Nuclear receptor signaling via NHR-49/MDT-15 regulates stress resilience and proteostasis in response to reproductive and metabolic cues

Ambre J. Sala,1,2 Rogan A. Grant,1,3 Ghania Imran,1 Claire Morton,1 Renee M. Brielmann,1 Szymon Gorgon,1 Jennifer Watts,4 Laura C. Bott,1 and Richard I. Morimoto1

1Department of Molecular Biosciences, Northwestern University, Evanston, Illinois 60208, USA; 2Institute for Integrative Biology of the Cell (I2BC), Commissariat à l’Énergie Atomique et Aux Énergies Alternatives (CEA), Centre National de la Recherche Scientifique (CNRS), Université Paris-Saclay, Gif-sur-Yvette 91190, France; 3Division of Pulmonary and Critical Care Medicine, Department of Medicine, Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611, USA; 4School of Molecular Biosciences, Washington State University, Pullman, Washington 99164, USA

The ability to sense and respond to proteotoxic insults declines with age, leaving cells vulnerable to chronic and acute stressors. Reproductive cues modulate this decline in cellular proteostasis to influence organismal stress resilience in Caenorhabditis elegans. We previously uncovered a pathway that links the integrity of developing embryos to somatic health in reproductive adults. Here, we show that the nuclear receptor NHR-49, an ortholog of mammalian peroxisome proliferator-activated receptor α (PPARα), regulates stress resilience and proteostasis downstream from embryo integrity and other pathways that influence lipid homeostasis and upstream of HSF-1. Disruption of the vitelline layer of the embryo envelope, which activates a proteostasis-enhancing intertissue pathway in somatic cells, triggers changes in lipid catabolism gene expression that are accompanied by an increase in fat stores. NHR-49, together with its coactivator, MDT-15, contributes to this remodeling of lipid metabolism and is also important for the elevated stress resilience mediated by inhibition of the embryonic vitelline layer. Our findings indicate that NHR-49 also contributes to stress resilience in other pathways known to change lipid homeostasis, including reduced insulin-like signaling and fasting, and that increased NHR-49 activity is sufficient to improve proteostasis and stress resilience in an HSF-1-dependent manner. Together, our results establish NHR-49 as a key regulator that links lipid homeostasis and cellular resilience to proteotoxic stress.

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Maintaining a properly folded and functional proteome is essential for cellular and organ ischemal health (Labbadia and Morimoto 2015a; Sala et al. 2017). The capacity of cells to prevent the formation and accumulation of misfolded and aggregated protein species, known as protein homeostasis (proteostasis), declines over time during aging, which is considered a major driver of age-dependent cellular dysfunction (Hipp et al. 2019). Abnormal protein aggregates are observed in many age-related disorders, including dementia, diabetes, and muscular dystrophy (Balch et al. 2008; Wilson et al. 2023). To counteract protein misfolding and aggregation, cells have evolved a sophisticated network of cellular machineries for protein synthesis, folding, and clearance, consisting mostly of molecular chaperones and protein degradation pathways (Labbadia and Morimoto 2015a; Sala et al. 2017). Proteostasis is constantly challenged by physiological and environmental stresses, and cells are also equipped with adaptive mechanisms that are activated during proteotoxic stress. Loss of proteostasis in the cytosol activates the heat shock response (HSR), which triggers the rapid and transient production of molecular chaperones and is mainly regulated by the evolutionarily conserved heat shock transcription factor 1 (HSF-1) (Li et al. 2017; Gomez-Pastor et al. 2018).
In addition to surveillance mechanisms at the cellular level, proteostasis regulation at the systemic level involves responses to physiological and metabolic cues that determine organismal health during aging [Hansen et al. 2013; Li et al. 2017; Sala et al. 2017]. Rewiring of metabolism toward survival is generally associated with improved proteostasis; for example, in conditions where insulin signaling is reduced [Hsu et al. 2003; Morley and Morimoto 2004; Hansen et al. 2013]. Mutations or treatments that impaire reproduction have also been shown to promote somatic resilience in several model systems [Antebi 2013]. Removal of germ cells in Caenorhabditis elegans and Drosophila increases life span and suppresses the age-dependent aggregation of disease model proteins in somatic tissues [Hsin and Kenyon 1999; Flatt et al. 2008; Shemesh et al. 2013]. In wild-type C. elegans, signals from germline stem cells [GSCs] initiate proteostasis collapse at reproductive maturity via generalized repression of the HSR, possibly to prioritize reproduction at the expense of somatic health [Shemesh et al. 2013; Labbadia and Morimoto 2015b]. However, somatic resilience and reproduction can be uncoupled, and specific molecular pathways rather than mere energetic trade-offs govern the regulation of proteostasis mechanisms by the reproductive system [Antebi 2013; Labbadia and Morimoto 2015b; Sala et al. 2020].

We recently identified a pathway in which signals emanating from fertilized eggs modulate proteostasis in the somatic tissues of reproductive mothers to promote maternal health when reproduction is compromised [Sala et al. 2020]. We demonstrated that perturbation of the fertilized egg in the uterus, induced by inhibition of cbd-1 or other genes encoding components of the vitelline layer of the eggshell, improves stress resilience and restores the HSR in maternal somatic tissues. This effect did not extend to genes that encode other components of the embryonic eggshell, such as proteins involved in the formation of the chitin layer, implying that specific signals linked to disruption of the vitelline layer, rather than the impairment of egg production, are responsible for the protective effects. The pathway involves a DAF-16/FOXO response specifically in the egg-laying apparatus [vulva], which may be involved in monitoring egg quality, and relies on HSF-1 in somatic tissues [Sala et al. 2020]. The beneficial effects observed in response to alteration of the embryonic vitelline layer may have evolved to promote maternal survival until conditions for optimal reproduction resume [Sala and Morimoto 2021]. This relationship between the health of the fertilized egg and the health of maternal somatic tissues provides a paradigm for how systemic regulation influences organismal robustness and resilience.

