrive at the conclusion that the gas-1 cest-2.2 oe animals extend lifespan because of enhanced lipid catabolism and not because of nutrient transfer to progeny. However I don't draw the same conclusion. I interpret this finding to suggest that somehow this lifespan extension requires vit-1. This never seems to figure into the various models that are proposed by the authors. Could it be that nutrient uptake is somehow compromised in these animals and this affects their lifespan?

fat-5 has only a few non-redundant roles in lipid biosynthesis, so if fat-5 RNAi suppresses the lifespan extension of these animals it has to involve the fat-5 delta 9 products, which are often found in membrane lipids. Once again, this is more consistent with signalling as opposed to energy mobilization.

If the lipids are critical for energy regeneration it should manifest as an increase in respiration, which is measurable. Do the animals respire more? Do they generate more heat? If the observed FAO required to extend the lifespan of these animals is due to increased generation of energy (which for whatever reason does not impact the overall levels of the lipids by TLC), it might be detectable by calorimetry. If the overexpression of cest-2.2 generates more reducing equivalents that might be limiting in the gas-1 mutants perhaps it will make various reactions more efficient without necessarily increasing respiration or energy/heat production.

Because the authors suggest that mdh RNAi works through the germ line can they rule out that cest-2.2 doesn't also work via the germ line? Just looking at the ORO stained animals the gonads of the o/e animals do not look quite normal.

The discussion is particularly long-winded and tends to overinterpret many of the observations made by the authors. Very few of the conclusions that the authors arrive at are strongly supported by their data, at least not enough to suggest any kind of single pathway that affects the lifespan of these animals. Stored lipid mobilization for example is blatantly incorrect and there seems to be no effect on the stored lipid levels. The TAGs are nearly identical! Overall, the lifespan extension by overexpressing this gene product is an interesting observation that is hard to explain based on the data presented here, and I am at odds with at least some of the conclusions that the authors make about readjusting energy stores and lipid mobilization.

In addition, that these animals exhibit any kind of benefit from these transgenic manipulations is once again an overinterpretation. Most of the known lifespan extending paradigms do just that, but they are nevertheless mutations that are selected against in the wild type population. This would indicate that they are NOT beneficial and DO NOT provide any kind of fitness advantage. Let's stick to the facts-these changes extend lifespan, but are not necessarily (and mostly not at all...) beneficial to the animal.

Figures and Legends
Figure 2B introduce a space Time (days) on the label of the X-axis. In H locomotor activity is quantified as well activity. What is well activity? How does this define numerically?

Figure 3H does not seem to have an associated heat map or at least I cannot understand which genes change in which direction. Perhaps this can be made more explicit.

Figure 5. The model that is shown adds little and is more confusing than clarifying. The first point, the TAGs, do not change in these animals, making everything else questionable and inaccurate.

Minor points

In the abstract...not "in" long-lived but rather "into"
Also "significantly determines longevity..." Would read better as "extends lifespan"

The rationale for choosing to examine cest-22 before the other candidates is a bit unclear. The DUF has 98% homology with its human counterpart if that is an important criterion for characterising a candidate. What is the major rationale for characterizing a carboxyesterase?

Page 5 More explanation for the rationale for using the HIS-72::GFP strain should be (ie...why not H3.3?) for those who are not aware of the H3.3 study previously performed by this group.

Page 6 The use of appropriate C. elegans genetic terminology should be used throughout the manuscript. Silencing can mean a lot of things RNAi means post-transcriptional silencing using dsRNA. Please use the term where and when necessary. Similarly, KO means nothing. If you have generated a CRISPR mediated null allele then show that it is null and use an appropriate allele designation.

Page 6. How can you begin to compare lifespans if you are not using the exact same conditions. Use the same bacterial strains throughout your work or you may be quantifying the effects of different qualities of bacterial strains. HT115 and OP50 have very different nutrient compositions, especially in terms of lipids.

Page 7 please define "enhanced spare respiratory capacity". I am not aware of this term.

Page 8, first paragraph-Did not...in neither gonads nor oocytes is awkward and should be corrected

Page 13 second to last paragraph "refills" would read better as "replenishes" or "regenerates"

Referee #3:

The study by Piazzesi et al. reveals the carboxylexterase CEST-2.2 to be involved in reversing the shortened lifespan of mitochondrial gas-1 mutant nematodes. The findings of CEST-2.2 regulating lipid catabolism in gas-1 mutants to extend lifespan is interesting and relevant. The authors have meticulously used complementary approaches to provide evidence for CEST-2.2's role in fatty acid oxidation and subsequent depletion of lipid stores that are sufficient to abrogate lifespan deficits in unhealthy gas-1 mutants. Although the authors demonstrate benefits on mitochondrial fragmentation and respiration rates, there is no mechanistic explanation about how mitochondrial defects are abrogated. Whether CEST-2.2 specifically contributes to mitochondrial rescue (maybe via lipid signaling) or simply extends lifespan in other short-lived mutants needs to be teased apart. Importantly, almost every lifespan analyses have off-the-chart levels of censoring, which is not highlighted as a major issue for

interpretation. Revisions are suggested below.

Specific Concerns:
1. CEST-2.2 did not have the strongest opposing signal for long- and short-lived mitochondrial mutants for H3.3 ChIP, yet it was selected for further studies owing to its involvement in metabolism. Is there a stronger rationale or previous reports related to mitochondrial function to select this gene? (E.g. Xu et al., 2021 Am J Physiol Gastrointest Liver Physiol).
2. To have a better understanding of the requirement of CEST-2.2 for Complex I-related lifespan changes, survival analysis with cest-2.2 KD in gas-1 mutants and cest-2.2 OE in nuo-6 to check for additive effects or epistasis would be informative. Especially since cest-2.2 mRNA levels are oppositely regulated in these mutants.
3. It would be interesting to note whether transcription of yolk receptors is altered in gas-1 mutants and gas-1;cest-2.2 OE strain and if that could contribute to the lack of gonadal lipid transfer.
4. On page 8, vitellogenins are bona fide lipid transporters (not "have lipid transporter activity") and their function is to carry lipids from the intestine to the progeny. The link to starvation in Halvard et al. 2017 is related to its impact on hlh-30 mutant survival during starvation. Please revise.
5. Increased lipid stores have been previously demonstrated in long-lived mutants such as daf-2 and glp-1 (O'Rourke et al., 2009 Cell Metab, Lapierre et al., 2013 Autophagy). It would be helpful to know the lipid accumulation status of nuo-6 worms and the effect of cest-2.2 KD on lipid content on these worms and how that correlates with lifespan to get a better understanding of lipid metabolism in mitochondrial-mediated longevity.
6. Since H3.3 has been used as an indicator of active transcriptional changes between gas-1 and nuo-6, the authors could comment on whether their later RNA seq DEGs (lipid metabolism) also had altered occupancy of H3.3 in gas-1 vs nuo-6. That would also give insights into whether a similar cest-2.2-mediated lipid catabolism is key in the long lifespan in nuo-6 or if the effects are additive.
7. Significant changes in median or mean lifespan can be indicated on the survival curves. Additional bar graphs for median lifespan seem unnecessary. Why has t-test or ANOVA been used for survival analysis and not Mantel-Cox log rank test?
8. The level of censoring across all lifespan analyses needs to be addressed, these numbers are unbelievably high.
9. What are the CEST-2.2 protein levels in the OE+KO strain (Fig. 2F). Is survival expected to be similar to only gas-1 mutants?
10. Are the respiration rates of gas-1;cest-2.2 OE similar to long-lived nuo-6 mutants? Is there a speculation about the link between partial restoration of respiration levels and lifespan? Having nuo-6 for comparison here would be useful.
11. The authors provide evidence for lipid oxidation as a mechanism for CEST-2.2-mediated rescue of mitochondrial mutant lifespan. However, is not clear to the reader how CEST-2.2 contributes to mitochondrial fragmentation, function (partial restoration of respiration), and how it counteracts mitochondrial lesions. Is the feeding of carbon into the TCA cycle from lipid breakdown beneficial only in mutants deficient in ETC? Is cest-2.2 mRNA downregulated in other short-lived mutants?
12. Could lipid breakdown contribute to enhanced lipid signaling as a general mechanism for lifespan extension?
13. Does blocking lipid catabolism enzymes in gas-1;cest-2.2 OE worms abrogate the restored respiration as well? (In addition to partial reversal of lifespan)
14. The heatmap seems to be missing a color scale in Fig. 3H
15. Please also correct grammatical errors in the manuscript

Reviewers' comments and point-by-point response

We would like to thank the Editor and the reviewers for taking the time to consider our manuscript. We are pleased to see that the reviewers thoroughly examined our study and found it of interest. We have carefully considered all the 68 points that the reviewers raised during their revision. We have done our best to accommodate all the reviewer's curiosities, although some of them went beyond the original scope of our study, for which we chose a format reporting "*the physiological and/or functional significance of a finding, rather than the mechanistic detail*". We hope that the three reviewers find our conclusions sufficiently strengthened by the additional experimental evidence that we have herein provided after 8 months of work.
Below, the Editor and the reviewers can find a point-by-point response to their questions.

Referee #1:

In this manuscript, Piazzesi and colleagues investigate whether enhanced metabolic flexibility is sufficient to modulate ageing in nematodes exhibiting bioenergetic abnormalities. More specifically, they assessed the epigenetic landscapes of two models of mitochondrial complex I deficiencies that differently affected *C. elegans* lifespan. To this end, they examined whether histone H3.3 variant, which was previously shown to impair the establishment of various lifespan extending programs, is differentially deposited into chromatin of long-lived and short-lived mitochondrial mutants. Indeed, by narrowing down the analysis of data collected from chromatin immunoprecipitation (ChIP) followed by deep sequencing and RNA seq analysis, the authors found that H3.3 was differentially loaded on the promoters of genes in long-lived and short-lived mitochondrial mutants. Indeed, H3.3 was enriched at the promoters of 179 genes in *nuo-6(qm200)* long-lived mutants. Analysis of RNA seq datasheets from these mutants identified 10 out of 179 genes that are consistently upregulated in *nuo-6(qm200)* long-lived animals while downregulated in *gas-1(fc21)* short-lived mutants. Further analysis was focused on the phylogenetically conserved *cest-2.2* that encodes a carboxylesterase. Knockdown or knockout of *cest-2.2* abrogates the extended lifespan of animals subjected to *cco-1* RNAi. By contrast, *cest-2.2* overexpression extends the lifespan of *gas-1(fc21)* mutants, improves their healthspan by rescuing locomotory defects and ameliorates mitochondrial fragmentation. Next generation RNA seq analysis in intestines isolated from *gas-1(fc21)* and *gas-1(fc21); cest-2.2 O/E* animals identified 354 dysregulated lipid metabolism genes. In particular, genes involved in fatty acid oxidation were downregulated in *gas-1(fc21)* animals compared to *gas-1(fc21); cest-2.2 O/E* animals. The longevity of the latter strain was partially reduced by knocking down the yolk protein gene *vit-1*, which is considered to have lipid transporter activity. Moreover, the composition of free fatty acids (FFA) was different between *gas-1(fc21)* animals and *gas-1(fc21); cest-2.2 O/E* animals, although their total lipid content was comparable. It was also shown that depletion of nuclear hormone receptors NHR-49 or NHR-80 shortens the lifespan of *gas-1(fc21); cest-2.2 O/E* animals. Downregulation of genes involved in lipid peroxidation in mitochondria such as *acs-2* and *ech-1* or in peroxisomes such as *acox-1* resulted in partial and significant reduction, respectively of lifespan in *gas-1(fc21); cest-2.2 O/E*. On the other hand, knockdown of *pck-1* did not affect the lifespan of these animals whereas mdh-2 deficiency reduced the lifespan of *gas-1(fc21); cest-2.2 O/E* while it enhanced the longevity of *gas-1(fc21)* mutants. Altogether, these findings suggest that fatty acid catabolism mediate the beneficial effects of *cest-2.2* overexpression on lifespan of certain mitochondrial mutants.

1. One of the key objectives of this study is to show the importance of the carboxylesterase CEST-2.2 in determining the survival threshold of complex I deficient nematodes through epigenetic changes that confer metabolic flexibility. However, despite the authors claim, CEST-2.2 depletion appears to adversely affect the lifespan of wild-type animals as well as long-lived mitochondrial mutants. Indeed, *cest-2.2* knockdown significantly shortens the lifespan of wild type worms, while only slightly influences the lifespan of *nuo-6(qm200)* mutants. The same is true for the *cest-2.2* null mutants which showed significantly reduced lifespan (P=0.0007) compared to wild type animals. Together, these findings suggest that CEST-2.2 depletion is detrimental in both wild type and mutant backgrounds.

We adjusted the text as following: *cest-2.2* deficiency inhibited *nuo-6(qm200)* longevity (Figure 1F-G and Appendix Table S1), while it slightly reduced the lifespan of wt nematodes (Figure 1H-I and Appendix Table S1). To further explore CEST-2.2 contribution to survival, we ablated the *cest-2.2* encoding region using CRISPR/Cas9-based gene editing (Figure EV1C). We found that *cest-2.2 loss-*

of-function (lf) mutants had a minor tendency toward a decreased median lifespan compared to wt animals.
For a comprehensive and transparent overview of our datasets, we are happy to provide each individual lifespan assay as well as statistical analyses of median lifespans.
The reviewer is surely aware that many genes have an effect in wt survival, as well as in long-lived mutants. Two examples:

(a) *daf-16* loss-of-function alters wt lifespan as well as the longevity of hypomorphic *daf-2* mutants;
(b) *atfs-1* deficient nematodes live less than wt animals. Similarly, *atfs-1* loss-of-function impairs the longevity of mitochondrial mutants.

Thus, even if there is a confounding effect in wt background, it does not mean that CEST-2.2 activity is not relevant in mitochondrial mutant nematodes.

2. The authors should examine whether their findings are relevant to other mitochondrial mutants or are mostly specific to complex I deficient mutants.

