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Corresponding Author:	Andrew Dillin University of California Berkeley Berkeley, California UNITED STATES
Corresponding Author's Institution:	University of California Berkeley
Corresponding Author E-Mail:	dillin@berkeley.edu
Order of Authors:	Raz Bar-Ziv Ashley E Frakes Ryo Higuchi-Sanabria Theodore Bolas Phillip A Frankino Holly K Gildea Melissa G Metcalf Andrew Dillin
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TITLE:

Measurements of Physiological Stress Responses in *C. elegans*

AUTHORS & AFFILIATIONS:

Raz Bar-Ziv*, Ashley E Frakes*, Ryo Higuchi-Sanabria*, Theodore Bolas[^], Phillip A Frankino[^], Holly K Gildea[^], Melissa G Metcalf[^], Andrew Dillin

Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA, USA

Corresponding Author:

Andrew Dillin: dillin@berkeley.edu

Other Contacts:

Raz Bar-Ziv: barziv@berkeley.edu

Ashley E Frakes: afrakes@berkeley.edu

Ryo Higuchi-Sanabria: ryo.sanabria@berkeley.edu

Theodore Bolas: bolas@berkeley.edu

Phillip A Frankino: phillip_frankino@berkeley.edu

Holly K Gildea: holly_gildea@berkeley.edu

Melissa G Metcalf: melissa.metcalf@berkeley.edu

* These authors contributed equally to the work; authors listed alphabetically.

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KEYWORDS:

Stress, *C. elegans*, unfolded protein response, endoplasmic reticulum, mitochondria, heat shock response, transcriptional reporter, oxidative stress, protein homeostasis.

SUMMARY:

Here, we characterize cellular proteotoxic stress responses in the nematode *C. elegans* by measuring the activation of fluorescent transcriptional reporters and assaying sensitivity to physiological stress.

ABSTRACT:

Organisms are often exposed to fluctuating environments and changes in intracellular homeostasis, which can have detrimental effects on their proteome and physiology. Thus, organisms have evolved targeted and specific stress responses dedicated to repair damage and maintain homeostasis. These mechanisms include the unfolded protein response of the endoplasmic reticulum (UPR^{ER}), the unfolded protein response of the mitochondria (UPR^{MT}), the heat shock response (HSR), and the oxidative stress response (OxSR). The protocols presented here describe methods to detect and characterize the activation of these pathways and their physiological consequences in the nematode, *C. elegans*. First, the use of pathway-specific fluorescent transcriptional reporters is described for rapid cellular characterization, drug screening, or large-scale genetic screening (e.g., RNAi or mutant libraries). In addition,

complementary, robust physiological assays are described, which can be used to directly assess sensitivity of animals to specific stressors, serving as functional validation of the transcriptional reporters. Together, these methods allow for rapid characterization of the cellular and physiological effects of internal and external proteotoxic perturbations.

INTRODUCTION:

The ability of an organism to respond to changes in the intra- and extracellular environment is crucial for its survival and adaptation. This is accomplished on a cellular level through numerous protective pathways that ensure the integrity of the cell. While numerous cellular components are subject to stress-associated damage, one major involvement of cellular stress responses is to repair and protect the homeostasis of the cellular proteome. However, the compartmentalization of proteins into special structures, called organelles, poses a challenge for the cell, as it cannot rely on one centralized form of protein quality control to ensure that all the proteins within the cell are properly folded and functional. Therefore, to deal with perturbations to their proteins, organelles have evolved dedicated quality control mechanisms, which can sense misfolded proteins and activate a stress response in an attempt to alleviate the stress within that compartment. For example, the cytosol relies on the heat shock response (HSR), while the endoplasmic reticulum (ER) and mitochondria rely on their compartment-specific unfolded protein responses (UPR). The OxSR serves to alleviate the toxic effects of reactive oxygen species (ROS). Each stress response is triggered in the presence of cellular challenges and environmental insults and induces a tailored transcriptional response. The hallmarks of these responses include synthesizing molecules that re-fold misfolded proteins (such as chaperones) targeted to the proper organelle, or alternatively, remove damaged proteins by protein degradation. Failure to activate these stress responses results in accumulation of damaged proteins, cellular dysfunction propagated to systemic failure of tissues, and eventually death of the organism. The function and regulation of the different stress responses are reviewed elsewhere¹.

Many insights regarding the regulation and activity of cellular stress responses have been attributed to the nematode, *Caenorhabditis elegans*, a multicellular model organism in genetic research. Nematodes not only allow studying the activation of stress responses on the cellular level, but also on the organismal level; nematodes have been used to study the effects of genetic perturbations or exposure to drugs and pollutants on their growth and survival. Their quick generation time, isogeny, transparency, genetic tractability, and ease of use during experimentation make them ideal for such studies. Additionally, the relatively quick physiological response to stress (between hours and a few days) and the evolutionary conservation of cellular pathways make nematodes a prominent tool in studying stress resistance.

There are two commonly used *E. coli* strains used as a food source to grow *C. elegans*: standard OP50, a B strain in which most experimentation has been historically performed² and HT115, a K-12 strain that is used for almost all RNAi experiments^{3,4}. It is important to note that there are significant differences between OP50 and HT115 bacterial diets. Growth on these different bacterial sources has been shown to cause major differences in metabolic profile, mitochondrial DNA copy number, and several major phenotypes, including lifespan⁵. Some of these differences are attributed to Vitamin B12 deficiency associated with growth on OP50 bacteria, which can

result in defects in mitochondrial homeostasis and increased sensitivity to pathogens and stresses. All of these phenotypes have been shown to be alleviated by growth on HT115 bacteria, which have higher levels of Vitamin B12⁶. Therefore, it is recommended that all experiments on physiological stress responses be performed on HT115 bacteria, regardless of the necessity of RNAi conditions. However, due to the ease of maintaining animals on OP50, all standard growth (i.e., maintenance and amplification of animals) can be performed on OP50, as significant differences in the experimental paradigms described here were not detected in worms maintained on OP50 as long as they were moved to HT115 post synchronization (i.e., from hatch post-bleaching with or without L1 arresting) until experimentation.

