

SHORT COMMUNICATION

Nuclear hormone receptors as mediators of metabolic adaptability following reproductive perturbations

Ramesh Ratnappan^a, Jordan D. Ward^b, Keith R. Yamamoto^b, and Arjumand Ghazi^a

^aDepartment of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA; ^bDepartment of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA, USA

ABSTRACT

Previously, we identified a group of nuclear hormone receptors (NHRs) that promote longevity in the nematode *Caenorhabditis elegans* following germline-stem cell (GSC) loss. This group included NHR-49, the worm protein that performs functions similar to vertebrate PPAR α , a key regulator of lipid metabolism. We showed that NHR-49/PPAR α enhances mitochondrial β -oxidation and fatty acid desaturation upon germline removal, and through the coordinated enhancement of these processes allows the animal to retain lipid homeostasis and undergo lifespan extension. NHR-49/PPAR α expression is elevated in GSC-ablated animals, in part, by DAF-16/FOXO3A and TCER-1/TCERG1, two other conserved, pro-longevity transcriptional regulators that are essential for germline-less longevity. In exploring the roles of the other pro-longevity NHRs, we discovered that one of them, NHR-71/HNF4, physically interacted with NHR-49/PPAR α . NHR-71/HNF4 did not have a broad impact on the expression of β -oxidation and desaturation targets of NHR-49/PPAR α . But, both NHR-49/PPAR α and NHR-71/HNF4 were essential for the increased expression of DAF-16/FOXO3A- and TCER-1/TCERG1-downstream target genes. In addition, *nhr-49* inactivation caused a striking membrane localization of KRI-1, the only known common upstream regulator of DAF-16/FOXO3A and TCER-1/TCERG1, suggesting that it may operate in a positive feedback loop to potentiate the activity of this pathway. These data underscore how selective interactions between NHRs that function as nodes in metabolic networks, confer functional specificity in response to different physiological stimuli.

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Introduction

Different stages of an animal's life, such as development, sexual maturity, peak fertility, and reproductive senescence require distinct metabolic programs. During these transitions, physiological homeostasis needs to be maintained, so a metabolic network must be both robust and flexible. This is brought about by the precise orchestration of numerous gene-expression networks. Understanding how these networks operate across multiple cells, tissues and organ systems to alter feeding, energy expenditure, lipid storage and length of life, depending on reproductive cues, is a significant undertaking. This is an important problem because the ability to respond to the persistent adaptive challenges, such as those created by changing procreational demands, is critical for an organism's survival. Indeed, multiple aging-associated debilitations and diseases in humans are attributed to loss of metabolic homeostasis with age.¹

Recently, research in the nematode *Caenorhabditis elegans* has revealed novel insights into the relationship between metabolic status, reproduction and aging.²⁻⁴ In worms, as in many other organisms, mating has damaging consequences for the female, including reducing its size, stress-resistance and lifespan.^{5,6} Alternatively, removing the germline increases worm lifespan significantly,⁷ in accordance with the observation that reduced fertility is often associated with increased lifespan in many species.⁸⁻¹¹ However, recent studies into the molecular basis of these phenomena have shown that the relationship between reproduction and aging is far more nuanced than previously appreciated reviewed in reference 12. For instance, while it is true that in some animals mating is detrimental to the health of the female, it is also well documented that in many others, males transfer 'nuptial gifts' along with the sperm that provide direct

CONTACT Arjumand Ghazi  arjumand.ghazi@chp.edu  Department of Pediatrics, University of Pittsburgh School of Medicine, 7129 Rangos Research Center, 4401 Penn Avenue, Pittsburgh, PA 15224, USA.

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benefits to the recipient female. These ‘nuptial gifts’ vary from energy supplies to glandular products that increase longevity.¹³ Similarly, simply making *C. elegans* sterile (by removing the differentiated gametes alone, for instance) does not make them live longer. It is only upon elimination of germline-stem cells (GSCs), a population of totipotent proliferative cells that give rise to the entire adult germline, that lifespan is extended.¹⁴ Little is understood about what causes some reproductive signals to impede health and others to improve it. However, it is evident that reproductive transitions require metabolic adaptability, and this in turn may determine whether procreative events produce beneficial or detrimental consequences to the animal.

Removal of GSCs in *C. elegans* is accompanied by the activation of a group of transcription factors that collectively mediate gene-expression changes resulting in the enhancement of protective cellular processes such as proteasomal function, autophagy and stress resistance (reviewed in²). These conserved proteins are essential for the longevity of GSC-less animals and include DAF-16/FOXO3A, TCER-1/TCERG1, PHA-4/FOXA, HLH-30/TFEB, HSF-1/HSF1, SKN-1/NRF2.^{7,15-19} Additionally, three members of the nuclear hormone receptor (NHR) family of transcription regulators, DAF-12/FXR, NHR-80/HNF4 and NHR-49/PPAR α are important for GSC-less longevity.^{7,20-22} Multiple lines of evidence have indicated that these factors link lipid metabolism to reproductive stimuli and aging. DAF-16/FOXO3A, TCER-1/TCERG1, SKN-1/NRF2 and DAF-12/FXR targets include lipid-metabolic genes,^{15,19,20,23-25} PHA-4/FOXA- and HLH-30/TFEB-mediated enhancement of autophagy contributes to increased lipase activity^{17,18} and NHR-80/HNF4 promotes fatty-acid desaturation in long-lived, GSC-less adults.²¹ However, the gene-regulatory network (GRN) in which these factors operate is poorly defined and it remains unclear how their activities allow an animal to not only survive the physiological disruption created by loss of GSCs but to convert it into a beneficial enhancement of health and lifespan.

