

Research Paper

Genetic Dissection Reveals New Components of the NHR-49 Multi-Stress Resistance and Longevity Pathway

Kelsie R. S. Doering^{1,2,3,4,5,*} Glafira Ermakova^{1,2,3,4} Brendil Sabatino^{1,2,3} Rachel Cheng^{1,2,3,6}
Luxcia Kugathasan^{1,2,3,7} Zoe DeBoer^{1,2,3} and Stefan Taubert^{1,2,3,4,*}

¹Centre for Molecular Medicine and Therapeutics, The University of British Columbia, Vancouver, BC, Canada

²Edwin S.H. Leong Centre for Healthy Aging, The University of British Columbia, Vancouver, BC, Canada

³British Columbia Children's Hospital Research Institute, Vancouver, BC, Canada

⁴Department of Medical Genetics, The University of British Columbia, Vancouver, BC, Canada

⁵Department of Biological and Health Sciences, Kwantlen Polytechnic University, Surrey, BC, Canada

⁶Now at AbCellera Biologics Inc., Vancouver, BC, Canada

⁷Now at Department of Medicine, Division of Nephrology, Toronto General Hospital, University of Toronto, Toronto, ON, Canada

*Corresponding authors: Kelsie.doering@kpu.ca, taubert@cmm.ubc.ca

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Cells, tissues, and organisms encounter many environmental and endogenous stresses, and the ability to mount specific responses to these stresses is critical to achieve homeostasis and survival. Stress also occurs in many pathological states and is therefore of great biomedical relevance, both as a cause or contributor to diseases and as a potential therapeutic target. Pathways that regulate the responses to oxidative stress, hypoxia, and starvation are evolutionarily conserved from the nematode *Caenorhabditis elegans* to humans and involve master regulators of gene expression. One such stress response pathway engages *C. elegans* NHR-49, an orthologue of mammalian PPAR α , which is required to survive tBOOH-induced oxidative stress, hypoxia, and starvation, and is also a key effector in many genetic longevity pathways. However, in contrast to other stress response pathways such as SKN-1/Nrf2 and HIF-1/HIF signaling, many aspects of NHR-49 signaling remain poorly understood. To map new components of this pathway, we performed reverse genetic screens, uncovering 80 candidate kinases, transcription factors, and transcriptional coregulators required for NHR-49-controlled gene activation in tBOOH, hypoxia, and/or starvation. Further analysis revealed that the kinase *hpk-1/HIPK* is required in the *nhr-49* pathway for hypoxia and oxidative stress survival, that pseudokinase *nipi-3* is required with *nhr-49* for hypoxia survival, and that transcription factor *nhr-80* is required with *nhr-49* for hypoxia and oxidative stress survival and for the normal lifespan of wild-type *C. elegans*. Collectively, our data identify numerous new potential regulators in an important stress response and thus substantially enhance our understanding of stress adaptation and longevity regulation.

Introduction

The ability to survive in the face of stress is fundamental for cells, tissues, and organisms in their natural surroundings, and altered stress responses are also a feature of many pathological states in humans, such as cancer. Common stresses include oxidative stress, hypoxia, and starvation. Oxidative stress occurs when reactive oxygen species (ROS), obligate and ubiquitous byproducts of aerobic respiration, accumulate within the cell to toxic levels. ROS are highly electrophilic and cause cellular damage by oxidizing DNA, proteins, and lipids, which promotes the activation of cytoprotective and damage repair pathways¹⁻³. Hypoxia occurs when cellular oxygen levels are too low for normal physiological functions, resulting in major metabolic and developmental rewiring^{4,5}. Finally, starvation causes major changes in cellular

metabolism, including depletion of energy stores via lipolysis, autophagy, and fatty acid β -oxidation. Thus, organisms rewire their physiology and metabolism in the face of limited nutrient availability⁶⁻⁸.

The pathways that regulate the response to these stresses are evolutionarily conserved and typically require master regulators of gene expression to orchestrate changes in developmental and physiological gene programs. Oxidative stress activates evolutionarily conserved transcription factors of the nuclear factor erythroid 2-related factor 2 (Nrf2) family. In the animal model *Caenorhabditis elegans*, the Nrf2 family member SKIN-1 (SKN-1) is modified by several kinases and translocates to the nucleus, where it activates the expression of cellular detoxification and antioxidant genes, reestablishing homeostasis and promoting survival⁹⁻¹⁴. Similarly, transcription factors of the

hypoxia-inducible factor (HIF) family play a critical role in hypoxia response in animals. For example, *C. elegans* typically activates the Egg-laying defective 9 (*egl-9*)-von Hippel-Lindau (*vhl-1*)-*hif-1* pathway to promote metabolic changes in hypoxia, thus enabling survival^{5,15}. Finally, the transcription factor EB (TFEB) family is critical for the response to starvation^{6,11,16–19}. In starving *C. elegans*, the TFEB ortholog HLH-30 translocates to the nucleus to regulate the expression of many lysosomal and autophagy genes^{6,8,17,20}. In addition to their role in acute stress responses, these genes also play essential roles in maintaining the normal lifespan of wild-type *C. elegans* and the extended lifespan of many long-lived mutants²¹, highlighting the importance of stress response pathways in longevity.

Although the *C. elegans* SKN-1/Nrf2, HIF-1, and HLH-30/TFEB pathways are critical for the oxidative stress, hypoxia, and starvation response, respectively, other pathways of equal importance to stress survival have been discovered. Several studies show that the nuclear hormone receptor (NHR) transcription factor NHR-49, originally identified as a regulator of fatty acid metabolism, is necessary for the resistance to hypoxia, oxidative stress, infection with pathogens, and for the normal lifespan of wild-type and long-lived *C. elegans* strains^{22–30}. Notably, *nhr-49* acts in parallel to *hif-1* in hypoxia and regulates *skn-1*-independent gene responses in oxidative stress^{31,32}, highlighting that several parallel pathways have evolved to provide *C. elegans* with complementary and optimal protection in the face of stress.

The activation and implementation of SKN-1-, HIF-1-, and HLH-30-driven signaling in *C. elegans* is complex and involves numerous factors, including kinases, transcriptional coregulators, and other transcription factors. For example, SKN-1 activity is controlled by post-translational modification by kinases such as P38 Map Kinase 1 (PMK-1), Glycogen Synthase Kinase 3 (GSK-3), AKT-1/-2, Serum- and Glucocorticoid-inducible Kinase 1 (SGK-1), and No Induction of Peptide after *Drechmeria coniospora* Infection 3 (NIPI-3)^{10,14,33,34}, by the proteasome via WD Repeat protein 23 (WDR-23) and Skp1 Related 1 and 2 (SKR-1/-2)^{35,36}, and by interactions with transcriptional coregulators such as cAMP response element-binding protein (CREB)-binding protein 1 (CBP-1) and Mediator subunit MDT-15^{37,38}. Collectively these factors fine-tune SKN-1 activity in various settings related to stress, pathogen infection, and longevity.

Less is known about how the *nhr-49* pathway is regulated, especially in conditions of stress. NHR-49 can dimerize with other NHRs^{39–41}, and some such partnerships (e.g., with NHR-66, -79, and -80) may regulate specific sets of lipid metabolism genes^{42–44}. However, how NHR-49 promotes certain stress response programs is unclear. Furthermore, although we and others showed that *mdt-15* is a coregulator required for the induction of *nhr-49*-dependent stress response genes³², NHR-49 is likely to engage additional coregulators to achieve specialized stress responses. AMP-activated kinase 2 (*aak-2*) may regulate NHR-49^{45,46}, and NHR-49 may also be controlled by fatty acids that could act as NHR-49 ligands⁴⁷, but overall, little is known about how NHR-49 is activated by stress. Thus, a substantial gap in knowledge remains on whether and how transcription factors, coregulators, and kinases function in NHR-49-controlled stress responses.

To discover new genes acting with NHR-49 to regulate stress responses, we conducted a reverse genetic screen using a flavin-containing monooxygenase 2 transcriptional reporter (*fmo-2p::gfp*) as a readout. *fmo-2p::gfp* reporter induction depends on *nhr-49* in oxidative stress caused by tert-butyl hydroperoxide

(tBOOH), hypoxia, and starvation, but is independent of *skn-1* in tBOOH and only weakly depends on *hif-1* in hypoxia and *hlh-30* in starvation^{31,32}. Our screen identified genes unique to one stress, genes involved in two stresses, and a set of 29 genes required for the upregulation of *fmo-2p::gfp* in all three stresses. The latter set included the known NHR-49 coregulator *mdt-15* and homeodomain interacting protein kinase 1 (*hpk-1*), which acts with *nhr-49* in the hypoxia response³¹, validating our screen. Follow-up studies on *hpk-1*, the pseudokinase *nipi-3*, and the transcription factor *nhr-80* revealed new potential partners of *nhr-49*-dependent stress responses with specific requirements in some, but not other, stresses. Our work has thus uncovered factors that may delineate stress-specific or general signaling branches of the NHR-49 stress response pathway.

Materials and Methods

Nematode strains and growth conditions

We cultured *C. elegans* strains on nematode growth media (NGM) plates using standard techniques. To avoid background effects, each mutant was crossed into our lab wild-type N2 strain at least six times. *E. coli* strain OP50 was the food source in all experiments except for RNAi experiments, for which we used *E. coli* strain HT115. All experiments were carried out at 20 °C. *C. elegans* strains used in this study are listed in Table 1. For synchronized *C. elegans* growths, embryos were isolated by standard sodium hypochlorite treatment⁴⁸. Isolated embryos were allowed to hatch overnight on unseeded NGM plates until the population reached a synchronized halted development at L1 stage via short-term fasting (12–24 hours). Synchronized L1 stage larvae were then transferred to OP50-seeded plates and grown to the desired stage.

