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

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Abstract

Mitochondrial dysfunction can either extend or decrease Caenorhabditis elegans lifespan, depending on whether transcriptionally regulated responses can elicit durable stress adaptation to otherwise detrimental lesions. Here, we test the hypothesis that enhanced metabolic flexibility is sufficient to circumvent bioenergetic abnormalities associated with the phenotypic threshold effect, thereby transforming short-lived mitochondrial mutants into long-lived ones. We find that CEST-2.2, a carboxylesterase mainly localizes in the intestine, may stimulate the survival of mitochondrial deficient animals. We report that genetic manipulation of cest-2.2 expression has a minor lifespan impact on wild-type nematodes, whereas its overexpression markedly extends the lifespan of complex I-deficient gas-1(fc21) mutants. We profile the transcriptome and lipidome of cest-2.2 overexpressing animals and show that CEST-2.2 stimulates lipid metabolism and fatty acid beta-oxidation, thereby enhancing mitochondrial respiratory capacity through complex II and LET-721/ETFDH, despite the inherited genetic lesion of complex I. Together, our findings unveil a metabolic pathway that, through the tissue-specific mobilization of lipid deposits, may influence the longevity of mitochondrial mutant C. elegans.

Keywords Caenorhabditis elegans; carboxylesterase CEST-2.2; epigenetics; lipid metabolism; mitochondria

Introduction

Mitochondria contribute to energy production and host biosynthetic reactions that supply molecules for metabolic and signaling processes. Through iterative oxidation of nutrients (e.g., sugars, amino acids, and fatty acids), mitochondria generate reducing equivalents in the form of metabolic intermediates, NADH, and FADH2 (Spinelli & Grigis, 2018; Martinez-Reyes & Chandel, 2020). The exothermic transfer of electrons from these reduced molecules to the electron transport chain (ETC) and then to oxygen is used by the respiratory complexes (i.e., complex I, complex III, and complex IV) to drive protons from the matrix to the intermembrane space. The subsequent dissipation of the electrochemical gradient across the inner membrane promotes ATP biosynthesis and thermogenesis, ROS production, and ion transport (Martinez-Reyes & Chandel, 2020; Sies & Jones, 2020). All these processes influence cell division, differentiation, maintenance, and demise, thereby contributing to animal development, growth, and survival.

A large literature has described an increasing number of genetic lesions and environmental toxins that alter mitochondrial oxidative phosphorylation (OXPHOS) and can elicit human metabolic disorders and neurodegenerative conditions, further emphasizing the relevance of mitochondria in human pathophysiology (Schon & Przedborski, 2011; Koopman et al, 2012; Gorman et al, 2016; Bano & Prehn, 2018; Wallace, 2018; Frazier et al, 2019). Despite profound clinical importance, currently available therapeutic interventions are mostly symptomatic, since they may improve the life quality of the patients without substantially modifying the progression of the disease (Gorman et al, 2016; Russell et al, 2020). Other challenges include a better understanding of the molecular mechanisms that can counteract mitochondrial dysfunction, which may help to single out novel targets potentially relevant for therapeutic purposes. In this regard, preclinical studies in recent years have
described innovative genetic and pharmacological approaches that improve organismal fitness by stimulating transcriptional programs that buffer toxic species and enhance the usage of available resources. Consistent with this concept, it was shown that inhibition of mTOR signaling ameliorates aberrant processes linked to mitochondrial dysfunction by shifting metabolism toward catabolism (Johnson et al, 2013; Ising et al, 2015; Peng et al, 2015; Zheng et al, 2016; Khan et al, 2017; Siegmund et al, 2017; Wischhof et al, 2018; Gioran et al, 2019). Similarly, chronic exposure to moderate hypoxia alleviates metabolic defects and triggers adaptive pathways that limit the damage due to impaired mitochondrial bioenergetics, thereby stimulating the survival of animals carrying mitochondrial genetic lesions (Jain et al, 2016, 2019, 2020; Ast et al, 2019; To et al, 2019; Grange et al, 2021). As additional examples, supplementation of nicotinamide mononucleotide, and pharmacological and genetic manipulations of the redox state improve the metabolic derangement due to NAD+ depletion in mitochondrial deficient cells (Canto et al, 2012; Karamanlidis et al, 2013; Mouchiroud et al, 2013; Cerutti et al, 2014; Pirinen et al, 2014; Cracan et al, 2017; Liu et al, 2021).

Based on these converging lines of evidence, eukaryotic cells are capable of responding to defective mitochondrial energy production by establishing transcriptional activities and broad metabolic reprogramming that channel resources to build up protective mechanisms against long-lasting systemic changes. In the context of human physiology, it is crucial to decode this stress response network, since it may provide molecular targets that are therapeutically valuable for chronic and/or inherited diseases associated with mitochondrial lesions (Lardenoije et al, 2015; Riera et al, 2016; Mottis et al, 2019).

The mitochondrial threshold effect theory assumes that phenotypic presentations of mitochondrial dysfunction occur when a critical state is reached and functional compensation is irretrievably compromised (Rossignol et al, 2003), however, it remains elusive, which are the key metabolic processes that dictate the trade-off between growth, reproduction, and somatic maintenance during adverse conditions. The nematode Caenorhabditis elegans has proven to be a useful in vivo model organism to investigate the mitochondrial threshold effect in stress resilience and lifespan-extending programs (Riera et al, 2016; Shpilka & Haynes, 2018). During C. elegans development (Feng et al, 2001; Dillin et al, 2002; Rea et al, 2007), mild disruption of the mitochondrial ETC activity impairs OXPHOS and evokes a profound transcriptional reprogramming that alleviates the chronic energy crisis. Along with a considerable shift toward catabolic processes (Gioran et al, 2019), transcriptional activation of unfolded protein response (UPR) and expression of detoxifying enzymes (e.g., SOD-3 and GST-4) prevent the detrimental collapse of cellular proteostasis (Lin & Haynes, 2016; Tian et al, 2016b; Shpilka & Haynes, 2018). In a cell-autonomous fashion, secretion of signals (e.g., ROS, neurotransmitters, and hormones) from mitochondrial deficient cells can communicate adverse conditions throughout the whole organism. In this regard, the enhanced responsiveness to stressful states presumably elicits global protective mechanisms that have beneficial effects on animal survival (Durieux et al, 2011). These broad physiological changes require a permissive chromatin landscape for the engagement and establishment of transcriptional programs that control homeostatic processes (Benedetti et al, 2006; Haynes et al, 2010; Nargund et al, 2012; Merkwirth et al, 2016; Piazzesi et al, 2016; Tian et al, 2016a). In this regard, we previously reported that aberrant chromatin remodeling due to the loss of replication-independent histone H3.3 (i.e., the main H3 variant that is expressed in postmitotic cells and accumulates during aging) impairs the establishment of lifespan-extending programs, including those dependent on mitochondria (Troulinaki & Bano, 2012; Piazzesi et al, 2016; Bano et al, 2017). Consistently, altered histone H3 methylation compromises the transcriptional activation of mitochondrial unfolded protein response (UPR) and, consequently, shortens the lifespan of mitochondrial C. elegans mutants (Merkwirth et al, 2016; Tian et al, 2016a, 2016b). Thus, these lines of evidence suggest that epigenetic plasticity can confer physiological fitness to multicellular organisms. If so, this concept raises a very basic question: can we transform sick, short-lived mitochondrial mutants into long-lived ones?