Discovery of the role of NHR-49/PPAR α in lipid homeostasis and longevity of germline-less animals

NHRs are of particular relevance in the context of lipid metabolism and aging since many of them are

lipid-sensing factors whose ligands are fatty-acid moieties and steroid signals, and because they have pivotal roles in metabolic homeostasis.²⁶ In *C. elegans*, the NHR family has expanded to include 284 members, many of which are derived from a single ancestor, the hepatocyte nuclear factor 4 (HNF4).^{27,28} In a recent study, we identified a group of NHRs that are essential for the longevity of germline-ablated worms. Of these, NHR-49 produced the most striking lifespan phenotypes.²² NHR-49 is sequentially similar to HNF4 α but performs functions undertaken by peroxisome proliferator activated receptor α (PPAR α) in vertebrates.^{29,30} The degree of equivalence between NHR-49 and PPAR α is currently unclear, but in view of their similar activities, and for clarity and convenience, NHR-49 is referred to as the functional homolog of PPAR α , here and in other studies.^{27,31-33} PPAR α is a key regulator of fatty-acid β -oxidation and a member of the PPAR family that also includes other modulators of energy metabolism.³⁴ *nhr-49* was first identified as a regulator of mitochondrial β -oxidation genes during development and for inducing the expression of some β -oxidation while repressing others during short-term fasting.^{29,30} In subsequent studies, it was found to be essential for adult reproductive diapause (ARD), an adaptive mechanism by which sexually mature adults delay reproduction and retain a small group of GSCs that are used to repopulate the gonad and continue reproduction when feeding is resumed.³⁵ Our study disclosed that NHR-49/PPAR α was critical for any lifespan increment following GSC removal.²² Based on its previously-described functions, it is plausible that NHR-49/PPAR α facilitates endurance under diverse conditions that pose physiological challenges to the animal such as nutritional limitation or loss of reproductive capacity.

Using quantitative PCRs (Q-PCRs) and a transgenic strain expressing GFP-tagged NHR-49/PPAR α protein under control of its endogenous promoter, we showed that NHR-49/PPAR α was transcriptionally upregulated upon germline loss, in part through the activities of DAF-16/FOXO3A and TCER-1/TCERG1. Strikingly, in an independent genomic study, we identified *nhr-49*, as well as multiple β -oxidation and desaturation genes, as being upregulated by DAF-16/FOXO3A- and/or TCER-1/TCERG1 in GSC-less adults (Amrit et al.³⁶). Based on this evidence, and the

previously-documented regulation of these processes by NHR-49/PPAR α , we asked if NHR-49/PPAR α enhanced mitochondrial β -oxidation and/or fatty-acid desaturation in germline-less animals. Indeed, the expression levels of 12 genes predicted to function in mitochondrial β -oxidation that we tested were all elevated in long-lived, germline-less adults. Of these, the upregulation of seven genes was NHR-49/PPAR α dependent. These genes encode enzymes that together represent all the individual steps of mitochondrial β -oxidation necessary for the breakdown of fatty acids into acetyl CoA. RNAi-mediated knockdown of many of these genes shortened the longevity of germline-ablated animals. Our observations indicated that NHR-49/PPAR α enhances the breakdown of fatty acids through mitochondrial β -oxidation following germline loss, and this augmentation is important for the consequent adaptation and longevity.²²

nhr-49 mutants exhibit altered expression of enzymes that mediate fatty-acid desaturation, during development as well as fasting.^{29,30} Fatty-acid desaturation is also regulated by NHR-80 that is structurally similar to HNF4³⁷ (we refer to NHR-80 as NHR-80/HNF4 in keeping with the terminology used in worm literature^{21,27}). In a previous study, Hugo Aguilaniu's laboratory reported that, in germline-less mutants too, NHR-80/HNF4 elevates the expression of enzymes responsible for converting a saturated fatty acid (SFA), stearic acid (SA, C16:0) into a mono-unsaturated fatty acid (MUFA), oleic acid (OA, C18:1n9).²¹ We found that NHR-49/PPAR α similarly controlled the expression of these desaturases in germline-less adults. Through lipidomic analyses, we further discovered that GSC-depleted adults showed an increase in unsaturated fatty acid (UFA) levels and a concomitant decrease in SFAs.^{22,36} The conversion of SA to OA, as well as that of many other SFAs to MUFAs, was prevented and the overall levels of MUFAs and PUFAs were demonstrably reduced in the absence of NHR-49/PPAR α .²² Thus, it appears that NHR-49/PPAR α mediates the simultaneous enhancement of mitochondrial β -oxidation as well as widespread desaturation of fatty acids following GSC removal. How it modulates disparate biochemical pathways and what other functions are undertaken by the protein upon germline removal remain unknown.