Here, the characterization of the activity of cellular stress responses using two functional methods is described. It should be noted that the protocols presented are primarily focused on cellular stress responses and their impact on protein homeostasis. First, fluorescent transcriptional reporters are utilized, which are regulated by endogenous gene promoters that are specifically activated in response to different cellular stresses. These fluorescent transcriptional reporters are based on the transcriptional induction of specific genes that are natively part of the stress response. For example, HSP-4, a heat shock protein orthologous to the human chaperone HSPA5/BiP, is activated upon ER-stress and localizes to the ER to alleviate the stress. In conditions of ER stress (e.g., exposure to tunicamycin), a green fluorescent protein (GFP), placed under the regulation of the *hsp-4* promoter, is synthesized in high levels as can be assessed by fluorescent microscopy or quantitatively measured using large-particle flow cytometry of nematodes⁷. Similarly, the promoter of a mitochondrial chaperone, *hsp-6* (orthologous to mammalian HSPA9), is utilized to monitor the activation of the UPR^{MT8}, and the promoter of the cytosolic chaperone *hsp-16.2* (orthologous to the human crystallin alpha genes) is used for assessing the activity of the HSR⁹. These reporters allow a rapid characterization of the pathways activated in response to various perturbations.

Often, the reporters presented here are imaged using microscopy, which provides a qualitative output of the activation of stress responses. However, while imaging techniques provide both information on intensity and tissue location of the reporters described above, its quantification is not always accurate or robust. While it is possible to quantify fluorescent activation using imaging analysis tools, these methods are relatively low throughput and sample size is small, due to the relatively low number of animals imaged. The ease and ability to obtain large quantities of animals quickly make *C. elegans* an ideal model system to assay the activation of fluorescent stress reporters through the use of a large particle flow cytometer. A large-particle flow cytometer is capable of recording, analyzing, and sorting based on size and fluorescence from many live animals. Using this method, it is possible to get the fluorescent intensity, size, and also spatial (2D) information for thousands of worms. The system is controlled using FlowPilot, which allows for real-time data acquisition and analysis of the measured parameters. Here, methods for both microscopic imaging and quantitative analysis using a large-particle flow cytometer are offered as methods to measure the activation of stress responses.

Beyond reporter analysis, the sensitivity or resistance of animals to stress can be measured using physiological stress assays. This is achieved by exposing animals to stressful environments that

activate specific cellular stress pathways. Here, several methods are provided to measure sensitivity of whole animals to specific types of stressors.

ER stress is applied to *C. elegans* by using the chemical agent, tunicamycin, which blocks N-linked glycosylation, causing accumulation of misfolded proteins in the ER¹⁰. In *C. elegans*, growth upon exposure to tunicamycin results in major perturbations in ER function, and a significantly decreased lifespan¹¹. By measuring the survival of animals on tunicamycin-containing plates, ER stress sensitivity of animals can be quantified. For example, animals with ectopic UPR^{ER} induction and thus increased resistance to protein misfolding stress in the ER have an increased survival upon tunicamycin exposure compared to wild-type animals¹².

Oxidative and mitochondrial stress is applied to *C. elegans* by exposing animals to the chemical agent, paraquat. Paraquat is a commonly used herbicide, which causes superoxide formation specifically in the mitochondria¹³. Due to the specific localization of mitochondria-derived reactive oxygen species (ROS), paraquat assays are often used as a “mitochondrial” stress assay. However, superoxide is rapidly converted into hydrogen peroxide by mitochondrial superoxide dismutases (SODs)¹⁴. Hydrogen peroxide can subsequently diffuse out of the mitochondria and cause oxidative stress in other compartments of the cell. Therefore, we describe paraquat survival assays as measuring sensitivity to both mitochondrial and oxidative stress (other oxidative stress assays can be found¹⁵).

Thermotolerance assays are performed in *C. elegans* by placing animals in elevated temperatures. Ambient temperatures for nematodes are ~15–20 °C and thermal stress is induced at temperatures above 25 °C^{16,17}. Thermotolerance assays are generally performed at temperatures ranging from 30–37 °C, as animals exhibit major cellular defects at this temperature, and survival assays are completed within 24 hours^{16,18}. Here, two alternative methods are provided for performing thermotolerance assays: growth at 34 °C and growth at 37 °C. Together, the protocols presented here can be utilized to perform large-scale screens when combined with standard gene knock-down using RNA interference or chemical drug libraries.

The protocol can be broken into 4 broad procedures- growth of *C. elegans* and preparation for imaging (sections 1 and 2), imaging of transcriptional reporters using fluorescent microscopy (sections 3–5), quantitative measurements of reporters using a large-particle flow cytometer (section 6), and physiological assays to measure stress sensitivity in *C. elegans* (section 7).

PROTOCOL:

1. Standard growth conditions of temperatures & OP50 vs HT115

1.1. Standard growth and expansion

1.1.1. Grow a culture of OP50 in LB (**Table 1**) or equivalent media of choice for 24–48 h at ambient temperature (~22–25 °C). Grow bacteria at room temperature as OP50 is an uracil auxotroph and

there is a higher incidence of revertants (e.g., suppressor mutants) when grown at 37 °C. Long-term storage of OP50 cultures is not recommended (max 1 week at 4 °C).

1.1.2. Seed a volume of ~100–200 µL of saturated OP50 culture onto a 60 mm NGM plate (**Table 1**) for maintenance of worms and 1 mL of saturated OP50 culture onto a 100 mm plate for expanding animals for experimentation.

1.1.3. Let plates dry overnight on a benchtop.

NOTE: 100 mm plates may need more time to dry, especially if using non-vented plates. Store all *C. elegans* plates in air-tight containers stored at 4 °C. Do not use plates past 6 months old as desiccation of plates will occur, which will change animal physiology on plates due to different osmotic pressure and stiffness of plates.

1.1.4. For standard maintenance, move 10–15 young animals (eggs, L1, or L2 stages) onto a 60 mm plate, although more can be moved if dealing with mutants or transgenic animals with lower fecundity. For animals with developmental or fecundity defects, chunk a more variable pool of animals to prevent loss of the stock. If kept at 15 °C, move animals every week; for 20 °C, move animals every 3–4 days.

1.1.5. Perform a fresh thaw of animals every 25–30 passages (~ 6 months if animals are moved once a week and kept at 15 °C).

1.1.6. For expansion, chunk a full 60 mm plate onto 100 mm plates for expansion. As a frame of reference, animals with wild-type fecundity can have a full 60 mm plate cut into 4ths-6ths and be chunked onto a large plate at 20 °C for 2 days or 15 °C for 3 days to create a full 100 mm plate without reaching starvation.

2. Staging/synchronization of worms using bleaching

2.1. Bleaching protocol to synchronize worms

2.1.1. Wash gravid worms (adults full of eggs) off agar plates using M9 (**Table 1**). Start from a non-synchronized population of worms (i.e., chunked worms from step 1.1.6) to bleach for experiments, as significant genetic drift may occur upon successive rounds of synchronization via bleaching.