Here, we report that the nuclear receptor NHR-49, a transcription factor orthologous to mammalian peroxisome proliferator-activated receptor α (PPARα), together with its coactivator, the mediator subunit MDT-15, is an important regulator of lipid metabolism and stress resilience in response to alteration of the embryonic vitelline layer. Furthermore, we found that NHR-49 plays a role in multiple paradigms of stress resilience that also alter lipid homeostasis, including reduced insulin signaling and fasting, and that increased NHR-49 activity is sufficient to improve stress resilience, the HSR, and proteostasis in an HSF-1-dependent manner. Together, our results demonstrate that NHR-49 has a pervasive role as a regulator that connects lipid homeostasis and cellular resilience to proteotoxic stress.

Results

Reduced function of the vitelline gene cbd-1 has systemic effects on gene expression and upregulates lipid metabolism genes

We previously uncovered that alteration of the vitelline layer of fertilized embryos in the uterus initiates a transcellular pathway that results in improved maternal proteostasis and stress resilience [Sala et al. 2020]. This pathway can be activated by reducing cbd-1 expression with RNA interference or using the cbd-1(ok2913) hypomorph mutation, which compromises eggshell integrity [Johnston et al. 2010; González et al. 2018] and simultaneously leads to protective effects on maternal stress resilience from the onset of reproduction on the first day of adulthood through at least day 4 of adulthood [Sala et al. 2020]. The protective effects of cbd-1(ok2913) are shown in Figure 1A as increased survival compared with wild-type animals following exposure to a lethal heat stress at 35°C on day 2 of adulthood. To gain a better understanding of the systemic effects of vitelline inhibition, we asked how the cbd-1(ok2913) mutation affects gene expression in basal conditions and following acute heat stress using RNA sequencing in extracts from whole animals [Supplemental Fig. S1A]. In order to avoid contamination of samples with eggs and progeny, this analysis was performed in day 4 adults when both the cbd-1(ok2913) and wild-type animals had laid most of their eggs [Supplemental Fig. S1B].

Differential gene expression analysis identified 811 upregulated and 855 downregulated genes [q < 0.05, Wald test] in cbd-1(ok2913) compared with wild-type animals [Fig. 1B], indicating a broad remodeling of transcription. Tissue enrichment analysis revealed that the differentially expressed genes were enriched for transcripts preferentially expressed in the intestine and other somatic tissues, confirming that although expression of the cbd-1 gene is restricted to the reproductive system [Johnston et al. 2010; González et al. 2018; Sala et al. 2020], modulating its activity has widespread organismal consequences [Supplemental Fig. S1C]. Gene ontology (GO) analysis indicated an enrichment of genes related to metabolism (in particular, lipid metabolism and secondary metabolism), ribosome biogenesis, and innate immunity in the set of differentially upregulated genes in cbd-1(ok2913) [Fig. 1C]. Downregulated genes were characterized by GO terms mostly related to innate immunity and defense responses [Fig. 1D]. Analysis of genes that were differentially induced in cbd-1(ok2913) specifically during heat shock revealed that they were similarly characterized by GO terms related to lipid metabolism and innate immunity [Supplemental Fig. S1D]. Importantly, the canonical heat
shock gene hsp-70 was among the genes more highly induced by heat stress in the cbd-1(ok2913) mutant in our RNA-seq data set (Supplemental Fig. S1E), which was also confirmed by real-time quantitative PCR (qPCR) (Supplemental Fig. S1F). This is in agreement with our previous findings that cbd-1 RNAi maintains the inducibility of heat shock genes in reproductive animals without affecting basal levels (Sala et al. 2020).

To gain insight into the functional significance of differential gene regulation in the cbd-1 mutant, we asked which phenotypes had previously been reported for the differentially expressed gene set using the WormBase Enrichment suite to identify enriched worm phenotype ontology (Schindelman et al. 2011; Angeles-Albores et al. 2018). Phenotypes related to cadmium stress resistance and stress-induced lethality were enriched [Supplemental Fig. S1G], in agreement with the stress-resilient phenotype of the cbd-1(ok2913) mutant (Fig. 1A; Sala et al. 2020). In addition, genes differentially regulated in cbd-1 mutants were associated with fat content increase, suggesting that changes in lipid metabolism may be another consequence of perturbation of the vitelline layer in these animals.

**Perturbation of the embryonic vitelline layer increases fat storage and triglyceride levels**

Lipid metabolism has been shown to be altered in animals lacking GSCs and in conditions of reduced insulin signaling that are also associated with elevated proteostasis and
stress resilience [Wang et al. 2008; O’Rourke et al. 2009]. In both cases, the mutants accumulate fat stores compared with wild-type animals [O’Rourke et al. 2009]. To assess whether fat storage is altered in response to inhibition of the embryonic vitelline layer, we used the lysochrome diazo dye oil red O (ORO) to stain neutral triglycerides and lipids. From this point on, most of our experiments were performed in day 2 adults, in order to focus on events that occur right after the onset of the pathway when reproduction begins. We consistently observed an accumulation of fat stores in adult \textit{cbd-1} mutant compared with wild-type animals [Fig. 1E,F]. We observed a similar increase in ORO staining in \textit{cbd-1} RNAi-treated animals compared with the empty vector control [Supplemental Fig. S2A,B]. Next, we measured triglyceride levels in whole-animal lysates and found an increase of ∼1.8-fold in \textit{cbd-1} mutant compared with wild-type animals [Fig. 1G]. Elevated fat storage is associated with a remodeling of fatty acid (FA) composition in animals lacking GSCs [Ratnappan et al. 2014; Amrit et al. 2016] and mutants with reduced insulin signaling [Shmookler Reis et al. 2011; Shi et al. 2013]. To determine whether excess fat storage in the \textit{cbd-1(ok2913)} mutant is also accompanied by changes in FA content, we examined FA composition in whole animals using gas chromatography/mass spectrometry (GC/MS). We found that \textit{cbd-1(ok2913)} and wild-type animals had a similar FA composition profile both at the peak of the reproductive period (day 2) and toward the end [day 4] (Supplemental Fig. S2C,D). Together, these data indicate that changes in the expression of lipid metabolism genes in response to inhibition of the embryonic vitelline layer are accompanied by an increase in fat storage and total triglyceride levels without remodeling of FA composition.