Results

NHR-49/PPAR α potentiates DAF-16/FOXO3A and TCER-1/TCERG1 activity in germline-less adults

NHRs have modular ligand-binding domains (LBDs) that can be used to bind co-factors, to homodimerize or to heterodimerize with other NHRs. Through these dimerizations, NHRs, including NHR-49/PPAR α , control the transcription of different subsets of targets.³⁷⁻³⁹ Since our screen identified multiple NHRs that were essential for germline-less longevity, we asked if NHR-49/PPAR α exhibited similar heterodimeric interactions with the other NHRs too. Using yeast 2 hybrid (Y2H) assays, we tested the potential interactions of 6 NHRs (genes encoding 5 of these, *nhr-60*, *nhr-71*, *nhr-81*, *nhr-212* and *nhr-213*, were identified in our screen²²) with NHR-49/PPAR α , DAF-16/FOXO3A and two of five TCER1/TCERG1 transcripts, TCER-1a and TCER-1d). We found that one of the NHRs we previously identified, NHR-71, (Fig. 1A) interacted with NHR-49/PPAR α (Fig. 1B). NHR-71 shows structural homology to HNF4 although its precise function has not been described so far, so we refer to it as NHR-71/HNF4. Interestingly, NHR-71/HNF4 and NHR-49/PPAR α were reported to exhibit reciprocal interactions with each other in a previous report that characterized transcriptional networks in *C. elegans*.³⁹ To address the mechanistic relevance of this interaction in GSC-less animals, we asked if NHR-71/HNF4 is also required for any of the molecular events controlled by NHR-49/PPAR α upon germline ablation, i.e., β -oxidation, and desaturation. In temperature-sensitive *glp-1* mutants, a widely used genetic surrogate for the longevity caused by GSC removal,¹⁴ *nhr-71* RNAi moderately impaired the expression of *acs-2* (Fig. 2A), but did not cause a consistent reduction in the expression of other β -oxidation genes upregulated by NHR-49/PPAR α upon germline loss (eg., *acs-17*, *cpt-5*, *ech-7* and *acdh-11*; data not shown). The upregulation of the desaturase genes, *fat-5* and *fat-6*, was not hampered by *nhr-71* RNAi either (Fig. 2B, C). This suggested that NHR-71/HNF4 may not partner with NHR-49/PPAR α to regulate the expression of the latter's β -oxidation and desaturation target genes upon GSC loss.

Since both *nhr-49* and *nhr-71* were identified in our screen based on a necessity for upregulation of a