2.1.2. Move worm/M9 mixture into a 15 mL conical tube using a glass pipette; several plates of worms can be collected into a single 15 mL conical tube.

2.1.3. Spin animals down for 30 s at 1,000 x *g*. Aspirate M9 supernatant. It is unnecessary to wash off residual bacteria unless there is an egregious amount of contamination or large clumps of bacteria. If this is the case, perform several washes with M9.

2.1.4. Prepare a fresh stock of bleaching solution (**Table 1**). Bleaching solution can be kept for several days at 4 °C, but use freshly made bleaching solution as inefficient bleaching can result in uneven bleaching, which will cause damage to eggs before eliminating all worm carcasses.

2.1.5. Add 2–10 mL of bleaching solution to the animals (~1 mL of bleach solution for every ~0.1 mL of animal pellet). Invert the bleach and worm mixture for ~5 min (do not exceed 10 min; note that bleaching times may vary and should be titrated specifically for each lab). Vigorously shake to help dissolve worm carcasses faster and for optimal preservation of eggs. Periodically look under a dissection microscope or put the conical tube to a light to observe when the adult worm carcasses have fully dissolved and only eggs remain in the tube.

2.1.6. Pellet the eggs by spinning at 1,000 x *g* for 30 s and then aspirate the supernatant. Eggs can be centrifuged faster than worms without disrupting their integrity, so if unsure of centrifuge speed, eggs can be spun down at up to 2,500 x *g* without affecting their physiology.

2.1.7. Add M9 up to 15 mL and invert the tube to clear bleach off the eggs.

2.1.8. Repeat steps 2.1.6–2.1.7 (wash/pellet process) 2–3 more times to remove bleach.

2.1.9. Pipet egg/M9 mix onto plates and grow at 15–20 °C for experimentation. To get a measure of how many worms to use, perform approximate egg counts by pipetting 5 µL of egg mixture onto an agar plate or slide and dividing the egg count by 5 to determine the number of eggs per 1 µL of volume. Averaging of 3 independent counts may improve accuracy. To avoid starvation, a table of recommended egg counts per plate is available in **Table 2**.

2.1.10. If needed, L1 arrest animals for tighter temporal synchronization by placing egg/M9 mix in a rotator at 20 °C for up to 24 h. For wild-type animals, no defects in animal physiology were detected when L1 arresting for up to 48 h. However, mutants that are sensitive to starvation (e.g., lysosome or autophagy mutants) do very poorly with L1 arresting, and thus it is not recommended to perform this synchronization method for mutants that are known to be sensitive to starvation. If tighter synchronization is required for animals that cannot be L1 arrested, use the egg-lay method described in section 2.2.

2.2. Egg-lay protocol for synchronizing worms

NOTE: As an alternative method to bleaching, an egg-lay assay can be performed. Egg-lay is used when bleaching of animals does not provide a close enough synchronization, as eggs within the egg sac of adult animals can be as different as 8–12 h apart. For experimental paradigms where it is critical for animals to be as closely staged as possible, but where L1 arresting is not possible (e.g., in starvation mutants), egg-lay assays are recommended. However, it should be noted that due to the labor involved in the egg-lay protocol, it is less feasible to perform high-scale experiments.

2.2.1. Place 4–12 gravid adults (see note after step 2.2.2) onto a standard OP50 or HT115-seeded NGM plate (see section 1 above for recommendations on bacterial strains). Depending on the scale of experiments, multiple plates can be used. Be sure to carefully document how many animals are on each plate for step 2.2.

2.2.2. Place animals at desired temperature for experiments (15–20 °C) for 4–8 h.

NOTE: The number of hours animals are left on the plate will determine how closely synchronized the first egg laid and the last egg laid will be, so the timing can be adjusted as needed. Since shorter incubation times will mean each animal has less time to lay eggs, more animals should be placed onto plates to ensure enough eggs are laid. While the actual egg-laying rate is not fully normalized due to the tendency of animals to go through short bursts of egg-laying rather than a normalized rate of egg-lay, the average rate of eggs laid per animal can be estimated at ~5 eggs/hour for animals exhibiting wild-type fecundity¹⁹. When trying to grow animals to day 1 adult stage, time the egg-lay to have <100 eggs per plate to avoid starvation (refer to **Table 2** for more details on recommended animals per plate).

2.2.3. Remove adult animals from plates. Ensure that all adult animals are removed from plates, as animals will continue to lay eggs and result in an unsynchronized population and/or starvation of the plate.

3. Growth conditions of worms for imaging of transcriptional reporters

3.1. Growth of worms

3.1.1. Inoculate bacterial culture of HT115 harboring pL4440 RNAi plasmid (EV) and/or carrying an RNAi cassette against desired target gene(s) into LB media supplemented with 100 µg/mL carbenicillin and 20 µg/mL tetracycline.

3.1.2. Grow culture overnight (~16 hours) to saturation in a shaking 37 °C incubator.

3.1.3. Spot 60 mm NGM RNAi plates with 200 µL of saturated bacterial culture and 100 mm NGM RNAi plates with 1,000 µL of saturated bacterial culture. Let dry at ambient temperature (~22 °C) overnight in the dark (covered loosely with aluminum foil).

3.1.4. Place synchronized population of transgenic worms carrying fluorescent reporters (see **Table 3** for complete list) onto NGM RNAi plates seeded with bacteria of choice.

3.1.5. Grow at 15–20 °C to required stages for specific reporters as outlined below.

3.2. Considerations for staging of worms for experimentations

3.2.1. Perform experiments for transcriptional reporters at day 1 of adulthood, with the exception of assays that require L4 animals. According to WormAtlas, L4 animals are obtained approximately 2.5 days (~56 h) of growth at 20 °C from the egg stage (www.wormatlas.org).

3.2.2. For “day 1 adults,” use animals at approximately 3–4 days (~65–96 h) of growth at 20 °C after plating eggs.

NOTE: This wide range is explained as follows: ~65 h at 20 °C is when animals reach “egg-laying,” which is the true “adulthood” state. 96 h is when animals enter what WormAtlas describes as “egg-laying maximal,” which is when the adult is a gravid adult and has a full egg sac. This is when animals would be described as being older than day 1 and may start to display differences, and thus the protocols described here are all recommended to start in this “day 1” stage starting as early as 65 h and as late as 96 h. For the assays described here, major differences were not observed when using animals in this ~65–96 h window.

3.2.3. For replicates of a single experiment, use a similar time point for more robust reproducibility (e.g., perform all experiments at ~65 h or ~96 h after plating eggs).