Here, we report that overexpression of a single enzyme is sufficient to potentiate the residual respiratory capacity of complex I-deficient nematodes by redirecting lipid usage toward fatty acid beta-oxidation. Through multomics analyses coupled with conventional epistatic studies, we demonstrate a previously unknown metabolic network that can extend C. elegans lifespan, with molecular aspects that may be partially conserved in some disease settings in higher metazoa. More broadly, our findings emphasize the strength of adaptive mechanisms in promoting epigenetic reprogramming that sustains survival of animals carrying mitochondrial lesions.

Results

CEST-2.2 expression influences the lifespan of complex I mutant nematodes

We previously reported that H3.3 expression establishes lifespan-extending programs in C. elegans by supporting transcriptional plasticity underlying stress resilience (Piazzesi et al, 2016). Based on that, we hypothesized that H3.3 may be differentially loaded onto chromatin in long-lived mitochondrial mutants compared with short-lived ones. To test this hypothesis, we employed animals carrying either nuo-6(qm200) or gas-1(fc21) alleles, both altering complex I activity, but extending or reducing C. elegans lifespan, respectively (Fig EV1A and Appendix Table S1). Using wild-type (wt) and mitochondrial mutant animals expressing GFP-tagged HIS-72 (Ooi et al, 2006; Piazzesi et al, 2016), we performed chromatin immunoprecipitation (ChIP) followed by deep sequencing (Fig 1A), focusing our analysis exclusively on promoter regions. We found that HIS-72::GFP was loaded onto the promoters of 996 genes and reproducibly expressed in wild-type nematodes by redirecting lipid usage toward fatty acid beta-oxidation. Through multomics analyses coupled with conventional epistatic studies, we demonstrate a previously unknown metabolic network that can extend C. elegans lifespan, with molecular aspects that may be partially conserved in some disease settings in higher metazoa. More broadly, our findings emphasize the strength of adaptive mechanisms in promoting epigenetic reprogramming that sustains survival of animals carrying mitochondrial lesions.
Figure 1.
conserved proteins (Fig 1D). One of these proteins is ZC376.2/CEST-2.2, which is a carboxylesterase phylogenetically related to the serine hydrolase family and orthologous to human carboxylesterases and carboxyl ester lipases (Chen et al, 2019; Le et al, 2020). As recently described (Le et al, 2020), CEST-2.2 localizes to intestinal granules and contributes to the biosynthesis of ascorbic acids, including ascr#8, ascr#81, and ascr#82. 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 performed quantitative real-time PCR (qRT-PCR) and confirmed that cest-2.2 was downregulated in gas-1(fc21) mutants compared with wt animals, whereas this was upregulated in a H3.3-dependent manner in nnuo-6(qm200) mutant nematodes (Figs 1E and EV1B). To test the role of cest-2.2 in longevity, wt and nnuo-6 mutant nematodes were grown on bacteria expressing double-strand RNA against cest-2.2. We observed that cest-2.2 RNAi inhibited nnuo-6(qm200) longevity (Fig 1F and G and Appendix Table S1) as well as also slightly (yet significantly) reduced the survival of wt nematodes (Fig 1H and I, 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 (Fig EV1C). We found that cest-2.2 loss-of-function (lf) mutants had a minor tendency toward a decreased median lifespan compared with wt animals, whereas cest-2.2(lf) inhibited the lifespan extension due to the RNAi against complex IV subunit COO-1 (Figs 1J and EV1D, Appendix Table S1). When we attempted to generate nnuo-6, cest-2.2 double mutants, we could obtain only hemaphrodites carrying the two mutant alleles in heterozygosity, further highlighting the contribution of CEST-2.2 to the survival of complex I-deficient nematodes. However, cest-2.2 downregulation had no effect on the survival of long-lived sod-2(ok1030) and complex III tsp-1(qm150) mutants (Fig EV1E and F, Appendix Table S1). Consistent with the metabolic heterogeneity of mitochondrial diseases (Lake et al, 2016; Wallace, 2018; Frazier et al, 2019; Russell et al, 2020), these data suggest that CEST-2.2 influences the lifespan of mitochondrial mutant nematodes depending on the genetic lesions in question.

Next, we reasoned that cest-2.2 upregulation could ameliorate the survival of short-lived complex I mutants. We therefore generated nnuo-6, cest-2.2 double mutants, which could obtain only hemaphrodites carrying the two mutant alleles in heterozygosity, further highlighting the contribution of CEST-2.2 to the survival of complex I-deficient nematodes. However, cest-2.2 downregulation had no effect on the survival of long-lived sod-2(ok1030) and complex III tsp-1(qm150) mutants (Fig EV1E and F, Appendix Table S1). Consistent with the metabolic heterogeneity of mitochondrial diseases (Lake et al, 2016; Wallace, 2018; Frazier et al, 2019; Russell et al, 2020), these data suggest that CEST-2.2 influences the lifespan of mitochondrial mutant nematodes depending on the genetic lesions in question.

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cest-2.2 O/E strongly stimulated the lifespan of short-lived complex I-deficient animals, with a median lifespan that was approximately 38% longer than that of gas-1(fc21) mutant nematodes (Fig 1N and Appendix Table S1). Both cest-2.2 downregulation and (lf) mutation abrogated the lifespan extension of gas-1(fc21); cest-2.2 O/E mutant nematodes, while cest-2.2 RNAi had no effect on gas-1 survival (Figs 1N–O and EV1G–J, Appendix Table S1). These lifespan assays further emphasize that the increased survival is specifically linked to CEST-2.2 activity and not to other confounding effects (e.g., integration site of the transgene). Of note, cest-2.2 O/E had no influence on nuo-6 lifespan, whereas it moderately extended the survival of complex II-deficient mev-1 mutants (Fig EV1K and L, Appendix Table S1).

Phenotypically, we noticed that cest-2.2 O/E had negligible effect on body size and locomotory activity, although cest-2.2(lf) animals were larger than wt (Fig EV1M and N). Nevertheless, cest-2.2 O/E led to a significant increase in body size as well as to complete rescue of locomotory defects caused by the gas-1(fc21) lesion (Fig 1P and Q). Furthermore, cest-2.2 O/E did not alter the pharyngeal pumping rate and egg laying of gas-1 mutants (Fig 1R and S), ruling out any confounding effect on survival due to fertility or food intake. Together, our findings show that CEST-2.2 overexpression shifts the survival threshold of complex I-deficient animals toward longevity.