Yolk lipoproteins accumulate in \textit{cbd-1(ok2913)} animals

Lipid-rich yolk is synthesized in the intestine, transported into the germline, and endocytosed by oocytes [Grant and Hirsh 1999]. Reproductive defects in the \textit{cbd-1} mutant could therefore lead to an accumulation of yolk lipids and underlie the increase in fat storage in these animals. To test this possibility, we examined yolk content and distribution using fluorescently labeled VIT-2, a major yolk lipoprotein or vitellogenin [Grant and Hirsh 1999]. As expected, we detected VIT-2::GFP mostly in the intestine, oocytes, and embryos of wild-type day 2 animals. In contrast, \textit{cbd-1(ok2913)} mutants exhibited elevated VIT-2::GFP signal throughout the body [Fig. 1H]. Quantification of the fluorescence from VIT-2::GFP revealed an almost twofold increase in \textit{cbd-1(ok2913)} compared with wild-type animals [Fig. 1I], suggesting that compromised egg production in the mutant leads to mislocalization and buildup of yolk lipoprotein. Therefore, accumulation of yolk-associated lipids could account for the elevated fat stores observed in \textit{cbd-1(ok2913)} animals.

Peroxisomal FA β-oxidation contributes to stress resilience in \textit{cbd-1(ok2913)}

To further understand the changes in lipid metabolism that occur when the embryonic vitelline layer is altered, we performed a KEGG pathway enrichment analysis on the gene set upregulated in \textit{cbd-1(ok2913)} and found that FA degradation, peroxisomes, and lysosomes were among the top 10 enriched pathways [Fig. 2A]. Two lysosomal lipases that break down triglycerides into free FA, as well as 10 genes encoding key enzymes of the peroxisomal FA β-oxidation pathway involved in the breakdown

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**Figure 2.** The peroxisomal FA β-oxidation gene \textit{daf-22} contributes to stress resilience in \textit{cbd-1(ok2913)}. (\textit{A}) KEGG pathway analysis of genes differentially upregulated in \textit{cbd-1(ok2913)}. The top 10 terms are shown. (\textit{B}) Relative expression of selected lipid catabolism genes in wild-type (WT) and \textit{cbd-1(ok2913)} animals. Columns correspond to different biological samples analyzed by RNA sequencing. (\textit{C}) Survival of wild-type (WT) or \textit{cbd-1(ok2913)} animals grown on the indicated RNAi until day 2 of adulthood, subjected to a 4 h heat stress at 35°C, and scored following 72 h of recovery at 20°C (\(n = 3\)). (\textit{D}) Heat stress survival of wild-type or \textit{cbd-1(ok2913)} animals grown on either control or \textit{daf-22} RNAi (\(n = 4\)). Error bars represent SEM. Statistical significance was based on two-way ANOVA followed by Dunnett correction with comparison with the control condition within each group [strain] \(\mid\) or two-way ANOVA \(D\). ∗∗∗ \(P < 0.01\), ∗∗ ∗ \(P < 0.001\), [ns nonsignificant] \(P > 0.05\).
of FA molecules, are consistently upregulated in *cbd-1 (ok2913)* compared with wild-type animals [Fig. 2B]. This suggests that mobilization of FA from triglycerides could be higher in the fat store-accumulating *cbd-1 (ok2913)* animals.

We asked whether these changes in lipid catabolism are important for the beneficial effects of inhibition of the embryonic vitelline layer on stress resilience. We used RNAi to knock down the expression of lysosomal lipase and peroxisomal FA β-oxidation genes and analyzed the effects on the survival of animals exposed to heat stress on day 2 of adulthood and scored for survival 72 h later. We found that among the lipid catabolism genes tested, only *daf-22* RNAi suppressed the elevated stress resilience of *cbd-1 (ok2913)* animals, while it had no effect in wild-type animals [Fig. 2C]. We confirmed these observations in a full time-course survival assay in wild-type and *cbd-1 (ok2913)* animals exposed to *daf-22* RNAi [Fig. 2D]. Of note, while most steps of the peroxisomal FA β-oxidation pathway are controlled by redundant enzymes, *daf-22* encodes the unique peroxisomal thiolase in *C. elegans*, the last enzyme in the pathway. We confirmed that *daf-22* RNAi leads to an increase in ORO staining in *cbd-1 (ok2913)* compared with the empty vector control, indicating that the peroxisomal FA β-oxidation pathway indeed influences fat stores in these animals [Supplemental Fig. S3A,B]. Therefore, increased lipid catabolism via the peroxisomal FA β-oxidation pathway contributes to elevated maternal stress resilience when the embryonic vitelline layer is compromised.