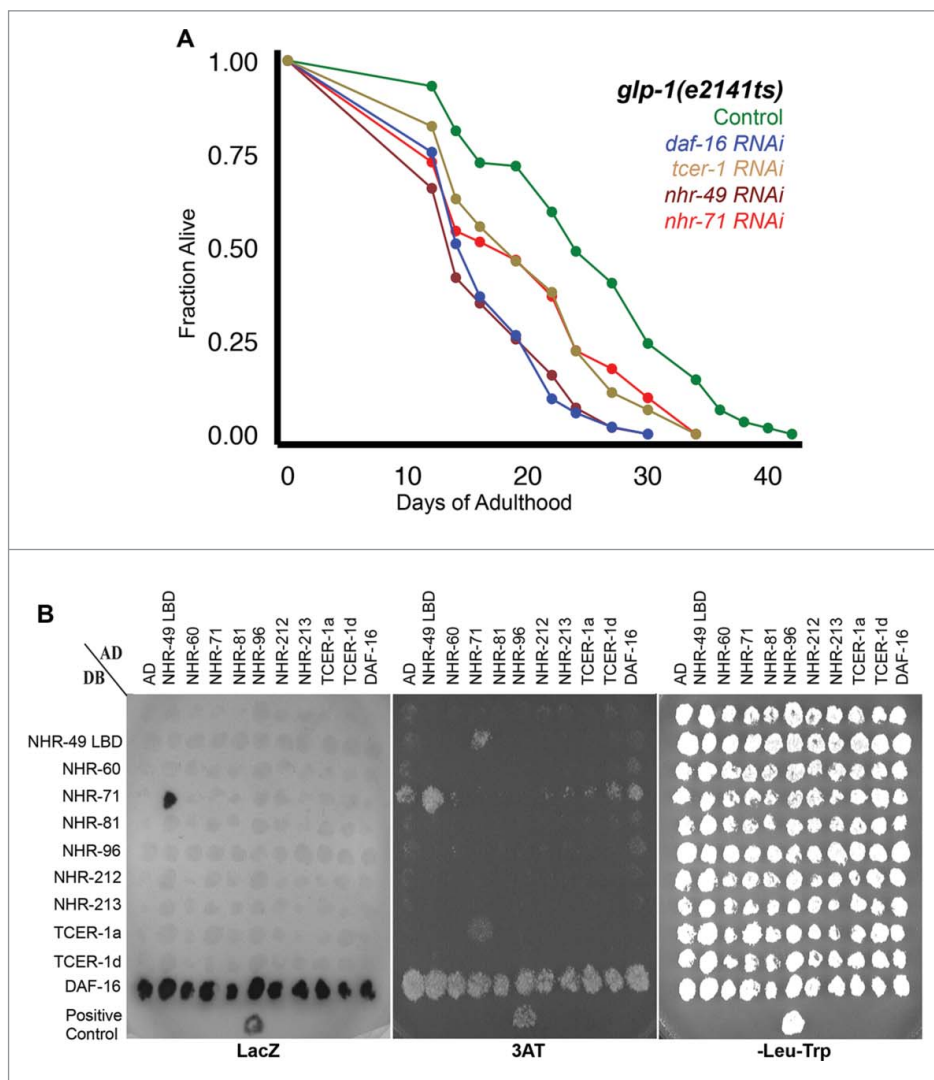


Figure 1. NHR-49/PPAR α collaborates with NHR-71/HNF4 to promote longevity of germline-less animals. **A:** Effect of *nhr-71* RNAi on the longevity of *glp-1* mutants. Adult *glp-1* mutants were subjected to feeding RNAi by culturing them on bacteria expressing dsRNA targeting *daf-16* (blue; $m = 16.8 \pm 0.2$; $n = 106/106$; P vs. control <0.0001), *tcer-1* (brown; $m = 19.9 \pm 0.7$; $n = 108/108$; P vs. control <0.0001), *nhr-49* (maroon; $m = 16.5 \pm 0.1$; $n = 116/117$; P vs. control <0.0001) and *nhr-71* (red; $m = 19.8 \pm 0.5$; $n = 103/103$; P vs. control 0.0001). Lifespan curve of control animals grown on empty vector is shown in green ($m = 25.1 \pm 0.1$; $n = 94/117$). Y-axis shows the fraction of animals alive at any time point, X-axis the days of adulthood. Data is shown as mean lifespan in days (m) \pm standard error of the mean (SEM). 'n' refers to the number of worms analyzed divided by total number of worms tested in the experiment. P values were calculated using the log rank (Mantel Cox) method. Similar results were obtained in 2 additional trials. **B:** Interaction of NHR-49/PPAR α and NHR-71/HNF4 in Yeast-Two-Hybrid (Y2H) assay. Small scale matrix to test Y2H interactions between NHRs that influence *glp-1* longevity as well as DAF-16/FOXO3A and TCER-1/TCERG1, by growth on 20 mM 3AT and by LacZ expression. Growth on permissive -Leu -Trp plates is provided as a control. Gal4-activation domain (AD) and DNA-binding domain fusions (DB) for the NHR-49/PPAR α ligand binding domain (LBD) and the indicated full-length NHRs, a DAF-16/FOXO3A full-length, TCER-1/TCERG1a full length and a C-terminally truncated isoform (TCER-1/TCERG1d) were tested pairwise. AD and DB are empty vector controls and an NHR-25-DB-SMO-1 AD interaction was used as a positive control. The NHR-49 ligand-binding domain (LBD) bait included amino acids 120-474 encoded by the *nhr-49a* transcript. All other baits were full-length proteins. As seen here, DAF-16/FOXO3A shows auto-activation that has not been reported before. The NHR-49 LBD bait did not exhibit homo-dimerization, in keeping with previous reports where the full-length protein was found to be essential for homodimerization^{38,39}

DAF-16/FOXO3A and TCER-1/TCERG1 target gene, *stdh-1/dod-8*,¹⁵ we tested if these factors may also influence the expression of other DAF-16/FOXO3A and TCER-1/TCERG1 targets. *nhr-49;glp-1* mutants

were significantly impaired in the induction of *sod-3*, a well-known, direct DAF-16/FOXO3A target^{40,41} as well as *mdt-15*⁴² and *lipl-4*,²⁴ two other genes upregulated by DAF-16/FOXO3A^{25,43} in *glp-1* mutants^{23,24}