**CEST-2.2 O/E stimulates mitochondrial respiration in complex I-deficient nematodes**

We next explored whether cest-2.2 O/E could influence mitochondrial bioenergetics in gas-1(fc21) nematodes. Using a previously adapted Seahorse protocol (Troulinaki et al., 2018; Gioran et al., 2019), we observed that cest-2.2 O/E was not sufficient to rescue the basal respiration defects of gas-1 mutants; however, it did significantly increase their maximal respiration (Fig 2A–C), indicating that the mitochondria of gas-1; cest-2.2 O/E animals possessed more metabolic flexibility, possibly due to an increased availability of substrates and tricarboxylic acid cycle (TCA) activity as shown in other biological contexts (van der Windt et al., 2012; Pfleger et al., 2015). The increased maximal respiration was associated with a visible trend toward more ATP levels in gas-1; cest-2.2 O/E animals compared with gas-1 mutants (Fig 2D). As expected, cest-2.2 RNAi almost abrogated the enhanced mitochondrial plasticity observed in gas-1; cest-2.2 O/E nematodes (Fig 2E and F). Both cest-2.2 O/E and cest-2.2(lf) animals had mitochondrial respiration profiles comparable to wt nematodes (Fig 2G), further indicating an exclusive effect on complex I-deficient mitochondria. We performed high-resolution confocal microscopy and observed that CEST-2.2 O/E was also sufficient to ameliorate the aberrant mitochondrial fragmentation in the gut of gas-1 mutant nematodes (Fig 2H), indicating a better maintenance of the mitochondrial network. Remarkably, CEST-2.2 O/E counteracted mitochondrial fragmentation in the body wall muscle cells of gas-1(fc21) mutants (Fig 2I), indicating a beneficial effect across different tissues.

Since lifespan extension of mitochondrial mutants often depends on a transcriptional upregulation of defensive mechanisms (Lin & Haynes, 2016; Quirós et al., 2016; Riera et al., 2018; Shpilka & Haynes, 2018; Mottis et al., 2019), we set out to explore whether CEST-2.2 O/E could stimulate mitochondrial UPR (UPRmt) and other stress response pathways involved in the detoxification of detrimental molecules, such as reactive oxygen species (ROS). To do so, we first assessed the expression of the zcsIs13[hsp-6p::gfp] transgene, a widely accepted UPRmt marker (Benedetti et al., 2006) under the transcriptional regulation of the bZip transcription factor ATFS-1 (Nargund et al., 2012). We found that cest-2.2 O/E significantly enhanced zcsIs13[hsp-6p::gfp] expression in gas-1 mutants (Fig 2J), suggesting that UPRmt was strongly induced. Moreover, we observed that atfs-1(lf) did not modify the maximal respiration of gas-1(fc21); cest-2.2 O/E animals (Fig 2K and L). Of note, cest-2.2

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**Figure 2. CEST-2.2 O/E stimulates mitochondrial respiration in complex I-deficient nematodes.**

A Representative oxygen consumption rate (OCR) curve of 5-day-old wt, gas-1(fc21) and gas-1(fc21); cest-2.2 O/E mutant nematodes as measured with a Seahorse XF24 Analyzer. Injection time of FCCP and Na2N is indicated with arrows (n = 9 biological replicates, from three independent experiments).

B, C Indices of (B) normalized basal OCR and (C) maximal respiration of wt, gas-1(fc21) and gas-1(fc21); cest-2.2 O/E mutant nematodes across (n = 9, one-way ANOVA with the Tukey’s multiple comparison test, n = 9 biological replicates, from three independent experiments).

D Relative ATP content of gas-1(fc21) and gas-1(fc21); cest-2.2 O/E nematodes (unpaired t-test, n = 3 biological replicates).

E, F Representative [E] Seahorse experiment and statistical analysis of [F] basal OCR and maximal respiration of 5-day-old gas-1; cest-2.2 O/E mutant C. elegans fed with control and cest-2.2 RNAi since hatching (ordinary one-way ANOVA with the Tukey’s multiple comparisons test, n = 7–9 biological replicates, from three independent experiments).

G OCR profiles of wt, cest-2.2 O/E and cest-2.2(lf) nematodes upon sequential treatment with FCCP and Na2N (n = 9–10 biological replicates, from three independent experiments).

H Representative images of control, gas-1(fc21) and gas-1(fc21); cest-2.2 O/E nematodes expressing mitochondria-targeted GFP in the intestine (scale bar = 5 μm).

I Representative high-resolution confocal images of C. elegans expressing mitochondria-targeted GFP in the body wall muscle cells (scale bar = 10 μm).

J Quantification of mitochondrial morphology (elongation) is reported in the right panel (one-way ANOVA with Holm-Sidak’s correction, n = 37–47 animals from three independent experiments).

K, L Representative [K] Seahorse profile and [L] statistical analysis of basal OCR and maximal respiration of mutant nematodes (unpaired t-test, n = 12–13 biological replicates, from three independent experiments).

M Immunoblot and statistical analysis of zcsIs13[hsp-6p::GFP] expression in control, nuo-6(qm200) animals fed with empty vector (C RNAi) and cest-2.2 RNAi bacteria (unpaired t-test, n = 3 biological replicates).

Data information: In A, E, G, K, points represent mean ± SEM; in B, C, D, F, J (right), L and M (right), bars represent mean ± SEM; in I (right), middle line represents the median, and the upper and lower lines represent the upper and lower quartiles, respectively. Across experiments, P-value summary is ns = not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Figure 2.
downregulation was not sufficient to alter zcs13[hsp-6p::gfp] expression in nuo-6(qm200) (Fig 2M), possibly because UPRmt was already efficiently engaged at an early larval stage and before an efficient RNAi effect. We went on to measure the expression of SOD-3 and GST-4, two detoxifying enzymes that are transcriptionally regulated in response to oxidative stress. We found that genetic manipulation of cest-2.2 did not alter sod-3 and gst-4 expression in neither nuo-6 nor gas-1 mutants (Fig EV2A–H), indicating that CEST-2.2 does not influence transcription factors targeting sod-3 and gst-4 promoters. To further rule out any role of oxidative stress on the observed lifespan extension, we exposed gas-1; cest-2.2 O/E animals to the free-radical scavenger ascorbate/vitamin C. Contrary to other long-lived mitochondrial mutants (Yang & Hekimi, 2010), vitamin C did not alter the survival of wt, gas-1(fc21) and gas-1(fc21); cest-2.2 O/E nematodes (Fig EV2J). Taken together, our data suggest that UPRmt is engaged and may contribute to gas-1; cest-2.2 O/E stress resilience.

**CEST-2.2 overexpression stimulates lipid metabolism in complex I-deficient C. elegans**

We set out to investigate the biological processes underlying the lifespan extension of gas-1; cest-2.2 O/E mutants. To do so, we initially assessed the expression pattern of cest-2.2 in C. elegans by generating a construct with the annotated sequence of the cest-2.2 promoter and a GFP cassette. We obtained a cest-2.2p::GFP extrachromosomal array by microinjection and, consistent with previous evidence (Lee et al., 2003; McKay et al., 2003), we reasoned that CEST-2.2 might have two functions: to drive fatty acid β-oxidation and/or to generate signalling molecules (Fig 3B), such as ascarosides (Ludewig & 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 (Fig 3C). Furthermore, ascr#8 treatment from hatching did not alter gas-1 (fc21) survival (Fig 3D and Appendix Table S1).

To investigate CEST-2.2 biology in the context of mitochondrial dysfunction, we dissected adult intestines, extracted total RNA, and performed next-generation RNA sequencing (NGS). After confirming the higher expression of cest-2.2 in gas-1 (fc21); cest-2.2 O/E animals compared with wt and gas-1 (fc21) mutant animals (Fig 3E), we analyzed our NGS data and found that 560 genes were differentially regulated in gas-1 (fc21) compared to wt animals, 376 of which have either a known or putative function (Fig 3F and G and Appendix Table S3). As expected with intestinal tissue and consistent with our previous study (Gioran et al., 2019), most of these genes are specifically involved in metabolic processes (Fig 3H and Appendix Table S3). When we compared the transcriptional profiles of gas-1 (fc21); cest-2.2 O/E animals and gas-1 mutants, we found that a total of 354 genes were significantly dysregulated (Fig 3F and G), with many of these encoding enzymes involved in lipid metabolism (Fig 3H). 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 (Fig 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.