NOTE: Some transgenic animals and mutants display slowed growth rates. For this, two recommendations exist. 1) Use staggered synchronization, where animals can be bleached at different times in order for experimentation to be performed at the same time. This is recommended when technical variability in the assay may be larger than the technical variabilities that may arise from the synchronization assay (e.g., survival assays, which run for long durations may suffer if they are not performed simultaneously). 2) Use staggered analysis, where animals can be bleached at the same time, but the assay itself is performed at different times. This is recommended for very simple assays that do not have inherent variability (e.g., RNAi-induction of stress responses).

4. Induction of stress responses

4.1. Using *hsp-4p::GFP* as a readout for the activation of the UPR^{ER}

4.1.1. Inducing ER stress using RNAi

4.1.1.1. Prepare plates spotted with RNAi bacteria targeting the gene of interest onto NGM RNAi plates (**Table 1**) as in section 3. Use EV as a control for basal UPR^{ER} levels and RNAi knockdown of *tag-335* (enzyme for N-linked glycosylation of ER resident proteins; RNAi knockdown has similar effects to tunicamycin treatment) as a positive control for activation of the UPR^{ER} under ER stress.

4.1.1.2. Synchronize *hsp-4p::GFP* reporter animals using methods described in section 2.

4.1.1.3. Plate eggs onto RNAi plates using criteria recommended in **Table 2**.

4.1.1.4. Incubate eggs at 20 °C for approximately 3–4 days (~65–96 h) to perform experiments at day 1 of adulthood. For the UPR^{ER} experiments, there are differences in basal fluorescence of the *hsp-4::GFP* reporter when grown at different temperatures. Therefore, perform all experiments at 20 °C.

4.1.2. Inducing ER stress using a chemical agent

4.1.2.1. Synchronize *hsp-4p::GFP* reporter animals using methods described in section 2 and grow at 20 °C until animals reach the L4 stage. Transfer animals to a tube of M9. Allow worms to settle (~2–3 min by gravity or a 30 s spin at 1,000 x g), and then remove M9.

4.1.2.2. Dilute tunicamycin to 25 ng/mL in M9 (a 1:40 dilution from 1 mg/mL stock). As a control, also dilute an equivalent volume of DMSO in M9.

4.1.2.3. Add 25 ng/mL tunicamycin/M9 or control DMSO/M9 solution to worms (use 400–500 mL for a 1.5 mL tube or 2 mL for a 15 mL conical tube). Incubate at 20 °C on a rotating platform for 3–4 hours.

NOTE: Tunicamycin can also be diluted directly into worm/M9 mix.

4.1.2.4. Allow worms to settle, remove M9/TM solution, and wash with 1 mL of M9.

4.1.2.5. Transfer animals to NGM plates or NGM RNAi plates and allow to recover overnight (or ~15–20 h) and reach Day 1 of adulthood at 20 °C prior to performing fluorescent microscopy (section 5). An overnight recovery is performed to allow for a detectable level of GFP to accumulate.

4.1.2.6. Alternatively, induce *hsp-4p::GFP* by moving L4 animals to agar plates containing 25 ng/μL tunicamycin for 16–24 h. This has a much more robust induction of *hsp-4p::GFP* due to the longer duration of stress, and thus the dynamic range is much lower than the assay described above.

4.2. Using *hsp-6p::GFP* as a readout for the activation of the UPR^{MT}

4.2.1. Inducing mitochondrial stress using RNAi

4.2.1.1. Activate UPR^{MT} following the same protocol as section 4.1.1, except using RNAi knockdown of mitochondrial genes, such as *cox-5b/cco-1* (cytochrome c oxidase subunit 5B; knockdown inhibits electron transport chain activity). For UPR^{MT} activation through perturbations in electron transport chain function, RNAi knockdown needs to be performed during early development²⁰. Therefore, perform RNAi from hatch for these experiments.

4.2.2. Inducing mitochondrial stress using a chemical agent

4.2.2.1. Prepare plates spotted with RNAi bacteria targeting the gene of interest as in section 3. Use HT115 bacteria even in experiments not involving RNAi knockdown (see section 1). Ensure that both NGM RNAi plates (or NGM RNAi + DMSO0.2) and NGM RNAi + antimycin A plates (**Table 1**) are both prepared for step 4.2.2.5.

4.2.2.2. Synchronize *hsp-6p::GFP* or *hsp-60p::GFP* reporter animals using methods described in section 2.

4.2.2.3. Plate eggs onto seeded plates of choice using criteria recommended in **Table 2**. Since antimycin A is dissolved in DMSO, grow the animals on NGM RNAi + DMSO0.2 plates from hatch.

4.2.2.4. Incubate eggs at 20 °C for 2 days (~56 h) to the L4 stage. Alternatively, grow animals at 15 °C for 3 days (~75 h) instead.

4.2.2.5. Move worms from NGM RNAi + DMSO0.2 plates to NGM RNAi + antimycin A plates or NGM RNAi + DMSO0.2 plates as a control. Worms can be moved manually with a pick for small-scale experiments, but for large-scale experiments, we recommend washing animals with M9, settling with centrifugation, aspirating M9, and then plating to NGM RNAi + antimycin A plates.

4.2.2.6. Incubate worms for an additional ~20 h and image at day 1 adult (section 5).

4.3. Using *gst-4p::GFP* as a readout for the oxidative stress response.

4.3.1. Inducing OxSR using RNAi

4.3.1.1. Alternatively, induce *gst-4p::GFP* using RNAi-knockdown of *wdr-23* (it encodes a negative regulator of *skn-1*; thus, its knockdown induces OxSR) using the protocol described in section 4.1.1.

4.3.2. Inducing the OxSR using exposure to the chemical oxidant Tert-butyl hydroperoxide (TBHP)

4.3.2.1. Prepare plates spotted with RNAi bacteria targeting the gene of interest as in section 3. Use HT115 bacteria even in experiments not involving RNAi knockdown (see section 1).

4.3.2.2. Synchronize *gst-4p::GFP* reporter animals using methods described in section 2.

4.3.2.3. Plate eggs onto seeded plates of choice using criteria recommended in **Table 2**. Be sure to prepare more plates than necessary as the drug treatment protocol results in loss of >10-20% of animals. For imaging, start with >100 animals, and for sorting, start with >1,000 animals.

4.3.2.4. Incubate eggs in 20 °C for 2 days (~56 h) to the L4 stage. Alternatively, grow animals at 15 °C for 3 days (~75 h) instead.