We appreciate the reviewer's comment and performed additional experiments using long-lived *sod-2(ok1030)*, *isp-1(qm150)* and *clk-1(e2519)* mutants as well as short-lived *mev-1(kn1)* mutant nematodes. While *cest-2.2 (lf)* inhibited the survival of *cco-1* deficient nematodes (Figure EV1D), *cest-2.2* downregulation did not alter the lifespan of *sod-2(ok1030)*, *isp-1(qm150)* (Figure EV1E-F) and *clk-1* mutants (Supplementary figure R1A-B). Furthermore, we found that CEST-2.2 O/E extended *mev-1(kn1)* survival of a few days (Figure EV1L). Despite 8 months of efforts, we could not obtain homozygous *nuo-6(qm200); cest-2.2 (lf)*, while we could easily generate other mitochondrial mutants lacking CEST-2.2 (see below). Together, these data underlie the peculiar contribution of CEST-2.2 to the survival of complex I deficient nematodes. Following the relevant comment of the reviewer and the evidence obtained during the revision, we toned down our claims and adjusted the text accordingly.

3. The authors state that CEST-2.2 O/E transgene ameliorates the aberrant mitochondrial fragmentation in *gas-1* mutant animals and provide some images to support this statement. To strengthen their findings, they could provide a quantification of fragmented versus healthy tubular mitochondria as well as measurements of ATP production and mitochondrial membrane potential as evidenced either by direct measurements of this parameter of indirectly by TMRE staining.

As the strains in question express mitochondrially-targeted GFP in the gut, we found that the natural autofluorescence in the gut provided enough background to make these kinds of quantifications trickier than usual and open to a certain amount of bias. In order to accommodate the reviewer's request, we generated *gas-1; cest-2.2 O/E* animals expressing mitochondrially-targeted GFP in the muscle and assessed mitochondrial morphology. Remarkably, we found that CEST-2.2 O/E counteracted mitochondrial fragmentation in the body wall muscle cells of *gas-1(fc21)* mutants (Figure 2I). Thus, our assessment goes beyond the reviewer's request and demonstrates the cell-nonautonomous contribution of CEST-2.2 to mitochondrial homeostasis in *gas-1(fc21)* mutant animals.

We have already provided evidence that CEST-2.2 O/E stimulate mitochondrial respiration by using a Seahorse XF24 Analyzer (Figure 2A-B). Respectfully, we do not believe that TMRE staining would be helpful in this context. Our scientific rationale is as follows:

(a) Based on our experience and that of others, there is no reliable method for measuring mitochondrial membrane potential *in vivo* in *C. elegans*. TMRE staining has been used as a method for staining mitochondria rather than for measuring mitochondrial membrane potential (Sarasija and Norman, 2018).
(b) We and other have described the limitations and the technical requirement for proper TMRE measurements (Connolly et al., 2018). In *C. elegans*, TMRE staining has been performed with limited success, because current methodologies do not allow for the necessary dissipation of TMRE staining with either FCCP or other mitochondrial toxins.
(c) Other fluorescent-based methods for measuring mitochondrial membrane potential (Gaskova et al., 2007) provide weak staining limited to the bulb of the pharynx.

Because of these arguments, we did not feel that attempting to measure mitochondrial membrane potential in the bulb of the pharynx will be sufficient to fully characterize the effect of *cest-2.2 O/E* on mitochondrial membrane potential.

As requested by the reviewer, we measured ATP levels in *gas-1* and *gas-1; cest-2.2 O/E* animals using commercially available kits (Figure 2D).

4. The authors state that *cest-2.2* null mutation inhibited the lifespan extension due to *cco-1* RNAi. First, were CRISPR-Cas9-generated mutant worms outcrossed for several generations to eliminate off target effects before being used to experiments?

The *cest-2.2(lf)* strain used in this manuscript was outcrossed twice.

Second, Figure EV1D (former 1O) shows that *cest-2.2* RNAi shortens the increased lifespan of wild type animals subjected to *cco-1 RNAi*, suggesting that CEST-2.2 is required for the lifespan extension conferred by *cco-1* knockdown in wild type background. On the other hand, *cco-1* RNAi significantly increases the lifespan of *cest-2.2* mutants (Figure EV1D and Table S1). These results are somehow confusing and need further investigation. Please, explain which strains /treatments were compared in Figure EV1D. In other words, the different P values (asterisks) to which comparisons correspond. This information should be included in Figure legend.

In EV1D (former 1O), we did not use *cest-2.2* RNAi. The experiment was a comparative lifespan analysis of wt and *cest-2.2(lf)* on control (solid lines) and *cco-1* RNAi (dotted lines). The representative figure as well as the summary lifespan Appendix Table S1 showed that

- wt nematodes grown on *cco-1* RNAi lived longer than controls. Consistent with previous evidence (Dillin et al., 2002), these data prove that our RNAi treatment had an effect on *C. elegans* survival;
- *cest-2.2(lf)* inhibited the lifespan extension induced by *cco-1* RNAi, indicating that CEST-2.2 expression is required to support the lifespan-extending properties of CCO-1 deficiency.

Since we almost doubled the number of panels during the revision, we removed former Figure 1P. However, data and statistics relative to our lifespan assays are comprehensively reported in Appendix Table S1 and are available to the readership.

5. While it is apparent that *cest-2.2* predominantly affects the lifespan of mitochondrial mutants, it is not obvious whether/how *cest-2.2* itself affects mitochondrial homeostasis or function per se. Towards this direction, the authors could assess OCR, mitochondrial morphology and other functional mitochondrial parameters in wild-type, *cest-2.2* knockout and *cest-2.2 O/E* animals.

Following the reviewer's concerns, we performed Seahorse experiments in wt, *cest-2.2 O/E* and *cest-2.2 (lf)* (Figure 2G). Furthermore, we assessed locomotor activity in wt, *cest-2.2 O/E* and *cest-2.2 (lf)* mutants, as an indirect measure of mitochondrial function (Figure EV1N). Our data indicate that *cest-2.2 O/E* and *cest-2.2 (lf)* had limited effects on mitochondrial respiration and behavior performance compared to controls.

6. Additionally, activation of mitochondrial unfolded protein response (UPR^{mt}) is often indispensable for the extended lifespan of mitochondrial mutants (Durieux and Dillin, Cell, 2011). Does *cest-2.2* overexpression employ UPR^{mt} to extend lifespan?

We thank the reviewer to point this important aspect associated with mitochondrial dysfunction and longevity. To address this request, we generated *gas-1(fc21)* and *gas-1(fc21); cest-2.2 O/E* animals carrying the *zcIs13[hsp-6p::gfp]* transgene, a widely used marker of UPR^{mt} (Benedetti et al., 2006). Surprisingly, we found that *zcIs13[hsp-6p::gfp]* was highly induced by CEST-2.2 O/E (Figure 2J). Since bZip transcription factor ATFS-1 is the major regulator of UPR^{mt} (Nargund et al., 2012), we generated *unc-119(ed3)III; atfs-1(tm4525)V; gas-1(fc21)X; cest-2.2 O/E* animals. When we set up lifespan assays, we immediately noticed that these animals were relatively sick and many underwent bagging. Because of that and the time constrain due to almost 8 months of revision, we could not provide reliable lifespan analyses of these mutants. Nevertheless, we were able to run some Seahorse experiments and showed that ATFS-1 loss did not influence mitochondrial respiration of

gas-1(fc21); cest-2.2 O/E animals (Figure 2K-L). These data indicate that ATFS-1 is engaged and may contribute to lifespan of *gas-1(fc21); cest-2.2 O/E* animals, although it does not influence OCR.

7. In Fig. 3A, an adult animal expressing a *cest-2.2p::GFP* extrachromosomal array is shown. The age of the animal should be defined. Also, a non-transgenic age-matched control should be included in the Figure in order to confirm that the green signal is not due to autofluorescence.

We included an age-matched negative control as per the reviewer's request. The age of the animals is indicated in the caption.

8. Based on the finding that *vit-1* knockdown partially reduced the lifespan of *gas-1(fc21); cest-2.2 O/E* animals, the authors propose that these animals have less lipid droplets because of enhanced lipid catabolism rather than as a consequence of a preferential nutrient transferring to the developing progeny. It is not clear how this conclusion is drawn from the above mentioned data.

It is known that lipid-rich particles are used to synthesise yolk particles in the intestine that are then transferred to developing oocytes (Perez and Lehner, 2019, Kimble and Sharrock, 1983, Chen et al., 2020). However, *gas-1(fc21); cest-2.2 O/E* and *gas-1(fc21)* had a comparable ORO staining in gonads and eggs (Figure EV4A), which rules out an increased lipid transfer to produce viable progeny. Since vitellogenin proteins have lipid transporter activity and mobilize lipids during starvation (Harvald et al., 2017), we further explored this biological aspect and assessed genes associated with yolk production. We found that vitellogenin genes were not differentially regulated in *gas-1(fc21); cest-2.2 O/E* mutants compared to *gas-1(fc21)* (Figure EV4B). Moreover, while *vit-1* downregulation slightly extended *C. elegans* lifespan (Figure EV4C and Appendix Table S1) as previously described (Seah et al., 2016), *vit-1* deficiency did not alter the survival of *gas-1(fc21)* nematodes and consistently reduced the lifespan extension of *gas-1(fc21); cest-2.2 O/E* mutants (Figure EV4C and Appendix Table S1). Together, these data support the hypothesis that *gas-1(fc21); cest-2.2 O/E* animals have less lipid stores because of enhanced lipid catabolism (see Figure 3 and EV3), rather than as a consequence of a preferential nutrient transferring to the developing progeny.
We hope that the reviewer agrees with our conclusions, which should be sufficiently supported by our previous and new experimental evidence.

9. The authors show that *cest-2.2* overexpression is sufficient to extend lifespan of *gas-1* mitochondrial mutants, which are defective for the ETC complex I. Can *cest-2.2* overexpression extend the lifespan of *mev-1* mutants, which are also inherently short-lived due to ETC complex II deficiency? Moreover, how does *cest-2.2* silencing affects the lifespan of long-lived *isp-1* mutants?

We addressed these questions in point 2.

10. The finding that *cest-2.2* overexpression extends the lifespan of short-lived *gas-1* mutants, while it does not affect the lifespan of wt animals should be adequately explained. Is lipid catabolism induced in wild-type animals upon *cest-2.2 O/E*?

As discussed in our manuscript, because wt animals lay significantly more eggs than *gas-1(fc21)* and *gas-1(fc21); cest-2.2 O/E* (Figure 1S), we excluded them from further lipid analysis to avoid the confounding effect of fat stores in the developing embryos. As this question cannot be answered with the current dataset, we would have needed additional NGS and lipidomics, which are time-consuming and budgetary demanding. From the scientific standpoint, while we agree that an in-depth characterization of CEST-2.2 function could be of interest to the scientific community, we do not believe that this is pertinent to our key biological question: how does *cest-2.2* overexpression counteracts mitochondrial dysfunction? We tried our best to address as many curiosities as possible, however we focused on those that were necessary to strength our story. We hope that the reviewer understands the limitation of a small laboratory.

Are free fatty acid pools exhausted, similarly to *gas-1* mutants? If *cest-2.2* overexpression is accompanied by similar metabolic changes, why don't wt animals live longer?

We performed both ORO and LipidTOX Green stainings in wild type, *cest-2.2 O/E* and *cest-2.2 (lf)* mutants in order to provide more information regarding the role of *cest-2.2* on lipid content. While the mean intensity of ORO staining was not significantly increased in *C. elegans* carrying *cest-2.2 (lf)*

alleles, many animals did present with large, mislocalized lipid droplets (Figure EV3H), as well as having a slight yet significantly larger body size when compared to wt worms (Figure EV1M). To further support our lines of evidence, we stained nematodes with the neutral lipid stain LipidTOX Green (Klapper et al., 2011) and obtained that *cest-2.2 O/E* animals had a tendency toward less lipids as shown by the ORO staining (Figure 3G-H, EV3J). Of note, *cest-2.2 (lf)* exhibited more LipidTOX Green staining compared to wt (Figure EV3J). Together, these results indicate that CEST-2.2 O/E can influence lipid deposits in both wt and complex I deficient nematodes.
Regarding the survival of *cest-2.2 O/E* animals compared to wt, we provide individual lifespan assays in Appendix Table S1. We show that wt nematodes have a median lifespan of 26.3±0.4 days, whereas *cest-2.2 O/E* animals live on average 28.0±0.7 days.

11. Figures denoted in the legend of former Fig. S2, are missing. More specifically, lifespan curves of *gas-1(fc21)* and *gas-1(fc21); cest-2.2 O/E* animals are not shown although mentioned in the legend. Regarding this point, it would be helpful to include survival curves of wild type animals upon *acox-1* RNAi or *mdh-2* RNAi in the curves of mutant worms presented in former Fig. 5H and J, respectively for better comparison.

In the submitted revised version, we implemented text and included the missing details.

12. The authors claim that CEST-2.2 enhances longevity in *gas-1(fc21); cest-2.2 O/E* animals by refilling intermediates through oxaloacetate production, ultimately buffering the deleterious loss of complex 1 activity. To support this statement, they can test whether exogenous supplementation of oxaloacetate increases the lifespan of *gas-1(fc21)* mutants.

Following the reviewer's comment, we assessed mitochondrial respiration in *gas-1* mutants fed with oxaloacetate. We found that oxalacetate supplementation was sufficient to potentiate mitochondrial respiration of *gas-1(fc21)* animals (Figure 5F-G), whereas it slightly altered the maximal respiration of wt nematodes (EV5D-E)

13. The authors state that "CEST-2.2 defines the survival threshold of complex I deficient nematodes, since it balances potentially deleterious mitochondrial lesions by engaging catabolic pathways that enable the usage of alternative resources to generate energy and sustain longevity". Indeed, a possible increase in beta-oxidation which feeds into the TCA cycle could increase oxygen consumption rate as well as ATP levels of *gas-1; cest-2.2* OE strain compared to *gas-1* mutants. In former figure 2J-L, maximum respiration is indeed increased in *cest-2.2 O/E* animals. However, basal OCR is not affected, which suggests that the basal supply of ETC is not affected upon *cest-2.2 O/E*.