To experimentally support our NGS data-based IPA predictions, we initially explored eventual changes of whole body fat using an Oil Red O (ORO) staining. We found that gas-1 (fc21); cest-2.2 O/E animals had significantly reduced lipid stores in the intestine when compared to both wt and gas-1 (fc21) mutant nematodes (Fig 4A–F), further implying that gas-1 (fc21); cest-2.2 O/E animals may have enhanced lipid catabolism as predicted by IPA (Fig 3I). There was no correlation between the ORO staining and body size (Fig 4D),

![Figure 3. CEST-2.2 overexpression induces transcriptional changes in gas-2 mutant nematodes.](image-url)

**Data information:** In D, the median lifespan ± SEM is reported underneath the graphs and additional information (e.g., n numbers) are reported in Appendix Table S1; in E, points represent mean ± SEM. Across experiments, P-value summary is ns – not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
ruling out the confounding effect of the larger body mass of gas-1 (fc21); cest-2.2 O/E animals (Fig 1P). As a side note, cest-2.2 O/E nematodes also had slightly less ORO staining in the hindgut when compared to wt ones (Fig 4E and F), though the effect was not as pronounced as the effect of CEST-2.2 O/E in the context of complex I deficiency (Fig 4A–C). 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
Figure 4.
droplets (Fig 4E), as well as having a significantly larger body size when compared to wt nematodes (Fig EV1M). To further support our lines of evidence, we stained nematodes with the neutral lipid stain LipidTOX Green (Klapper et al., 2011) and observed that cest-2.2 O/E animals had a tendency toward less lipids as shown by the ORO staining (Fig 4G–J). Of note, cest-2.2 (lf) exhibited more LipidTOX Green staining compared with wt (Fig 4I and J). Furthermore, we noticed that cest-2.2 downregulation in nuo-6 mutant animals also resulted in a significant increase in LipidTOX staining (Fig EV3A and B). Together, these findings indicate that CEST-2.2 O/E can influence lipid deposits in both wt and complex I-deficient nematodes.

It is known that lipid-rich particles are used to synthesize yolk particles in the intestine that are then transferred to developing oocytes (Kimble & Sharrock, 1983; Perez & Lehner, 2019; 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 (Fig EV3C), 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 with gas-1(fc21) (Fig EV3D). Moreover, while vit-1 downregulation slightly extended C. elegans lifespan (Fig EV3E and Appendix Table S1) as previously described (Seah et al., 2016), vit-1 deficiency had no effect on gas-1(fc21) survival and robustly reduced the lifespan extension of gas-1(fc21); cest-2.2 O/E mutants (Fig EV3E 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.

To identify individual lipid species and gain knowledge of the underlying molecular processes, we set off to refine our studies using more quantitative methods. Because wt animals lay significantly more eggs than gas-1(fc21) and gas-1(fc21); cest-2.2 O/E (Fig 1S), we excluded them from further lipid analysis to avoid the confounding effect of fat stores in the developing embryos. First, we performed a qualitative thin layer chromatography (TLC) in samples from gas-1(fc21) and gas-1(fc21); cest-2.2 O/E animals (Fig 4K). Among all the detected lipids, our TLC analysis showed that free fatty acids (FFAs) tended to be less in cest-2.2 O/E animals (Fig 4K and L). We then performed a quantitative mass-spec analysis of FFAs and found comparable FFA levels between gas-1(fc21) and gas-1(fc21); cest-2.2 O/E animals (Fig 4M and N). However, further investigation of the individual FFAs showed that, compared to gas-1 (fc21) mutants, gas-1(fc21); cest-2.2 O/E animals had a dramatically altered FFA composition with undetectable levels of C-14, C-15, C-18, and C-21 fatty acids (Fig 4O). Of note, total lipid content was comparable between gas-1(fc21) and gas-1(fc21); cest-2.2 O/E animals, with only a handful of fatty acids that were slightly altered (Fig EV3F and G), possibly due to the confounding effect of the plentiful lipids present in cell membranes. Overall, these results strongly indicate that CEST-2.2 O/E alters stored lipid composition, stimulating the mobilization of certain FFAs in complex I-deficient animals.

**CEST-2.2 overexpression stimulates lifespan via fatty acid beta-oxidation**

Our multiomics indicated that CEST-2.2 can influence lipid deposits by enhancing fat catabolism. Consistent with our IPA predictions (Fig 3I), we found that key enzymes involved in lipid metabolism were upregulated in gas-1(fc21); cest-2.2 O/E animals compared with gas-1(fc21) mutants (Fig 5A and B). Since these are described targets of nuclear hormone receptors NHR-49 and NHR-80 (Van Gilst et al., 2005; Brock et al., 2006), 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
Figure 5.
animals (Fig 5C–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 (Fig 5E 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 (Fig 5F and Appendix Table S1). To our surprise and in contrast to previous evidence (Brock *et al*, 2006), both *nhr-49* and *nhr-80* RNAi had an impact on wt lifespan (Fig 5D 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.

The delta-9 fatty acid desaturase FAT-5 contributes to the biosynthesis of monounsaturated fatty acids (MUFA) from palmitic acid (16:0) mostly coming from *E. coli* digestion (Brock *et al*, 2006, 2007; Watts & Ristow, 2017). Although *fat-5* deficiency can be partially compensated by *fat-6* expression because of functional redundancy of delta-9 desaturases in *C. elegans* (Brock *et al*, 2006), we found that *fat-5* downregulation significantly compromised the lifespan extension of *gas-1*(fc21); *cest-2.2* O/E nematodes and partially reduced *gas-1*(fc21) survival (Fig 5G–I and Appendix Table S1).

The lifespan reduction was associated with an increased LipidTOX staining (Fig EV4A), suggesting that loss of FAT-5-dependent fatty acid biosynthesis undermines the lifespan-extending properties of CEST-2.2 because of the inability to efficiently mobilize fatty acid content stored in the intestine. To challenge this hypothesis, we assessed the contribution of fatty acid beta-oxidation, knowing that short chain fatty acid oxidation occurs primarily in mitochondria, whereas longer fatty acids are usually oxidized in peroxisomes (Watts & Ristow, 2017). Since C-21 and C-19 fatty acids were not detected in our quantitative mass-spec analysis of FFAs (Fig 4O), we initially focused on the peroxisomal acyl-CoA oxidase ACOX-1/AOCX1. We found that *acox-1* downregulation significantly reduced the lifespan of *gas-1*(fc21); *cest-2.2* O/E animals, while having only a slight effect on the survival of wt nematodes (Fig 5J and AK and Appendix Table S1). We then downregulated the expression of mitochondrial acyl-CoA synthetase family member 2 ACS-2/ACSF2 and enoyl-CoA hydratase ECH-1.1/HADHA, both significantly upregulated in *gas-1*(fc21); *cest-2.2* O/E mutants compared with *gas-1*(fc21) animals (Fig 5A). We observed that downregulation of both *acs-2* and *ech-1.1* led to a partial lifespan reduction of *gas-1*(fc21); *cest-2.2* O/E and wt animals, whereas the survival of *gas-1*(fc21) mutants remained unaffected (Fig 5L–P and Appendix Table S1).