**Vitelline disruption alters lipid metabolism genes through NHR-49 and its coactivator, MDT-15**

To identify mediators of the transcriptional changes observed in *cbd-1 (ok2913)* mutants, we examined known regulators of lipid catabolism. Many of the genes that are differentially regulated in *cbd-1 (ok2913)* are also responsive to the loss of the NHR-49 nuclear receptor, orthologous to human PPARs, and are similarly involved in the regulation of lipid metabolism and FA β-oxidation [Van Gilst et al. 2005a,b]. Indeed, we observed that among the genes upregulated in *cbd-1 (ok2913)*, 62 genes are also downregulated in the loss-of-function mutant *nhr-49 (nr2041)* [Fig. 3A; Waterson et al. 2022]. Of note, this data set was generated in L4 larvae under basal conditions in which NHR-49 is likely only marginally active. The overlapping gene set primarily contains genes involved in FA metabolic processes, as shown by GO enrichment analysis [Fig. 3B], including genes encoding key enzymes of the peroxisomal FA β-oxidation pathway (*maoc-1, dhs-28, and daf-22*). We thus tested whether NHR-49 and its known interactor and coactivator, the mediator subunit MDT-15 [Taubert et al. 2006; Goh et al. 2018], regulate the
expression of lipid metabolism genes in *cbd-1* mutants. For this, we selected two lipid metabolism genes that were among the top upregulated genes in *nhr-49(nr2041)*, namely, *far-3*, which encodes a fatty acid- and retinol-binding protein, and the cytochrome P450-encoding gene *cyp-29A2*. qPCR analysis confirmed that mRNA levels of *far-3* and *cyp-29A2* were upregulated >10-fold and threefold, respectively, in *cbd-1(ok2913)* compared with wild-type animals [Fig. 3C,D]. Knockdown of *nhr-49* or *mdt-15* with RNAi blocked the induction of *far-3* [Fig. 3C] and *cyp-29A2* [Fig. 3D], indicating that the upregulation of these genes in *cbd-1(ok2913)* is strictly dependent on both NHR-49 and MDT-15. We then asked whether NHR-49 signaling also mediates the changes in fat stores and found that elevated fat storage in *cbd-1(ok2913)* was not affected by the *nhr-49* loss-of-function mutation [Fig. 3E], indicating that NHR-49 activity is dispensable for this phenotype. Together, these results indicate that the NHR-49/MDT-15 nuclear receptor complex acts downstream from the increase in fat storage to mediate changes in lipid metabolism gene expression in response to inhibition of the embryonic vitelline layer.

**NHR-49 and MDT-15 are required for enhanced stress resilience in response to vitelline disruption**

In addition to its role in regulating lipid metabolism, NHR-49 has previously been shown to mediate resistance to oxidative stress, hypoxia, and pathogens [Goh et al. 2018; Wani et al. 2021; Doering et al. 2022]. We thus asked whether NHR-49/MDT-15 nuclear receptor signaling also has a role in the elevated heat stress resilience induced by impairment of the vitelline layer. We found that RNAi knockdown of *mdt-15* completely suppressed and *nhr-49* significantly reduced the prolonged survival of *cbd-1(ok2913)* following a lethal heat stress [Fig. 3F; Supplemental Fig. S3C]. The partial suppression of *nhr-49* was likely due to incomplete penetrance of the RNAi [Supplemental Fig. S3D] because the loss-of-function allele *nhr-49(nr2041)* nearly completely abrogated the beneficial effects of *cbd-1(ok2913)* on heat stress survival [Fig. 3G]. However, *nhr-49* loss of function or RNAi had no significant effect on heat stress survival in the wild-type background [Fig. 3G; Supplemental Fig. S3C], in agreement with previous reports [Watterson et al. 2022] and in contrast to what has been observed for resistance to oxidative stress and hypoxia [Goh et al. 2018; Doering et al. 2022]. Therefore, regulation by the NHR-49/MDT-15 nuclear receptor complex is required for the beneficial organismal effects in response to signals from the reproductive system when embryo integrity is compromised.

**NHR-49 is important in multiple paradigms of stress resilience**

Considering the function of NHR-49 as a lipid-sensing factor that regulates gene expression, we hypothesized that this nuclear receptor may have a general role in promoting stress resilience in diverse contexts where lipid homeosta-

Figure 4. NHR-49 is important in multiple paradigms of stress resilience. [A] Heat stress survival of wild type and *daf-2(e1370)* mutants crossed into *nhr-49(nr2041)* [n = 3]. [B] Heat stress survival of wild type and *nhr-49(nr2041)* mutants grown on OP50 bacteria [fed] or deprived of food source for 16 h prior to the heat stress [starved] [n = 3]. [C] Heat stress survival of wild type and *glp-1(e2141)* temperature-sensitive mutants crossed into *nhr-49(nr2041)*. Animals were grown at 25°C prior to heat stress and transferred back to 25°C [n = 3]. Error bars represent SEM. Statistical significance was based on two-way ANOVA. (**) P < 0.01, (***) P < 0.001, [ns [nonsignificant]] P > 0.05.
Increased NHR-49 activity enhances organismal stress resilience and proteostasis

Since NHR-49 mediates stress resilience in several contexts, we asked whether increasing the activity of this nuclear receptor was sufficient to induce protective effects. To this test, we first used the gain-of-function mutation \textit{nhr-49(et7)}, which results in a single amino acid substitution near the ligand binding domain of NHR-49 and was shown to upregulate putative targets [Lee et al. 2016]. We found that \textit{nhr-49(et7)} animals exhibit increased survival to lethal heat stress compared with wild-type animals, at a level comparable with that of \textit{cbd-1(ok2913)} [Fig. 5A]. We did not observe any additive effect on heat stress survival in \textit{nhr-49(et7);cbd-1(ok2913)} double mutants [Fig. 5A], suggesting that these two mutations act within the same genetic pathway to promote stress resilience. We also found that, similar to the \textit{cbd-1} mutant, the elevated survival of \textit{nhr-49} gain of function following a lethal heat stress was hampered by \textit{daf-22} RNAi [Supplemental Fig. S5A], highlighting the importance of the peroxisomal FA \(\beta\)-oxidation pathway downstream from NHR-49.