Feeding RNA interference

RNAi was performed on NGM plates supplemented with 25 µg/mL carbenicillin (BioBasic CDJ469), 1 mM IPTG (Santa

Table 1. *C. elegans* strains used in this study.

Strain	Genotype	Reference
N2	Wild type	83
VE40	<i>eavEx20[fmo-2p::gfp + rol-6(su1006)]</i>	32
STE117	<i>nhr-49(et13) I; eavEx20[fmo-2p::gfp + rol-6(su1006)]</i>	32
STE68	<i>nhr-49(nr2041) I</i>	28
EK273	<i>hpk-1(pk1393) X</i>	84
STE132	<i>nhr-49(nr2041) I; hpk-1(pk1393) X</i>	31
AGP25f	<i>glmEx5 (nhr-49p::nhr-49::gfp + myo-2p::mCherry)</i>	27
STE142	<i>hpk-1(pk1393) X; glmEx5 (nhr-49p::nhr-49::gfp + myo-2p::mCherry)</i>	31
AVS310	<i>artEx27[Phpk-1::hpk-1::GFP + pRF4(rol-6(su1006))]</i>	54
STE197	<i>nhr-49(nr2041) I; artEx27[Phpk-1::hpk-1::GFP + pRF4(rol-6(su1006))]</i>	This study
IG544	<i>nipi-3(fr4) X</i>	58
STE198	<i>nhr-49(nr2041) I; nipi-3(fr4) X</i>	This study
STE70	<i>nhr-80(tm1011) III</i>	43
STE199	<i>nhr-49(nr2041) I; nhr-80(tm1011)</i>	This study

Cruz CAS 367-93-1), and 12.5 µg/mL tetracycline (BioBasic TB0504; NGM-RNAi plates) and seeded with appropriate HT115 RNAi bacteria. RNAi sub-libraries covered 1,543 genes total, including 332 kinases, 768 transcription factors, and 443 coregulators^{40,49}. RNAi clones were from the Ahringer and Vidal libraries^{50,51}; for a complete list, see **Supplementary Table 1H**.

fmo-2p::gfp RNAi screens

Egg lays were performed for 3–4 hours on 12-well NGM-RNAi plates with VE40 *eavEx20[fmo-2p::gfp + rol-6(su1006)]* animals (hereafter *fmo-2p::gfp* animals) until there were approximately 60 eggs per well. Adults were then removed, and synchronized progeny (which include transgenic *fmo-2p::gfp* animals displaying the Roller phenotype and non-transgenic, non-Roller siblings) were grown for three days at 20 °C to the L4/young adult stage. At that time, animals were transferred to 10 mM tBOOH RNAi plates seeded with heat-inactivated HT115 bacteria for 5 hours for the tBOOH screen; transferred to 0.5% O₂ for 4 hours followed by 1 hour in 21% O₂ for the hypoxia screen; or washed at least five times with M9 to remove all excess bacteria and transferred to unseeded plates for 8 hours for the starvation screen. For the *nhr-49(et13)* screen, *fmo-2p::gfp;nhr-49(et13)* animals synchronized as described above were grown to the L4/young adult stage. Two control wells were always included during the screen: L4440 empty vector (EV) RNAi as a negative control and *nhr-49* RNAi as a positive control (on *fmo-2p::gfp* or *fmo-2p::gfp;nhr-49(et13)* animals, as appropriate).

As a screen readout, we performed semi-quantitative scoring of *fmo-2p::gfp* fluorescence. Specifically, approximately 20 L4/young adult stage *C. elegans* were assessed for whole-body GFP fluorescence levels under a Leica M205FA fluorescence microscope, and fluorescence levels were scored as high, medium, low, or off for each animal in each well. In the tBOOH screen, wells with at least 40% of animals showing medium, low, or off GFP fluorescence were counted as “hits” (**Supplementary Table 1A–C**). For all subsequent screens, wells with at least 30% of animals showing low or off GFP fluorescence were counted as “hits” (**Supplementary Table 1A, 1D–G**). Following one repeat of all appropriate RNAi clones in each screen, all initial “hits” were re-screened. Only RNAi clones which yielded “hits” in all three repeats (original screen and two re-screens) were considered validated hits (see **Supplementary Fig. 1A**). Any culture that impaired animal development was subsequently removed from the total number of cultures tested for that screen, regardless of hit status. This included removal of 37 cultures from the tBOOH screen and removal of 2 cultures from the hypoxia screen (**Supplementary Fig. 1A**). All 80 RNAi cultures representing final hits were sequenced to verify that the correct target gene was targeted.

For the tBOOH screen, we used the high, medium, low, or off designations to calculate an “activity score” (AS) for each RNAi clone, as follows: $AS = (0 * [\text{number of “off” animals} / [\text{number of total transgenic animals}]] + 1 * [\text{number of “low” animals} / [\text{number of total transgenic animals}]] + 2 * [\text{number of “medium” animals} / [\text{number of total transgenic animals}]] + 0 * [\text{number of “high” animals} / [\text{number of total transgenic animals}]]$. Thus, the minimum AS possible is 0, and the maximum possible AS is 3. We then used unpaired, two-tailed Student’s T-tests to calculate the significance of AS for each hit versus EV control (**Supplementary Table 1C**).

Analysis of fluorescent reporter lines via differential interference contrast (DIC) and fluorescence microscopy

To analyze *fmo-2p::gfp* fluorescence in the RNAi screens, *C. elegans* were grown and treated in the appropriate condition as described above. For NHR-49::GFP experiments, adult animals were transferred to 10 mM tBOOH plates for 3 hours. For microscopy, *C. elegans* were collected into M9 buffer containing 0.06% levamisole (Sigma L9756) for immobilization on 2% (w/v) agarose pads. We captured images at 10x magnification on a CoolSnap HQ camera (Photometrics) attached to a Zeiss Axioplan 2 compound microscope, followed by MetaMorph Imaging Software with Autoquant 3D digital deconvolution. All images for the same experiment were captured at the same exposure time. Images were analyzed using ImageJ software (<https://imagej.nih.gov/ij/download.html>), with fluorescence calculated by taking the difference of the background fluorescence (area beside the animal) from the mean whole animal fluorescence. For each experiment, at least three independent trials were performed with a minimum of 30 *C. elegans* per condition.

tBOOH survival assay

Synchronized *C. elegans* were grown to the late L4 stage and then transferred to plates containing 6 mM tBOOH seeded with heat-inactivated OP50. Dead and surviving animals were quantified twice a day for 96 hours or until all animals were dead, whichever came first. Death was assessed by prodding the animals with a platinum wire. Animals that crawled off the plate or died due to rupturing or internal hatching were censored. GraphPad Prism was used to generate survival curves and calculate statistical significance using the log-rank (Mantel–Cox) test, and statistics are listed in the accompanying tables.

Hypoxia sensitivity assays

Hypoxic conditions were maintained using continuous flow chambers, as previously described⁵². Compressed gas tanks (5000 ppm O₂ balanced with N₂) were certified as standard to be within 2% of indicated concentration from Praxair Canada (Delta, BC). Oxygen flow was regulated using Aalborg rotameters (Aalborg Instruments and Controls, Inc., Orangeburg, NY, USA). Hypoxic chambers (and room air controls) were maintained in a 20 °C incubator for the duration of the experiments.

For embryo survival assays, gravid first-day adult animals (picked as L4 the previous day) were allowed to lay eggs for 1–4 hours on plates seeded with 15 µL OP50 the previous day. Adults were removed, and eggs were exposed to 0.5% O₂ for 24 hours. After being placed back into normoxia (21% O₂) for 65 hours, animals were scored for developmental success, as defined as reaching at least L4 stage. For all normoxia comparison experiments, methods were as described above except plates were kept in room air (21% O₂) for the duration (instead of being exposed to 0.5% O₂).

L1 starvation recovery assay

Gravid day 1 adult *C. elegans* were picked onto agar plates seeded with *E. coli* (OP50), with 15–20 animals per plate and 2–3 plates per strain, and maintained at 20 °C. Four days later, a first sodium hypochlorite treatment was conducted to obtain synchronized eggs (see above). The bleached eggs were incubated on a rotator in 15 mL tube in S basal solution with antibiotics/antimycotics at 20 °C overnight. On the following day, hatched

L1 stage animals were plated onto seeded plates and then kept in a 20 °C incubator. When the *C. elegans* reached day 1 adulthood (96 hours post first bleach), a second sodium hypochlorite was conducted, after which the bleached eggs were incubated on a rotator in 15 mL tube in S basal solution with antibiotics/antimycotics at 20 °C overnight. On the following day, hatched L1 animals were diluted to achieve a concentration of 1 *C. elegans*/μL in the 15 mL tubes. On the same day, 150 μL of *C. elegans* suspended in S Basal were plated onto plates seeded with OP50 for day 0. Recovery counts were performed 48 hours after plating to assess the percentage of animals that reached the L4 stage. Subsequent plating and survival counting were carried out on days 2 and 4, and continued every other day until day 26 or until all animals failed to recover. Statistical analysis and graphical representation of % recovery from starvation were done with GraphPad Prism using one-way ANOVA of the AUC of the individual recovery curves. To eliminate effects due to intrinsic developmental delays of some mutants, we normalized baseline viability of each strain to 100%.