That is correct and our new Figure 5 explain how CEST-2.2 O/E stimulates mitochondrial respiratory flexibility. In this regard, it is assumed that mitochondrial respiratory capacity is a phenomenon linked to fatty acid beta-oxidation and to an enhanced efficiency in NADH and $FADH_2$ oxidation, which may be in part due to the ETC forming higher-order respiratory supercomplexes (Calvo et al., 2020, van der Windt et al., 2012, Brand and Nicholls, 2011, Bertan et al., 2021). To gain further biological understanding of the enhanced mitochondrial respiratory capacity exhibited by *gas-1(fc21); cest-2.2 O/E*, we set up additional Seahorse experiments and explored the contribution of complex II, knowing that acetyl-CoA is condensed with oxaloacetate to be then oxidized via the TCA cycle. We found that *mev-1* downregulation significantly diminished the mitochondrial respiratory capacity in wt, *gas-1(fc21)* and *gas-1(fc21); cest-2.2 O/E* animals (Figure 5I-J, EV5H-I), further highlighting the importance of complex II in the flexibility of mitochondrial bioenergetics (van der Windt et al., 2012, Brand and Nicholls, 2011). Next, we assessed the importance of transfer flavoprotein (ETF) and electron transfer flavoprotein dehydrogenase (ETFDH), which functionally couple fatty acid beta-oxidation to the ETC by transferring electrons from $FADH_2$ to coenzyme Q (Eaton, 2002, Wang et al., 2019). While downregulation of LET-721/ETFDH did not alter the Seahorse profile of *gas-1(fc21)* mutants, it significantly reduced the flexibility of mitochondrial respiration in *gas-1(fc21); cest-2.2 O/E* nematodes (Figure 5K-N). Taken together, our data suggest that CEST-2.2 stimulates mitochondrial bioenergetics via complex II and LET-721/ETFDH.

What are the ATP levels of control, *gas-1* and *gas-1; cest-2.2 O/E* animals?

Following the reviewer's request, we sought to measure ATP levels in *C. elegans* homogenates. We found that the increased respiratory capacity correlated with a clear trend toward higher ATP levels in *gas-1; cest-2.2 O/E* animals compared to *gas-1* mutants (Figure 2D).

14. Fatty acid levels are the net result of fatty acid dietary intake, de novo fatty acid synthesis and fatty acid catabolism. The authors believe that *cest-2.2 O/E* leads to increased FAO, based on prediction from RNA seq data. To verify whether this is indeed the case the authors could either measure FAO upon *cest-2.2 O/E* directly (PMID: 24862277) or try increasing FAO by other means and test whether it can still rescue *gas-1* short lifespan (i.e PMID: 32597196).

We claim that *cest-2.2 O/E* leads to increased fatty acid beta-oxidation because of our RNA sequence data, ORO staining, targeted lipidomics and epistatic analyses. Together, these four complementary approaches point toward a differentially regulated fatty acid beta-oxidation.

Biochemical analysis in *C. elegans* is notoriously tricky and unfortunately one of the biggest drawbacks of this model. We (i.e., Frank Schroeder) attempted to run a full metabolomics in wt, *gas-1(fc21)* and *gas-1(fc21); cest-2.2 O/E* animals using established protocols in his laboratory. Despite being one of leading scientists in the field, he could not grow enough material for this experiment. Although we understand the suggestion of the reviewer, we regret to be not in the position to accommodate the reviewer's request, because we have already attempted to run this assessment.
Furthermore, available fluorescent-based kits to assess fatty acid beta-oxidation are only validated for cells and not for tissue homogenates, let alone for *C. elegans*. Given these drawbacks, we regretfully believe that there is no way to address the reviewer's comment within a reasonable timeframe.

As for stimulating fatty acid beta-oxidation in *gas-1* mutants, we encounter the same problem as mentioned above. Specifically, to our knowledge there is no validated method for stimulating fatty acid beta-oxidation in *C. elegans.* Following a suggestion by referee #2, we attempted to generate an extrachromosomal array expressing *cest-2.2p::cpt-1::HA::SL2::GFP*, knowing that carnitine palmitoyltransferase critically regulates fatty acid beta-oxidation. While we were able to obtain a few mosaic F1 clearly expressing GFP, we could not retrieve lines with proper germline transmission, despite the multiple attempts. While we appreciate the valuable suggestions of the referees, it is evident that CPT-1 O/E in *C. elegans* remains a big challenge for the field.

15. Do *gas-1* mutants exhibit reduced FAO, which is reversed upon CEST-2.2 O/E?

Pathway Analysis on the transcriptome of wt, *gas-1* and *gas-1; cest-2.2* O/E nematodes predicts that fatty acid beta-oxidation is reduced in *gas-1* mutants and enhanced in *gas-1; cest-2.2 O/E* worms (Figure 3D). As mentioned in the previous comment, we do not have a method for measuring fatty acid beta-oxidation directly, so we cannot confirm this prediction by biochemical assay.

16. Some lifespan results are puzzling. The most prominent example is the purple-colored curve in former figure 2B. Survival drops steeply within a very narrow time frame (between day 28 and day 32), while no death event has been recorded till day 25. I recommend that the authors carefully consider the validity of these lifespan results, so that they are consistent and meaningful.

We performed four independent assays in which we assessed *cest-2.2 O/E* lifespan (Figure 1M and Appendix Table S1). Each biological replicate consisted of 80-108 death events, were consistent with each other, and well exceeded the widely accepted parameters for lifespan analyses in the field. We believe that these lifespan results were reliable and meaningful. We are happy to provide the reviewers with the lifespan curves of the other three biological replicates for further scrutiny (Supplementary figure R1C).

17. In the transcriptomic analysis, shown in former figure 3H, wild type control would also be needed for a more direct association of the differences in the expression profile and a potential role in longevity of the presented genes.

As shown in Figure 3B-D, EV3E and Appendix Table S3, as well as indicated in the text, wt animals were included in the transcriptome analysis. All results are presented as comparisons, first of *gas-1* compared to wt, then as *gas-1; cest-2.2 O/E* compared to *gas-1* worms.

18. Former figure 5 (now figure 4): to my understanding, the contribution of NHR-49 and NHR-80 on the beneficial effects of *cest-2.2 O/E* is not easily deduced by the data, as depletion of each of those key lipid metabolism players severely shortens lifespan of wt animals. In order to conclude that a gene mediates the pro-longevity effects of *cest-2.2 O/E*, ideally it should not affect the lifespan of wt animals. This criterion is only met for *acs-2* gene, which corroborates the proposed hypothesis.

This is a comment that we sometime receive from colleagues outside the field and/or less familiar with epistatic analyses in model organisms (e.g., *C. elegans*, Drosophila, mice). While in an ideal scenario the downregulation of these genes should not have an effect on wt lifespan, that is not the case. As discussed before (see point 1), DAF-16 and ATFS-1 are two excellent examples. If necessary, we can also provide additional examples in *C. elegans* as well as in mice. While we understand the reviewer's comment, an effect on wild type lifespan is generally accepted in the scientific community. We hope that our explanation is sufficient to address the reviewer's concern.

19. Several controls are missing throughout the manuscript. The authors compare wt, *gas-1(fc21)* and *gas-1(fc21); cest-2.2 O/E* animals, without presenting or mentioning the effects of *cest-2.2* overexpression in wt animals' physiology and lipid metabolism. Does CEST-2.2 overexpression result in increased lipid catabolism in wt nematodes similar to *gas-1(fc21)* mutants? Do CEST-2.2 overexpressed animals display reduced lipid droplets compared to wt nematodes?

Our study focuses on mitochondrial dysfunction. The role of CEST-2.2 in the context of ascaroside signaling has been already described elsewhere (Le et al., 2020). As discussed at point 10, we described lipid deposits in *cest-2.2 (lf)* and *cest-2.2 O/E* animals. We noticed that *cest-2.2 O/E* nematodes also had slightly less ORO staining in the hindgut when compared to wt ones (Figure EV3H-I), though the effect was not as pronounced as the effect of CEST-2.2 O/E in the context of complex I deficiency (Figure 3F, EV3F). While the mean intensity of ORO staining was not significantly increased in *C. elegans* carrying *cest-2.2 (lf)* alleles, many animals did present with large, mislocalized lipid droplets (Figure EV3H), as well as having a slight yet significantly larger body size when compared to wt nematodes (Figure EV1M). A further characterization of CEST-2.2 function in wt nematodes may be of interest, however it is a curiosity that may be the central focus of another study.

20. A growing body of evidence suggest the several longevity pathways, including caloric restriction, low insulin signaling and germline ablation among others, converge on lipid catabolism and metabolic rewiring. Does CEST-2.2 depletion or overexpression affect the lifespan of other long-lived mutants, such as *daf-2*, *eat-2* and *glp-1*?

Definitely, the reviewer's questions are relevant in biology, but they are beyond the aim of our investigation. Since our study focuses on mitochondrial threshold effect, future studies will be able to address the role of CEST-2.2 in other lifespan extending programs.

To kindly meet the demands of the reviewer and provide preliminary evidence, we performed 2 independent lifespan assays of *glp-1* mutants fed with control and *cest-2.2* RNAi (Supplementary figure R1D-F). We report that *cest-2.2* deficiency does not affect the lifespan extension of *glp-1* mutants grown at 25 °C. We hope that these results are sufficient to satisfy the reviewer's curiosity. Although these data will be available to the scientific community through the transparent revision process of *EMBO Reports*, it is not our intention to include them in our manuscript because we do not want to dilute its message. Again, we would like to emphasize that we have never claimed a role of CEST-2.2 in other lifespan-extending paradigms.

21. The authors suggest that "The differences in median lifespan between *cest-2.2(lf)* and *cest-2.2* silenced animals are probably due to the confounding effect of the two bacteria strains (i.e., OP50 and HT-115, respectively) used in the two experimental settings". To validate their hypothesis, they should use the OP50-RNAi strain (Xiao et al., Cell Rep 2015), which is available in CGC, and knockdown *cest-2.2* by utilizing the same bacterial background in their assay.

We realize that we did not properly elaborate this concept. All lifespan assays were strictly conducted with their appropriate controls: *cest-2.2(lf)* and wt nematodes were fed with exactly the same OP50 bacteria, while wild type nematodes were fed with HT115 bacteria (empty vector) and compared with

wild type animals grown on HT115 bacteria and expressing dsRNA against *cest-2.2*. The sentence in question was simply included to address any potential concerns regarding the differences in median lifespan across different experimental setups. Following the reviewer's comment, we adjusted the text, so that it is clear that we do not draw any conclusions by comparing animals fed with different bacterial strains. We apologize for the confusion.

22. In Figure EV3C the authors investigate whether supplementation of ascaroside-8 extends the lifespan of *gas-1* mutants. However, they did not observe any significant difference. Have they checked the effects of supplementation with other ascarosides? Is ascr#8 efficiently absorbed by animals? It has been reported in the literature that ascr#2 supplementation extends the median lifespan of wt animals. In those studies, animals were exposed to ascr#2 at the L4 developmental stage, and not from the egg stage (Ludewig, Izrayelit et al. PNAS, 2013). The authors should repeat this experiment by supplementing *gas-1* mutants with ascr#8 at L4, to avoid possible developmental side-effects. They should also use wt animals as controls in former Fig EV3C.

CEST-2.2 contributes to the biosynthesis of ascr#8 and ascr#81 (Le HH et al, 2020). Because of this scientific evidence and the link with CEST-2.2, we tested ascr#8 effect on *gas-1(fc21)* survival and observed no obvious changes. Respectfully, we do not see the rationale to assess other ascarosides, apart from a curiosity toward other signaling processes. Based on his/her comment, we can conclude that the reviewer appreciates our effort to assess various aspects associated with CEST-2.2 activity. However, he/she knows how difficult is to work with ascarosides, how rare they are and how costly such an experiment can be.
Regarding whether the compounds have been absorbed: we can confirm that we followed a well-established protocol from one of the leading scientists (i.e., Frank Schroeder) in the field.

Referee #2:

The manuscript submitted for review by Piazzesi et al. describes a novel approach in identifying gene products that distinguish lifespan extending vs lifespan shortening effects of mitochondrial complex I mutants using an interesting ChIP based strategy as a follow up of a previous study published in *Cell Reports*. One of 5 differentially targeted genes they identified was used to evaluate if its overexpression might be responsible for the different lifespan affecting outcomes of these complex I mutants. The manuscript delineates how a carboxyesterase CEST-2.2 extends the lifespan of *gas-1* mutants while is also required for the lifespan extension of *nuo-6* and another unrelated complex IV mutant (*cco-1*). Although these observations are in and of themselves very interesting, the authors' attempts to understand how the overexpression this enzyme results in the observed lifespan extension take the manuscript into several metabolic analyses that yield very little concrete data that can explain how this enzyme, when overexpressed, actually extends lifespan. The genetic analysis is quite reasonable, leaving the reader with a solid foundation that suggests that *vit-1*, some aspects of fatty acid oxidation, and *nhr-49/80* are likely required for the prolonged lifespan. How these genes function together is not clear from the work however. The authors rely on some systems biology approaches to suggest that many of the observed changes are dependent on alterations to the animals' lipid metabolism, but this is not reflected by the TLC analysis of the various lipid species that are separated using this method. There are VERY few differences, although the authors focus on the very small changes (abundance) in free fatty acids. In fact, there are a few changes probably in phospho or more polar lipids, but that are not identified in the analysis, that are equally changed, perhaps more convincingly than the free fatty acids, the molar difference of which is really minimal. The rest of the work pushes down these avenues that are weak and unsupported leaving the authors with a model to explain all the data that is jumbled, confusing and mostly unsubstantiated-barring the genetic data, which is, in my opinion convincing. I have listed below my major concerns and elaborated on some of the points that I have already brought up. In addition I have pointed out some other minor issues in the text and the figures that are more editorial in nature.

Major points of concern and comments:

23. If an allele is generated it is much more valuable than the RNAi analysis. This data should simply be replaced with similar curves using the CRISPR allele (give it an allele name and do not call it KO). RNAi experiments are notoriously inconsistent and difficult to control. In addition, this would also remove any inconsistencies associated with the differences in food. HT115 and OP50 may have very

different lipid compositions and are likely to contribute quite differently to the TAG levels and the TAG composition. Stay consistent and use only one strain throughout the study.

We agree that loss-of-function alleles are valuable for epistatic studies, that is why we generated a *cest-2.2 null* allele. However, his/her view is not shared by all other colleagues and experts in the field, who consider RNAi also a valuable complementary approach. To avoid a never-ending-discussion, we would recommend to include both sets of data. We kindly ask the reviewer if he/she agrees.