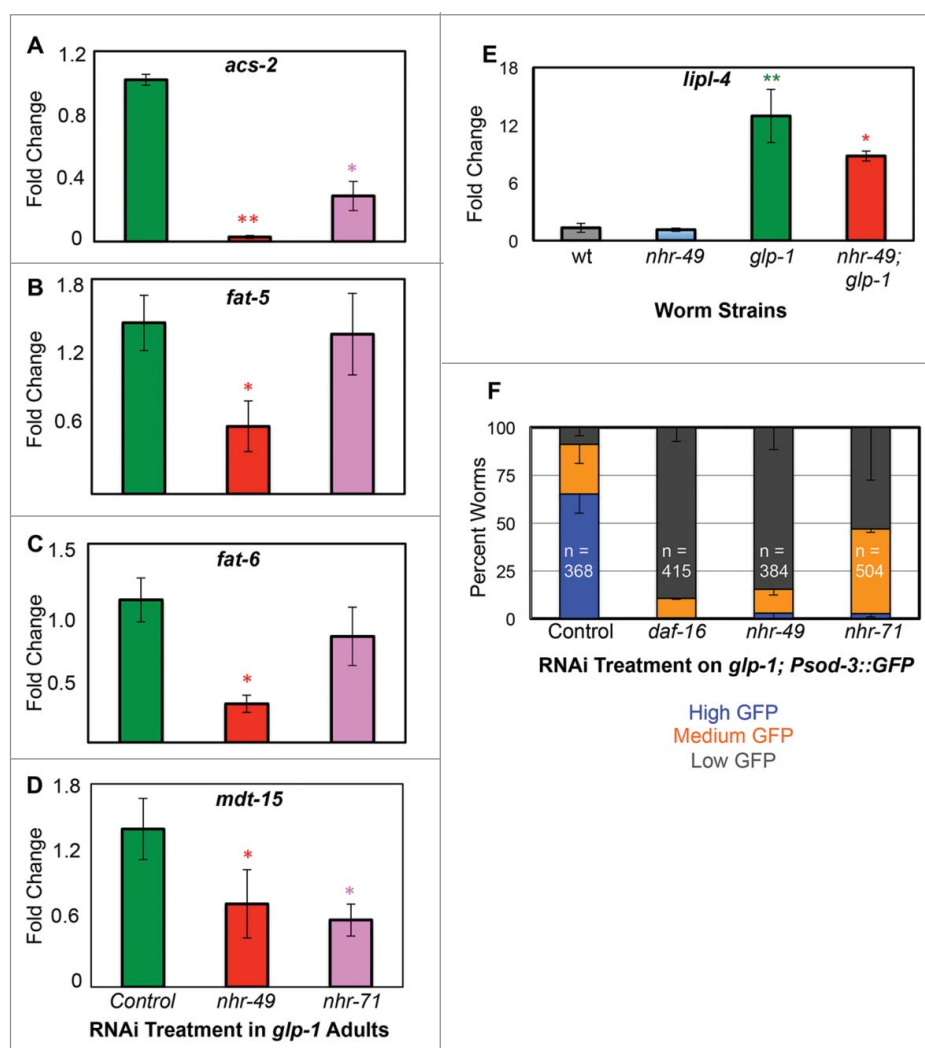


Figure 2. *nhr-49* and *nhr-71* are required for upregulation of DAF-16/FOXO3A target genes in germline-less adults. A-D: mRNA levels of *acs-2* (A), *fat-5* (B), *fat-6* (C) and the NHR-49/PPAR α co-regulator and DAF-16/FOXO3A target gene, *mdt-15* (D), were measured in day 2 *glp-1* adults grown on control vector (green) and those fed RNAi bacteria targeting *nhr-49* (red) and *nhr-71* (pink). X-axis represents the RNAi treatments and Y-axis the fold change in expression (normalized to 'housekeeping' gene *rpl-32*). (E) Quantitative PCR (Q-PCR) analysis of mRNA levels of DAF-16/FOXO3A target *lipi-4* compared between wild-type worms (wt, gray) and *nhr-49* (blue), *glp-1* (green) and *nhr-49; glp-1* (red) day 2 adults. In A-E, error bars display standard error of the mean, and asterisks depict the statistical significance of the observed differences in an unpaired, 2-tailed t-test with P values 0.05 (*) and 0.005 (**). (F) Expression of DAF-16/FOXO3A direct target *sod-3* examined using a transcriptional GFP reporter in *glp-1* mutants (*glp-1; Psod-3::gfp*). Day 2 adults subjected to RNAi treatments shown on the X-axis were classified as high (blue), medium (orange) and low (gray) based on the level of intestinal GFP. 'n' signifies the total number of worms examined in three independent trials. Error bars represent the standard error of the mean

(Fig. 2D-F) suggesting that NHR-49/PPAR α feeds back positively to potentiate the DAF-16/TCER-1 pathway. The upregulation of *mdt-15* and *sod-3* was also attenuated upon *nhr-71* RNAi (Fig. 2D, F).

The only known common upstream regulator of DAF-16/FOXO3A and TCER-1/TCERG1 is KRI-1, an intestinal Ankyrin-repeat protein that is essential for DAF-16/FOXO3A nuclear localization and TCER-1/TCERG1 upregulation in germline-less adults.^{15,44}

Based on the strong influence of *nhr-49* inactivation on upregulation of DAF-16/FOXO3A and TCER-1/TCERG1 targets, we asked if NHR-49/PPAR α acts in a positive feedback loop to potentiate the activities of these factors. We reasoned that it is likely to do so through KRI-1 regulation, which was also identified as being downregulated in *nhr-49* mutants in a previous genomic study.³⁸ Indeed, we found that a GFP-tagged KRI-1 protein reporter that shows diffuse cytoplasmic and nuclear expression in intestinal cells of germline-