Lifespan assay

Gravid day 1 adult *C. elegans* were picked onto agar plates seeded with *E. coli* OP50, with 15–20 animals per plate and 2–3 plates per strain, and maintained at 20 °C. Four days later, hypochlorite treatment was conducted to obtain synchronized eggs (see above). The bleached eggs were incubated on a rotator in 15 mL tube in S basal solution with antibiotics/antimycotics at 20 °C overnight. On the following day, hatched L1 stage animals were plated onto seeded plates and then kept in a 20 °C incubator. Ninety-six hours after sodium hypochlorite treatment, adult longevity experiments commenced by picking synchronized adult animals onto freshly seeded plates. Animals were scored as alive/dead/censored and moved to newly seeded plates every day for the first 10 days. Animals were scored every other day until day 30 or until all animals were dead. Statistical analysis

and graphical representation of the *C. elegans* lifespan were done with GraphPad Prism using Kaplan-Meier survival analysis.

Results

Reverse genetic screen reveals 80 genes required to activate *fmo-2* in oxidative stress, hypoxia, and/or starvation

To identify new genes in the NHR-49-regulated tBOOH oxidative stress response pathway, we prepared RNAi sub-libraries covering 1,543 genes, including 332 kinases, 768 transcription factors, and 443 coregulators (see the [Materials and Methods](#) section, [Supplementary Table 1H](#)). We then tested each RNAi clone for its requirement to induce the NHR-49-activated *fmo-2p::gfp* promoter reporter after exposing *C. elegans* to 10 mM tBOOH for 3 hours, that is, testing for *nhr-49* phenocopy ([Fig. 1A](#)). Thirty-seven cultures caused animal development to become impaired and were subsequently removed from the total number of cultures tested for the tBOOH screen, bringing the total number of genes tested to 1,506. Screening all clones leading to healthy animals one time revealed 194 potential hits, including 35 kinases, 77 transcription factors, and 82 coregulators. These 194 initial hits were then screened again in the same conditions, resulting in 122 hits. These 122 hits were then screened one more time in the same conditions, which resulted in a final total of 80 hits, including 9 kinases, 16 transcription factors, and 55 coregulators ([Table 2](#), [Supplementary Table 1A–C](#), [Supplementary Fig. 1A](#)).

In addition to its role in the oxidative stress response, *nhr-49* is required to induce genes in hypoxia and for survival in and recovery from transient hypoxia stress and from starvation^{31,32}. We therefore tested if the genes required for *fmo-2p::gfp* induction in oxidative stress are also required for its induction in hypoxia and starvation ([Fig. 1A](#)). Screening 78 candidates from the oxidative stress screen in hypoxia conditions revealed 49 potential hits, which were then screened twice more with the same assay,

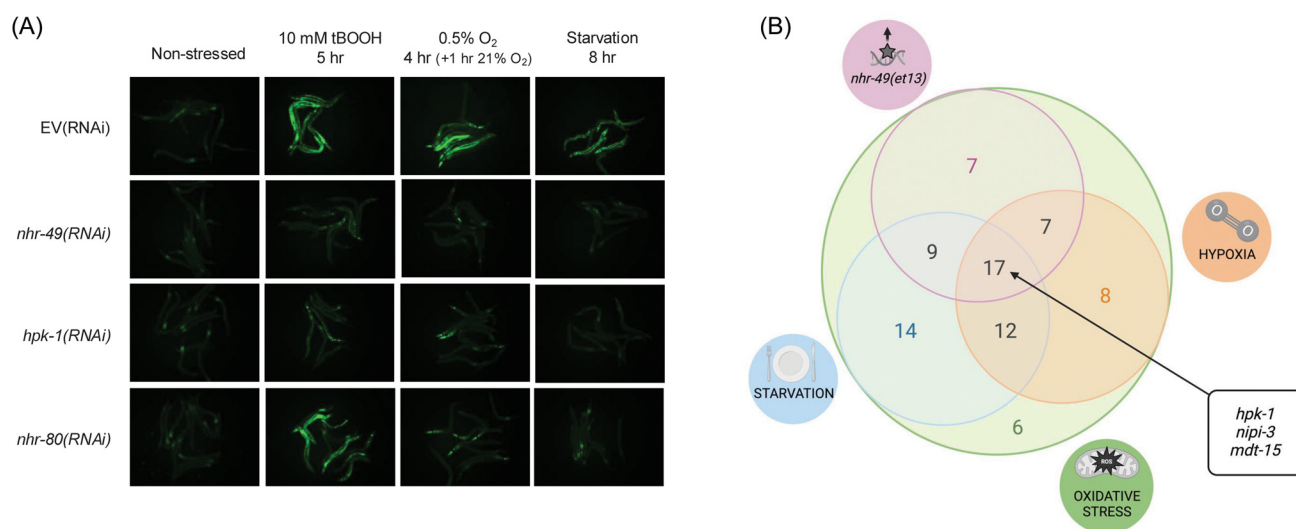


Figure 1. Reverse genetic RNAi screen reveals new genes that may function in the *nhr-49* stress response pathway. (A) Representative micrographs of *fmo-2p::gfp* adult animals fed empty vector (EV) or *nhr-49* RNAi (negative and positive controls, respectively) or RNAi of *hpk-1* (screen hit) or *nhr-80* in non-stressed conditions, after 5 hours exposure to 10 mM tBOOH, after 4 hours exposure to 0.5% O₂ followed by 1 hour recovery in 21% O₂, and after 8 hours without food. (B) The Venn diagram shows the overlap of hits from RNAi screens for genes required to induce *fmo-2p::gfp* in oxidative stress, hypoxia, starvation, or in the *nhr-49*(*et13*) background (see [Supplementary Table 1G](#) for gene lists). Three genes that were hits in all screens are highlighted: *mdt-15*, a known *nhr-49* coregulator^{32,41}, and *hpk-1* and *nipi-3*, which were further investigated in this study.

Table 2. Eighty genes are required to induce the *fmo-2p::gfp* reporter in tBOOH oxidative stress.

WormBase Gene ID	Gene Name	Human Homolog	Type	Hypoxia Hit	Starvation Hit	et13 Hit
Kinases						
C36B1.10	<i>gskl-2</i>	GSK3B	MAP kinase	X	X	X
F35C8.2	<i>sek-4</i>	MAP2K6	MAP kinase	X	X	X
W04B5.5		PDPK2	serine/threonine kinase		X	
C01C4.3		SBK1	serine/threonine kinase	X		
F20B6.8	<i>hpk-1</i>	HIPK	serine/threonine kinase	X	X	X
M79.1	<i>abl-1</i>	ABL1	tyrosine kinase		X	
K09A9.1	<i>nipi-3</i>	TRIB1	pseudokinase	X	X	X
T10D4.6			GTPase binding	X	X	X
B0024.6	<i>gcy-6</i>	NPR1	Guanylyl Cyclase	X		
Transcription factors						
C25A1.11	<i>aha-1</i>	ARNT	aryl hydrocarbon receptor associated protein	X	X	
T05A7.4	<i>hmg-11</i>	HMGA2	high mobility group AT-hook	X		X
C28A5.4	<i>ceh-43</i>	DLX1	homeobox	X		X
ZC247.3	<i>lin-11</i>	LHX1	homeobox		X	
F31E8.3	<i>tab-1</i>	BSX	homeobox	X	X	
W01D2.1		RPL37	ribosomal protein			X
T27A1.6	<i>mab-9</i>	TBX20	transcription factor	X		
C01G12.1	<i>madf-5</i>		transcription factor	X	X	X
T22E7.2	<i>dhhc-11</i>	ZDHHC11	zinc-finger		X	
F13D11.2	<i>hbl-1</i>		zinc-finger			X
C27A12.3	<i>let-391</i>	ZFP2	zinc-finger	X		
K01H12.3	<i>nhr-38</i>		zinc-finger	X	X	
K12H6.1	<i>nhr-119</i>		zinc-finger	X		
Y40B1A.4	<i>sptf-3</i>	SP3	zinc-finger	X	X	
ZC123.3	<i>zfh-2</i>	ZFHX3	zinc-finger	X		X
F52F12.6	<i>ztf-11</i>	MYT1L	zinc-finger	X		X
Coregulators						
Y48B6A.11	<i>jmjd-2</i>	KDM4	demethylation			
F42A6.5			DNA repair	X	X	X
C06E1.4	<i>glr-1</i>	GRIA2/3	glutamate receptor			
F33H2.1	<i>dog-1</i>	BRIP1	helicase		X	X
C26D10.2	<i>hel-1</i>	DDX39A	helicase			X
W08D2.7	<i>mtr-4</i>	MTREX	helicase	X	X	X
Y48G8AL.6	<i>smg-2</i>	UPF1	helicase		X	
F55F8.2		DDX24	helicase		X	
F35H10.1	<i>his-30</i>	H2AX	histone	X		X
F17E9.10	<i>his-32</i>	H3C	histone		X	X
R06F6.1	<i>cdl-1</i>	SLBP	histone binding protein	X	X	
F54A5.1	<i>hmbx-1</i>	HMBX1	homeobox			
C01H6.9	<i>hasp-1</i>	HASPIN	kinase		X	
F47A4.2	<i>dpy-22</i>	MED12L	mediator complex	X	X	X
Y54E5B.3	<i>let-49</i>	MED7	mediator complex			X
T09A5.6	<i>mdt-10</i>	MED10	mediator complex		X	X
R12B2.5	<i>mdt-15</i>	MED15	mediator complex	X	X	X
C55B7.9	<i>mdt-18</i>	MED18	mediator complex	X	X	

(Continued on next page)

Table 2. Continued.