While partial downregulation of genes can provoke inconsistencies, we have clearly demonstrated that *cest-2.2* RNAi results in a very strong and consistent downregulation in all strains and biological replicates. Across experiments, validated *cest-2.2* qRT-PCRs clearly indicate that our RNAi protocol is robust and very consistent.

In the original version, we wrote *cest-2.2 KO* because we felt that KO was easier to follow than the allele name, especially for scientists that are not familiar with *C. elegans* nomenclature. However, we see the reviewer's concern as an appropriate and valid point. Therefore, in the revised version of our ms, *cest-2.2 KO* is now *cest-2.2 (lf)*.

We realize that we did not properly elaborate this concept. All lifespan assays were always strictly conducted with their appropriate controls: *cest-2.2(lf)* and wt nematodes were fed with exactly the same OP50 bacteria, while wild type nematodes were fed with HT115 bacteria (empty vector) and compared with wild type animals grown on HT115 bacteria and expressing dsRNA against *cest-2.2*. The sentence in question was simply included to address any potential concerns regarding the differences in median lifespan across different experimental setups. Following the reviewer's comment, we adjusted the text, so that it is clear that we do not draw any conclusions by comparing animals fed with different bacterial strains. We apologize for the confusion.

24. What is the result of the *nuo-6; cest-2.2* double mutant? Does it suppress the lifespan extension similar to the RNAi treatment?

This is an excellent question. For more than a year, we tried to generate *nuo-6(qm200)I; cest-2.2(bon52)V* double mutants. However, we could not obtain homozygous double mutant animals. Given the relevance of this point, we attempted this experiment again during the revision, however we could obtain only hermaphrodites carrying the two mutant alleles in heterozygosity, further underlying the peculiar contribution of CEST-2.2 to the healthspan of complex I deficient nematodes. We tried crosses at 15 °C as well as to balance *cest-2.2(lf)V* with *nT1[qIs51]IV;V*. Our efforts were futile.

To rule out any possible experimental bias, we tried to recapitulate the toxic effect of *cest-2.2(lf)* in another mitochondrial mutant nematodes that we recently generated. Since *cest-2.2* is on chromosome V, we employed a line carrying a genetic lesion that compromises the expression of mitochondrial calcium uptake protein (MICU-1) (Tang et al., 2020). Based on our unpublished data, *micu-1/Y67H2A.4 (null)V* allele causes mitochondrial dysfunction and germline loss (paper under revision). The loss-of-function allele can be maintained using *nT1[qIs51]IV;V* balancer. Contrary to our previous disappointing experience with *nuo-6* mutants, we easily generated *micu-1(lf)V; cest-2.2(lf)V/ nT1[qIs51]IV;V* and could perform lifespan assay (Supplementary figure R1G-H)[Figure R1G for referees not shown.] . Our data indicate that CEST-2.2 is dispensable for some mitochondrial mutants, whereas it crucially regulates development and survival of *nuo-6(qm200)*. Thus, the reasonable request of the reviewer (i.e., generation of *nuo-6; cest-2.2* double mutants) cannot be addressed because experimentally not feasible.

25. If *cest-2.2* RNAi can suppress the prolonged lifespan of a complex IV mitochondrial specific mutant then is it specific? How does it affect *clk-1* mutants or other non-mitochondrial based long-lived mutants that have been described ie...*eat-5*; *daf-2* or germ line ablated mutants. Does it also affect lifespan in animals treated with weak complex I inhibitors (ie..metformin) or animals treated with compounds that affect other complexes?

We appreciate the reviewer's comment and performed additional experiments using long-lived *sod-2(ok1030)*, *isp-1(qm150)* and *clk-1(e2519)* mutants as well as short-lived *mev-1(kn1)* mutant nematodes. While *cest-2.2 (lf)* inhibited the survival of *cco-1* deficient nematodes (Figure EV1D), *cest-*

2.2 downregulation did not alter the lifespan of *sod-2(ok1030)*, *isp-1(qm150)* (Figure EV1E-F) and *clk-1* mutants (Supplementary figure R1A-B). Furthermore, we found that CEST-2.2 O/E extended *mev-1(kn1)* survival of a few days (Figure EV1L). Despite 8 months of efforts, we could not obtain homozygous *nuo-6(qm200); cest-2.2 (lf)*, while we could easily generate other mitochondrial mutants lacking CEST-2.2 (see above). Together, these data underlie the peculiar contribution of CEST-2.2 to the survival of complex I deficient nematodes. Following the relevant comment of the reviewer and the evidence obtained during the revision, we toned down our claims and adjusted the text accordingly.

To kindly meet the demands of the reviewer and provide preliminary evidence, we performed 2 independent lifespan assays of *glp-1* mutants fed with control and *cest-2.2* RNAi (Supplementary figure R1D-F). We report that *cest-2.2* deficiency does not affect the lifespan extension of *glp-1* mutants grown at 25 °C. We hope that these results are sufficient to satisfy the reviewer's curiosity. We are not going to include these data in our manuscript because we do not want to dilute its message. Again, we would like to emphasize that we have never claimed a role of CEST-2.2 in other lifespan-extending paradigms.

Respectfully, we cannot consider to use metformin as modulator of complex I, because of its pleiotropic effects as largely described in *C. elegans* (Cabreiro et al., 2013, Espada et al., 2020).

26. Does the *cest-2.2 O/E* extend the lifespan of *nuo-6 (lf)*?

We have generated this strain and showed that *cest-2.2 O/E* does not extend *nuo-6(qm200)* lifespan (Figure EV1K and Appendix Table S1).

27. The statement in the final sentence of paragraph 1-page 7 "Collectively, our findings show that CEST-2.2..." might be more accurate by simply stating that CEST-2.2 overexpression is sufficient to suppress the premature lethality and alternatively extend the lifespan of gas-1 mutants since no other complex 1 mutants that cause premature lethality were tested. With an n of 1 it is hard to make a general statement.

Based the data collected during the revision, we agree with the reviewer's comment and adjust the message of our manuscript accordingly.

28. The mitochondrial morphological analysis needs to be quantified. Images of several mitochondria must be subjected to accepted analysis using available algorithms that assess various aspects of mitochondrial states/morphology.

As mentioned earlier, we found that the natural autofluorescence in the gut provided enough background to make these kinds of quantifications trickier than usual and open to a certain amount of bias. In order to accommodate the reviewer's request, we generated *gas-1; cest-2.2 O/E* animals expressing mitochondrially-targeted GFP in the muscle and assessed mitochondrial morphology there (Figure 2I). Remarkably, we found that CEST-2.2 O/E counteracted mitochondrial fragmentation in the body wall muscle cells of *gas-1(fc21)* mutants (Figure 2I). Thus, our assessment goes beyond the reviewer's request and demonstrates the cell-nonautonomous contribution of CEST-2.2 to mitochondrial homeostasis in *gas-1(fc21)* mutant animals.

29. The ORO staining data is strange. Why do the authors focus on a small section of the animal (hind gut) and ignore the rest of the animal?

The reviewer might have overlooked at some of our data. Our ORO staining was quantified in the foregut, hindgut, whole body, gonad and eggs of the animals. Please see Figure 3E-F and EV3F-I. In order to further corroborate our findings, we have also included LipidTOX Green stainings in various stains, which provided results that were relatively comparable to our ORO staining (Figure 3G-H and EV3J-K).

30. The germ line looks strange in these animals-the embryos also look very advanced. Are they long lived because of the effects of *cest-2.2* on the germ line/gonad?

As shown in Figure 1S, *cest-2.2* overexpression had no effect on fertility in *gas-1* mutants. Furthermore, ORO staining in the gonad and eggs is comparable between *gas-1* and *gas-1; cest-2.2*

O/E animals (Figure 3E and EV4A). Finally, transcriptome analysis and lifespan analysis of *gas-1; cest-2.2 O/E* animals fed with *vit-1* RNAi (Figure EV4B-C) all point to *cest-2.2* overexpression affecting lifespan without affecting the germline. We hope that these experiments should be sufficient to address the reviewer's concerns.

31. Getting a better comparative picture of overall neutral lipid levels would be advantageous. In addition, these differences do not correlate with the TLC analysis whatsoever. The levels of all the neutral lipids were virtually indistinguishable by TLC, including the TAGs. The VERY small change in FFA was detectable but really, not convincing, and although composition may be altered the molar differences associated with these changes are unlikely to be significant.

We provided TLC analysis coupled with mass-spec analysis of FFAs, meaning a comprehensive analysis of the different FFAs. While it is true that the sum of all FFAs put together resulted in small differences (Figure 3K-L), mass spec analysis clearly showed that specific species of FFAs were completely absent in *gas-1; cest-2.2 O/E* worms, compared to *gas-1* mutants (Figure 3M). We believe that these changes in the composition of the FFA pool of *gas-1; cest-2.2 O/E* animals is of interest. To further address the reviewer's concern, we have included LipidTOX Green staining, which specifically stains neutral lipids and which also yielded results consistent with our previous conclusions (Figure 3G-H and EV3J-K).

32. If there is a role of lipid metabolism in this phenotype it is not due to gross changes in the lipidome, but potentially some link to an important signalling lipid. It is almost certainly not due to changes in total energy (J) lost or gained due to lipid catabolism or lack thereof. This is consistent with their observations obtained by removing *nhr-49*.

Respectfully, our experimental evidence points toward a different biological scenario. Lipid staining *via* two different methods clearly show alterations in total lipid content in *gas-1; cest-2.2 O/E* worms. Characterization of mitochondrial respiration via Seahorse strongly suggest differences in total energy. Epistatic analysis of key metabolic enzymes is also consistent with our hypothesis. Given all of these data, we believe that there is sufficient evidence to support the conclusion that *cest-2.2 O/E* leads to changes in lipid metabolism.
Having said that, we cannot exclude that *cest-2.2 O/E* may also lead to changes in one or a few signaling lipid(s), which might also play a role. Future studies will elucidate this possibility.

33. The alternative hypothesis might include consequent generation of reducing equivalents following FA oxidation. Small changes in the NADH levels might contribute to the suppression of *gas-1* and the observed extension. Can the authors extend lifespan in these mutants by blocking NADH consuming pathways and/or by manipulating an NADH generating system? How do the NAD:NADH ratios compare in these long-lived mutants?

Following the reviewer's question and as agreed with the Editor, we performed a large number of Seahorse experiments (see Figure 2, 5 and EV5). We found that oxaloacetate supplementation to *gas-1(fc21)* was sufficient to potentiate mitochondrial respiration (Figure 5F-G, EV5C-D), whereas feeding of the NAD^+-precursor nicotinamide mononucleotide (NMN) had no effect (Figure EV5E). We were not able to measure NAD^+/NADH ratio directly, as it is very complicated in cells, even more so in a whole organism.

34. Could *cest-2.2 O/E* animals somehow use ROS or generate more ROS to extend lifespan? Have you measured and compared ROS levels between *gas-1* and *gas-1; cest-2.2 O/E* lines?

To address this question, we manipulated ROS in *gas-1* and *gas-1; cest-2.2 O/E* nematodes by exposing to the free radical scavenger ascorbate/vitamin C. Contrary to other long-lived mitochondrial mutants (Yang and Hekimi, 2010), vitamin C did not alter the survival of both wt, *gas-1(fc21)* and *gas-1(fc21); cest-2.2 O/E* nematodes (Figure EV2I).

Following one of the requests of referee #1, we include a thorough analyses of stress response in *gas-1* vs *gas-1; cest-2.2 O/E* animals. Our data suggest that ATFS-1 dependent UPR^{mt} is engaged and may contribute to *gas-1; cest-2.2 O/E* pro-longevity programs (Figure 2J-L and EV2A-I).

35. C14, 15, 19 and C21 FAs are all absent or below the level of detection in the LC/MS analysis.

These appear to be FAs that are normally obtained from the diet, consistent with some dysfunction in uptake. It is not clear what the role of these lipids might be but if they are somehow directly associated with, or important for, the observed lifespan extension, adding them back to the medium should revert the o/e animals to a *gas-1* lifespan.

To be clear: C14, 15, 19 and C21 FAs were only undetectable in our five biological replicates of *gas-1; cest-2.2 OE* animals, while they were perfectly detected and measured in all five biological replicates of *gas-1* mutants. These two strains were always synchronized at the same time, on plates from the same batch, seeded with the same over-day culture of OP50 and collected at the same time. There was therefore absolutely no difference in the medium fed to *gas-1* and *gas-1; cest-2.2 O/E* nematodes. The fact that these FAs were absent only in *gas-1; cest-2.2 O/E* animals therefore points to a difference in their FA metabolism, rather than an absence in the medium or a defect in uptake.

To rule out the concern about food intake, we also performed pharyngeal pumping and observed no difference between *gas-1* and *gas-1; cest-2.2 O/E* nematodes (Figure 1R).

When I was referring to uptake I was thinking more in line of the *vit-1* result, and uptake from the intestine and not from the exterior via pumping. In the end, I think it is very important to reconcile all the results that this group has presented in a unifying model. Namely, there has to be some way to account for a role for *vit-1*, *nhr-49/80*, changes in lipid levels, TCA effects and finally b-oxidation. VIT-1 gets lost in the wash somehow and the authors do not really account for its role in the suppression of the o/e lifespan extension. It may be important for moving lipids around organelles including the droplets as the authors say in their rebuttal. This needs to be teased out or discussed in this model.

It is known that lipid-rich particles are used to synthesise yolk particles in the intestine that are then transferred to developing oocytes (Perez and Lehner, 2019, Kimble and Sharrock, 1983, Chen et al., 2020). However, *gas-1(fc21); cest-2.2 O/E* and *gas-1(fc21)* had a comparable ORO staining in gonads and eggs (Figure EV4A), which rules out an increased lipid transfer to produce viable progeny. Since vitellogenin proteins have lipid transporter activity and mobilize lipids during starvation (Harvald et al., 2017), we further explored this biological aspect and assessed genes associated with yolk production. We found that vitellogenin genes were not differentially regulated in *gas-1(fc21); cest-2.2 O/E* mutants compared to *gas-1(fc21)* (Figure EV4B). Moreover, while *vit-1* downregulation slightly extended *C. elegans* lifespan (Figure EV4C and Appendix Table S1) as previously described (Seah et al., 2016), *vit-1* deficiency did not alter the survival of *gas-1(fc21)* nematodes and consistently reduced the lifespan extension of *gas-1(fc21); cest-2.2 O/E* mutants (Figure EV4C and Appendix Table S1). Together, these data support the hypothesis that *gas-1(fc21); cest-2.2 O/E* animals have less lipid stores because of enhanced lipid catabolism, rather than as a consequence of a preferential nutrient transferring to the developing progeny.