We next asked whether overexpression of NHR-49 in individual somatic tissues was sufficient to impact stress resilience. For this, we used animals that overexpress the NHR-49::GFP protein from extrachromosomal arrays under the control of different tissue-specific promoters—corresponding to \textit{gly-19p} [intestinal], \textit{col-12p} [hypodermis], or \textit{myo-3p} [muscle]—in an otherwise \textit{nhr-49(nr2041)} mutant background [Naim et al. 2021]. We measured the ability of these strains to survive following a lethal heat stress and found that overexpression of NHR-49 in each of the three tissues tested was sufficient to improve survival compared with the \textit{nhr-49(nr2041)} control, with the strongest effect being observed for intestinal overexpression [Fig. 5B].

We then sought to determine whether NHR-49 activity also has beneficial effects on organismal proteostasis. Aggregation-prone polyglutamine repeats fused to fluorescent proteins have been widely used to monitor age-dependent protein aggregation and its modulation by cellular factors [Morley et al. 2002; Gidalevitz et al. 2006; Silvia et al. 2011]. We asked whether gain of function or intestinal overexpression of NHR-49 affected the age-dependent aggregation of a polyglutamine model protein expressed in intestinal cells. For this, we used a strain that harbors an integrated array of a transgene encoding a stretch of 35 glutamines fused to mCherry fluorescent protein [Q35::mCherry] and expressed from an intestine-specific \textit{vha-6} promoter. The animals exhibited mCherry fluorescence in intestinal cells that, upon aging, changed from diffuse distribution to visible foci that corresponded to protein aggregates. Focus formation began around day 3 of adulthood and increased progressively as the animals aged [Supplemental Fig. S4A,B]. We found that the \textit{nhr-49(et7)} gain-of-function mutation or \textit{gly-19p::nhr-49::gfp} intestinal overexpression significantly decreased Q35::mCherry aggregate formation, with a 30% reduction in polyglutamine foci at day 5 of adulthood compared with the wild-type control [Fig. 5C,D; Supplemental Fig. S4C].

Therefore, increased NHR-49 activity is sufficient to improve organismal stress resilience and to reduce age-associated protein aggregation. This indicates that NHR-49 drives a transcriptional program that directly or indirectly promotes cellular resilience during both acute and chronic proteotoxic stress.

\textit{NHR-49 enhances the HSR and acts via HSF-1 to improve stress resilience}

We have previously shown that damage to the embryonic vitelline layer induced by \textit{cbd-1} RNAi requires HSF-1 for its beneficial effects on organismal stress resilience [Sala et al. 2020]. Here, we show that the \textit{cbd-1(ok2913)} mutant also relies on HSF-1 for elevated stress resilience using RNAi to knock down its expression [Supplemental Fig. S5B]. HSF-1 is also known to be regulated downstream from insulin and GSC signaling [Hsu et al. 2003; Morley and Morimoto 2004; Shemesh et al. 2013; Labbadia and Morimoto 2015b]. If HSF-1 activity is increased by NHR-49, we would expect higher levels of induction of the HSR. We tested this by monitoring transcripts
corresponding to the canonical heat shock genes hsp-70 (C12C8.1) and hsp-70(F44E5.4), both encoding members of the Hsp70 family, in nhr-49(et7) gain-of-function mutants following a mild heat stress for 1 h at 33°C. We found that the induction of both Hsp70-encoding genes was enhanced in nhr-49(et7) compared with wild-type animals by 1.7-fold and 2.7-fold, respectively (Fig. 5E). This effect was specific to heat stress conditions, as we did not observe any effect of nhr-49(et7) gain of function on the basal levels of hsp70 mRNA, in contrast to putative NHR-49 targets far-3, cyp-29A2, and daf-22, which were upregulated fourfold, 10-fold, and 3.5-fold, respectively (Supplemental Fig. S5C). This indicates that increased NHR-49 activity potentiates the ability of adult animals to induce the HSR in response to acute heat stress. We next asked whether increased NHR-49 activity acts via HSF-1 to promote stress resilience. We observed a complete suppression of the elevated heat stress survival of the nhr-49(et7) gain-of-function mutant when the animals were exposed to hsf-1 RNAi [Fig. 5F], indicating that the beneficial effects of NHR-49 on stress resilience are dependent on HSF-1. Together, the data presented in this study suggest that NHR-49, through its lipid-sensing and gene regulatory function, represents a link between lipid metabolism and regulation of the HSR via modulation of HSF-1 activity (Fig. 5G).

Discussion

In this study, we show that NHR-49 facilitates the adaptation to conditions that modify lipid homeostasis by regulating the expression of genes involved in lipid metabolism but also by rewiring the activity of the master regulator of the HSR, HSF-1, to enhance cellular resilience toward both acute and chronic proteotoxic stress (Fig. 5G). While the HSF-1-mediated HSR is an ancient pathway conserved throughout the eukaryotic kingdom, nuclear hormone receptors are only present in metazoans. We posit that regulation of the HSR by NHR-49 serves to coordinate programs of survival as organismal strategies based on certain metabolic and reproductive cues that converge on lipid homeostasis.