WormBase Gene ID	Gene Name	Human Homolog	Type	Hypoxia Hit	Starvation Hit	<i>et13</i> Hit
Y17G7B.2	<i>ash-2</i>	ASH2L	methylation			
R06A4.7	<i>mes-2</i>	EZH1/2	methylation	X		
F46A9.6	<i>mec-8</i>	RBPMS	mRNA processing	X		
F37E3.1	<i>ncbp-1</i>	NCBP1	nuclear cap binding protein	X	X	X
F26A3.2	<i>ncbp-2</i>	NCBP2	nuclear cap binding protein		X	X
F20D12.2	<i>mcm-3AP</i>	MCM3AP	nuclear export	X	X	X
R12C12.2	<i>ran-5</i>	RANBP3	nuclear export		X	
K07F5.13	<i>npp-1</i>	NUP54	nucleoporin	X		X
T01G9.4	<i>npp-2</i>	NUP85	nucleoporin	X	X	
Y54E5A.4	<i>npp-4</i>		nucleoporin	X	X	X
F56A3.3	<i>npp-6</i>	NUP160	nucleoporin		X	X
T19B4.2	<i>npp-7</i>	POM121	nucleoporin	X	X	X
C03D6.4	<i>npp-14</i>	NUP214	nucleoporin	X	X	
C29E4.4	<i>npp-15</i>	NUP133	nucleoporin	X	X	
Y56A3A.17	<i>npp-16</i>	NUP50	nucleoporin		X	X
Y43F4B.4	<i>npp-18</i>	SEH1L	nucleoporin			X
R06F6.5	<i>npp-19</i>	NUP35	nucleoporin	X	X	
Y77E11A.13	<i>npp-20</i>	SEC13	nucleoporin		X	
F36A4.7	<i>ama-1</i>	POLR2A	RNA polymerase			X
C26E6.4	<i>rpb-2</i>	POLR2B	RNA polymerase	X	X	X
C41G7.4	<i>set-32</i>		SET domain containing protein		X	
C48B6.2		IMP3	snoRNA		X	
Y54E2A.3	<i>tac-1</i>		spindle-associated	X	X	
W02D3.11	<i>hrpf-1</i>		splicing		X	
C50C3.6	<i>prp-8</i>	PRPF8	splicing		X	X
W02B12.2	<i>rsp-2</i>	SRSF5/6	splicing			
C08B11.5	<i>sap-49</i>	SF3B4	splicing		X	
C50D2.5	<i>sftb-6</i>	SF3B6	splicing	X		
Y49E10.15	<i>snr-6</i>	SNRPE	splicing			X
Y71F9B.4	<i>snr-7</i>	SNRPG	splicing		X	
Y116A8C.35	<i>uaf-2</i>	U2AF1	splicing		X	X
C23H3.3	<i>ascc-1</i>	ASCC1	transcription coregulator	X	X	
K03H1.10	<i>cbp-2</i>	CBP/p300	transcription coregulator		X	X
C25H3.6	<i>mdt-26</i>	TCEANC2	transcription elongation	X	X	X
F46F11.4	<i>ubl-5</i>	UBL5	ubiquitin-like	X		X
ZK637.4				X	X	X
F01G12.5	<i>let-2</i>	COL4A	collagen			

The table lists each hit, their predicted mammalian homolog, the type of protein it represents, and results of follow-up screens in hypoxia, starvation, and in the *nhr-49(et13)* background ("X" indicates hit). Hits are organized in groups of similar predicted molecular function/type. All 80 RNAi cultures were sequenced to verify that the correct target gene was knocked down.

resulting in 44 validated hits, including 7 kinases, 12 transcription factors, and 25 coregulators (**Table 2**, **Supplementary Fig. 1A**, **Supplementary Table 1A,D**; note: two of 80 RNAi clone cultures identified in the tBOOH screen impaired animal development and were removed from the hypoxia screen analysis, resulting in 78 tested genes). Screening 80 candidates from the oxidative stress screen in starvation revealed 52 potential hits, which were then screened twice more with the same assay,

resulting in 52 validated hits, including 7 kinases, 7 transcription factors, and 38 coregulators (**Table 2**, **Supplementary Fig. 1A**, **Supplementary Table 1A,E**). In total, of the genes studied in these screens, 13 genes only regulate *fmo-2p::gfp* activity in tBOOH; 15 genes regulate *fmo-2p::gfp* activity in tBOOH and hypoxia; 23 genes regulate *fmo-2p::gfp* activity in tBOOH and starvation; and 29 genes regulate *fmo-2p::gfp* activity in all three stresses, including 5 kinases, 5 transcription factors, and 19

coregulators (Supplementary Fig. 1B, Table 2, Supplementary Table 1G). Several of these 29 genes have known roles in stress responses and/or longevity, including the kinases *hpk-1*, *nipi-3*, and *gskl-2*, and the transcription factor *aha-1*^{31,34,53–58}. As expected, the known NHR-49 binding transcriptional coregulator *mdt-15*^{32,41} was also in this list, validating our approach.

Forty genes are required for *fmo-2* induction in the *nhr-49(et-13)* background

The stress conditions studied above likely engage numerous regulators that cooperatively activate *fmo-2*. To identify genes required for NHR-49 activity in the absence of stress, we crossed the *fmo-2p::gfp* reporter into a strain bearing the *nhr-49(et13)* gain-of-function allele. The *et13* mutation affects the ligand binding domain of NHR-49, is sufficient to induce *fmo-2* in the absence of stress, and promotes hypoxia resistance^{31,59}. These properties make it a useful tool to study factors acting directly with NHR-49. Screening of the 80 genes required to induce *fmo-2p::gfp* in oxidative stress in the *nhr-49(et13)* background revealed 53 potential hits, which were then screened twice more with the same assay, resulting in 40 hits, including 5 kinases, 7 transcription factors, and 28 coregulators (Supplementary Fig. 1A, Table 2, Supplementary Table 1A,F). Comparing the list of genes required for *fmo-2p::gfp* reporter induction in the *nhr-49(et13)* background with the lists of genes required for induction in different stresses revealed that 17 genes are essential in all four

conditions, including 5 kinases, 1 transcription factor, and 11 coregulators; these genes most closely phenocopy *nhr-49*'s requirement (Fig. 1B).

HPK-1 functions within the NHR-49-controlled hypoxia and oxidative stress response pathways

Our screens revealed that the kinase *hpk-1* is required for *fmo-2p::gfp* induction in oxidative stress, hypoxia, starvation, and in the *nhr-49(et13)* background. We previously showed that *hpk-1* functions upstream of NHR-49 in the hypoxia response pathway, with *hpk-1(pk1393)* mutant animals being sensitive to hypoxia and epistasis experiments using the *nhr-49(nr2041);hpk-1(pk1393)* double mutant showing that *hpk-1* acts in the same pathway as *nhr-49*. Furthermore, an overexpressed NHR-49::GFP fusion protein is regulated post-translationally in hypoxia, and *hpk-1* is required for increased NHR-49::GFP protein levels in low oxygen³¹. To determine if *hpk-1* acts in the same pathway as *nhr-49* to promote oxidative stress survival, wild-type, *nhr-49(nr2041)*, *hpk-1(pk1393)*, and *nhr-49(nr2041);hpk-1(pk1393)* L4 animals were exposed to 6 mM tBOOH. All strains were sensitive to tBOOH compared to wild type (Fig. 2A). Epistasis analysis revealed that survival of the *nhr-49(nr2041);hpk-1(pk1393)* double mutant is not different from that of the single mutants, demonstrating that these two genes act in the same pathway to promote tBOOH resistance (Fig. 2A).