Together, these epistatic analyses suggest that fatty acid beta-oxidation is required for the lifespan extension of *gas-1*(fc21); *cest-2.2* O/E mutants.

**Oxaloacetate levels correlate with complex II- and LET-721/ETFDH-dependent mitochondrial respiratory capacity of *gas-1*(fc21); *cest-2.2* O/E mutants**

Our data indicate that CEST-2.2 overexpression can stimulate *gas-1*(fc21) lifespan extension and the associated mitochondrial plasticity (Figs 1N and 2A–C), despite the inherent genetic lesion of complex I. To better understand the correlation between longevity and mitochondrial bioenergetics, we downregulated *nhr-49* and *nhr-80* in nematodes and measured OCR. Consistent with their essential role in survival (Fig 5D and E), we found that *nhr-49* and, to a lesser extent, *nhr-80* expression was required for the proper mitochondrial respiratory profiles of wt, *gas-1*(fc21) and *gas-1*(fc21); *cest-2.2* O/E animals (Fig 6A–C).

It is assumed that mitochondrial respiratory capacity is a phenomenon linked to fatty acid beta-oxidation and to an enhanced efficiency in NADH and FADH2 oxidation, which may be in part due to the ETC forming higher order respiratory supercomplexes (Brand & Nicholls, 2011; van der Windt *et al*, 2012; Calvo *et al*, 2020; Bertan *et al*, 2021). Since oxaloacetate is required to sustain the tricarboxylic acid (TCA) cycle and the oxidation of carbon units generated during fatty acid beta-oxidation (Fig 6D), we tested the consequence of oxaloacetate depletion by growing animals on RNAi expressing bacteria against malate dehydrogenase MDH-2. We found that *mdh-2* downregulation reduced the brood size and the lifespan extension of *gas-1*(fc21); *cest-2.2* O/E nematodes, while having a moderate effect on wt survival (Figs 5J, 6E and EV4B, Appendix Table S1). Of note, *mdh-2* RNAi partially extended the lifespan of *gas-1*(fc21) mutants (Fig 6E and Appendix Table S1), possibly because of complete germline loss (Fig EV4C), which can occur as a consequence...
Figure 6.
of TCA cycle impairment (Rahman et al., 2014). Given the well-described lifespan-extending properties of germline loss in C. elegans (Arantes-Oliveira et al., 2002; Wang et al., 2008), it is likely that mdh-2 RNAi extended the lifespan of gas-1(fc21) mutants due to this secondary effect. Of note, CEST-2.2 O/E partially preserved the germline and egg production of gas-1(fc21) mutants grown on mdh-2 RNAi bacteria (Fig EV4C). In line with our hypothesis, oxaloacetate supplementation to gas-1(fc21) was sufficient to potentiate mitochondrial respiration (Figs 6F and G and EV4D and E), whereas feeding of the NAD⁺-precursor nicotinamide mononucleotide (NMN) had no effect (Fig EV4F).

It is known that oxaloacetate can be used to synthesize phosphoenolpyruvate (Fig 6D), which may be redirected to generate glucose or lactate, the latter deriving from NADH-dependent pyruvate reduction. To test this hypothesis, we downregulated the expression of ETFDH, which functionally couple fatty acid beta-oxidation to the ETC by transferring electrons from FADH₂ to ubiquinone/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 maximal respiration of gas-1(fc21); cest-2.2 O/E nematodes (Fig 6K–N). Taken together, our data suggest that CEST-2.2 stimulates mitochondrial bioenergetics by shuttling electrons to complex II and LET-721/ETFDH (Fig 6O).

**Discussion**

Here, we report a genetic manipulation that transforms short-lived gas-1(fc21) nematodes into long-lived ones. Moreover, we describe how the usage of lipid deposits can be redirected to generate metabolites and reducing equivalents that stimulate the residual mitochondrial respiration despite an intrinsic complex I lesion.

Building upon our previous studies (Piazzesi et al., 2016; Gioran et al., 2019), we assessed the epigenetic landscapes of two models of complex I deficiencies that differently affect C. elegans lifespan. Specifically, we compared histone H3.3 deposition at the promoters of transcriptionally dysregulated genes in long-lived nuo-6(qm200) mutants and short-lived gas-1(fc21) animals. We herein report that H3.3 is indeed differentially loaded on promoters in short-lived and long-lived mitochondrial mutants, indicating that H3.3 deposition can be affected by the disease state of the organism. We found that H3.3 deposition on cest-2.2 promoter correlates with its expression levels in gas-1(fc21) and nuo-6(qm200) mutants. We ultimately show that the recently described carboxylesterase CEST-2.2 influences 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. In this regard, our NGS data in gas-1 mutants demonstrate that CEST-2.2 O/E
stimulates the regulation of genes that are primarily involved in lipid mobilization and catabolism, whereas it suppresses the accumulation of storage lipids.

The contribution of lipid metabolism in age-related processes has attracted much attention because of its relevance in human illnesses (Johnson & Stolzing, 2019). In the context of aging, a tightly regulated lipid mobilization can sustain lifespan-extending programs by coupling reproduction, energy availability, and somatic aging (Wang et al., 2008). Some of these events may be transgenerationally inherited, since they depend on chromatin remodeling and require an epigenetically regulated transcription of enzymes involved in the accumulation of distinct classes of lipids (e.g., MUFAs) (Greer et al., 2011; Han et al., 2017). In this regard, lipolysis is often an adaptive response to environment changes and nutritional state that may influence energy homeostasis and, as a consequence, longevity (O’Rourke & Ruvkun, 2013). Interestingly, lipid remodeling in distinct tissues may even have a paracrine function, since it induces cell-nonautonomous signals that promote epigenetic regulation of transcriptionally controlled prolongevity pathways (Imanikia et al., 2019; Schmeisser et al., 2019). In our work, we demonstrate that CEST-2.2 expression sustains the longevity of nuo-6 (qm200) nematodes and, more importantly, is sufficient to improve the survival of sick mitochondrial gas-1 mutants. The significant lifespan extension of gas-1(fc21); cest-2.2 O/E animals further emphasizes the essential nature of this tissue-specific hydrolase in the mobilization of available resources that can counteract mitochondrial lesions. With the recently acquired knowledge that CEST-2.2 is involved in very long-chain fatty acid oxidation and ascarosides biosynthesis (Le et al., 2020), we suggest that an increase of lipolysis and/or fatty acid beta-oxidation seems to provide 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 either partially or completely ablated the lifespan extension of gas-1(fc21); cest-2.2 O/E animals. Our current experimental evidence indicates that malate dehydrogenase MDH-2/MDH significantly supports the lifespan extension of gas-1(fc21); cest-2.2 O/E animals, while PCK1/PCK1 contribution is negligible. Thus, it seems plausible that MDH-2/MDH is required to maintain levels of oxaloacetate that are compatible with an enhanced TCA cycle, which mediates the oxidation of carbon units deriving from fatty acid beta-oxidation. In support of this, 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 propose 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 feed 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 heterogeneous nature of disease associated with mitochondrial dysfunction.