4.3.2.5. Wash L4 animals off the plates and split into two 15 mL conical tubes per condition. Aspirate volume down to at least 1 mL and add an equal volume of freshly made 2 mM TBHP. Use a total liquid volume to at least 2 mL, as lower volumes can cause significant death of worms when spinning. Do not wash prior to drug treatment, as residual bacteria from plates will help to ensure worms to not overstarve during incubation.

4.3.2.6. Incubate worms on the rotator for 4 h at 20 °C.

4.3.2.7. Wash worms by spinning at 1,000 x *g*, aspirating M9 + TBHP mix, and replacing with 15 mL of M9. Repeat for a second wash.

4.3.2.8. Plate worms on EV RNAi to recover overnight (~16–24 h) at 20 °C. Worms can be recovered on matching RNAi of choice, but no significant differences were seen when recovered on EV RNAi, so this can be done for ease of experimental set-up. Take images of day 1 adults after recovery.

NOTE: An overnight recovery is performed to allow for a detectable level of GFP to accumulate. Without this recovery, there is no detectable GFP signal. If a shorter-term recovery is desired, it is possible to use the assay described in section 4.3.3.

4.3.3. Inducing the OxSR using exposure to the chemical oxidant paraquat (PQ)

4.3.3.1. Repeat all steps in section 4.2.2 to get 2 batches of L4 *gst-4p::GFP* animals in 15 mL conical tubes per condition.

4.3.3.2. Aspirate volume down to at least 1 mL and add an equal volume of freshly made 100 µM PQ. Similar to 4.3.2.5, a minimum volume of 2 mL is recommended.

4.3.3.3. Incubate worms on the rotator for 2 h at 20 °C.

4.3.3.4. Wash worms by spinning at 1,000 x *g*, aspirating M9 + PQ mix, and replacing with 15 mL of M9. Repeat for a second wash.

4.3.3.5. Plate worms on EV RNAi to recover for 2 h at 20 °C, and then take images immediately after recovery.

NOTE: 2 h of recovery was the minimal recovery required to visualize the GFP induction.

4.4. Using *hsp-16.2p::GFP* and *hsp-70p::GFP* as a readout for HSR activation.

4.4.1. Inducing HSR using exposure to elevated temperatures

4.4.1.1. Prepare plates spotted with RNAi bacteria targeting the gene of interest as in section 3. Use HT115 bacteria, even in experiments not involving RNAi knockdown (see introduction).

4.4.1.2. Synchronize *hsp-16.2p::GFP* or *hsp-70p::GFP* reporter animals using methods described in section 2.

4.4.1.3. Plate eggs onto seeded plates of choice using criteria recommended in **Table 2**. Be sure to prepare 2x the number of plates necessary as half of the sample will be exposed to elevated temperatures for heat-shock induction, and the other half will serve as a non-heat-shocked control.

4.4.1.4. Incubate eggs at 20 °C for approximately 3–4 days (~65–96 h) to perform experiments at day 1 of adulthood. Do not grow worms at 15 °C for heat-shock experiments as there are only minor differences between animals experiencing heat-shock out of 15 °C versus 20 °C.

4.4.1.5. Move experimental groups of animals to a 34 °C incubator for 2 h. Place plates in the incubator as a single layer (i.e., no stacking of plates) to ensure the fastest and most equal distribution of heat across the plates.

4.4.1.6. Move heat-shocked animals to a 20 °C incubator to recover for 2 h and then image immediately (see section 5). Animals can be recovered longer if necessary for higher GFP induction.

NOTE: 2 h of recovery was the minimal recovery required to visualize the GFP induction.

5. Imaging using a stereo microscope or low-magnification wide-field/compound microscope

5.1. Preparation of worms for fluorescent microscopy

5.1.1. Pipette 5–10 µL of 100 mM sodium azide on top of a standard NGM plate (i.e., no bacteria).

NOTE: Sodium azide concentration can be brought down as low as 10 mM, although the most robust immobilization was observed at 100 mM sodium azide with no detectable effect on fluorescent signal.

5.1.2. Under a dissecting microscope, pick 10–20 animals from experimental plates, and transfer into the spot of sodium azide. Animals should cease movement shortly after landing in sodium azide, and sodium azide itself will evaporate within seconds.

5.1.3. Once sodium azide has evaporated, line up animals to desired imaging setup. Move the animals side by side with anterior and posterior sides in the same orientation for all animals. Image animals immediately.

NOTE: No changes in reporter signal were observed for any of the transcriptional reporters in **Table 3** for up to 15 min after paralyzing in sodium azide.

5.2. Image acquisition using a stereomicroscope

NOTE: For this protocol, a Leica M205FA microscope equipped with a Leica DFC3000G monochromatic CCD camera, standard Leica GFP filter (ex 395-455, EM 480 LP), and LAS X software was used. Recommended settings for exposure times can be found in **Table 4**.

5.2.1. Launch LAS X program.

5.2.2. Start a new project: Open the acquisition tab, click on **Open Projects** and click **Folder** to open a new project. This project can be renamed by right-clicking the folder and scrolling down to **Rename** or clicking **F2**. In the acquisition tab, there are also options to adjust exposure time and zoom to desired settings.

5.2.3. Position the worm sample under the microscope objective and locate the correct focal point of worms using the bright-field setting to minimize fluorescent bleaching. The center of the sample is where the line of eggs is clearly visible and not fuzzy. Set the exposure time, zoom, focus, and bright-field condensers to desired settings.

5.2.4. Acquire an image using the **Capture Image** button.

5.2.5. Save the image in .lif (Leica Image File) format as this saves all raw images and metadata. A TIFF can also be exported by right clicking the image (or project) and under the **Save as** option, click **TIFF**. This will store all channels (e.g., bright-field and GFP) with any modifications (e.g., if contrast was adjusted, this will be saved into the TIFF).

5.3. Quantitative analysis of fluorescent images

5.3.1. If quantitative analysis will be performed, take 3-D images. This is performed by clicking the z-section option labelled with “z” at the top right. Z-sections will be active if this box is red.

5.3.2. Optimize z-sections by selecting the range and slice thickness in the bottom left of adjustable options. Whenever possible, use the **System Optimized** button for optimal settings.

5.3.3. Capture the image and store the image as described above in section 5.2. Line up worms with spaces between them for easier measurements.

5.3.4. Import TIFF images into the imaging software of choice (e.g., ImageJ).

5.3.5. For ImageJ, from the **Analyze** menu, choose **Set Measurements**. Check the following: area, mean gray value, integrated density, display label.