Regarding NHR-49/80, we performed epistatic analyses of animals grown on double strand RNA against either *nhr-49* or *nhr-80*. We found that *nhr-49* downregulation significantly decreased the survival of *gas-1(fc21); cest-2.2 O/E* animals (Figure 4C-E and Appendix Table S1). Downregulation of *nhr-49* did not affect the median lifespan of *gas-1(fc21)* mutants, although it reduced their maximal lifespan (Figure 4E and Appendix Table S1). Consistently, *nhr-80* deficiency also inhibited the survival of *gas-1(fc21); cest-2.2 O/E* nematodes without affecting the median lifespan of *gas-1(fc21)* mutants (Figure 4F and Appendix Table S1). These data suggest that CEST-2.2 extends *gas-1(fc21)* lifespan by engaging NHR-49- and NHR-80-dependent pathways. To better understand the correlation between longevity and mitochondrial bioenergetics, we downregulated *nhr-49* in nematodes and measured OCR. Consistent with its essential role in survival (Figure 4D-E), we found that NHR-49 expression was required for the proper mitochondrial respiratory profiles of wt, *gas-1(fc21)* and *gas-1(fc21); cest-2.2 O/E* animals (Figure 5A-C).

We hope that these lines of evidence are sufficient to address the reviewer's concerns.

36. Page 8. The authors arrive at the conclusion that the *gas-1; cest-2.2 O/E* animals extend lifespan because of enhanced lipid catabolism and not because of nutrient transfer to progeny. However, I don't draw the same conclusion. I interpret this finding to suggest that somehow this lifespan extension requires *vit-1*. This never seems to figure into the various models that are proposed by the authors. Could it be that nutrient uptake is somehow compromised in these animals and this affects their lifespan?

Please, see reply above.

37. FAT-5 has only a few non-redundant roles in lipid biosynthesis, so if *fat-5* RNAi suppresses the lifespan extension of these animals it has to involve the fat-5 delta 9 products, which are often found in membrane lipids. Once again, this is more consistent with signalling as opposed to energy mobilization.

To the best of our understanding, the delta-9 fatty acid desaturase FAT-5 contributes to the biosynthesis of monounsaturated fatty acids (MUFAs) from palmitic acid (16:0) mostly coming from *E. coli* digestion (Watts and Ristow, 2017, Brock et al., 2007, Brock et al., 2006). As the reviewer correctly pointed, FAT-5 may also take part in the synthesis of membrane lipids, while its function in signaling needs to be further elucidated. It was previously shown that, along with FAT-6 and FAT-7, FAT-5 is required for the fatty acid composition and maintenance in C. elegans (Brock et al., 2007). Based on these considerations, our data suggest that loss of FAT-5-dependent fatty acid biosynthesis undermines the lifespan extending properties of CEST-2.2, possibly because of the very fast depletion and/or inefficient mobilization of organismal fatty acid content.

The main point of my concern here has to do with the products of the FAT-5 reactions. If the authors see an effect by eliminating *fat-5* that means they are affecting the production of only a few unsaturated C16 or C18 derivatives. All other reactions that FAT-5 might be involved in are redundantly maintained by other desaturases in *fat-5 lf*. So if the phenotype is dependent on FAT-5 function, there must be some role of these FAT-5 products in the final lifespan extension. Its levels may also be maintained by NHR-49/80.

We apologize with the reviewer, but we have some difficulties to follow his/her line of arguments, which may be extremely relevant. The purpose of the *fat-5* RNAi experiments was based on three considerations: (a) *fat-5* is a target of NHR49/80; (b) *fat-5* is upregulated in *gas-1* vs wt and partially upregulated in *gas-1; cest-2.2 O/E* vs *gas-1* (Figure 3C and 4A); (c) FAT-5 contributes to lipid biosynthesis. Consistent with the other RNAi experiments (i.e., *nhr-49* and *nhr-80* RNAi), we showed that *fat-5* RNAi compromised the survival of *gas-1; cest-2.2 O/E*. As explained above, our interpretation is that fast depletion of organismal fatty acid content undermines the survival of *gas-1; cest-2.2 O/E*. We do not make any claim about C16 or C18 derivatives.

38. If the lipids are critical for energy regeneration it should manifest as an increase in respiration, which is measurable. Do the animals respire more? Do they generate more heat? If the observed FAO required to extend the lifespan of these animals is due to increased generation of energy (which for whatever reason does not impact the overall levels of the lipids by TLC), it might be detectable by calorimetry. If the overexpression of CEST-2.2 generates more reducing equivalents that might be limiting in the *gas-1* mutants perhaps it will make various reactions more efficient without necessarily increasing respiration or energy/heat production.

We performed a series of Seahorse experiments that hopefully address these points. Please, see Figure 2, 5 and EV5D-F.

39. Because the authors suggest that *mdh-2* RNAi works through the germ line, can they rule out that *cest-2.2* doesn't also work via the germ line? Just looking at the ORO-stained animals the gonads of the o/e animals do not look quite normal.

We do not claim that *mdh-2* RNAi affects metabolism through the germline, we merely observe that *mdh-2* completely abrogates the proliferating germline of *gas-1* mutants, while having a lesser effect on the germline of wild-type and *gas-1; cest-2.2 O/E* animals.
Given that our single representative image of a *gas-1; cest-2.2 O/E* mutant seems to be causing the reviewer some concerns, we have provided additional images of age-matched *gas-1* and *gas-1; cest-2.2 O/E* nematodes (Supplementary figure R2A).

40. The discussion is particularly long-winded and tends to overinterpret many of the observations made by the authors. Very few of the conclusions that the authors arrive at are strongly supported by their data, at least not enough to suggest any kind of single pathway that affects the lifespan of these animals. Stored lipid mobilization for example is blatantly incorrect and there seems to be no effect on

the stored lipid levels. The TAGs are nearly identical! Overall, the lifespan extension by overexpressing this gene product is an interesting observation that is hard to explain based on the data presented here, and I am at odds with at least some of the conclusions that the authors make about readjusting energy stores and lipid mobilization.

Our conclusions are based on ORO and LipidTOX Green stainings, TLC coupled to mass spec analysis, epistatic analyses with multiple RNAi and Seahorse profiling of OCR for various mutants. During the revision, we addressed all the experiments requested by the referees and agreed with the Editor.
Following the reviewer comment, we adjusted the Discussion and tone down some of our conclusions. We hope that our efforts are sufficient to convince the referee.

41. In addition, that these animals exhibit any kind of benefit from these transgenic manipulations is once again an overinterpretation. Most of the known lifespan extending paradigms do just that, but they are nevertheless mutations that are selected against in the wild type population. This would indicate that they are NOT beneficial and DO NOT provide any kind of fitness advantage. Let's stick to the facts-these changes extend lifespan, but are not necessarily (and mostly not at all...) beneficial to the animal.

We agree that these are sick animals and would not survive the natural selection in the wild. We modified the text according to the reviewer's input. However, other comments should be carefully toned down, as they may be negatively received by a whole field that has tried to address the contribution of different molecular components in longevity pathways in the context of modeling disease. In this regard, we feel appropriate to emphasize that many of these cascades, although identified in experimental models, have been mechanistically linked to age-related processes across the animal kingdom. We respect the reviewer opinion and agree with some of his/her arguments. However, other personal considerations are more appropriate within a broad discussion on the limitations of animal models in biomedical research, rather than in the context of a manuscript under revision. Otherwise, it becomes a rhetorical statement that goes beyond the scope of the current revision process.

With our work in nematodes, we have been trying to identify mechanisms that can counteract mitochondrial dysfunction. We are not trying to make general statements about which mutations might confer selective advantages or disadvantages to populations of *C. elegans* in the wild. To solve this impasse, we carefully rephrased some of our statements.

Figures and Legends
42. Former figure 2B introduce a space Time (days) on the label of the X-axis. In former 2H (now 1Q), locomotor activity is quantified as well activity. What is well activity? How does this define numerically?

We measure locomotor activity with a WMicrotracker, which in turn measures locomotor activity as an arbitrary unit defined as "well activity" by the developers. We have included this explanation in the materials and methods.

43. Former figure 3H (now 3C) does not seem to have an associated heat map or at least I cannot understand which genes change in which direction. Perhaps this can be made more explicit.

We have added the color bar to the left and clarified the caption.

44. Former figure 5. The model that is shown adds little and is more confusing than clarifying. The first point, the TAGs, do not change in these animals, making everything else questionable and inaccurate.

Former figure 5A was intended to give an overview of how the genes that were targeted by RNAi are connected to each other and to metabolic processes. We apologize if the reviewer found this confusing, but the schematic was not intended to make any biological claims, but rather is simply a reflection of what is known in the literature, and thus cannot be interpreted as inaccurate.
Following the reviewers' comment, we generated three new schemes (Figure 4B, 5D and5O) that will hopefully help to better understand the scientific rationale of our experimental investigation.

Minor points

45. In the abstract...not "in" long-lived but rather "into". Also "significantly determines longevity..." Would read better as "extends lifespan".

Thanks for the comment. We modified the abstract accordingly.

46. The rationale for choosing to examine *cest-2.2* before the other candidates is a bit unclear. The DUF has 98% homology with its human counterpart if that is an important criterion for characterizing a candidate. What is the major rationale for characterizing a carboxyesterase?

The text is a bit confusing and we hope to correctly interpret the reviewer's questions. As written in the manuscript, ZC376.2/CEST-2.2 is a carboxylesterase phylogenetically related to the serine hydrolase family and orthologous to human carboxylesterases and carboxyl ester lipases. Since CEST-2.2 is the most likely to play a role in metabolism, we decided to make this candidate the focus of our study. NDUF-2.2 is a complex I subunit of the electron transport chain, therefore it would not have helped us to understand how metabolic flexibility can contribute to the phenotypic threshold effect observed in invertebrates carrying complex I lesions.

47. Page 5 More explanation for the rationale for using the HIS-72::GFP strain should be (ie...why not H3.3?) for those who are not aware of the H3.3 study previously performed by this group.

We used HIS-72::GFP because the GFP antibody was validated for ChIP. We included this information in materials and methods. Other antibodies would have required time-consuming validation.

48. Page 6 The use of appropriate C. elegans genetic terminology should be used throughout the manuscript. Silencing can mean a lot of things RNAi means post-transcriptional silencing using dsRNA. Please use the term where and when necessary. Similarly, KO means nothing. If you have generated a CRISPR mediated null allele then show that it is null and use an appropriate allele designation.

We have modified the figures and text to *cest-2.2 (lf).*

49. Page 6. How can you begin to compare lifespans if you are not using the exact same conditions. Use the same bacterial strains throughout your work or you may be quantifying the effects of different qualities of bacterial strains. HT115 and OP50 have very different nutrient compositions, especially in terms of lipids.

As we mentioned several times before, we realize that we did not properly elaborate this concept. All lifespan assays were always strictly conducted with their appropriate controls: *cest-2.2(lf)* and wt nematodes were fed with exactly the same OP50 bacteria, while wild type nematodes were fed with HT115 bacteria (empty vector) and compared with wild type animals grown on HT115 bacteria and expressing dsRNA against *cest-2.2*. The sentence in question was simply included to address any potential concerns regarding the differences in median lifespan across different experimental setups. Following the reviewer's comment, we adjusted the text, so that it is clear that we do not draw any conclusions by comparing animals fed with different bacterial strains. We apologize for the confusion.

50. Page 7 please define "enhanced spare respiratory capacity". I am not aware of this term.

At page 12, we wrote the following: it is assumed that mitochondrial respiratory capacity is a phenomenon linked to fatty acid beta-oxidation and to an enhanced efficiency in NADH and $FADH_2$ oxidation.

51. Page 8, first paragraph-Did not...in neither gonads nor oocytes is awkward and should be corrected.

We adjusted the text accordingly.

52. Page 13 second to last paragraph "refills" would read better as "replenishes" or "regenerates".

We adjusted the text accordingly.

53. When the authors examined total ORO levels there was indeed a small but significant difference between *gas-1* and the o/e line. This is presumably the molar difference in total neutral lipid between the two genotypes. I am still not quite sure what the regional analysis of ORO staining contributes to the paper. The TLCs analysis is very complementary because it give a quantitative and qualitative representation of these differences are however great for that because this is quantitatively and qualitatively representative of all the lipid that is present in these samples. There are some minor differences in the neutral lipids including the triacylglycerides, which the authors claim is the source of energy driving the extension. The differences in the PL and other polar entities seem more apparent, although this seems to be ignored. The MS analysis of the FFA indicates some species are completely lacking in the o/e strain. So in the end, the differences in total lipid are not great, but are measurable, and these may include these FFA that appear to be preferentially utilized in the o/e strain. The abundance of the more common FFA remain more or less identical. Therefore, in the end, to recapitulate, based on genetic analysis which I think is sound, the authors claim that b-oxidation is required for this phenotype and presumably to generate energy which is responsible for the extension. They therefore claim that this extra energy then must come from the FFAs and presumably those species that are missing are preferentially utilized in these o/e strains. But, with the exception of perhaps the C21s, these FFA do not seem to correspond to the typical very long chain FFA substrates that CEST-2.2 is stated to utilize (Le et al. elife 2020- from the references). So if there is a preference or some specificity for utilizing these substrates (C14, C15, C19, C21) the authors might need to account for this specificity. If it is simply due to an increase in b-oxidation, perhaps intestine-specific disruption of *pod-2* (acc1/2) might increase *cpt-1* and increase b-oxidation in mitochondria. If this extended the lifespan of gas-1 mutants then that would lend significant support to this model.