The “phenotypic threshold effect” is a multifactorial phenomenon that tries to explain the complex pathobiology of mitochondrial diseases (Rossignol et al., 2003). Based on this theory, clinical outcomes become evident when the existing compensatory mechanisms, which were initially sufficient to buffer mitochondrial deficiency, cannot longer satisfy extraordinary energy demands or sustain an additional environmental pressure. The subsequent energy crisis overcomes the established adaptation and triggers an irreversible cascade of detrimental events that eventually lead to pathology onset. In this complex scenario, it may appear counterintuitive that mild mitochondrial deficiency during development can stimulate C. elegans survival and healthspan (Lakowski & Hekimi, 1996; Felkai et al., 1999; Dillin et al., 2002; Rea et al., 2007; Riera et al., 2016; Shpilka & Haynes, 2018). This evidence further emphasizes the capacity of a biological system to adjust its metabolic activities adequately to overcome the lack of an efficient ATP generation primarily based on aerobic OXPHOS. To establish processes that promote stress resilience and longevity, cells must stimulate mitochondria-to-nucleus stress signaling that efficiently builds up an adaptive resistance to a potentially lethal metabolic state as well as to other detrimental insults, such as poor nutritional conditions and hostile environmental challenges. In our study, we show that CEST-2.2 overexpression specifically induces UPR(l), indicating that the transcriptional engagement of distinct longevity pathways, rather than others (e.g., ROS-dependent signaling), may act as powerful disease modifiers.

Taken together, our findings imply that metabolic flexibility contributes to the phenotypic threshold effect observed in invertebrates carrying mitochondrial complex I lesions. As an added value, our study suggests that stimulation of lipid mobilization, through a transcriptionally regulated expression of a distinct intestine-specific hydrolase, is sufficient to circumvent complex I deficiency in C. elegans, with beneficial consequence on survival.

Materials and Methods

ATP quantification

Synchronized nematodes (100 mg) were collected, flash-frozen in liquid nitrogen and stored at −80°C. Pellets were homogenized in ATP buffer and deproteinized in 4 M perchloric acid and 2 M KOH as per the manufacturer’s instructions (abcam ATP Assay Kit ab83355). Relative ATP content was measured with a microplate fluorescence reader at Ex/Em 535/587.

Caenorhabditis elegans strains and maintenance

All experiments were performed at 20°C, and all strains were cultured following standard methods. The following strains were used in this study: wild-type N2 (Bristol), BAN125 gas-1(fc21)X; zuls178[his-72p::his-72::GFP], BAN126 zuls178[his-72p::his-72::GFP], BAN129 nuo-6(qm200); zuls178[his-72p::his-72::GFP], BAN141 nuo-6(qm200); his-72(tm2066)III; his-71(ok2289)X, BAN150 gas-1 (fc21); zcls13[hsp-6p::GFP; lin-15(+)], BAN198 unc-119(ed3)III, BAN328 unc-119(ed3)III; bonl455[cest-2.2p::cest-2.2; unc-119(+)], BAN329 unc-119(ed3)III; gas-1(fc21); bonl455[cest-2.2p::cest-2.2; unc-119(+)], BAN332 nuo-6(qm200); zcls13[hsp-6p::GFP; lin-15(+)],
BAN335 bonEx49[cest-2.2p::GFP myo-2p::mCherry], BAN351 cest-2.2 (bon52)V, BAN363 gas-1(fc21)X; zcls17[ges-1p::GFP(mat)], BAN366 gas-1(fc21); zcls14[myo-3p::GFP(mat)], BAN367 unc-119(ed3)III; gas-1(fc21)X; bonls45[cest-2.2p::cest-2.2; unc-119(+)], ges-1p::GFP(mat), BAN455 unc-119(ed3)III; cest-2.2(bon52)V; gas-1(fc21)X; bonls45[cest-2.2p::cest-2.2; unc-119(+)], BAN466 nuo-6(qm200)i; unc-119 (ed3)III; bonls45[cest-2.2p::cest-2.2; unc-119(+)], BAN480 mev-1 (kn1)III; unc-119(ed3)III; bonls45[cest-2.2p::cest-2.2; unc-119(+)], BAN485 unc-119(ed3)III; gas-1(fc21)X; bonls45[cest-2.2p::cest-2.2; unc-119(+)]; zcls14[myo-3p::GFP(mat)], BAN499 unc-119(ed3)III; atfs-1(tm4525); gas-1(fc21)X; bonls45[cest-2.2p::cest-2.2; unc-119(+)], CW152 gas-1(fc21)X, MQ887 isp-1(qm150)IV, MQ1333 nuo-6(qm200)i, RB1702 sod-2 (ok1030)i, SJ4100 zcls13[hisp-6p::GFP; lin-15(+)]; SJ4103 zcls14[myo-3p::GFP(mat)], SJ4143 zcls17[ges-1p::GFP(mat)], and TK22 mev-1 (kn1)III. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

ChiP-Seq

Chromatin extraction and chromatin immunoprecipitation followed by MeDIP sequencing (ChiP-Seq) was performed by Active Motif Inc using a previously validated antibody against GFP. L4 nematodes were collected in M9, flash-frozen in liquid nitrogen, and stored at –80°C. Chromatin was extracted as previously described (Askjaer et al., 2014). Approximately 10 μg of chromatin was then used for immunoprecipitation with 5 μl of a ChIP-validated rabbit polyclonal anti-GFP antibody (Abcam, ab290, Lot No. GR278073-1). Sequence reads were generated by illumina sequencing and mapped with the BWA algorithm. Peaks were called using MACS and SICER. Only peaks > 120 were used in the comparative analysis.

Confocal imaging analysis

In vivo super-resolution confocal imaging of mitochondria was performed in young adult anesthetized (levamisole) nematodes carrying ges-1p::gfp(mat), and adults fixed with 4% PFA in nematodes carrying myo-3p::gfp(mat). Images were taken using the Zeiss Airyscan 2 LSM 900 equipped with a 63× oil objective. Images were deconvoluted using Zen blue. Analysis of mitochondrial morphology was performed using Fiji 2.0.0 (open source).

CRISPR/Cas9 genome editing

A customized injection mix including sgRNAs targeting the coding sequence of cest-2.2, Cas9 protein, and a plasmid encoding myo-2p::GFP was generated by NemaMetrix Inc. (Eugene OR, USA). Adult wild-type worms were microinjected with this custom mix, and the progeny was screened for GFP expression in the pharynx. GFP(+) worms were singled out and genotyped for the mutation until homozygosity was reached. The cest-2.2(lf) strain was then outcrossed twice before use in this study.

DNA constructs

The cest-2.2p::cest-2.2p::cest-2.2 3′UTR plasmid was generated by ATG:biosynthetics GmbH (Merzhausen, Germany). The unc-119 rescue cassette was cloned into the plasmid by digesting it with Apal and SacII restriction enzymes followed by ligation, in order to make the plasmid compatible with biolistic transformation. A Gateway-compatible plasmid with the annotated sequence of the cest-2.2 promoter was obtained from Dharmacon, Inc (Colorado, USA). The cest-2.2 promoter was then cloned into a Gateway-compatible plasmid containing a gfp::let-858 3′UTR cassette with the 2-fragment Gateway method as per the manufacturer’s instructions (Thermo Fisher Scientific, USA).