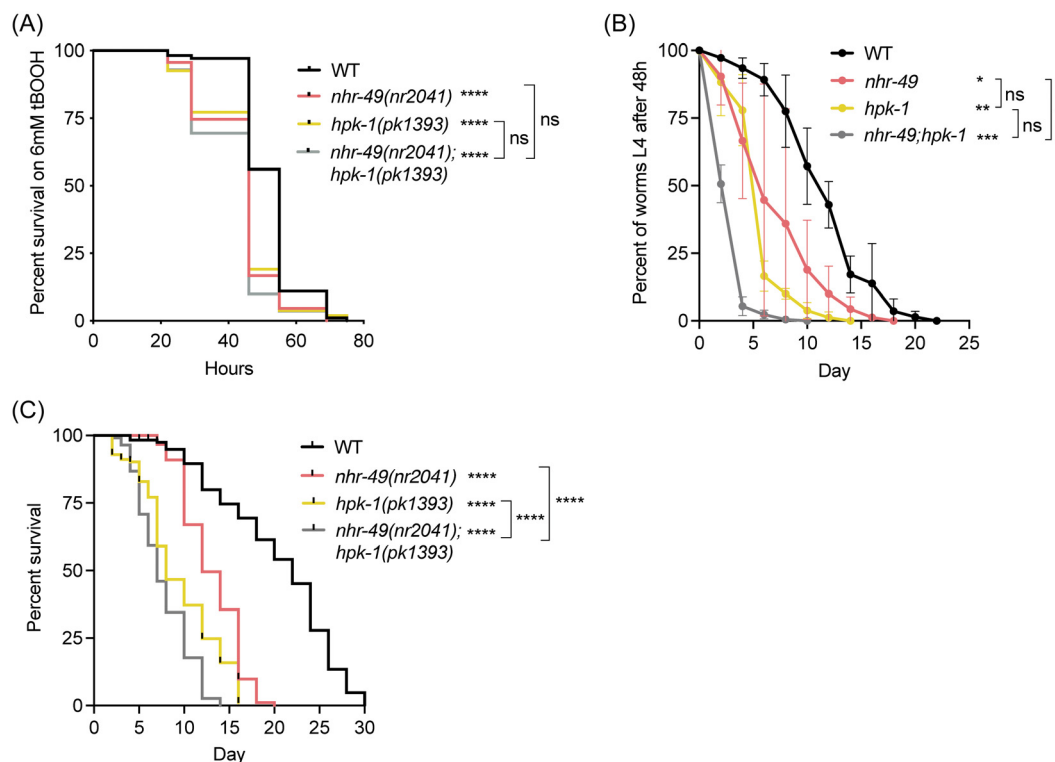


Figure 2. *hpk-1* is required for resistance to oxidative stress and starvation and for normal lifespan, acting partly in the *nhr-49* pathway. (A) Survival plot of wild-type, *nhr-49(nr2041)*, *hpk-1(pk1393)*, and *nhr-49(nr2041);hpk-1(pk1393)* L4 animals exposed to 6 mM tBOOH. p-values were derived using the log-rank (Mantel-Cox) test. ****p < 0.0001 versus wild-type animals; ns versus *nhr-49(nr2041);hpk-1(pk1393)*. Data and statistical analysis for additional repeats in Supplementary Table 2. (B) Fractional population recovery to L4 after L1 starvation (in percent, y-axis) over time (days of starvation, x-axis) of wild-type, *nhr-49(nr2041)*, *hpk-1(pk1393)*, and *nhr-49(nr2041);hpk-1(pk1393)* animals. Error bars represent SD from at least three independent experiments. p-values were derived by calculating area under the curve (AUC) for each strain using one-way ANOVA with Dunnett's multiple comparisons test. *p < 0.05, **p < 0.01, ***p < 0.001 (n = 3, >100 animals per time point, normalized to 100% viability for each strain). (C) Lifespan of wild-type, *nhr-49(nr2041)*, *hpk-1(pk1393)*, and *nhr-49(nr2041);hpk-1(pk1393)* animals. p-values were derived using the log-rank (Mantel-Cox) test. ****p < 0.0001. Data and statistical analysis for additional repeats in Supplementary Table 3. ns = not significant.

To determine if *hpk-1* acts in the same pathway as *nhr-49* to promote recovery from starvation, wild-type, *nhr-49(nr2041)*, *hpk-1(pk1393)*, and *nhr-49(nr2041);hpk-1(pk1393)* mutant L1 stage *C. elegans* were starved and tested for their ability to reach at least the L4 stage after recovering on food for 48 hours. Similar to *nhr-49*, *hpk-1* animals were sensitive to starvation compared to wild type (Fig. 2B). Interestingly, epistasis analysis revealed that *nhr-49(nr2041);hpk-1(pk1393)* double mutant animals are significantly more starvation-sensitive than each single mutant, suggesting that these two genes act in separate pathways to promote recovery from starvation (Fig. 2B).

nhr-49 and *hpk-1* are both required for normal lifespan in *C. elegans*^{27,28,53,54}, but whether they interact genetically in this context is unknown. To test if *hpk-1* acts in the same pathway as *nhr-49* to promote lifespan, we analyzed population survival of wild-type, *nhr-49(nr2041)*, *hpk-1(pk1393)*, and *nhr-49(nr2041);hpk-1(pk1393)* animals (Fig. 2C). Consistent with previous findings, the *nhr-49* and *hpk-1* single mutants are short-lived compared to wild type. Interestingly, the *nhr-49;hpk-1* double mutant displayed an additive defect, suggesting that these genes act in parallel pathways to promote normal *C. elegans* lifespan (Fig. 2C).

NHR-49 is regulated post-translationally during tBOOH-induced oxidative stress³² and in hypoxia³¹, and *hpk-1* is required for the elevated levels of an overexpressed NHR-49::GFP protein in hypoxia³¹. To determine if *hpk-1* is required for induction of this NHR-49::GFP protein during tBOOH-induced oxidative stress, we exposed *C. elegans* bearing the *nhr-49p::nhr-49::gfp* translational reporter (encoding overexpressed NHR-49::GFP)²⁷ to 10 mM tBOOH for 3 hours in the wild-type and *hpk-1(pk1393)* mutant background. *hpk-1* mutation reduced NHR-49::GFP levels in the wild-type background; in addition, tBOOH exposure significantly induced NHR-49::GFP levels in the wild-type background, but this induction was abrogated in the *hpk-1(pk1393)* background (Fig. 3A,B). Thus, *hpk-1* is required for NHR-49::GFP protein induction during tBOOH oxidative stress, as seen in hypoxia³¹.

To determine if this requirement for *hpk-1* in NHR-49 protein induction affected animal survival on tBOOH, we exposed NHR-49::GFP overexpressing animals in the wild-type and *hpk-1* mutant backgrounds to 6 mM tBOOH. As negative controls, non-GFP siblings from each strain were used. Similar to previous findings, overexpressing NHR-49::GFP slightly enhanced the survival of *C. elegans* on tBOOH compared to the non-GFP sibling controls³² (Fig. 3C). Importantly, loss of *hpk-1* abolished this extension (Fig. 3C), suggesting that *hpk-1* is required for the increased stress resistance due to NHR-49::GFP overexpression.

In addition, we tested if stress resilience caused by HPK-1 overexpression was dependent on *nhr-49*. We studied *C. elegans* bearing an *hpk-1p::hpk-1::gfp* transgene (hereafter HPK-1::GFP)⁵⁴ in wild-type and *nhr-49(nr2041)* mutant backgrounds. HPK-1::GFP overexpression increased the population survival of animals on tBOOH compared to non-GFP siblings (Fig. 3D). Critically, loss of *nhr-49* abolished this enhanced stress resistance (Fig. 3D). Taken together, our results suggest that, like in hypoxia, HPK-1 acts together with NHR-49 in tBOOH-induced oxidative stress, possibly upstream of NHR-49, to induce NHR-49 protein levels, thus activating NHR-49 to promote animal survival (Fig. 3E).

NIPI-3 acts in the NHR-49 pathway to promote hypoxia survival

Our RNAi screens revealed the pseudokinase *nipi-3* as a new gene required for *fmo-2p::gfp* induction in tBOOH-induced

oxidative stress, hypoxia, starvation, and in the *nhr-49(et13)* background (Table 2, Supplementary Table 1A–F). To determine if *nipi-3* is required to promote oxidative stress survival, wild-type, *nhr-49(nr2041)*, *nipi-3(fr4)*, and *nhr-49(nr2041);nipi-3(fr4)* L4 animals were exposed to 6 mM tBOOH. Unexpectedly, loss of *nipi-3* enhanced survival on tBOOH compared to wild-type (Fig. 4A,E). Epistasis analysis revealed that *nipi-3* loss restored the oxidative stress sensitivity of the *nhr-49* mutant to wild-type levels (Fig. 4A,E). This suggests that these two factors act in separate tBOOH survival pathways, with *nhr-49* promoting and *nipi-3* inhibiting survival.

To determine if *nipi-3* is required to promote hypoxia survival, wild-type, *nhr-49(nr2041)*, *nipi-3(fr4)*, and *nhr-49(nr2041);nipi-3(fr4)* embryos were exposed to 0.5% O₂ for 24 hours and then allowed to recover to the L4 or later stage after being placed back in normoxia (21% O₂) for 65 hours. We found that *nipi-3* is required for embryo survival and recovery in hypoxia (Fig. 4B,E). Epistasis analysis revealed that the *nhr-49(nr2041);nipi-3(fr4)* double mutant is not significantly more sensitive to hypoxia than the *nhr-49* single mutants, suggesting that these two genes act in the same hypoxia response pathway (Fig. 4B,E).

To determine if *nipi-3* acts in the same pathway as *nhr-49* to promote recovery from starvation, wild-type, *nhr-49(nr2041)*, *nipi-3(fr4)*, and *nhr-49(nr2041);nipi-3(fr4)* L1 animals were starved and tested for their ability to reach the L4 stage after recovering on food for 48 hours. Unlike *nhr-49*, *nipi-3* animals were not sensitive to starvation (Fig. 4C). Similarly, *nipi-3* loss does not affect lifespan in the wild-type background on its own (Fig. 4D). Curiously, however, *nhr-49(nr2041);nipi-3(fr4)* double mutant animals live significantly shorter than *nhr-49(nr2041)* single mutants (Fig. 4D). This suggests a potential epistatic effect of *nipi-3* in the absence of *nhr-49*.

NHR-80 acts in the NHR-49 pathway to promote oxidative stress and hypoxia survival and normal lifespan

NHR-49 likely dimerizes to exert its function, but NHR-49's dimerization partner(s) in the control of stress responses are unknown. *nhr-80* is required for the normal and extended lifespans of wild-type and *glp-1* mutant *C. elegans*, respectively^{42,60}, dimerizes with NHR-49, and both genes are required to activate fatty acid desaturase genes⁴³, which are implicated in longevity¹³. Thus, although *nhr-80* was not a hit in our tBOOH screen (Fig. 1A), its link to *nhr-49* function made it an interesting candidate for further investigation. Indeed, we found that *nhr-80* is required for *fmo-2* induction in both hypoxia and starvation (Fig. 1A). To determine if *nhr-80* is required to promote oxidative stress survival, wild-type, *nhr-49(nr2041)*, *nhr-80(tm1011)*, and *nhr-49(nr2041);nhr-80(tm1011)* L4 animals were exposed to 6 mM tBOOH. All mutant animals were sensitive to tBOOH compared to wild-type animals, and *nhr-49* mutant animals were more sensitive than *nhr-80* mutant animals (Fig. 5A). Epistasis analysis revealed that there is no additive effect when both *nhr-49* and *nhr-80* are lost compared to the *nhr-49* single mutant, suggesting that these two factors act in the same pathway in the tBOOH response (Fig. 5A,E). Thus, although *nhr-80* is not required to induce *fmo-2* in response to oxidative stress, it may regulate other important stress response genes to promote survival to tBOOH with *nhr-49*.