We found that the cbd-1 vitelline mutants accumulate fat stores and have elevated levels of triglycerides. These mutants also exhibit defective transport and accumulation of yolk lipoproteins in somatic tissues as a result of impaired reproduction. We posit that these changes in

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**Figure 5.** NHR-49 is sufficient to enhance stress resilience, proteostasis, and the HSR and acts in an HSF-1-dependent manner. (A) Heat stress survival of wild type and cbd-1(ok2913) mutants crossed into the nhr-49(et7) gain-of-function allele (n = 3). (B) Heat stress survival of nhr-49(m2041) and tissue-specific NHR-49::GFP overexpression strains (n = 3). (C) Representative confocal images of day 5 adults expressing Q35::mCherry in intestinal cells, with the corresponding bright-field images shown in the insets. (D) Quantification of Q35::mCherry aggregates in day 5 adults. Wild type, n = 112; nhr-49(et7), n = 106. (E) Expression of heat shock genes relative to cdc-42 in wild-type animals exposed for 1 h to 33°C heat shock (n = 5). (F) Heat stress survival of wild-type or nhr-49(et7) animals grown on either control or hsf-1 RNAi (n = 3). (G) Model for regulation of organismal stress resilience and proteostasis by NHR-49. Embryo damage, reduced insulin signaling, or food scarcity can activate NHR-49, which, together with its coactivator, MDT-15, induces the expression of genes involved in lipid catabolism. This includes daf-22, which contributes to stress resilience. NHR-49 activation also leads to potentiation of HSF-1 activity and enhancement of the HSR, resulting in improved stress resilience and proteostasis. The beneficial effects of daf-22 may occur via HSF-1 or through a parallel pathway. Error bars represent SEM. Statistical significance was based on two-way ANOVA (A,B,F) or unpaired t-test (D,E). [*] P < 0.05, [**] P < 0.01, [***] P < 0.001, [ns [nonsignificant]] P > 0.05.
lipid content lead to an activation of genes related to lipid catabolism driven at least in part by NHR-49, which also has a positive impact on the activity of the HSR and organismal stress resilience. Importantly, we and others have previously shown that impaired reproduction does not systematically elicit protective organismal effects. This implies that accumulation of yolk proteins in somatic tissues and the resulting changes in lipid homeostasis are not sufficient to increase stress resilience and proteostasis in somatic tissues, but that there may be additional signals specific to vitelline layer impairment. This issue of specificity was brought up previously in the case of GSC-less animals, in which accumulation of yolk and alteration of lipid metabolism activate SKN-1 to mediate increased stress resilience and longevity (Steinbaugh et al. 2015).

We have previously shown that embryo-to-mother signaling in response to an impaired vitelline layer involves a DAF-16-dependent response in the egg-laying apparatus (vulva). Therefore, we speculate that both fat accumulation and the vulva signal may be required to activate the protective downstream pathway.

The role of NHR-49 in lipid homeostasis is complex, as it is required to maintain lipid stores in adults but also promotes FA breakdown via β-oxidation (Van Gilst et al. 2005a,b, Ratnappan et al. 2014). We found that NHR-49 is important for stress resilience in conditions that increase fat stores [e.g., embryo damage and reduced insulin signaling] but also in conditions of food deprivation that result in depletion of lipid stores [Fig. 4]. This indicates that stress resilience and the activity of NHR-49 are not directly influenced by the level of stored fat. Rather, we propose that fat mobilization induced by NHR-49 via activation of daf-22 and other targets involved in FA β-oxidation, which can occur as a result of either elevated fat stores or food scarcity, contributes to elevated stress resilience. Beneficial effects of fat mobilization on longevity have been reported for insulin and germline signaling, as well as overexpression of the lysosomal lipase LIPL-4 (Wang et al. 2008; Ratnappan et al. 2014; Folick et al. 2015). Our results further link lipid catabolism and resilience by establishing a role for NHR-49-mediated fat mobilization in organismal stress resilience and proteostasis.

NHR-49 was recently shown to also be responsive to changes in protein homeostasis during loss of HSF-1, affecting the expression of lipid metabolism genes and life span but not thermotolerance (Watterson et al. 2022). This is consistent with our findings that nhr-49 loss of function has little impact on stress resilience in wild-type animals and that its activation in conditions that change lipid homeostasis promotes cellular resilience to proteotoxic stress. Since we show that NHR-49 is dependent on HSF-1 for this effect, it is then expected that during loss of HSF-1, the protective effects of NHR-49 on stress resilience were not observed (Watterson et al. 2022). Taken together, the findings of Watterson et al. (2022) and this study establish NHR-49 as a two-way regulator connecting lipid homeostasis and proteostasis. Considering that NHR-49 has been shown to promote resilience to several types of stress, including oxidative (Goh et al. 2018), pathogenic (Wani et al. 2021), and hypoxic (Doering et al. 2022), as well as to promote life span (Ratnappan et al. 2014; Lee et al. 2016; Naim et al. 2021; Savini et al. 2022), it will be interesting to determine whether these effects are also dependent on HSF-1.

We found that the HSR is enhanced by nhr-49 gain of function specifically in stress conditions [Fig. 5E; Supplemental Fig. S5C], suggesting that NHR-49 does not activate the HSR but potentiates the intrinsic transcriptional activity of HSF-1 upon activation by stress. This idea is consistent with our observations in ced-1 mutants [Supplemental Fig. S1E,F] and RNAi treatment (Sala et al. 2020). The transcriptional activity of HSF-1 is modulated by post-translational modifications as well as chromatin remodeling factors that favor binding at heat shock gene promoters (Holmberg et al. 2001; Guerin and Lis 2010; Gomez-Pastor et al. 2018; Takii et al. 2019). An important step in the activation of the HSR is the recruitment of Mediator by HSF-1 at heat shock promoters [Park et al. 2001; Anandhakumar et al. 2016], which physically interacts with and recruits RNA polymerase II (Soutourina 2018). Direct interaction of NHR-49/MED-15 with HSF-1 or binding to a nearby motif may thus facilitate the recruitment of the transcription machinery to heat shock genes during proteotoxic stress. Future efforts to map the binding sites of NHR-49 in the C. elegans genome will shed light on its mechanism of cooperation with HSF-1 during proteotoxic stress. NHR-49 may also promote the activity of HSF-1 indirectly. We show that the elevated stress resilience of the ced-1 vitelline mutant and nhr-49 gain of function are hampered by daf-22 knockdown [Fig. 2E; Supplemental Fig. S5A], which suggests that changes in the peroxisomal β-oxidation pathway induced by NHR-49 may influence the activity of HSF-1. The molecular mechanism by which NHR-49 regulates the activity of HSF-1 remains an open question.