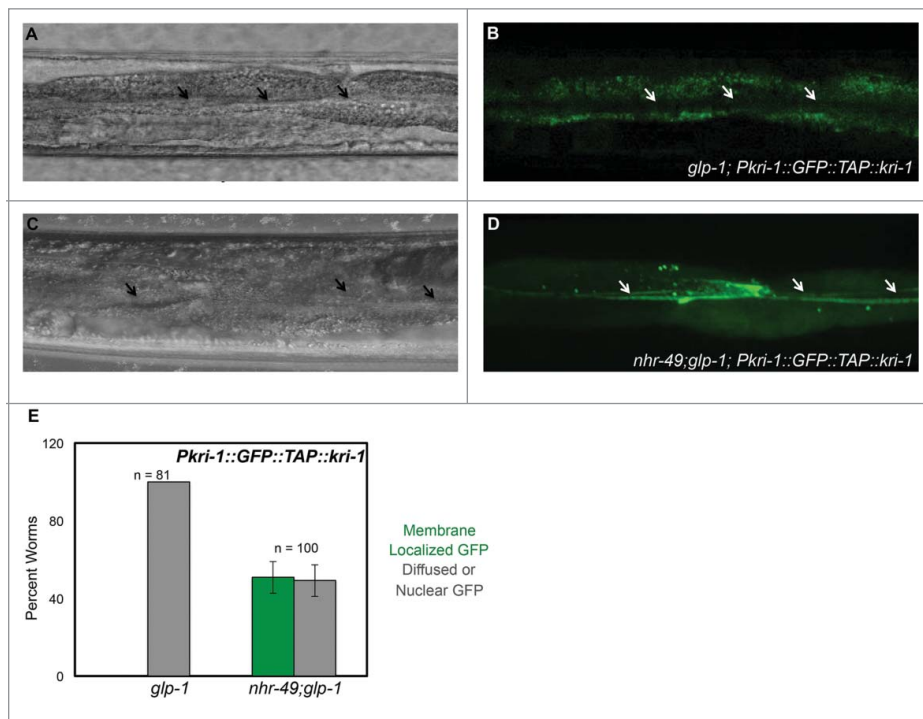


Figure 3. NHR-49/PPAR α potentiates the DAF-16/FOXO3A and TCER-1/TCERG1 pathway by influencing the sub-cellular localization of KRI-1. A-E: KRI-1 is predominantly membrane-restricted in intestinal nuclei of germline-less worms in the absence of NHR-49/PPAR α . *Pkri-1::GFP::TAP::kri-1* expression examined in day 2 adults of *glp-1* (A, B) and *nhr-49;glp-1* (C, D). GFP is largely diffuse in *glp-1* mutants, but in *nhr-49;glp-1* adults there is a distinct relocation to membranes, especially near the lumen (indicated by arrows). Quantification of the data is shown in E. Y-axis represents the percent of worms that showed membrane localization (green) or diffuse cytoplasmic or nuclear expression (gray) of GFP in one or more intestinal cells. 'n' indicates total number of worms examined

ablated animals was highly membrane-localized in germline-less *nhr-49* mutants (Fig. 3). These data support the possibility that NHR-49/PPAR α positively feeds back into the DAF-16/TCER-1 pathway by directing KRI-1 sub-cellular localization. It may do so by hetero-dimerizing with NHR-71/HNF4, but the possibility remains to be experimentally tested (Fig. 4).

Discussion

The coordination of disparate lipid-metabolic pathways by NHR-49/PPAR α

Germline loss not only increases lifespan, it also increases fat accumulation in intestinal cells of the sterile animals. Surprisingly, they also exhibit enhanced expression of mitochondrial β -oxidation genes, and these genes are required for lifespan extension. These observations suggest that fatty-acid β -oxidation is elevated following GSC removal and contributes to the consequent longevity.²² So, what purpose does enhanced β -oxidation serve in these animals? Similarly, why does GSC removal cause the

animal to simultaneously augment β -oxidation and desaturation- lipid-degradative and lipid-synthetic pathways, respectively? We posit that the simultaneous elevation of these antagonistic processes reflects increased lipid turnover, which in turn allows the organism to adapt to the physiological catastrophe of lost fertility by (a) breaking down fats that would have been utilized in reproduction, and (b) converting the lipids to unsaturated forms that are more amenable for storage and cellular maintenance.

This hypothesis, of enhanced lipid turnover as an adaptive response to germline loss, may also explain a dramatic and surprising fat loss displayed by GSC-less *nhr-49* mutants. Since β -oxidation is a lipolytic pathway, we expected *nhr-49;glp-1* mutants to exhibit further increase in fat levels. However, using the lipid-labeling dye oil red O (ORO) as well as gas chromatography/mass spectrometry (GC/MS), we found that *nhr-49;glp-1* mutants had lower fat levels than *glp-1* adults, and this fat was rapidly lost with age.²² This could be explained in part by the fact that NHR-49/PPAR α also contributed to *de novo* lipid