Egg-laying assay

Synchronized wt, gas-1(fc21) and gas-1(fc21); cest-2.2 O/E nematodes were placed on individual NGM plates seeded with OP50 at 3 days of age (L4 stage). Animals were transferred every day, and the eggs laid in each 24 h period were counted until the animal exited the egg-laying stage.

Fatty acid analysis

Synchronized 5-day-old nematodes were sorted with a COPAS Biosorter (~1,000 animals per samples), flash-frozen in liquid N2, and stored at –80°C. For total lipid analysis, samples were extracted overnight in 5 ml chloroform:methanol (1:1, v:v). An aliquot of 500 μl of each sample was transferred in precleaved glass vials, the solvent was evaporated, and the samples were transesterified for 2 h at 80°C adding 2 ml of 1 N methanolic:HCl. Each sample was spiked with dotriacontane as an internal standard. The transesterification reaction was stopped by adding 2 ml of a saturated NaCl solution, and lipid monomers were extracted three times with hexane. Samples were derivatized by adding 20 μl N,O-bis (trimethylsilyl)-trifluoroacetamide (BSTFA; Macher-Nagel, Duren, Germany) and 20 μl pyridine (Sigma Aldrich, Deisenhofen, Germany) for 45 min at 70°C. For free fatty acid analysis, the respective TLC bands were scratched out, extracted in 5 ml chloroform, and analyzed as described above. For quantification 1 μl of each sample was analyzed by on column injection and gas chromatography equipped with flame ionization detection (GC-FID; CG-Hewlett-Packard 5890 series H, Hewlett-Packard, Palo Alto, CA, USA). For substance identification, 1 μl of each sample was analyzed by GC-MS (mass spectrometry, quadrupole mass selective detector HP 5971, Hewlett-Packard, Palo Alto, CA, USA).

Lifespan assays

All lifespan assays were performed at 20°C and without 5′-fluorodeoxyuridine (FUDR) supplementation. Gravid nematodes were treated with hypochlorite solution and eggs were placed on NGM plates seeded with E. coli OP50. For RNAi experiments, eggs were placed on NGM plates supplemented with ampicillin and seeded with E. coli HT115 bacteria carrying either an empty vector (pL4400) or plasmids expressing dsRNA against target genes (Ahringer library, Source Bioscience LifeSciences). Expression of dsRNA was induced by 1 mM isopropyl-β-d-1-thiogalactopyranoside. Adults were transferred every other day until egg laying was complete. Subsequently, nematodes were transferred twice per week and scored every other day by gentle prodding, and those that did not respond were scored as dead. Nematodes, which died of internal
egg hatching, protruded vulvas, or drying on the edge of the plates were censored.

**Lipid staining**

**LipidTOX staining**
Adult nematodes were collected in M9 buffer, washed, and fixed in 4% PFA for 15 min. PFA was removed, and worm pellets were permeabilized with three freeze/thaw cycles in liquid nitrogen. Worm pellets were washed three times in M9 then stained with 1:200 HCS LipidTOX Green Neutral Lipid Stain solution (ThermoFisher) in M9 for 1.5 h at room temperature. Worms were washed three times with M9 and mounted onto glass slides in 100% glycerol. Images were taken using the Zeiss Airyscan 2 LSM 900 equipped with a 20× air objective and analyzed with Fiji 2.0.0 (open source).

**ORO lipid staining**
Adult nematodes were collected in M9 buffer, washed, and fixed in 1% PFA for 30 min. Fixed nematodes were then washed twice with M9 and incubated in 60% isopropanol for 15 min. Freshly prepared Oil Red O (ORO) stock solution (5mg/ml, in isopropanol) was diluted to 60% and used to stain the fixed nematodes overnight at RT. Samples were then washed three times in M9 and mounted onto glass slides in 100% glycerol. Brightfield images were acquired with an Axio Scan.Z1 slide scanner equipped with a 20× air objective (Zeiss). Images were analyzed with Fiji 2.0.0 (open source).

**Locomotor activity**
Nematodes were synchronized by NaClO/NaOH treatment and transferred every other day until they were 10 days old. At adulthood stage, 35 nematodes were manually transferred to a 96 well flat-bottom plate containing 100 µl of S medium and 6 mg/ml of filtered OP50, with a minimum of three wells analyzed per condition. The plate was placed into a WMicrotracker (Phylumtech S.A., Argentina) and the locomotory activity was recorded twice over 1 h.

**Microparticle bombardment**
Freshly starved unc-119(ed3) mutant nematodes were washed in M9 and placed on an unseeded NGM plate. Approximately 15 µg of plasmid DNA was added to 50 µl of 60 mg/ml gold particles, 20 µl of 0.1 M spermidine and 50 µl of 2.5 M CaCl2. The suspension was pelleted, washed twice, and then resuspended in 170 µl of 100% ethanol. DNA-gold complexes were then spotted on microparticle carrier disks, and nematodes were bombarded with a Biologic PDS/1000-He System (BIO-RAD). Bombarded animals were recovered in M9 and spotted on NGM plates with OP50. F1 animals were then screened and selected based on normal movement, and F2 animals were screened for homozygosity.

**Next-generation sequencing (NGS)**
We manually dissected 20 intestines from wt N2, gas-1(fc21) and gas-1(fc21); cest-2.2 O/E animals (5 replicates each), which were then placed in 1 ml of Qiazol and snap-frozen in liquid nitrogen. Total RNA was extracted using the miRNeasy Micro kit (Qiagen) according to manufacturer’s protocol. RNA was quantified and RNA integrity was determined using the HS RNA assay on a Tapestation 4200 system (Agilent). Smart-seq2 was used for the generation of non-strand-specific, full transcript sequencing libraries using standard reagents and procedures as previously described (Picelli et al., 2014). Briefly, 1 ng of total RNA was transferred to buffer containing 0.2% TritonX-100, protein-based RNase inhibitor, dNTPs, and oligo-dT oligonucleotides to prime the subsequent RT reaction on polyadenylated mRNA sequences. The SMART RT reaction was performed at 42°C for 90 min using commercial SuperScript II (Invitrogen) and a TSO. A preamplification PCR of 14 cycles was performed to generate double-stranded DNA from the cDNA template. At least 200 pg of amplified cDNA were used for tagmentation reaction and subsequent PCR amplification using the Nextera XT kit (Illumina) to construct sequencing libraries. Libraries were quantified using the Qubit hs dsDNA assay, and library fragment size distribution was determined using the D1000 assay on a Tapestation 4200 system (Agilent). Samples were pooled and clustered at 1.4 pM on a NextSeq500 system (Illumina) to generate ~10 M single end reads per sample using High Output v2.5 chemistry. Sequencing data were demultiplexed using bcl2fastq2 v2.20 and pseudo aligned to Caenorhabditis elegans, WBCel235.98 transcriptome using kallisto v.0.44.0. Data analysis was performed using Shiny-Seq.

**Oxygen consumption**
Synchronized adult nematodes were placed on NGM plate seeded with heat-killed OP50 for 2 h. We manually transferred 50 nematodes per well to a Seahorse XF24 cell culture microplate in 525 µl of M9 buffer. Measurements were performed by using a Seahorse XF24 Analyzer (Agilent Technologies, USA). Nematodes were monitored for basal oxygen consumption and maximal respiration following an injection of 25 µM FCCP. To control for nonmitochondrial oxygen consumption, 20 mM sodium azide was finally injected.