Following the reviewer's question, we agreed with the Editor to address this point by generating an extrachromosomal array expressing *cest-2.2p::cpt-1::HA::SL2::GFP*, knowing that carnitine palmitoyltransferase critically regulates fatty acid beta-oxidation. While we were able to obtain a few mosaic F1 clearly expressing GFP, we could not retrieve lines with proper germline transmission, despite the multiple attempts. Despite our effort, it seems that CPT-1 O/E in *C. elegans* remains a big challenge for the field. Through the transparent revision process of *EMBO Reports*, we are happy to inform the scientific community that our plasmid is available upon request.

Referee #3:

The study by Piazzesi et al. reveals the carboxylexterase CEST-2.2 to be involved in reversing the shortened lifespan of mitochondrial *gas-1* mutant nematodes. The findings of CEST-2.2 regulating lipid catabolism in *gas-1* mutants to extend lifespan is interesting and relevant. The authors have meticulously used complementary approaches to provide evidence for CEST-2.2's role in fatty acid oxidation and subsequent depletion of lipid stores that are sufficient to abrogate lifespan deficits in unhealthy *gas-1* mutants. Although the authors demonstrate benefits on mitochondrial fragmentation and respiration rates, there is no mechanistic explanation about how mitochondrial defects are abrogated. Whether CEST-2.2 specifically contributes to mitochondrial rescue (maybe via lipid signaling) or simply extends lifespan in other short-lived mutants needs to be teased apart. Importantly, almost every lifespan analyses have off-the-chart levels of censoring, which is not highlighted as a major issue for interpretation. Revisions are suggested below.

Specific Concerns:

54. CEST-2.2 did not have the strongest opposing signal for long- and short-lived mitochondrial mutants for H3.3 ChIP, yet it was selected for further studies owing to its involvement in metabolism. Is there a stronger rationale or previous reports related to mitochondrial function to select this gene? (E.g. Xu et al., 2021 Am J Physiol Gastrointest Liver Physiol).

We focused on CEST-2.2 because *"ZC376.2/CEST-2.2 is a carboxylesterase phylogenetically related to the serine hydrolase family and orthologous to human carboxylesterases and carboxyl ester lipases. Since CEST-2.2 is the most likely to play a role in metabolism, we decided to make this candidate the focus of our study."* We have emphasized that our focus is on an enzyme that, through

lipid metabolism, can influence the survival of complex I deficient animals, which may lead to the identification of processes relevant in diseases associated with mitochondrial dysfunction.

55. To have a better understanding of the requirement of CEST-2.2 for Complex I-related lifespan changes, survival analysis with *cest-2.2* KD in *gas-1* mutants and *cest-2.2 O/E* in *nuo-6* to check for additive effects or epistasis would be informative. Especially since *cest-2.2* mRNA levels are oppositely regulated in these mutants.

We performed the experiments as suggested by the reviewer (Figure EV1J-K).

56. It would be interesting to note whether transcription of yolk receptors is altered in *gas-1* mutants and *gas-1; cest-2.2 O/E* strain and if that could contribute to the lack of gonadal lipid transfer.

In Figure EV4, we tried to further elucidate these points. We quantified ORO staining in gonads and eggs of wt, *gas-1* and *gas-1; cest-2.2 O/E* mutant animals. Moreover, we measured the expression of *vit-1*, *vit-2*, *vit-3,4,5* and *vit-6* by qRT-PCR. Finally, we performed lifespan assays of animals grown on *vit-1* RNAi expressing bacteria. Together, these data support the hypothesis that *gas-1(fc21); cest-2.2 O/E* animals have less lipid stores because of enhanced lipid catabolism, rather than as a consequence of a preferential nutrient transferring to the developing progeny.

57. On page 8, vitellogenins are bona fide lipid transporters (not "have lipid transporter activity") and their function is to carry lipids from the intestine to the progeny. The link to starvation in Halvard et al. 2017 is related to its impact on *hlh-30* mutant survival during starvation. Please revise.

As mentioned earlier, the fact that *vit-1* RNAi suppressed the lifespan of *gas-1; cest-2.2 O/E* was very surprising, because multiple studies have shown that many different models of longevity usually suppress the expression of vitellogenins and it is actually the overexpression of vitellogenins that normally reduces the lifespan of long-lived mutants. In fact, it is widely established that fertility/fecundity is inversely correlated with longevity, and thus the transport of nutrients by VIT-1 to the developing progeny is detrimental to longevity. Our results in *gas-1; cest-2.2 O/E* worms is essentially the opposite of all other previously reported findings, with *vit-1* RNAi reducing the longevity of *gas-1; cest-2.2 O/E*. However, given that *gas-1; cest-2.2 O/E* worms do not have increased lipid staining in the gonads or eggs (Figure EV4A), nor does *cest-2.2 O/E* increase the number of eggs produced in gas-1 mutants (Figure 1S), we reason that VIT-1 may have lipid transporter activity that is important for *gas-1; cest-2.2 O/E* longevity independently of its role in lipid transfer to developing embryos. We clarified our interpretation of these data in the text.

58. Increased lipid stores have been previously demonstrated in long-lived mutants such as *daf-2* and *glp-1* (O'Rourke et al., 2009 Cell Metab, Lapierre et al., 2013 Autophagy). It would be helpful to know the lipid accumulation status of *nuo-6* worms and the effect of *cest-2.2* KD on lipid content on these worms and how that correlates with lifespan to get a better understanding of lipid metabolism in mitochondrial-mediated longevity.

We stained lipids in *nuo-6* mutants fed with control and *cest-2.2* RNAi (Figure EV3K).

59. Since H3.3 has been used as an indicator of active transcriptional changes between *gas-1* and *nuo-6*, the authors could comment on whether their later RNA seq DEGs (lipid metabolism) also had altered occupancy of H3.3 in *gas-1* vs *nuo-6*. That would also give insights into whether a similar *cest-2.2*-mediated lipid catabolism is key in the long lifespan in *nuo-6* or if the effects are additive.

We have overlaid the genes involved in lipid metabolism (Supplementary Table S2) with the ChIP-Seq data as suggested by the reviewer. Out of the 78 genes found to be involved in lipid metabolism, only 16 showed H3.3 enrichment in their promoter regions (Supplementary figure R2B). Given the very low overlap of genes being detected in both datasets, we do not feel confident to draw any conclusion as to whether or not H3.3 occupancy is correlated with *cest-2.2*-mediated lipid catabolism. Of note, *cest-2.2* could not be identified in our RNA-seq analysis on gut tissue, however we carefully confirmed its down- and upregulation in *gas-1* and *nuo-6,* respectively, via RT-PCR (Figure 1K and Figure EV1B).

60. Significant changes in median or mean lifespan can be indicated on the survival curves. Additional

bar graphs for median lifespan seem unnecessary. Why has t-test or ANOVA been used for survival analysis and not Mantel-Cox log rank test?

Median lifespans+/-SEM across replicates are reported under all lifespan curves. Furthermore, Appendix Table S1 reports the Mantel-Cox log rank tests for each replicate, as generally reported in the field. We have removed bar charts of pooled medians for lifespan analysis in order to avoid further confusion.

61. The level of censoring across all lifespan analyses needs to be addressed, these numbers are unbelievably high.

As we have done in our previous publications, we report all censored subjects, including animals that are censored before death events begin to occur in our lifespan. Given this, our levels of censored subjects are not out of the ordinary, especially for mutants which are prone to bagging, and especially considering that we do not use FUdR in our lifespans. We have explained our criteria for censoring more thoroughly in the materials and methods.

62. What are the CEST-2.2 protein levels in the OE+KO strain (former Fig. 2F). Is survival expected to be similar to only *gas-1* mutants?

We performed qRT-PCR of *cest-2.2* in *cest-2.2 KO; gas-1; cest-2.2 O/E* animals and compared to *gas-1; cest-2.2* mutants (Figure 1E, 1K, EV1G, EV3D). Moreover, we provide quantitative proteomics using two stable isotope-labelled (SIL) peptides, confirming a significant CEST-2.2 upregulation in our newly generated line compared to controls (Figure 1L and Appendix Table S2).

63. Are the respiration rates of *gas-1; cest-2.2 O/E* similar to long-lived *nuo-6* mutants? Is there a speculation about the link between partial restoration of respiration levels and lifespan? Having *nuo-6* for comparison here would be useful.

We have performed Seahorse experiments of wt, *gas-1* and *nuo-6* mutant animals in the past. In short, the respiration profile of *nuo-6* mutants does not resemble that of *gas-1; cest-2.2 OE* animals, as they do not have any spare respiratory capacity because they do not respond to sodium azide. This odd and to our knowledge unique profile opens the door to many questions, both technical and biological, which would need to be explained in the text for those who are not familiar with Seahorse experiments, and which we feared would distract from our main message. In brief, we cannot say if *nuo-6* worms have virtually no mitochondrial respiration, but consume substantial amounts of oxygen in a different manner (e.g., via hyperactive oxidases), or if this strain is somehow more resistant to the penetration of NaN_3 into the animal, for example because of differences in the cuticle. Without a clear response to FCCP and NaN_3, we cannot make any claim about mitochondrial respiration. Since we have no interest and benefit in hiding data, we provided Seahorse profiles of *nuo-6* mutants (Supplementary Figure R2C). However, we suggest to leave these data out of the revised manuscript because they may be misinterpreted and/or misleading.

64. The authors provide evidence for lipid oxidation as a mechanism for CEST-2.2-mediated rescue of mitochondrial mutant lifespan. However, is not clear to the reader how CEST-2.2 contributes to mitochondrial fragmentation, function (partial restoration of respiration), and how it counteracts mitochondrial lesions. Is the feeding of carbon into the TCA cycle from lipid breakdown beneficial only in mutants deficient in ETC? Is *cest-2.2* mRNA downregulated in other short-lived mutants?

Thanks to the comments of the three reviewers, we are able to provide a better interpretation of our data. Based on the new evidence, including Figure 5, we suggest that an increase of lipolysis and/or fatty acid beta-oxidation provides an optimal trade-off between energy production and long-term stress. In support of this scenario, our epistatic analysis demonstrates that downregulation of key enzymes involved in lipid catabolism all either partially or completely ablated the lifespan extension of *gas-1(fc21); cest-2.2* O/E animals. Our current experimental evidence indicates that MDH-2/MDH significantly supports the lifespan extension of *gas-1(fc21); cest-2.2 O/E* animals, while PCK-1/PCK1 contribution is negligible. Thus, it seems plausible that MDH-2/MDH is required to maintain levels of oxalacetate that are compatible with an enhanced TCA cycle, which mediates the oxidation of carbon units deriving from fatty acid beta-oxidation. In support of that, downregulation of complex II subunit MEV-1 as well as LET-721/ETFDH disrupts the flexibility of mitochondrial bioenergetics acquired by

CEST-2.2 overexpression. Based on these lines of experimental evidence, we suggest that the available lipids are directed to generate acetyl-CoA that, upon condensation with oxaloacetate, is oxidized via TCA cycle to generate reducing equivalents that subsequently fed into the ETC through complex II and LET-721/ETFDH, thereby partially overcoming the intrinsic complex I defects. While this CEST-2.2-dependent metabolic rewiring is sufficient for complex I deficient *gas-1* and *nuo-6* mutants, it has a much more modest effect in *cco-1* silenced animals and no consequence in other mitochondrial mutants, such as *sod-2* and *isp-1*. These findings indicate that lipid mobilization may represent a novel intervention for diseases associated with complex I defects, whereas it may be less attractive for genetic lesions affecting other respiratory complexes, further emphasizing the heterogenous nature of disease associated with mitochondrial dysfunction.

As requested by the reviewers, we performed qRT-PCR analyses (n=2) and found that *cest-2.2* expression has the tendency to be higher in *mev-1(kn1)* mutants compared to wt animals (Supplementary figure R2D). If the reviewer considers this experiment essential for the manuscript, we can refine this analysis and include a new additional panel, although we do not see a much gain to the already existing message.

65. Could lipid breakdown contribute to enhanced lipid signaling as a general mechanism for lifespan extension?

Consistent with the line of thoughts of the reviewer, we reasoned that CEST-2.2 might have two functions: to drive fatty acid β-oxidation and/or to generate signaling molecules (Figure EV3A), such as ascarosides (Ludewig and Schroeder, 2013). To rule out the lifespan-extending properties of the latter, we exposed *gas-1(fc21)* nematodes to ethanol (as a control), ascr#8 and ascr#81. We performed confocal analysis and found that ascaroside treatments did not rescue mitochondrial fragmentation due to complex I deficiency (Figure EV3B). Furthermore, ascr#8 treatment from hatching did not alter *gas-1(fc21)* survival (Figure EVS3C).

Although we cannot rule out that lipid signaling may contribute to some biological processes linked to CEST-2.2, we did not explore this possibility. The reason was because of the epistastic analyses demonstrating the importance of fatty acid-beta oxidation in the lifespan extension of *gas-1; cest-2.2* O/E animals. Surely, future studies may help to address this relevant point that the reviewer, correctly, raises.

66. Does blocking lipid catabolism enzymes in *gas-1;cest-2.2 O/E* worms abrogate the restored respiration as well? (In addition to partial reversal of lifespan).

We thank the reviewer for this question, since it helped to better understand the biological processes linked to CEST-2.2. All data are reported in Figure 5.

67. The heatmap seems to be missing a color scale in former Fig. 3H.

We adjusted the heatmap in figure 3C.

68. Please also correct grammatical errors in the manuscript.

Following the reviewer's comment, we have thoroughly revised the text of our manuscript.