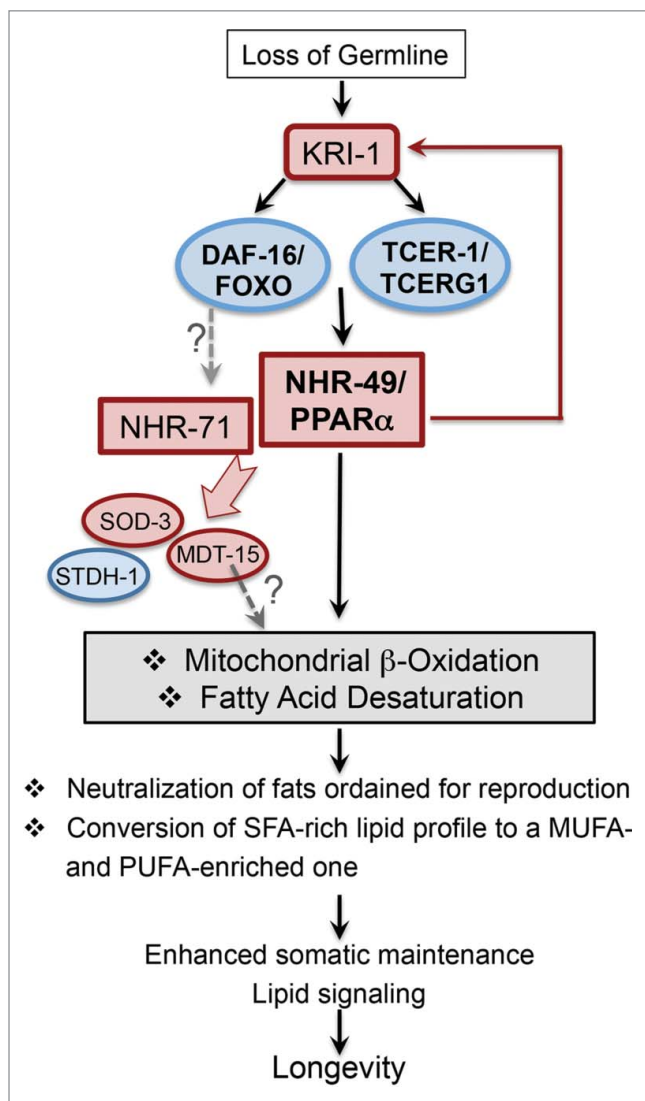


Figure 4. Schematic representation of the proposed model to explain NHR-49/PPAR α function in promoting the longevity of germline-less animals (modified from Ratnappan *et al.*,²²). Following germline loss, NHR-49/PPAR α is upregulated by the joint activity of DAF-16/FOXO3A and TCER-1/TCERG1. NHR-49/PPAR α , in turn, mediates the upregulation of genes involved in fatty-acid β -oxidation and desaturation. The synchronized enhancement of these processes allows the animal to adapt to loss of fertility and orchestrate a lipid-homeostasis profile that supports longevity. NHR-49/PPAR α and NHR-71/HNF4, another NHR essential for germline-less longevity, are required for the upregulation of DAF-16/FOXO3A targets previously shown to be elevated upon germline loss, such as *sod-3*, *stdh-1* and *mdt-15*. Loss of *nhr-49* causes membrane relocation of KRI-1, the only known common upstream regulator of DAF-16/FOXO3A and TCER-1/TCERG1, suggesting that NHR-49/PPAR α may operate in a feedback loop to potentiate the activity of this pathway by regulating KRI-1 sub-cellular localization. Our data suggest that NHR-71/HNF4, similar to NHR-49/PPAR α , may be upregulated by DAF-16/FOXO3A and TCER-1/TCERG1. MDT-15, a known co-regulator of NHR-49/PPAR α , may also contribute to the increased expression of β -oxidation genes. Regulatory steps for which evidence is provided by our data are indicated by red arrows. Putative relationships suggested by our observations are represented by gray, stippled arrows.

synthesis,²² but it does not account for the dramatic age-related fat depletion seen in the absence of NHR-49/PPAR α . We propose that this drastic fat depletion is a consequence of accumulation of free fatty acids (FFAs), due to simultaneous inhibition of β -oxidation and desaturation in the absence of NHR-49/PPAR α , which causes lipotoxicity. FFAs stimulate insulin release and serve as key signaling molecules. But their chronic accrual causes deregulated insulin secretion and apoptosis in pancreatic β -cells⁴⁵; in muscle and liver cells it leads to insulin resistance.⁴⁶ Impaired fatty-acid oxidation is a primary cause of lipotoxicity in human cells, and desaturation is critical for protecting β -cells and endothelial cells from toxic effects of SFA accumulation.^{47,48} Long-chain, non-metabolized SFAs are implicated as the primary causative agents underlying lipotoxicity.^{49,50} We observed significant elevation of these species, including SA, in germline-less *nhr-49* mutants. SA is also a poor substrate for triglyceride (TAG) synthesis and a potent inducer of pro-inflammatory responses and cell death in human cells.⁵¹ FFA accumulation also prevents *de novo* lipid synthesis by inhibiting the function of fat-synthesis enzymes.⁵² Hence, it is conceivable that in the absence of NHR-49/PPAR α , the lipid turnover that would facilitate adaptation to loss of fertility is prevented, and the resultant accumulation of toxic FFAs causes lipotoxicity that gets aggravated over a short time and causes lipid degeneration and death.

The healthy obesity of long-lived, germline-less animals

Germline-ablated animals are remarkable because they manifest an apparently paradoxical healthy obesity, along with longer life, as compared to their leaner, fertile counterparts.^{22,53} This discrepancy is not unique to *C. elegans* either. Similar observations have been made in other model organisms and in the natural world.^{11,54,55} Even in human populations, while obesity is generally associated with disease, ‘Metabolically Healthy Obese’ individuals are noteworthy because they retain excessive weight without developing metabolic disorders.⁵⁶ Hence, there is accumulating evidence that absolute fat level is not the deterministic factor for health and longevity. Instead, the composition of lipids has a significant bearing on metabolic health. Lipids with higher UFAs are

generally associated with improved cellular maintenance.⁵⁷ In hibernating animals, fatty acid composition of stored lipids affects the depth and duration of torpor. Diets rich in MUFAs and PUFAs have a beneficial impact on mammalian torpor, whereas diets rich in SFAs have a negative effect.^{58,59} Alternatively, SFAs are critical for reproductive health. They make up >70% of human oocytes, of which SA and PA are the most abundant, whereas, MUFAs make up <15%.⁶⁰⁻⁶² Thus, activation of NHR-49/PPAR α may help transform the SFA-rich, reproduction-oriented lipid profile of a fertile adult into a UFA-rich profile that is more conducive for fat storage and cellular maintenance.