5.3.6. Using the ROI (region of interest) tool, draw a ROI. Measure each worm individually. From the **Analyze** menu, choose **Measure** or press **M**. Draw a ROI in the background where there are

no worms, and then measure the background in the same manner. Copy or save measurements that appear in the **Results** window.

5.3.7. Subtract the background integrated density from the integrated density of each measured ROI. The background intensity for a ROI is defined as the product of the background mean gray value and the area of the ROI drawn.

5.4. Image acquisition using a compound/wide-field microscope

NOTE: For imaging of transcriptional reporters using a compound/wide-field microscope, this protocol uses a Revolve ECHO R4 microscope equipped with an Olympus 4x Plan Fluorite NA 0.13 objective lens, a standard Olympus FITC filter (ex 470/40; em 525/50; DM 560), and an iPad Pro for the camera and to drive the ECHO software. Recommended settings for exposure times can be found in **Table 4**.

5.4.1. Use the touchpad to launch the control program. Create a new album and file name.

5.4.2. Position plate under the objective lens. Set the exposure time and fluorescence intensity by using the baseline (EV/control treatment) and positive control, so that the signal is visible but not saturated.

5.4.3. Save a bright-field image and GFP/FITC image.

6. Quantitative measurements of reporters using a large-particle flow cytometer

NOTE: Growth and preparation of worms for large-particle flow cytometer analysis can follow the same paradigms as sections 1–5 for preparation of worms for fluorescent imaging, with the exception that a larger number of animals are required. Use >500 animals per condition, as some animals are lost during manipulation, not all animals pass the filtering criteria during quantification, and some animals are not properly read by the flow cytometer. Wash animals ready for sorting off plates in 5–10 mL of M9 solution into 15 mL conical tubes for subsequent sorting on the flow cytometer.

6.1. Sorting setup using a large particle flow cytometer

6.1.1. Before turning on the flow cytometer

6.1.1.1. Make sure the sheath liquid bottle is not empty. Prepare sheath liquid from the 250x stock at least a few hours prior to using the sorter, as there is a small amount of detergent in the sheath fluid, which can cause bubbles that can cause artifacts during acquisition.

6.1.1.2. Ensure that all waste containers are not full.

6.1.2. Turning on the flow cytometer

6.1.2.1. Turn on the air compressor. Turn it to **Auto**. Check the pressure gauge – it should be around 30 psi.

6.1.2.2. Turn on the instrument. Use the power switch that is next to the power cord on the left of the instrument.

6.1.2.3. Turn on lasers. A 488 nm light source is usually sufficient for most experiments, although a 561 nm light source needs to be used if higher excitation is required for red fluorescence.

6.1.2.4. Open the FlowPilot software; the instrument should make a series of clicks switching on the different valves.

6.1.2.5. Turn on the lasers in the software window by clicking **Start**. Initiate laser in the Argon laser control popup window by hitting **Run**. This should cause the laser to turn on and reach around 12 mW. The 488 light source level will go up to around 12.

6.1.2.6. Hit **Done** to close the window.

6.1.3. Checks on software before progressing

6.1.3.1. Check pressure gauges – Look at the 4 pressure values displayed in the bottom of the window. The values should be around the original setup (Sheath 5.5-5.7; Sample 5.7-6.0; Sorter 3.1-3.3; Clean 8.5-8.7). If it looks similar, check the box next to **Pressure OK**.

6.1.3.2. Check fluidics – To make sure there are no air bubbles and debris blocking the flow of sheath/sample through the flow cell, click **Clean** several times.

6.1.3.3. Check sheath flow rate – For this, collect sheath for 60 s. Switch **Off Sort**, then switch **On Sheath** in the manual controls to start the flow of sheath. Collect in a 15 mL tube for 60 s; the flow rate should be ~9–10 mL/min.

6.1.4. Cleaning before use of the flow cytometer

6.1.4.1. Put ~3–5 mL of 10% bleach solution into the collection ‘cup’ and hit **Acquire**. Let run for ~30 s, hit **Abort**, and remove excess with vacuum.

6.1.4.2. Rinse the collection ‘cup’ with deionized water and remove with vacuum. Repeat 2x.

6.1.4.3. Put ~3–5 mL of COPAS cleaning solution into the collection ‘cup’ and hit **Acquire**. Let run for ~30 s, hit **Abort**, and remove excess cleaning solution with vacuum.

6.1.4.4. Rinse the collection ‘cup’ with deionized water and remove with vacuum. Repeat 2x.

6.1.4.5. Put ~3–5 mL of M9 solution into the collection ‘cup’ and hit **Acquire**. Let run for ~30 s, hit **Stop**, and remove excess M9 solution with vacuum.

6.2. Running samples on sorter

6.2.1. Adjust laser PMT power and size gating based on the condition that causes the brightest activation of the transcriptional reporter of interest. Recommended settings can be found in **Table 4**.

6.2.2. Add prepared worms to ‘cup’. Hit **Acquire**. Watch to make sure that all the liquid is not taken up into the machine; this will cause the flow cytometer to take in air and create bubbles in the detector.

6.2.3. Hit **Abort** when the sample is low and/or enough animals have been collected.

6.2.4. Click **Setup | Data Storage | Gated Only**. This will save the data only based on the size constraints. Click **Store Gated** and save gated data. Click **Erase** to erase data.

6.2.5. Rinse the collection ‘cup’ with deionized water and remove with vacuum. Repeat 2x.

6.2.6. Repeat steps 6.2.1–6.2.5 with the rest of the samples.

6.3. Calibration/ Quality Control – if necessary

NOTE: This requires running control 42 μ M GYR fluorescent particles provided, to calibrate the 488 laser.

6.3.1. Press the metal lip on the top of the sample cup to remove the air tube. Unscrew the cap and use a syringe to remove liquid from the sample cup.

6.3.2. Mix the bottle of control particles well before use and add few milliliters into the sample cup. Close the cap and put the air back on by pressing it on till it clicks in place.

6.3.3. In the software, go to the **Tools** option and click **Run Control Particles**.

6.3.4. For control particles, reset the PMT values to: GREEN – 325; YELLOW – 365; RED – 575.

6.3.5. Click **Acquire**. The sheath should turn on followed by sample. Once the beads start to go through the flow cell, the flow rate will be seen at the bottom of the screen. Optimally, the flow rate should be between 5/s and 15/s. If the flow rate is too low or zero, turn the sample valve physically clockwise to increase flow rate. If the flow rate is too high, turn the sample valve physically anti-clockwise to decrease flow rate.

NOTE: Normally, under bead saver mode, 500 beads are read before switching off. The data can be erased and beads re-read.