To determine if *nhr-80* is required to promote hypoxia survival, wild-type, *nhr-49(nr2041)*, *nhr-80(tm1011)*, and *nhr-49*

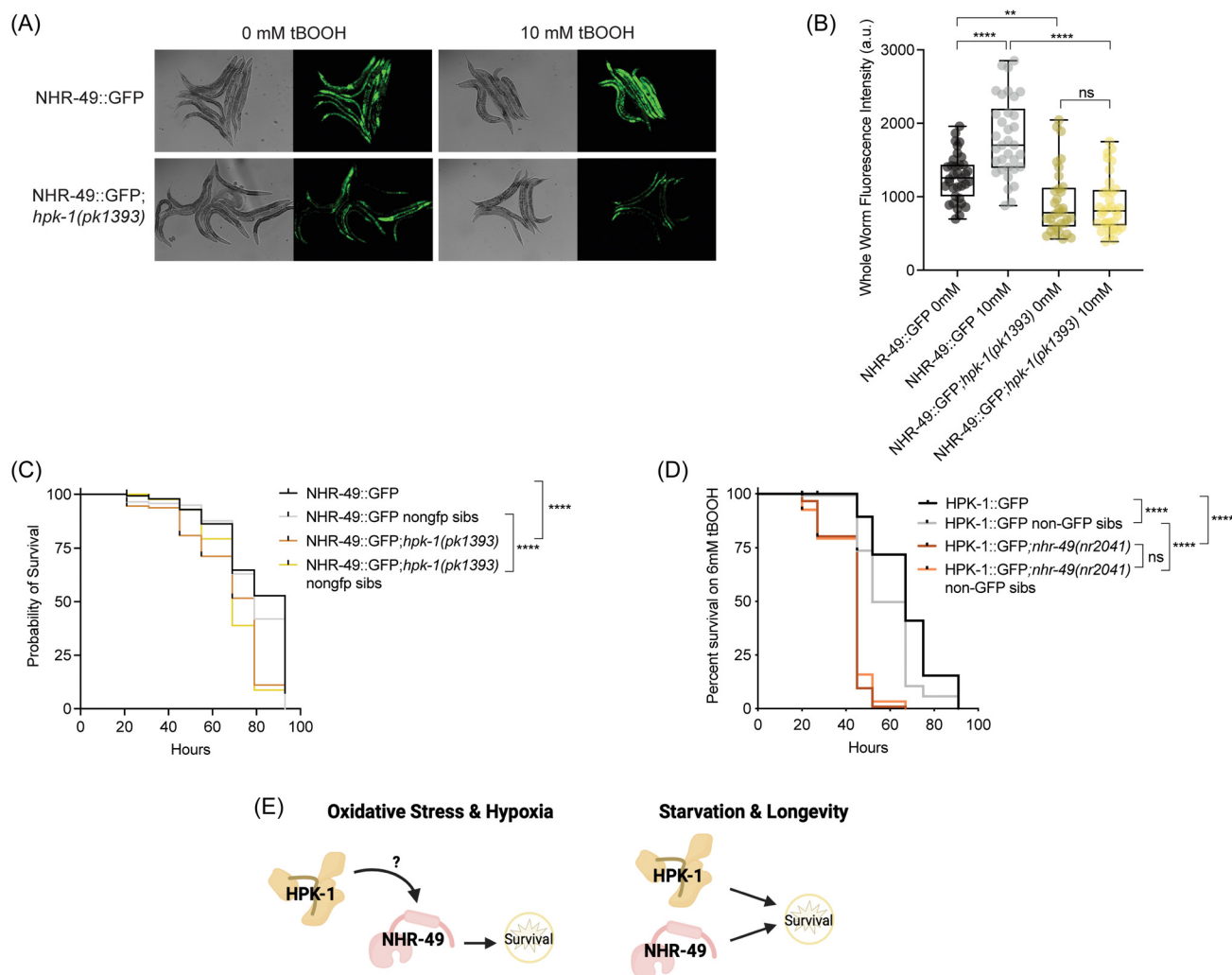


Figure 3. HPK-1 induces NHR-49::GFP in oxidative stress. (A) Representative micrographs show *nhr-49p::nhr-49::gfp* and *nhr-49p::nhr-49::gfp;hpk-1(pk1393)* adult animals following 3 hours exposure to 10 mM tBOOH. (B) The graph shows the quantification of whole animal GFP levels in *nhr-49p::nhr-49::gfp* and *nhr-49p::nhr-49::gfp;hpk-1(pk1393)* animals following 3 hours exposure 10 mM tBOOH (three repeats totaling > 30 individual animals per strain). ** $p < 0.01$, *** $p < 0.001$ (two-way ANOVA corrected for multiple comparisons using the Tukey method). (C) Survival plot of *nhr-49p::nhr-49::gfp* and *nhr-49p::nhr-49::gfp;hpk-1(pk1393)* and non-GFP sibling L4 animals exposed to 6 mM tBOOH. p-values were derived using the log-rank (Mantel-Cox) test. **** $p < 0.0001$. Data and statistical analysis for additional repeats in **Supplementary Table 4**. (D) Survival plot of *hpk-1p::hpk-1::gfp* and *hpk-1p::hpk-1::gfp;nhr-49(nr2041)* and non-GFP sibling L4 animals exposed to 6 mM tBOOH. p-values were derived using the log-rank (Mantel-Cox) test. **** $p < 0.0001$. Data and statistical analysis for additional repeats in **Supplementary Table 5**. (E) Working model of how NHR-49 and HPK-1 interact in response to oxidative stress, hypoxia, starvation, and for promotion of normal lifespan. ns = not significant.

(*nr2041*);*nhr-80(tm1011)* embryos were exposed to 0.5% O_2 for 24 hours and then allowed to recover to the L4 or later stage when placed back in normoxia for 65 hours. Similar to *nhr-49*, loss of *nhr-80* rendered animals sensitive to hypoxia (Fig. 5B). Loss of both *nhr-49* and *nhr-80* showed similar survival to each of the single mutants, suggesting that these two genes act in the same pathway to promote hypoxia survival (Fig. 5B,E).

To determine if *nhr-80* acts in the same pathway as *nhr-49* to promote recovery from starvation, wild-type, *nhr-49(nr2041)*, *nhr-80(tm1011)*, and *nhr-49(nr2041);nhr-80(tm1011)* L1 stage animals were starved and tested for ability to reach the L4 stage after recovering on food for 48 hours. Unlike *nhr-49*, *nhr-80* animals were not sensitive to starvation, recovering like wild type (Fig. 5C).

Both *nhr-49* and *nhr-80* are required for normal lifespan in *C. elegans*, but whether they interact genetically in this context is unknown^{27,28,42,60}. Thus, we carried out lifespan assays of wild-type, *nhr-49(nr2041)*, *nhr-80(tm1011)*, and *nhr-49*

(*nr2041*);*nhr-80(tm1011)* animals (Fig. 5D). Consistent with previous findings, the *nhr-49* and *nhr-80* single mutants showed a shortened lifespan compared to the wild-type control, with *nhr-49* mutant animals having a shorter lifespan than *nhr-80*. Interestingly, the *nhr-49;nhr-80* double loss-of-function mutant had a similar lifespan to the *nhr-49* single mutant animals, suggesting that these two genes act in the same genetic pathway to promote normal *C. elegans* lifespan (Fig. 5D,E).

Discussion

NHR-49 was first identified as an important regulator of lipid metabolism, through which it affects development and lifespan. More recently, additional roles of NHR-49 emerged in the control of stress responses, including in the response to tBOOH-induced oxidative stress, hypoxia, starvation, and innate immune