Feedback loops and NHR partnerships triggered by germline signals

Our observations, that NHR-49/PPAR α influences the subcellular localization of KRI-1, the common upstream regulator of DAF-16/FOXO3A and TCER-1/TCERG1, and is required for increased expression of DAF-16/FOXO3A and TCER-1/TCERG1 targets reveal the existence of a positive feedback loop between NHR-49/PPAR α and its upstream regulatory factors (Fig. 4). Since NHR-71/HNF4 is also required for the expression of some of these genes, and since NHR-49/PPAR α binds NHR-71/HNF4 in Y2H assays, it is plausible that the two NHRs partner with each other to accomplish this positive feedback regulation, however this remains to be experimentally tested. NHR-71:NHR-49 binding has been observed before, and in the same report NHR-49/PPAR α was also found to bind NHR-274, one of the pro-longevity NHRs identified in our screen, but that we could not test in the Y2H assays.³⁹ Partnerships between NHR-49/PPAR α and many NHRs have been reported in other contexts. It physically interacts with NHR-66 and both *nhr-49* and *nhr-66* are required for repression of 3 genes involved in sphingolipid metabolism. Similarly, it binds NHR-80/HNF4 and both genes facilitate lipid desaturation, possibly working together.³⁸ Additionally, NHR-49/PPAR α interactions with NHR-79 and NHR-234 have been described.³⁷ As observed in normal, fertile animals, NHR-49/PPAR α may dimerize with NHR-80/HNF4 to regulate desaturase gene expression in GSC-less adults too. However, the factors that it would interact with to regulate β -oxidation remain unknown. One or more of the

other pro-longevity NHRs we identified are likely candidates to fulfill these roles, though we did not notice binding in our Y2H assays. It is possible that heterodimerization is triggered specifically upon germline ablation that could not be recapitulated in a Y2H experimental setup. Regardless, these data provide insights that underscore how NHRs such as NHR-49/PPAR α , that function as key nodes of large metabolic networks, achieve functional specificity through selective interactions with other constituents of the network, depending on the physiological stimulus.

Perspective

PPAR proteins were the first identified genetic sensors for fat and their study has revolutionized our understanding of energy metabolism.³⁴ PPAR α is implicated in metabolic diseases such as dyslipidemia and CVD and PPAR α agonists like Fibrates are widely used lipid-lowering drugs.^{50,63} Interestingly, a role for PPAR α in mammalian female reproduction has been revealed recently,⁶⁴ and in ovariectomized rats Fenofibrate helps maintain bone-mass.⁶⁵ Given this context, and accruing evidence that reproductive signals alter lifespan of diverse species, it is enticing to speculate if PPAR α and other NHRs may influence vertebrate longevity. Since molecular dissection of metabolic pathways is challenging in mammalian systems, understanding how NHR-49/PPAR α and its partners promote *C. elegans* longevity is likely to provide new mechanistic insights and therapeutic targets for regulating lipid homeostasis and aging in humans.

Materials and methods

Worm culture and lifespan analysis

The following worm strains were used in this study: N2 (wild type), CF1903 *{glp-1(e2141)III}*, AGP12a *{nhr-49(nr2041)I}*, AGP22 *{nhr-49(nr2041)I;glp-1(e2141ts)III}*, CF1929 *{glp-1(e4141ts)III; muIs84 [Psod-3::GFP]}* AGP116 *muIs162 [Pkri-1::GFP::TAP::kri-1];nhr-49(nr2041)I;glp-1(e2141ts)V* and CF3352 *muIs162 [Pkri-1::GFP::TAP::kri-1];glp-1(e2141ts)III*. All strains were maintained by standard techniques at 20°C. Lifespan experiments were conducted as described previously and have been discussed in detail elsewhere.⁶⁶ For all experiments that involved the *glp-1* genetic background, eggs were incubated at 20°C

for 2–6 h, transferred to 25°C to eliminate germ cells, then shifted back to 20°C on day 1 of adulthood (72 h later) for the rest of their lifespan.

Q-PCRs

Worm RNA was isolated using the mirVana miRNA Isolation Kit (Ambion, AM1561), DNase treated (DNase kit, Sigma #AMPD1) and reverse transcribed into cDNA (Protoscript m-MuLV First Strand cDNA Synthesis kit, NEB #E6300S) according to the manufacturer's instructions. Quantitative real-time PCRs were performed using an Applied Bio Systems 7300 Real Time PCR System employing the Sybr Green chemistry (SensiMix SYBR Hi-ROX kit, Biorline #QT-605). Gene expression data were normalized to the housekeeping gene *rpl-32*. Data reported here were obtained by combining results from 3 biological replicates, each comprising 2–4 technical repeats.

GFP assays

Eggs were transferred to freshly-seeded *Escherichia coli* OP50 plates (or *E. coli* HT115 RNAi plates targeting the appropriate gene), incubated at 20°C for 2–6 h, transferred to 25°C (to eliminate germ cells in strains containing *glp-1* mutation), then shifted back to 20°C on day 1 of adulthood. GFP assays were conducted on day 2 of adulthood, using a Leica MZ16F stereomicroscope. All assays were performed blind after initial familiarization of GFP levels in control plates by the experimenter.

Yeast two-hybrid (Y2H) assay

The cDNAs used in yeast 2-hybrid experiments were Gateway cloned (Invitrogen) into pDONR221 vector to generate entry clones and then moved into pAD-dest and pDB-dest vectors, which contain the Gal4 activation domain and DNA binding domain, respectively.⁶⁷ Yeast transformations and Y2H assays were carried out as described by Deplancke *et al.*⁶⁸

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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