6.3.6. Once the reading is completed, check for clean single peaks for the 5 parameters as well as the CV values. The Coefficient of Variance (CV) should be <15%. Also, make sure the CV values for the three different fluorescent channels are close to each other.

6.3.7. Make a record of the QC check: under the **File** tab, click **Save as screen image**.

6.4. Cleaning and shutdown

6.4.1. Use a vacuum to remove the sample from the sample cup and repeat section 4.1.4.

6.4.2. Put ~3–5 mL of deionized water into collection ‘cup’ and hit **Acquire**, let run for ~ 30 s, and then hit **Abort**. Leave some distilled water behind in the sample cup.

6.4.3. Empty the sample recovery cup and the waste bottle.

6.4.4. Turn off software. Under the **File** tab, click **Exit**. In the pop-up menu, click **Turn Off Without Purging**.

6.4.5. Turn off the laser, turn off the instrument, and then turn off the air compressor. Close the hatch to cover the instrument.

7. Physiological assays to measure stress sensitivity in *C. elegans*

7.1. Measurement of ER stress sensitivity using tunicamycin exposure

7.1.1. Prepare NGM RNAi DMSO plates spotted with RNAi bacteria targeting the gene of interest as in section 3. Use HT115 bacteria even in experiments not involving RNAi knockdown (see section 1). Remember to also seed NGM RNAi TM plates (see **Table 1**). Seed a sufficient amount of plates: plan for ~5–7 sets of NGM RNAi DMSO plates and ~2–3 sets of NGM RNAi TM plates.

7.1.2. Synchronize animals of choice using methods described in section 2.

7.1.3. Plate eggs onto NGM RNAi DMSO seeded plates of choice using criteria recommended in **Table 2**. Be sure to prepare 2x the number of plates necessary as half of the sample will be transferred to NGM RNAi TM plates.

7.1.4. Incubate eggs at 20 °C for approximately 3–4 days (~65–96 h) to day 1 of adulthood.

7.1.5. At day 1, prepare lifespans by transferring animals onto separate plates. To conserve plates (as TM costs are high), use 8 plates of 15 animals per condition, for a total of 120 animals per

condition. This allows a manageable number of animals per plate for scoring and allows a sufficient amount of animals for statistical analyses, even with some censorship.

7.1.6. For the first 5–7 days, move adult animals away from progeny every day onto a new plate until progeny are no longer visible. During this stage, censor animals that are bagged, exhibit vulval protrusions/explosions, or crawling up the sides of the plates, as these are not deaths associated with ER stress sensitivity. Note that TM treatment causes arrest in animals, and thus only 1–2 moves of these animals every 2–3 days is sufficient to minimize the costs associated with producing TM-containing plates. Wild-type animals have an average survival of ~15–17 days on DMSO and 12–14 days on tunicamycin.

7.1.7. After animals have stopped producing progeny, score lifespans every 1–2 days until all animals are scored as dead or censored. TM-treat animals every day during day 6–14 of adulthood for higher resolution.

7.2. Measurement of mitochondrial and oxidative stress sensitivity using exposure to paraquat

7.2.1. Prepare plates spotted with RNAi bacteria targeting the gene of interest as in section 3. Use HT115 bacteria even in experiments not involving RNAi knockdown (see introduction).

7.2.2. Synchronize animals of choice using methods described in section 2.

7.2.3. Plate eggs onto NGM RNAi seeded plates of choice using criteria recommended in **Table 1**. The assay calls for ~60-100 animals per condition, so prepare accordingly.

7.2.4. Incubate eggs at 20 °C for approximately 3–4 days (~65–96 h) to day 1 of adulthood.

7.2.5. Prepare a fresh vial of 100 mM paraquat in M9 solution.

7.2.6. Pipette 50–75 µL of M9+paraquat into as many wells of a flat-bottom 96-well plate as desired. It is generally recommended to have ~8–10 wells per condition containing ~8–10 animals per well. This allows for an easily visible number of animals per well with ~80 animals per strain.

7.2.7. Pick 8–10 animals per condition and transfer them into each well containing M9+paraquat. Use a pick to transfer animals into the wells rather than pipetting to avoid differences in volume and unintended changes in paraquat concentrations.

7.2.8. Every 2 h, score for death of animals in each well. Tap the plates gently, which will cause live animals to thrash or bend. Note that it is possible that live animals are sometimes paralyzed long enough to be scored as dead. Therefore, if the number of live animals exceeds the number of live animals from a previous timepoint, it is likely that the animal was alive and should be unscored (e.g., if at hour 4, 2/10 animals are scored as dead, and at hour 6, only 1/10 animals are dead, hour 4 should be rescored as 1/10 animals dead).

7.3. Measurement of heat sensitivity using exposure to elevated temperatures

7.3.1. Prepare plates spotted with RNAi bacteria targeting the gene of interest as in section 3. Use HT115 bacteria even in experiments not involving RNAi knockdown (see section 1).

7.3.2. Synchronize animals of choice using methods described in section 2.

7.3.3. Plate eggs onto seeded plates of choice using criteria recommended in **Table 1**. Have 60–100 animals per condition for thermotolerance assays. For thermotolerance assays, use L1 arrest or egg lay assays for the best synchronization as there is major variability based on age of animals in the assay.

7.3.4. Incubate animals at 20 °C for approximately 3–4 days (~65–96 h) to perform experiments at day 1 of adulthood. Do not grow worms at 15 °C for heat-shock experiments as there is a minor difference between animals experiencing heat-shock out of 15 °C versus 20 °C.

7.3.5. At day 1, prepare animals by transferring them onto separate plates. It is generally recommended to have ~10–15 animals per plate with 4–6 plates, for a total of 60 animals. This allows a manageable number of animals per plate for scoring and allows for minimal time for animals to be pulled out of elevated temperatures.

7.3.6. Place animals into a 37 °C incubator and score every 2 h. Start scoring for thermotolerance at 37 °C at hour 5, as little to no death occurs prior to 5 hours. Median thermotolerance is accomplished at ~9 hours, so hour 7, 9, and 11 are critical timepoints, although due to incubator and lab-to-lab variability, this may need to be titrated per lab. Moreover, any methods to decrease variability will help (e.g., not stacking plates, placing plates in the same area within a single incubator, minimizing time the incubator is opened or closed, taking as few plates out of the incubator at a time to minimize time animals spend outside of 37 °C, etc. For a full guide, see²¹).

7.3.7. As an alternative to step 7.3.6, place animals at 34 °C instead of 37 °C. Median thermotolerance at 34 °C is at a little over 14 h, so time points 12, 14, and 16 are critical for 34 °C thermotolerance assays.