Considering the conservation of these transcription factors, our finding that NHR-49 regulates HSF-1 and the HSR may be relevant to higher organisms, including mammals. Interestingly, PPARα was shown to be required for the expression of a subset of heat stress-responsive genes in the mouse liver, including genes previously shown to be HSF-1-dependent (Vallanat et al. 2010). This suggests that the regulatory role of NHR-49/PPARα in the HSR may be conserved. Overall, our findings that the lipid-sensing nuclear receptor NHR-49 acts as a critical regulator of cellular resilience to proteotoxic stress link two major cellular processes and form the basis for future studies to further understand the interconnections between lipid homeostasis and regulation of the HSR as organismal strategies to optimize fitness and survival.

Materials and methods

C. elegans strains and maintenance

Standard C. elegans methods were used as described previously [Brenner 1974]. Worms were maintained on solid nematode growth medium (NGM) seeded with Escherichia coli OP50 or, for RNAi experiments, HT115
transformed with the appropriate plasmids. All experiments were performed at 20°C unless stated otherwise. Age synchronization was performed by timed egg laying in a 1–2 h period for all experiments.

The following strains were used in this study: wild type [N2 Bristol], VC2258 std-1(ok2913) IV, RT130 pwi23 [vict-2::GFP], ST68 4hr-49(nr2041) I, STE108 4hr-49 (et7) I, AGP65 4hr-49(nr2041) I, glmExpress9 [pgly-19::4hr-49::GFP+myo-2::mCherry], AGP56 4hr-49(nr2041) I, glmExpress11 [Pcol-12::4hr-49::GFP+myo-2::mCherry], and AGP63 4hr-49(nr2041) I, glmEx12 [pgly-3::4hr-49::GFP+myo-2::mCherry]. Strain VC2258 was generated by the C. elegans Gene Knockout Consortium and was backcrossed six times to N2 before use. STE108 was obtained from Stephan Taubert, and AGP65, AGP63, and AGP56 were obtained from Arjumand Ghazi. All other strains were either generated in our laboratory or obtained from the Caenorhabditis Genetics Center (CGC). RT130 was backcrossed three times to N2 before use to alleviate a slow-growth phenotype.

The VC2258 strain was crossed with RT130, STE68, and STE108 to generate strains AM1239, AM1238, and AM1237, respectively. Strain STE68 was crossed with CB4037 and CB1370, resulting in strains AM1243 and AM1244, respectively.

A plasmid construct for the expression of Q35::mCherry was generated by inserting the sequence encoding Q35 upstream of mCherry in pGH8 (Addgene plasmid 19359) and replacing the promoter with an 880 bp sequence corresponding to the vha-6 promoter using the NEBuilder HiFi DNA assembly cloning kit. The resulting plasmid was introduced into the gonads of adult N2 hermaphrodites at 10 ng/µL, and fluorescent progeny were isolated to establish extrachromosomal arrays. The array was integrated by UV irradiation, and the resulting strain was backcrossed five times to N2 animals to generate strain AM1240 rmIs406 [vha-6p::Q35::mCherry]. The rmIs406 allele was crossed with the 4hr-49(et7) allele to generate strain AM1241 and with the glmExpress9 array to generate strain AM1242.

**RNA extraction and sequencing**

Age-synchronized populations of at least 200 animals were grown until day 4 of adulthood at 20°C and transferred to fresh plates every day to avoid contamination with progeny. Heat shock was performed on NGM plates wrapped with parafilm and submerged in a water bath for 1 h at 33°C. Nontreated and heat-stressed samples were then collected into M9 media and snap-frozen in liquid nitrogen. RNA was extracted using Trizol [invitrogen] and purified with Qiagen RNeasy MinElute RNA extraction kit with on-column DNase I digestion according to the manufacturers’ instructions. Samples were prepared in biological triplicates.

Library preparation and sequencing were performed by Novogene. Briefly, 1 µg of RNA was used to generate sequencing libraries using NEBNext Ultra RNA library preparation kit for Illumina (NEB) following poly[A] selection. The library preparations were sequenced on an Illumina HiSeq 4000 instrument, and 150 bp paired-end reads were generated.

To facilitate reproducible analysis, samples were processed using the publicly available nf-core/rnaseq pipeline version 3.8.1 implemented in Nextflow 22.04.5.5708 using Singularity 3.8.1 with the minimal command nextflow run nf-core/rnaseq –v ‘3.8.1’ –profile nu_genomics --genome ‘WBcel235’. Briefly, lane-level reads were trimmed using trimGalore! 0.6.7 and aligned to the WBcel235 genome described above using STAR 2.6.1d. Gene-level assignment was then performed using salmon 1.5.2.