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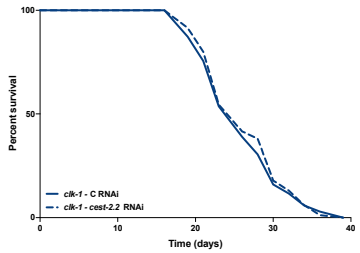
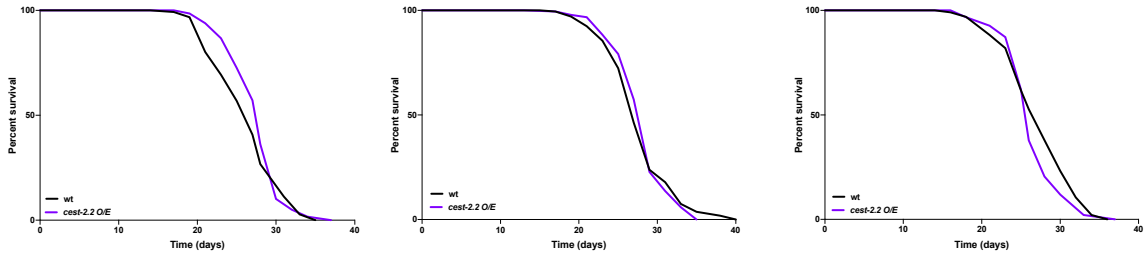
R1A



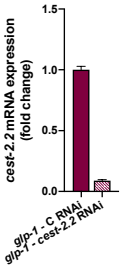
R1B

HT115					
Exp	**Strain**	**Dead (Cens)**	**Median**	**Max**	***p* value**
R1B1	*clk-1(e2519)* - C RNAi	83(63)	26	39	-
	clk-1(e2519) - cest-2.2 RNAi	95(88)	26	39	0.5323
R1B2	*clk-1(e2519)* - C RNAi	152(142)	26	40	-
	clk-1(e2519) - cest-2.2 RNAi	88(122)	24	38	0.0030
R1B3	*clk-1(e2519)* - C RNAi	43(62)	25	37	-
	clk-1(e2519) - cest-2.2 RNAi	92(118)	24	39	0.8811

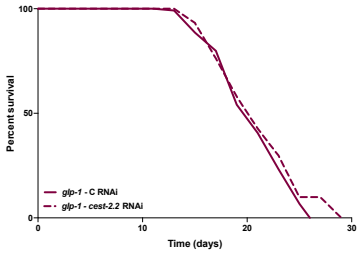
R1C



R1D



R1E



R1F

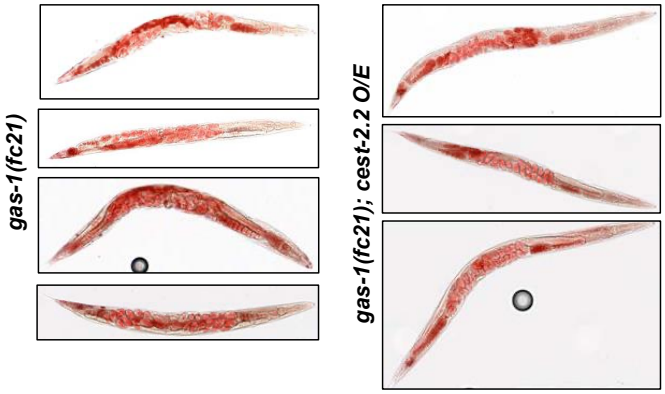
HT115					
Exp	**Strain**	**Dead (Cens)**	**Median**	**Max**	***p* value**
R1F4	*glp-1(bn18)* - C RNAi	59(62)	21	26	-
	glp-1(bn18) - cest-2.2 RNAi	74(103)	21	29	0.4688
R1F5	*glp-1(bn18)* - C RNAi	79(128)	21	30	-
	glp-1(bn18) - cest-2.2 RNAi	57(90)	21	27	0.0301

R1H

OP50					
Exp	**Strain**	**Dead (Cens)**	**Median**	**Max**	***p* value**
R1H6	*micu-1(bon20)*	146(57)	34	52	-
	micu-1(bon20); cest-2.2(bon52)	158(19)	36	54	0.0064
R1H7	*micu-1(bon20)*	195(125)	31	61	-
	micu-1(bon20); cest-2.2(bon52)	200(40)	32	61	0.0145

Point-by-point response, supplementary figures for the reviewers

R2A

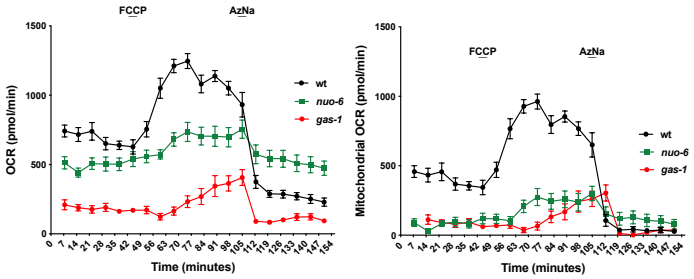


R2B

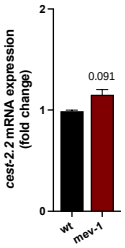
Genes involved in lipid metabolism			H3.3 loading		
Gene	gas-1 vs wt	gas-1;cest-2.2 vs gas-1	gas-1	nuo-6	wt
asit-2	↑	↓	-	-	-
atl-1	↑	ns	-	-	+
acoh-9	↓	↑	-	-	-
apm-3	ns	↑	+	+	+
ard-1	↑	ns	-	-	-
bgal-2	ns	↑	-	-	-
C24A3.4	↑	↓	-	-	-
C29E4.10	↑	ns	-	-	-
cdc-73	↓	ns	-	-	-
cmk-1	↓	↑	+	+	+
col-64	↑	ns	-	-	-
cpna-3	↓	ns	-	-	-
cyp-31A2	↑	ns	+	-	-
cyp-44A1	↑	ns	-	-	-
ech-9	↓	↑	-	-	-
elo-3	↑	ns	+	+	+
elt-7	↑	ns	-	-	-
F08F1.9	↓	ns	-	-	-
F09B12.3	↑	ns	-	-	-
F13H8.11	↑	ns	-	-	-
F32A5.8	↑	ns	-	-	-
F46H5.7	↑	↓	+	+	+
F52F10.2	↑	ns	-	-	-
F53B3.6	ns	↓	-	-	-
F56A4.4	↑	↓	-	-	-
F57A8.7	ns	↑	-	-	-
F58A6.1	↑	ns	-	-	-
fat-5	↑	ns	-	-	-
gst-1.1	↑	↓	-	-	-
gst-1.2	↑	↓	+	+	+
gpdh-2	ns	↓	+	+	+
gst-14	↑	ns	-	-	-
gst-15	ns	↑	-	-	-
gst-21	↑	ns	-	-	-
gst-22	↑	ns	-	-	-
hsp-12.2	↓	↑	-	-	-
iglg-1	↑	ns	-	-	-
K03H6.1	↓	↑	-	-	-
K11G12.6	↑	ns	-	+	-
kin-28	↑	↓	-	-	-
mak-1	↑	ns	-	-	-
mboa-2	↑	↓	-	-	-
mboa-6	↑	ns	+	+	+
mdh-17	↓	↑	-	-	+
mep-1	ns	↑	+	+	-
mg-1	↑	↓	-	-	-
nbx-1	↑	↑	-	-	-
nhr-168	↑	ns	-	-	-
nhr-175	↓	ns	-	-	-
nhr-20	↓	ns	-	-	-
nhr-219	ns	↓	-	-	-
nhr-232	↑	↓	+	+	+
nhr-48	↑	ns	+	+	-
nhr-52	ns	↓	-	-	-
nhr-65	↓	↑	-	-	-
oac-31	↑	↓	-	-	-
oac-9	↑	nd	-	-	-
obr-2	↑	↓	-	-	-
ohh-4	↑	ns	-	-	-
paf-1	↓	ns	-	-	-
pept-2	↓	↑	-	-	-
pdk-2	ns	↓	+	+	+
phi-1	↓	ns	-	-	-
plb-13	↑	↓	-	-	-
plb-21	↑	↓	+	+	+
R03H5.4	↑	ns	-	-	-
rho-1	ns	↑	+	+	+
sel-2	↑	ns	-	-	-
T06D8.3	↓	↑	-	-	-
T20B3.1	↑	ns	-	-	-
taf-7.2	↑	ns	-	-	-
tin-2	↑	ns	-	-	-
vgt-15	↓	↓	-	-	-
vgt-54	↑	ns	-	-	-
Y41E3.18	ns	↓	-	-	-
Y87G2A.18	ns	↓	-	-	-
ZK1073.1	↑	↓	-	-	-
ZK550.2	ns	↓	-	-	-

↑ significantly upregulated
↓ significantly downregulated
ns: not significant
nd: not detected

R2C



R2D



Point-by-point response, supplementary figures for the reviewers

Dear Daniele,

Thank you for submitting your revised manuscript. It has now been seen by two of the original referees.

My apologies for this unusual delay in getting back to you, which was due to personal circumstances of the referees.

The referees acknowledge that the study is significantly improved during revision. However, they also have some minor remaining concerns regarding missing controls and statements that need to be toned down. Although additional mechanistic insight is not required, we believe that these concerns need to be sorted out prior to publication. Please let me know if you have any questions/comments on any of these points.

Moreover, I need you to address the editorial points below before I can accept the manuscript.

• Please address the remaining referee concerns, provide a point-by-point response and mark the changes in the text (e.g. by leaving track changes on).
• We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Also, please rename the 'Conflict of Interests' section as 'Disclosure statement and competing interests'.
• EMBO Reports papers in Article format typically contain 6 main figures or more. Therefore, please consider converting one of the EV figures into a main figure.
• We note that Fig EV3D is currently not called out in the text.
• As mentioned earlier, we note that two traces were reused in Figures 4K and 5E. We understand that this is the clearest way to present data. However, please make a statement in the legends of Figures 4K and 5E that these panels are derived from the same experiment.
• Please clarify the nature of the replicates (technical or biological) in the figure legends after each n=x.
• Please split the source data as one file per figure. Moreover, we note that Fig EV3 J (left lower) and Fig EV3 J (right) need to be renamed as Fig EV3 H (left lower) and Fig EV3 H (right).
• We note that the title 'Expanded View Figure Legends' is currently missing.
• Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the paper (max 35 words) and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz

--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The revised manuscript by Piazzesi and colleagues is substantially improved. The authors performed additional experiments and answered many of the reviewers' comments. However, some of the reviewers' comments were not adequately answered mostly due to experimental difficulties (i.e. generation of nuo-6/cest-2.2 double mutants). Specific comments are summarized below:

One of the main findings of this study is that CEST-2.2 is responsible for lipidome alterations, which influence the lifespan specifically of mitochondrial complex I mutant animals. Therefore, the conclusion that "the intestine-specific hydrolase CEST-2.2 orchestrates the survival of mitochondrial deficient animals" is an overstatement.

Issues related to missing controls have not been sufficiently addressed (see for example, point 19 of the rebuttal letter). It is important to explore the effects of CEST-2.2 depletion and overexpression on lipid metabolism and metabolic rewiring not only in some mitochondrial mutants but also in wild-type animals.

A metabolomics analysis to detect increased fatty acid oxidation products upon cest-2.2 O/E (requested by Reviewe1) has not been performed due to practical difficulties ("could not grow enough material for this experiment").

The authors note that "cest-2.2 deficiency inhibited nuo-6(qm200) longevity, while it slightly reduced the lifespan of wt nematodes". However, from their graphs (Fig. 1I) and the respective statistical analyses (Appendix), it seems that the lifespan of wt animals is significantly (and not slightly) affected by cest-2.2(RNAi).

I understand the fact that gas-1(fc21); cest-2.2 O/E and gas-1(fc21) had a comparable ORO staining in gonads and eggs and both displayed reduced lipids compared to wt. However, if there is a decrease in ORO staining in hindgut, foregut, eggs and gonad then why is there an increase in total body for the gas-1 mutants and no difference in the total body for gas-1(fc21); cest-2.2 O/E? Moreover, the fact that vit-1 deficiency abolished gas-1(fc21); cest-2.2 O/E longevity suggests that yolk production and therefore lipid transfer in progeny is taking place in these animals and more importantly this is significant for their longevity. I believe that this should be discussed by the authors in the text.

The authors have performed seahorse analysis as well as ATP measurements to assess how mitochondrial function is changed in gas-1 mutants upon CEST-2.2 overexpression. They should also check whether mitochondrial mass changes upon CEST-2.2 overexpression, by using tissue-specific mitochondrial reporter strains, potential-independent dyes, or by determining mtDNA number. The trend towards higher ATP levels in gas-1; cest-2.2 O/E animals is not so clear, judging from the error bar as well as the statistics.

Regarding point 6, the authors have included in the revised manuscript data showing that UPRmt is significantly induced in CEST-2.2 overexpressing animals of the gas-1(fc21) mutant background, suggesting that UPRmt activation may mediate the lifespan extension conferred by CEST-2.2 overexpression. First, the authors should have included as controls wt animals in order to investigate whether CEST-2.2-dependent UPRmt induction is generally observed or is specific to complex I deficiency. Second, they should check whether UPRmt induction (genetically or through drug supplementation) recapitulates CEST-2.2 overexpression in the gas-1 mutant background.

Regarding point 21, the authors could simply perform lifespan assays by growing the cest-2.2 (lf) animals on HT115 strain, in order to make clear comparisons between wt and cest-2.2(RNAi) treated animals.

On page 8, the authors state "However, atfs-1(lf) did not modify the respiration profile of gas-1(fc21); cest-2.2 O/E animals (Figure 2K-L)." This is not true. Atfs-1(lf) increased OCR of gas-1(fc21;cest-2.2O/E animals (Figure 2K-L).

On page 9, the authors state "Ingenuity Pathway Analysis (IPA) predicted that gas-1(fc21) animals had decreased fatty acid oxidation and, as a consequence, an increased concentration of fatty acids (Figure 3D)." However, the predicted increase in fatty acid concentration in the intestine of gas-1 mutants was not confirmed in fig3F.

Lipid droplet formation (Figure EV3H) could be quantified in order to strengthen authors' findings.