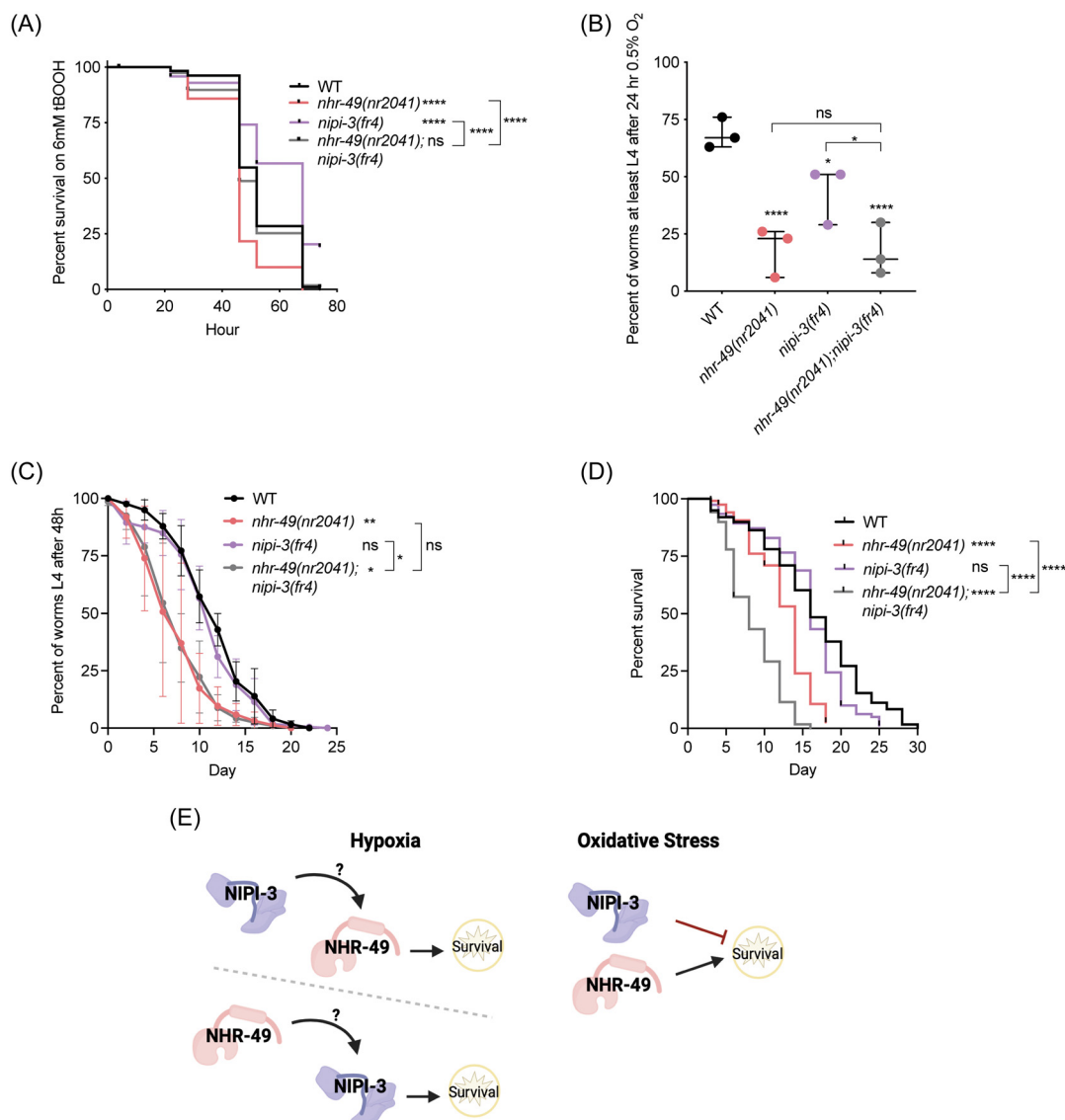


Figure 4. *C. elegans* *nipi-3* loss causes oxidative stress resistance and hypoxia sensitivity. (A) The graph shows population survival of wild-type, *nhr-49(nr2041)*, *nipi-3(fr4)*, and *nhr-49(nr2041); nipi-3(fr4)* L4 stage animals exposed to 6 mM tBOOH. p-values were derived using the log-rank (Mantel-Cox) test. ****p < 0.0001. Data and statistical analysis for additional repeats in **Supplementary Table 6**. (B) The graph shows the average population survival of wild-type, *nhr-49(nr2041)*, *nipi-3(fr4)*, and *nhr-49(nr2041); nipi-3(fr4)* embryos following 24 hours exposure to 0.5% O₂, then allowed to recover at 21% O₂ for 65 hours, and counted as the ability to reach at least L4 stage (n = 4, totaling > 100 individual animals per strain). p-values were derived using ordinary one-way ANOVA corrected for multiple comparisons using the Tukey method. *p < 0.05, ****p < 0.0001. (C) Fractional population recovery to L4 after L1 starvation (in percent, y-axis) over time (days of starvation, x-axis) of wild-type, *nhr-49(nr2041)*, *nipi-3(fr4)*, and *nhr-49(nr2041); nipi-3(fr4)* animals. Error bars represent SD from at least three independent experiments. p-values were derived by calculating AUC for each strain using one-way ANOVA with Dunnett's multiple comparisons test. *p < 0.05, **p < 0.01 (n = 4, >100 animals per time point, normalized to 100% viability for each strain). (D) Lifespans of wild-type, *nhr-49(nr2041)*, *nipi-3(fr4)*, and *nhr-49(nr2041); nipi-3(fr4)* animals. p-values were derived using the log-rank (Mantel-Cox) test. ****p < 0.0001. Data and statistical analysis for additional repeats in **Supplementary Table 7**. (E) Working model of how NHR-49 and NIPi-3 interact in response to oxidative stress and hypoxia. ns = not significant.

responses after pathogen infection²⁴. However, several aspects of NHR-49-mediated stress resilience remain poorly understood, including how it is activated in stress and how it achieves its effects on gene regulation, especially considering that it orchestrates different transcriptomic responses in different conditions. Here, we use a series of reverse genetic RNAi screens to identify 80 new genes that are required to induce the *nhr-49*-dependent reporter *fmo-2p::gfp* in at least one stress, including genes encoding for kinases, transcription factors, and coregulators. Functional follow-up studies of three such genes, the kinase *hpk-1*, the pseudokinase *nipi-3*, and the nuclear hormone receptor *nhr-80*,

revealed that they feature nonequivalent contributions to NHR-49-driven stress resistance and longevity. Collectively, our work identifies numerous new genes, some of which may act with NHR-49 to implement condition-specific gene programs, representing a substantial advance in uncovering the composition of important cytoprotective and pro-longevity pathways.

fmo-2 is variably required for stress resistance

We used the *fmo-2* promoter as a basis for our screen readout because it is highly induced by NHR-49 in various stresses^{23,30–32,61}. The FMO family is conserved and present in

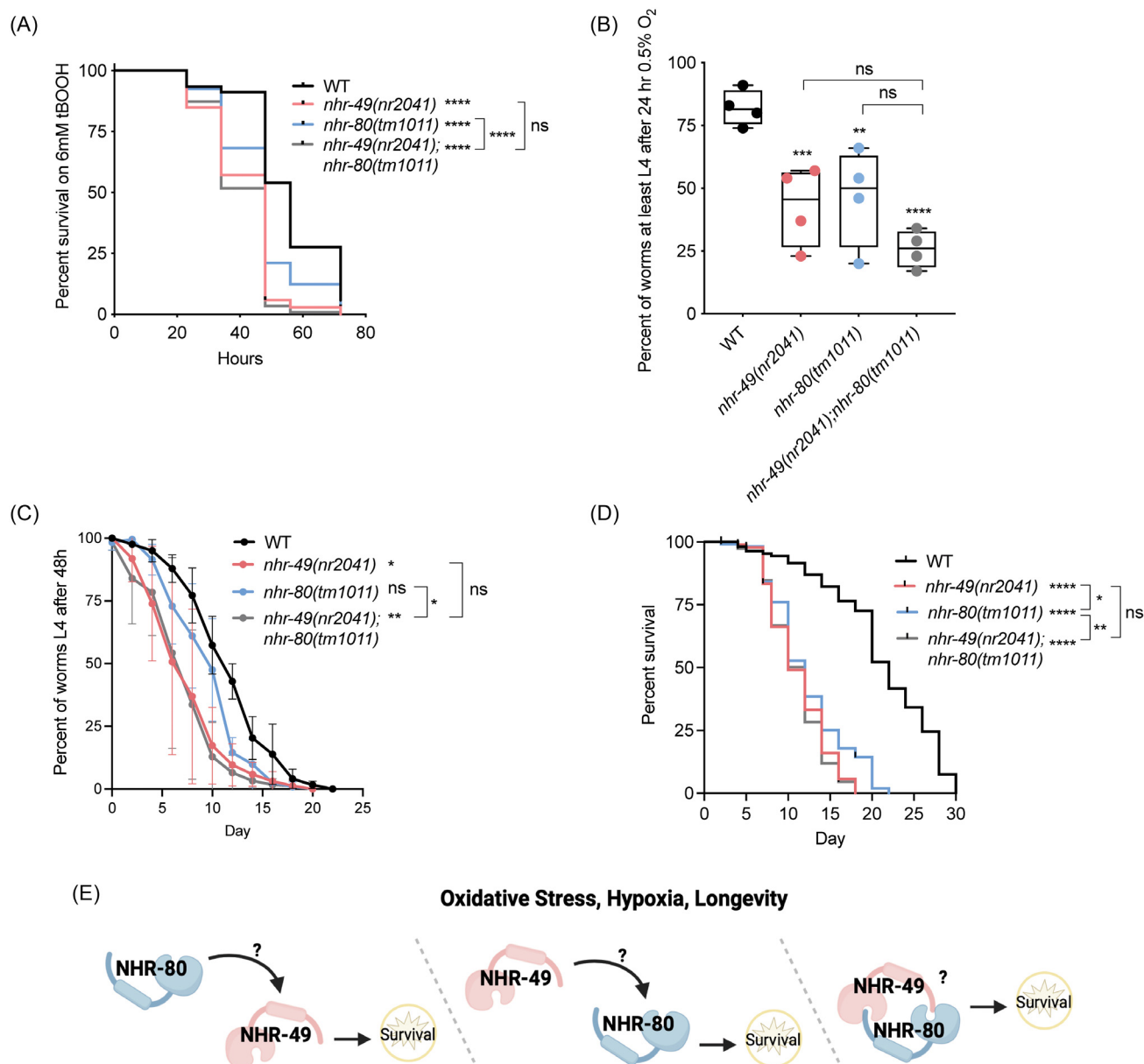


Figure 5. *nhr-80* is required for *C. elegans* oxidative stress survival, hypoxia survival, and normal lifespan. (A) Survival plot of wild-type, *nhr-49(nr2041)*, *nhr-80(tm1011)*, and *nhr-49(nr2041); nhr-80(tm1011)* L4 animals exposed to 6 mM tBOOH. p-values were derived using the log-rank (Mantel-Cox) test. ****p < 0.0001. Data and statistical analysis for additional repeats in **Supplementary Table 8**. (B) The graph shows the average population survival of wild-type, *nhr-49(nr2041)*, *nhr-80(tm1011)*, and *nhr-49(nr2041); nhr-80(tm1011)* embryos following 24 hours exposure to 0.5% O₂, then allowed to recover at 21% O₂ for 65 hours, and counted as the ability to reach at least L4 stage (n = 4, totaling > 100 individual animals per strain). p-values were derived using ordinary one-way ANOVA corrected for multiple comparisons using the Tukey method. **p < 0.01, ***p < 0.001, ****p < 0.0001. (C) Fractional population recovery to L4 after L1 starvation (in percent, y-axis) over time (days of starvation, x-axis) of wild-type, *nhr-49(nr2041)*, *nhr-80(tm1011)*, and *nhr-49(nr2041); nhr-80(tm1011)* animals. Error bars represent SD from at least three independent experiments. p-values were derived by calculating AUC for each strain using one-way ANOVA with Dunnett's multiple comparisons test. *p < 0.05, **p < 0.01 (n = 4, >100 animals per time point, normalized to 100% viability for each strain). (D) Lifespan of wild-type, *nhr-49(nr2041)*, *nhr-80(tm1011)*, and *nhr-49(nr2041); nhr-80(tm1011)* animals. p-values were derived using the log-rank (Mantel-Cox) test. *p < 0.05, **p < 0.01, ****p < 0.0001. Data and statistical analysis for additional repeats in **Supplementary Table 9**. (E) Working model of how NHR-49 and NHR-80 interact in response to oxidative stress, hypoxia, and for promotion of normal lifespan. ns = not significant.