**Differential expression analysis**

Differential expression analysis (DEA) was performed using custom scripts in R version 4.1.1 using the DESeq2 version 1.34.0 framework. A “local” model of gene dispersion was used, as this better fit dispersion trends without obvious overfitting, and pairwise comparisons were performed by treating genotype and treatment (heat shock) as independent factors [–genotype + treatment], as no significant interaction between these factors was observed for any gene. For pairwise comparisons, a combined factor of genotype and treatment was used [–combined]. For all DEAs, a was set at 0.05; otherwise, default settings were used. High-level analysis was performed using custom scripts available in the NU Pulmonary/utils GitHub repository. GO term enrichment was then determined using Fisher’s exact test [classic mode] with FDR correction in topGO version 2.46.0, with org.Ce.eg.db version 3.14.0 as a reference. For KEGG enrichment analysis, an analogous approach was used using manual Fisher’s exact tests with FDR correction against all annotated KEGG pathways in C. elegans using KEGGREST 1.34.0. Tissue and phenotype enrichments were determined using the gene set enrichment analysis tool available on WormBase with a q-value threshold of 0.1 [Angeles-Albores et al. 2016]. Transcriptomic data of 4hr-49(nr2041) mutants from Watterson et al. [2022] were obtained from the NCBI Gene Expression Omnibus (GEO) database (accession no. GSE199971) and analyzed using the same method and parameters.

**Heat stress survival assay**

Synchronized animals (~50 per plate) were grown at 20°C until day 2 of adulthood, transferred to new plates, sealed with parafilm, and incubated for 4 h in a water bath at 35°C. Worms were allowed to recover at 20°C, and survival was scored every 24 h until all animals were dead. Animals were scored as dead in the absence of pharyngeal pumping or touch response, and animals with prolapsed gonads were censored from the analysis. Scoring was performed in a blinded manner, and the assay was repeated at least three times per condition.

For stress survival assays following food deprivation, age-synchronized animals were grown to day 1 of adulthood (72 h after egg lay) and transferred to NGM plates without any bacterial food source. After 16 h of food
deprivation, the animals were transferred back onto OP50-seeded NGM plates and heat-shocked for 4 h as detailed above.

**Brood size**

Individual young adults were placed onto OP50-seeded plates and transferred to fresh plates every 24 h for 5 days. Following transfer, the plates containing the eggs were kept for 24 h at 20°C, and viable progeny were counted.

**Oil red O staining and quantification**

Oil red O staining of neutral lipids was performed in fixed animals as described previously (Escorcia et al. 2018). Briefly, age-synchronized day 2 adults were washed in PBS supplemented with 0.01% Triton X-100 (PBST) and incubated in 40% isopropanol for 3 min. Oil red O (Sigma O0625) resuspended in 60% isopropanol was added to each tube, and worms were rocked for 2 h at room temperature. After a 30 min wash in PBST, animals were mounted onto slides and imaged on an Olympus BX53 microscope using a Micropublisher color camera. Color images were quantified in ImageJ as described previously (O’Rourke et al. 2009) by measuring within each worm object the level of intensity in the red channel in areas with excess red intensity compared with blue and green signals and normalizing to the area of the object.

**Triglyceride quantification**

Approximately 200 animals were collected on day 2 of adulthood in water and washed extensively. The worms were resuspended in lysis buffer (25 mM Tris HCl at pH 7.4, 1 mM EDTA, 1% Triton X-100) and sonicated with a Bioruptor for 30 sec on and 30 sec off at maximum power for 10 min. The lysate was cleared of debris with a brief spin, and protein concentration was estimated using the Bradford assay. Lipids were dissolved by adding 5% NP40 to the samples and slowly heating them to 100°C, followed by cooling at room temperature. This was performed twice before the lysate was cleared by centrifugation. The supernatant was then used for the triglyceride quantification fluorometric assay (Sigma MAK266) per the manufacturer’s instructions. Experiments were performed in biological triplicates for each condition examined.

**Fatty acid composition analysis**

Fatty acid composition was determined through direct transesterification of worm extracts to generate fatty acid methyl esters (FAMES). Approximately 1000 age-synchronized worms were added to 1 mL of 2.5% sulfuric acid in methanol and heated for 1 h at 70°C. FAMES were extracted with hexane and injected into a gas chromatography/mass spectrometry (GC/MS) device equipped with an SP-2380 column for separation and analysis (Harrison and Watts 2022).

**RNAi treatments**

For RNAi-mediated knockdown, the indicated strains were synchronized by egg laying on *E. coli* strain HT115 [DE3] containing the appropriate RNAi vectors obtained from the Ahringer RNAi library, with L4440 as the empty vector control (Kamath et al. 2003). RNAi bacteria were grown overnight, and cultures were induced with 5 mM IPTG for 3 h.

**Real-time quantitative PCR**

RNA extraction was performed on populations of at least 100 animals per replicate using TRIzol (Invitrogen) and RNA purified with Qiagen RNeasy MinElute columns as described previously (Labbadia and Morimoto 2015b). mRNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad), and real-time qPCR was performed using iTaq SYBR Green Supermix (Bio-Rad) in a Bio-Rad CFX384 real-time PCR system. Relative expression was determined from cycle threshold values using the standard curve method, and the expression of genes of interest was normalized to *rpb-2*. The primers used are listed in Supplemental Table S1.

**Fluorescence microscopy**

Age-synchronized animals were mounted on 3% agarose pads in 2 mM levamisole in M9 buffer and imaged with a Zeiss LSM 800 confocal microscope using a 10× or 20× objective lens and Zen imaging software. Maximum intensity projection images were generated from Z stacks using ImageJ software.

**Quantification of polyglutamine aggregates**

Populations of 30 age-synchronized adult animals were transferred to fresh plates every day after reaching adulthood, and the number of Q35:mCherry aggregates was visually determined in live animals by counting large, bright fluorescent foci using a fluorescence stereomicroscope. Scoring was performed in a blinded manner, and experiments were repeated three times.

**Data availability**

Raw transcriptomic data files generated for this study have been deposited in the SRA database [http://www.ncbi.nlm.nih.gov/sra] under accession number PRJNA948034.0.

**Competing interest statement**

The authors declare no competing interests.

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References


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