Referee #2:

The revised manuscript by Piazzesi et al. is a description of how a lifespan shortening mitochondrial mutation is converted to a long lived strain through the manipulation of a differentially regulated gene called cest-2.2. The amount of data that have been generated to understand how this might work is quite impressive, yet none of these data provide a smoking gun/clear cut explanation of how this might occur. The most consistent explanation that the authors have painstakingly unveiled is through a change in lipid metabolism that is mediated by increase FFA catabolism fuelled presumably by CEST-2.2. This in turn generates increased acetyl-CoA units that feed the mitochondrial TCA cycle of the gas-1 mitochrondria-impaired mutants.
Their genetic analyses indicate that vit-1 is required for the lifespan extension afforded by cest-2.2 o/e as are nhr-49 and nhr-80, although there is no clear means of accounting for the roles of these key genes in the observed lifespan extension phenotype, while the explanations provided by the authors are speculative at best. Nevertheless, the authors have attempted to link these gene activities to their observations and have generated a model that is at least superficially consistent with their findings. I do agree with the author's model shown in Figure 4B, where nhr-49/80 are shown to have a direct effect on either acs-2, ech-1.1, or fat-5. From my understanding of the work presented in this manuscript there is no evidence to support this claim and is simply inferred from the effects on lifespan. At best this simple direct arrow should be shown with a question mark and/or elaborated upon in the figure legend. The authors really have not fully dissected how these receptors mediate the lifespan extension afforded by cest-2.2 o/e.
The main conclusion from all this work is that the increased hydrolysis of FFAs leads to an increase in acetyl-CoA to combine with oxaloacetate units generated by the TCA. Therefore, if this is correct, if the gas-1 animals are provided with additional units of acetyl CoA in the form of acetate, it should provide the same kind of conversion from short gas-1 lifespan to long gas-1; cest-2.2 o/e lifespan. If that makes sense, it should at least be tried and included in the final version of the manuscript.
The authors have responded to all my queries as best they can and I appreciate their attempts to make new genotypes to test some of their ideas more rigorously. That some of these are non-viable probably means something important and is potentially

consistent with their findings. Based on the preface that the authors indicated in their rebuttal; the philosophy of the journal per se, I guess I should not expect that the authors provide a mechanistic explanation of all their data, but accept that the authors' claims are sound, interesting and supported by their observations.
The text of the manuscript is substantially improved and many of the minor grammatical, syntax and formatting errors have been fixed, although there still are a few issues that could be ironed out following a thorough proofread.

EMBOR-2021-52606V2_point-by-point response

We would like to thank the Editor and the reviewers for their constructive inputs during the revision of our manuscript. Below, we provide a point-by-point response to their last concerns.

Referee #1:
The revised manuscript by Piazzesi and colleagues is substantially improved. The authors performed additional experiments and answered many of the reviewers' comments. However, some of the reviewers' comments were not adequately answered mostly due to experimental difficulties (i.e. generation of *nuo-6; cest-2.2* double mutants). Specific comments are summarized below:

8. One of the main findings of this study is that CEST-2.2 is responsible for lipidome alterations, which influence the lifespan specifically of mitochondrial complex I mutant animals. Therefore, the conclusion that "the intestine-specific hydrolase CEST-2.2 orchestrates the survival of mitochondrial deficient animals" is an overstatement.

To balance our statement, we adjusted the sentence as following: *"We find that CEST-2.2, a carboxylesterase mainly localizes in the intestine, may stimulate the survival of mitochondrial deficient animals."*

9. Issues related to missing controls have not been sufficiently addressed (see for example, point 19 of the rebuttal letter). It is important to explore the effects of CEST-2.2 depletion and overexpression on lipid metabolism and metabolic rewiring not only in some mitochondrial mutants but also in wild-type animals.

Point 19 was addressed in our previous point-by-point response. Our study focuses on mitochondrial dysfunction. The role of CEST-2.2 in the context of ascaroside signaling has been already described elsewhere (Le et al., 2020). We described lipid deposits in *cest-2.2 (lf)* and *cest-2.2 O/E* animals. A further characterization of CEST-2.2 function in wt nematodes may be of interest, however it is a curiosity that may be the central focus of another study.

10. A metabolomics analysis to detect increased fatty acid oxidation products upon *cest-2.2 O/E* (requested by Reviewe1) has not been performed due to practical difficulties ("could not grow enough material for this experiment").

That is correct. We are as disappointed as the reviewer. However, as mentioned in the previous response we could not grow enough material to run a proper metabolomics. Due to technical drawbacks, we regret to be not in the position to accommodate the reviewer's request, because we have already attempted to run this assessment.

11. The authors note that "*cest-2.2* deficiency inhibited *nuo-6(qm200)* longevity, while it slightly reduced the lifespan of wt nematodes". However, from their graphs (Fig. 1I) and the respective statistical analyses (Appendix), it seems that the lifespan of wt animals is significantly (and not slightly) affected by *cest-2.2(RNAi)*.

We are totally unbiased on this aspect and adjusted the sentence as following: *"We observed that cest-2.2 RNAi inhibited nuo-6(qm200) longevity (Figure 1F-G and Appendix Table S1) as well as also slightly (yet significantly) reduced the survival of wt nematodes (Figure 1H-I and Appendix Table S1)."*
We hope that the reviewer agrees with our changes.

12. I understand the fact that *gas-1(fc21); cest-2.2 O/E* and *gas-1(fc21)* had a comparable ORO staining in gonads and eggs and both displayed reduced lipids compared to wt. However, if there is a decrease

in ORO staining in hindgut, foregut, eggs and gonad then why is there an increase in total body for the *gas-1* mutants and no difference in the total body for *gas-1(fc21); cest-2.2 O/E*? Moreover, the fact that *vit-1* deficiency abolished *gas-1(fc21); cest-2.2 O/E* longevity suggests that yolk production and therefore lipid transfer in progeny is taking place in these animals and more importantly this is significant for their longevity. I believe that this should be discussed by the authors in the text.

Respectfully, we have difficulties to follow the line of arguments of the reviewer, especially in regard to his/her first question.
We showed that *vit-1* deficiency did not alter the survival of *gas-1(fc21)* nematodes, whereas it consistently reduced the lifespan extension of *gas-1(fc21); cest-2.2 O/E* mutants. If VIT-1 contributes to the nutrient transferring to the developing progeny, more VIT-1 expression would deplete resources in *gas-1(fc21); cest-2.2 O/E* mutants. If so, *vit-1* RNAi would be beneficial for the mother because it makes available more macromolecules for animal survival, as in the case of wt nematodes. In other words, we would have the exact opposite scenario of the one depicted by the reviewer, unless s/he hypothesizes that yolk production and transferring to the progeny stimulates the survival of the *gas-1(fc21); cest-2.2 O/E* hermaphrodites.

13. The authors have performed seahorse analysis as well as ATP measurements to assess how mitochondrial function is changed in *gas-1* mutants upon CEST-2.2 overexpression. They should also check whether mitochondrial mass changes upon CEST-2.2 overexpression, by using tissue-specific mitochondrial reporter strains, potential-independent dyes, or by determining mtDNA number.

Respectfully, these are newly raised suggestions that go beyond the scope of the revision.

The trend towards higher ATP levels in *gas-1; cest-2.2 O/E* animals is not so clear, judging from the error bar as well as the statistics.

We performed the experiment as requested by the reviewer. Since we did not reach statistical significance, we simply describe a possible trend in ATP levels: *"The increased maximal respiration was associated with a visible trend toward more ATP levels."* We believe that our previous effort sufficiently addressed this secondary point.

14. Regarding point 6, the authors have included in the revised manuscript data showing that UPRmt is significantly induced in CEST-2.2 overexpressing animals of the *gas-1(fc21)* mutant background, suggesting that UPRmt activation may mediate the lifespan extension conferred by CEST-2.2 overexpression. First, the authors should have included as controls wt animals in order to investigate whether CEST-2.2-dependent UPRmt induction is generally observed or is specific to complex I deficiency. Second, they should check whether UPRmt induction (genetically or through drug supplementation) recapitulates CEST-2.2 overexpression in the *gas-1* mutant background.

The reviewer previously asked whether *cest-2.2* overexpression employs UPR^{mt} to extend the lifespan of mitochondrial mutants. Thus, these are newly raised suggestions that would require experimental work beyond a reasonable timeframe.

15. Regarding point 21, the authors could simply perform lifespan assays by growing the *cest-2.2 (lf)* animals on HT115 strain, in order to make clear comparisons between wt and *cest-2.2(RNAi)* treated animals.

This point was already addressed. As clearly stated in the previous point-by-point response, we explained that we did not properly elaborate our message in the original draft. Throughout all our investigation, all lifespan assays were strictly conducted with their appropriate controls: *cest-2.2(lf)* and wt nematodes were fed with exactly the same OP50 bacteria, while wild type nematodes were fed with

HT115 bacteria (empty vector) and compared with wild type animals grown on HT115 bacteria and expressing dsRNA against *cest-2.2*. The sentence in question was simply included to address any potential concerns regarding the differences in median lifespan across different experimental setups. Following the reviewer's comment, we adjusted the text, so that it is clear that we do not draw any conclusions by comparing animals fed with different bacterial strains.
We believe that the suggested comparison would not add anything to the existing dataset and current conclusions.

16. On page 8, the authors state "However, atfs-1(lf) did not modify the respiration profile of *gas-1(fc21); cest-2.2 O/E* animals (Figure 2K-L)." This is not true. *atfs-1(lf)* increased OCR of *gas-1(fc21); cest-2.2 O/E* animals (Figure 2K-L).

We modified the sentence according to the reviewer's comment: *"we observed that atfs-1(lf) did not modify the maximal respiration of gas-1(fc21); cest-2.2 O/E animals (Figure 2K-L)."*

17. On page 9, the authors state "Ingenuity Pathway Analysis (IPA) predicted that *gas-1(fc21)* animals had decreased fatty acid oxidation and, as a consequence, an increased concentration of fatty acids (Figure 3D)." However, the predicted increase in fatty acid concentration in the intestine of *gas-1* mutants was not confirmed in fig 3F.

We thank the reviewer for the note. We adjusted the text as following: *"Ingenuity Pathway Analysis (IPA) predicted that gas-1(fc21) animals had decreased fatty acid oxidation and, as a consequence, potential changes in stored fatty acids (Figure 3I). Together, our data indicate that gas-1(fc21) mutants have impaired fatty acid metabolism, whereas gas-1(fc21); cest-2.2 O/E nematodes may be able to mobilize more efficiently lipid stores and to induce lipid catabolism."*

18. Lipid droplet formation (Figure EV3H) could be quantified in order to strengthen authors' findings.

In this newly raised suggestion, we assume that the reviewer asks for lipid droplet size (rather than formation, since that would require *in vivo* time-course imaging analysis). If so, we attempted to address this point, however the droplets are too overlapping and quantification becomes bias.
Despite the lack of these additional data, we believe that the already submitted evidence sufficiently supports our claims.

Referee #2:

19. The revised manuscript by Piazzesi et al. is a description of how a lifespan shortening mitochondrial mutation is converted to a long-lived strain through the manipulation of a differentially regulated gene called *cest-2.2*. The amount of data that have been generated to understand how this might work is quite impressive, yet none of these data provide a smoking gun/clear cut explanation of how this might occur. The most consistent explanation that the authors have painstakingly unveiled is through a change in lipid metabolism that is mediated by increase FFA catabolism fuelled presumably by CEST-2.2. This in turn generates increased acetyl-CoA units that feed the mitochondrial TCA cycle of the gas-1 mitochrondria-impaired mutants. Their genetic analyses indicate that *vit-1* is required for the lifespan extension afforded by *cest-2.2 O/E* as are *nhr-49* and *nhr-80*, although there is no clear means of accounting for the roles of these key genes in the observed lifespan extension phenotype, while the explanations provided by the authors are speculative at best. Nevertheless, the authors have attempted to link these gene activities to their observations and have generated a model that is at least superficially consistent with their findings. I do agree with the author's model shown in Figure 4B, where *nhr-49/80* are shown to have a direct effect on either *acs-2*, *ech-1.1*, or *fat-5*. From my understanding of the work presented in this manuscript there is no evidence to support this claim and is simply inferred from the

effects on lifespan. At best this simple direct arrow should be shown with a question mark and/or elaborated upon in the figure legend.

Figure 4B (now Figure 5B) is a simplified schematic representation of NHR-49/NHR-80 participation to fatty acid beta-oxidation based on the current knowledge. We employed this scheme as a simple way to graphically depict the pathway under investigation. It is not a schematic summary of our findings. To accommodate the reviewer's comment, we substitute solid lines with dotted ones. In the caption, we wrote the following: *"Green dotted arrows indicate potential transcriptional regulation by NHR-49/NHR-80."*

20. The authors really have not fully dissected how these receptors mediate the lifespan extension afforded by *cest-2.2 O/E*.

To address the contribution of NHR-49 and NHR-80, we performed conventional epistatic analyses and Seahorse experiments as outlined in our initial experimental proposal. We believe that a full characterization of NHR-49/NHR-80 role in *gas-1(fc21); cest-2.2 O/E* lifespan would go beyond the original purpose of the submitted work and its revision.

21. The main conclusion from all this work is that the increased hydrolysis of FFAs leads to an increase in acetyl-CoA to combine with oxaloacetate units generated by the TCA. Therefore, if this is correct, if the *gas-1* animals are provided with additional units of acetyl CoA in the form of acetate, it should provide the same kind of conversion from short *gas-1* lifespan to long *gas-1; cest-2.2 O/E* lifespan. If that makes sense, it should at least be tried and included in the final version of the manuscript.

The experiment with acetate is a newly raised suggestion and potentially a valuable proof-of-concept, however it has been already partially addressed by some of our experimental evidence provided during the lengthen revision of the submitted manuscript.

22. The authors have responded to all my queries as best they can and I appreciate their attempts to make new genotypes to test some of their ideas more rigorously. That some of these are non-viable probably means something important and is potentially consistent with their findings. Based on the preface that the authors indicated in their rebuttal; the philosophy of the journal per se, I guess I should not expect that the authors provide a mechanistic explanation of all their data, but accept that the authors' claims are sound, interesting and supported by their observations.

We would like to thank the referee for his/her understanding.

23. The text of the manuscript is substantially improved and many of the minor grammatical, syntax and formatting errors have been fixed, although there still are a few issues that could be ironed out following a thorough proofread.

We have seriously taken in consideration the reviewer's comment and thoroughly revised grammar and syntax of our manuscript.

2nd Revision - Editorial Decision **22nd Feb 2022**

Dear Daniele,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore, I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Kind regards,

Deniz
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

--

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Corresponding Author Name: Dr. Daniele Bano
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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 $\chi 2$ 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.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Samples size was chosen based to obtain robust and reproducible statistical analyses.
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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.	N/A
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Is the variance similar between the groups that are being statistically compared?	N/A

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).	Catalog numbers are provided for the antibodies used in this study in the Materials and Methods.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All information has been provided

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