**Pharyngeal pumping**
Pharyngeal pumping assay was performed using 5-day-old nematodes, which were placed on an NGM plate seeded with OP50 and left to acclimatize for at least 1 h. Pharyngeal pumps of individual animals were counted in a 60-s interval.

**RNA extraction and quantitative real-time PCR**
RNA was extracted from nematodes stored at –80°C with a QIAGEN RNeasy Kit as per the manufacturer’s instructions. RNA was then retrotranscribed with qScript (Quantabio), and qRT-PCR was performed with SYBERgreen master mix (Applied Biosystems, USA) on an Applied Biosystems qRT-PCR Thermocycler. The following primers were used in this study:

- caco-1.1 F: 5’-CTTCCAGCAAAGACCCTCGT-3’
- caco-1.1 R: 5’-TCAGTTGGGCCTAGTTCC-3’
- acs-2 F: 5’-CTATGTTCACACAAATGCTTGAGG-3’
- acs-2 R: 5’-TGGGATTTGATGCTTCACAA-3’
- cest-2.2 F: 5’-TGCACTGGAAACAATTGAGG-3’
Characterization of Stable isotope Labeled (SIL) Peptides

Synthetic PEPotec isotope-labeled c-terminal lysine (K) or arginine (R) crude peptides were purchased (Thermo Scientific), and an equimolar amount of each heavy peptide was mixed together at a final concentration of 1 pmol/μl in 0.1% FA to generate a pool of CEST-2.2 SIL peptides for subsequent nanoLC-MS/MS analysis to determine their intensities.

Liquid chromatography and Survey MS analyses

Endogenous and SIL peptides were mixed and injected with a flow rate of 300 nl/min, at starting conditions of 95% eluent A (0.1% FA in water) and 5% eluent B (0.1% FA in 80% ACN) for analysis on a Dionex Ultimate 3000 RSLC nanosystem coupled to an Orbitrap Exploris 480 MS. They were loaded onto a trap column cartridge (Acclaim PepMap C18 100 Å, 5 mm × 300 μm i.d., #160454, Thermo Scientific) and separated by reverse-phase chromatography on an Acclaim PepMap 100 C18 75 μm × 25 cm (both columns from Thermo Scientific) using a 35-min linear increasing gradient from 5% to 25% of eluent B followed by a 5-min linear increase to 50% eluent B. The mass spectrometer was operated in data-dependent and positive ion mode with a spray voltage of 1.9 kV, no sheath or auxiliary gas flow, heated capillary temperature of 300°C, MS1 spectra recorded at a resolution of 120K, mass scan range of 300–1,500, automatic gain control (AGC) target value of 300% (3 × 10⁶) ions, maximum injection time (maxIT) of 50 ms, and a default charge state of 2. For every scan, the top 40 most intense ions on the inclusion list (if above a 1e5 intensity threshold) were isolated within a width of 1.0 m/z and fragmented with a normalized collision energy (NCE) of 28% by higher energy collisional dissociation (HCD), scan range of 100–1,700 m/z, maximum IT of 10 ms, AGC target value of 1,000%, and resolution of 7,500.

SureQuant-based quantitation of selected CEST-2.2 tryptic peptides

SureQuant analysis was performed as previously described (Stopfer et al., 2021). Briefly, data acquisition was performed using a modified SureQuant template with 3 branches for the +2 (R, K), +3 (K) charge states of SIL lysine and arginine residues. Peak area ratios of endogenous light peptides and corresponding heavy IS peptides for the 6 selected product ions were exported from Skyline software v21.1.0.278 (MacLean et al. 2010) and peptides filtered according to the following criteria: First, only IS peptides with an AUC > 0 for n ≥ 5 product ions were considered. Second, peak area values of the 3 highest intensity product ions from both the light/heavy peptides were summed, and their light/heavy ratios were used to quantify peptide signals across samples. Quantitation was based on 3 selected product ions to balance specificity with the ability to retain low abundant targets.

Thin layer chromatography

Animals at 5 days of age were sorted with a COPAS BioSorter, collected (1,000 animals per sample), flash-frozen, and stored at −80°C. Lipids were extracted by homogenizing pellets in 5 ml ExMi (chloroform–methanol–water 10:5:1 by volume) and then incubated at 48°C for 48 h. Denaturated proteins were then filtered from extract with cotton, and lipids were dried under an N₂ stream. Dried lipids were resuspended in ExMi and spotted on a silica gel plate.
(Merck). Half of the plate was developed twice with chloroform–
methanol–water (60:30:5 by volume), and then, the full plate was
developed in hexane–diethyl ether–acetic acid (80:20:1.5 by vol-
ume). Lipids were visualized by charcoaling or alternatively by
iodine vapors.

Treatments with oxaloacetate, NMN, ascorbate/vitamin C,
and ascarosides

Standard NGM was prepared, supplemented with either 2 mM oxalo-
acetate or 1 mM nicotinamide mononucleotide (NMN) in aqueous
solution, and the pH was adjusted with NaOH before pouring. Plates
were seeded with OP50, and gas-1(fk21) mutants were grown on
supplemented plates from hatching. Alternatively, plates were seeded
with heat-killed OP50, and 4-day-old gas-1(fk21) mutants were placed
on these plates for 24 h before collection. L-ascorbate/vitamin C was
dissolved in MilliQ water and then filtered to obtain a sterile 10 mg/ ml stock solution. Stock solution was added into the freshly prepared
NGM to achieve a final concentration of 1 mg/ml. After pouring,
plates were kept at 4°C until their use. Ascaroside ascr#8 and ascr#81
were synthesized and resuspended in 100% ethanol, then diluted to
50 μM solutions in dH2O for a final ethanol concentration of 1.7%.
Both ascr#8 and ascr#81 (200 μl) or 1.7% ethanol (as control) were
spread on the surface of 55 mm NGM plates and allowed to dry over-
night (final concentration: 1 μM). The following day the plates were
seeded with OP50, dried overnight and, gas-1(fk21) eggs were added.

Western blot analysis

Adult nematodes were washed three times with sterile water and
the frozen at −80°C until protein extraction. Pellets were sonicated
in ice-cold RIPA buffer (Sigma) with protease and phosphatase
inhibitors. Lysates were cleared by centrifugation, total protein was
determined by Bradford Assay (Sigma) and boiled in Laemmli buffer
for 5 min. Approximately 20–40 μg of total protein was resolved on
10–15% polyacrylamide gels and subsequently transferred to nitro-
cellulose membranes using semi-dry transfer Trans-Blot Turbo™
(Bio-Rad). Following antibody incubation, membranes were devel-
oped in ECL and imaged using Chemidoc imaging system (Bio-Rad).

Data availability

The ChIP-Seq and the RNA-Seq in the gut have been deposited to
NCBI GEO database under the common accession number
GSE168502: https://www.ncbi.nlm.nih.gov/geo/experiment?query=sample&series=168501

The RNA-Seq in nuo-6(qm200) versus wt and nuo-6(qm200);
his-72(tm2066); his-71(ok2289) versus nuo-6(qm200) has been
published in Piazzesi et al (2016). The RNA-Seq of gas-1(fk21) ver-
sus wt has been published in Gioran et al (2019).

Expanded View for this article is available online.

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Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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