all eukaryotes examined so far⁶². Although *S. cerevisiae* has only one *FMO* gene, *C. elegans*, mice, and humans all contain five due to evolutionary duplication events, possibly due to selection pressure caused by increased xenobiotic exposure⁶³. The five *C. elegans* *fmo* genes are paralogs and are most closely related to mammalian *FMO5*⁶⁴. Here, we exploit the strong induction of *fmo-2* by using the transgenic *fmo-2p::gfp* strain as a

transcriptional reporter in oxidative stress, hypoxia, starvation, and in the *nhr-49(et13)* background to identify potential genes acting within NHR-49-controlled stress response pathways. However, although *fmo-2* expression is strongly induced in many stresses, its functional role is not entirely clear. Interestingly, *FMOs* regulate metabolism, and overexpression of any of five mouse *Fmo* genes render cells resistant to multiple stressors

and affect cellular metabolism by increasing mitochondrial respiration and decreasing glycolytic activity⁶². In *Fmo5* knockout mice, glycolysis and fatty acid metabolism were downregulated, and mice were leaner and exhibited higher energy expenditure, suggesting roles for *Fmo5* in regulating metabolism and energy balance^{65,66}. In addition, *Fmo3* regulates cholesterol, glucose, and lipid metabolism in mice⁶⁷. Interestingly, there is a significant overlap between metabolism genes regulated by *Fmo3* and those regulated by Peroxisome proliferator-activated receptor alpha (PPAR α), a mammalian NHR-49 homolog, suggesting that *Fmo3* may regulate cellular metabolism via this pathway⁶⁷. Thus, it is possible that the *C. elegans fmo* genes act with NHR-49 to regulate both cellular detoxification and metabolism. However, although it is highly induced by stress, loss of *fmo-2* causes only weak sensitivity to starvation, doesn't cause sensitivity to hypoxia, and causes resistance to tBOOH³². Additional *C. elegans fmo* genes are induced in stress, including *fmo-1* and *fmo-3* during starvation, and *fmo-1*, *-3*, and *-4* during oxidative stress^{68–70}. Perhaps these genes act redundantly, with multiple *fmo* genes cooperating to achieve stress resistance. Functional analysis of combinatorial mutants in the *fmo* family genes may elucidate if this family of genes is important for stress resistance in *C. elegans*. In addition, we highlight that candidates from our regulatory screen will require detailed further analysis for functional roles in various stress and longevity contexts, as done herein for *hpk-1*, *nipi-3*, and *nhr-80*.

Several candidate *nhr-49* pathway genes play known roles in stress responses and/or longevity

Notably, the 80 screen hits include many genes with known roles in stress responses or the regulation of animal life span, often associated with stress response programs. *abl-1* is a tyrosine kinase required for apoptosis during oxidative stress⁷¹. Another kinase, *gskl-2*, becomes transcriptionally induced during dietary restriction by the transcription factor BLMP-1, which also regulates a HIF-1-independent hypoxia response pathway^{55,72}. Whether these genes are in the same pathway in the hypoxia (or any other) response will be interesting to test in the future. In addition, many of the 55 coregulator hits are part of the transcription-export (TRES) complex, which couples transcription to nuclear mRNA export and ensures stress response gene transcripts are delivered to the cytoplasm for translation⁷³. Interaction of NHR-49, and possibly of other cytoprotective transcription factors, with this machinery may represent an important mechanism of ensuring preferential export of stress resilience transcripts.

Homeodomain interacting protein kinase HPK-1 emerges as key contributor in NHR-49-regulated stress response pathways

Our screen identified the nuclear serine/threonine protein kinase *hpk-1* as a regulator of *fmo-2p::gfp* in tBOOH, hypoxia, starvation, and in the *nhr-49(et13)* background. Expanding on our earlier work on *nhr-49* and *hpk-1* in hypoxia³¹, we show that *hpk-1* is required in the *nhr-49* pathway for survival on tBOOH as well as for the stress-induced increase in NHR-49::GFP levels (Figs. 2 and 3). These findings agree with previous reports showing that *hpk-1* is required for *C. elegans* survival in the presence of the cytochrome oxidase inhibitor sodium azide, which causes oxidative stress⁵³. Furthermore, *hpk-1* regulates a suite of Phase I and Phase II detoxification genes in response to oxidative stress, including cytochrome P450s, short-chain dehydrogenases, and

glutathione S transferases, which *nhr-49* also regulates to promote redox homeostasis^{32,53}. Interestingly, HPK-1 does not seem to be induced in tBOOH, but expression was only examined in the neurons and hypodermis⁵⁴. It is therefore possible that HPK-1 acts in a different tissue, potentially the intestine, during this stress to exert its functions.

Notably, the function of HIPKs in redox responses is conserved in mammals, where HIPK2 is de-SUMOylated during ROS accumulation, which relieves it from proteasomal degradation. HIPK2 is then acetylated by p300/CBP, which increases cellular survival⁷⁴. Interestingly, HIPK2 is regulated at the transcriptional level by Nrf2, a master regulator of redox homeostasis⁷⁵, which induces expression of *HIPK2* when cells are exposed to hydrogen peroxide. Downstream, HIPK2 promotes Nrf2 nuclear localization (potentially directly or indirectly) to promote expression of antioxidant genes such as NAD(P)H dehydrogenase [quinone] 1 (NQO1) and heme oxygenase 1 (HO-1)⁷⁵. Future work may assess whether HIPK2 similarly regulates the NHR-49 homologs PPAR α or hepatocyte nuclear factor 4 alpha (HNF4 α) during stress in mammals.

Our data show that *nhr-49* and *hpk-1* function in the same response pathway to tBOOH and hypoxia, but whether or how they physically interact is not known. As HPK-1 phosphorylates transcription factors, such as p53 and CREB^{76,77}, it will be interesting to determine if HPK-1 binds to and/or phosphorylates NHR-49.

The pseudokinase NIPI-3 interacts with the NHR-49 pathway differently in various stress responses

nipi-3 is required for *fmo-2p::gfp* induction in tBOOH, hypoxia, starvation, and in the *nhr-49(et13)* background. *nipi-3* is a homolog of the highly conserved tribbles family of pseudokinases, which contain structural elements of canonical kinases but cannot phosphorylate substrates⁷⁸. Instead, tribbles pseudokinases likely act as “decoys” to disrupt normal kinase function or adaptors that facilitate protein degradation⁷⁹. Interestingly, *nipi-3/TRIB3* has conserved roles in many stress response pathways, including starvation, hypoxia, and following pathogen infection^{34,58,80,81}. In *C. elegans*, *nipi-3* is required for survival upon pathogen infection, including by the fungus *D. coniospora* and the bacteria *P. aeruginosa* and *E. faecalis*; the latter two responses are also controlled by NHR-49^{23,26,34,57,58}. *nipi-3* functions upstream of SKN-1 during these infections, controlling activation of the MAPK pathway^{34,58}. This function is conserved, as human TRIB3 functions upstream of MAPK in humans, too⁸². As the MAPK pathway appears to play a minor role in NHR-49-controlled gene expression³², future experiments may uncover if NIPI-3 acts upstream to regulate both NHR-49 and SKN-1 in these stress responses, or if NIPI-3 controls pathways in parallel to NHR-49.

NHR-80 may regulate a subset of NHR-49-dependent metabolism genes to selectively promote stress resistance

In vitro assays show that NHR-49 can interact with numerous other transcription factors, including many NHRs; in addition, NHR-49 also homodimerizes in these assays^{40,41,43}. *In vivo*, *nhr-49* and *nhr-80* both regulate fatty acid desaturase genes to promote longevity^{28,60}, suggesting that they interact genetically in this context. Overall, this raises the interesting hypothesis that NHR-49 engages in specific NHR dimerization partnerships to implement the regulation of specific gene sets and thus biological

processes. In support of this model, NHR-79 appears to regulate peroxisomal genes together with NHR-49^{43,44}, where as NHR-66 may cooperate with NHR-49 to repress sphingolipid metabolism genes. Our data indicate that *nhr-80* and *nhr-49* lie in one pathway, not only in the regulation of longevity but also in the hypoxia and oxidative stress response. In contrast, *nhr-80* was dispensable for starvation resistance. As such, it is possible that, although *nhr-80*, like *nhr-49*, regulates lipid metabolism genes essential for longevity, such as the fatty acid desaturases, the *nhr-49*-dependent genes promoting starvation survival (e.g., *acs-2*) are not dependent on *nhr-80*. Further fine mapping of how various *nhr* genes contribute to *nhr-49*-dependent regulation may reveal additional specificity determinants in the future.

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Author Contributions

Design of research: K.R.S.D., G.E., S.T.; Performed experiments: K.R.S.D., G.E., B.S., R.C., L.K., Z.B.; Analyzed data: K.R.S.D., G.E.; Wrote the manuscript: K.R.S.D., G.E., S.T.; Edited the manuscript: All authors.

Conflict of Interest statement

The authors declare that they have no actual or apparent conflict of interest between authorship of this study and any other activities.

Supplementary Materials

Supplemental information can be found here: [Supplementary](#).

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