REPRESENTATIVE RESULTS:

Using transcriptional reporters to measure activation of stress responses

Here, fluorescent transcriptional reporters are used, which serve as robust tools to measure activation of most stress responses in *C. elegans*. GFP expression is driven under the promoter of canonical targets of master transcriptional regulators involved in responding to compartment-specific stresses. A comprehensive list of commonly used transcriptional reporters is available in **Table 3**.

Perturbing ER homeostasis via unfolded or misfolded proteins or lipid bilayer stress causes the activation of the unfolded protein response of the ER (UPR^{ER}) to restore ER quality and function.

The UPR^{ER} consists of 3 distinct branches defined by the transmembrane sensors: inositol-requiring protein 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase RNA (PKR)-like ER kinase (PERK), all of which are conserved in *C. elegans*^{7,22,23}. The most common tool to monitor activation of the UPR^{ER} in the nematode, is the transcriptional reporter strain expressing GFP under the control of the *hsp-4* promoter (*hsp-4p::GFP*)⁷. The gene *hsp-4* encodes an orthologue of mammalian Hsp70, HSPA5 (or BiP/Grp78). In times of ER stress when the UPR^{ER} is activated, the *hsp-4p::GFP* reporter strain expresses GFP. This reporter has minimal basal expression in the absence of stress, but exhibits robust GFP expression when animals are exposed to tunicamycin (**Figure 1A**). These differences can also be quantified using a large-particle flow cytometer (**Figure 1B**). Moreover, the induction of *hsp-4p::GFP* under ER stress can be completely suppressed by RNAi-knockdown of *xbp-1*, as the activation of this transcriptional reporter is dependent on the transcription factor, XBP-1¹².

Similar to the UPR^{ER}, the mitochondria houses its own protective mechanism against proteotoxic stress. This mechanism, termed the mitochondrial UPR (UPR^{MT})²⁴, is mainly regulated by the transcription factor, ATFS-1, which fails to enter the mitochondria under stress due to decreased import efficiency, resulting in entry of ATFS-1 into the nucleus²⁵. Interestingly, different perturbations to mitochondrial processes can activate this response, including protein aggregation, knock down of electron transport chain (ETC) complexes subunits, mitochondrial DNA replication stress, and mitochondrial protein translation^{8,26}. The activation of the UPR^{MT} has been monitored by using worms expressing a transgenic construct in which GFP was placed under the regulation of the promoters of the mitochondrial chaperone genes, *hsp-6* and *hsp-60*⁸. Similar to *hsp-4p::GFP* animals, *hsp-6p::GFP* animals exhibit minimal basal signal in the absence of stress. The most robust method to induce the UPR^{MT} is through RNAi-knockdown of the following mitochondrial proteins: *cox-5B*, the cytochrome c oxidase subunit Vb/COX4 (Complex IV)²⁰, *nuo-4*, the NADH dehydrogenase protein (Complex I)²⁷, or *mrps-5*, a mitochondrial ribosomal protein²⁸, activated the *hsp-6p::GFP* reporter. GFP expression through this reporter is robustly activated and can be easily visualized and quantified under these conditions (**Figure 2A-B**). The UPR^{MT} can also be triggered through chemical inhibition of the electron transport chain (ETC), such as with antimycin A, which inhibits cytochrome c reductase (complex III). Similar to RNAi-knockdown of ETC components, antimycin A treatment causes a robust induction of *hsp-6p::GFP* (**Figure 2C-D**).

The ability for organisms to sense and respond to oxidative stress is a conserved process present from bacteria to humans²⁹. In *C. elegans*, the NRF2 homologue, SKN-1, serves as an important transcription factor, which is sensitive to redox changes due to reactive cysteines throughout the protein. SKN-1 serves as one of the transcriptional activator of the OxsR through binding to conserved consensus sequence highly resembling the antioxidant response elements bound by NRF2³⁰. In humans, NRF2 is negatively regulated by KEAP1, which is thought to be sensitive to redox changes due to reactive cysteines throughout the protein³¹. While there is no direct ortholog of KEAP1 in worms, SKN-1 is negatively regulated by the WD-repeat protein, WDR-23, in a manner that is mechanistically distinct from KEAP1/NRF2 inhibition³². Upon oxidative stress, such as tert-butyl hydroperoxide (TBHP), SKN-1 activates detoxification and antioxidant genes such as *gst-4*, a Glutathione S-Transferase. To measure oxidative stress, GFP expression is placed

under the promoter of *gst-4*, a glutathione S-transferase³³. Unlike the other transcriptional regulators presented here, *gst-4p::GFP* has high basal expression. However, this expression can still be robustly activated under conditions of oxidative stress, which can be performed both genetically and chemically. To genetically induce oxidative stress, we knockdown *wdr-23*, which encodes a protein that plays a role in proteasomal degradation of SKN-1³⁴. *wdr-23* knockdown results in robust activation of *gst-4p::GFP*. Moreover, treatment of worms with the chemical oxidant, TBHP, results in a milder, but still significant, activation of *gst-4p::GFP* (**Figure 3**). Both chemical and genetic activation of *gst-4p::GFP* can be almost completely suppressed by RNAi knockdown of the *skn-1*, the gene encoding the master transcriptional regulator of the OxSR.

Most cellular proteins are translated in the cytoplasm and reside there, even if only temporarily before being targeted elsewhere. Thus, the cytoplasm hosts a diverse array of chaperones that promote proper protein folding and function, as well as enzymes and proteins responsible for degrading damaged, dysfunctional, or excess proteins. To protect this complex protein landscape of the cytoplasm, the cell has evolved several cytoplasmic stress response pathways, including the heat-shock response (HSR)^{35,36}. The HSR is a pathway dedicated to promoting protein homeostasis under conditions of heat stress and is modulated by the master transcriptional regulator, HSF-1³⁷. Under steady state conditions, HSF-1 is bound by cytoplasmic chaperones, HSP90 and HSP70/40, which keeps it locked in a monomeric, inactive state. Under conditions of heat or similar stress, an increase in misfolded proteins results in titration of chaperones away from HSF-1, allowing it to trimerize and translocate to the nucleus to activate the HSR^{38,39}. Perhaps the most-studied downstream targets of HSF-1 under HSR activation are the heat-shock proteins (HSPs), such as HSP70, HSP90, DNAJ, and HSP60^{17,40}. In *C. elegans*, transcriptional reporters for HSR have been synthesized by driving the expression of GFP under the promoters of canonical HSPs, *hsp-16.2* and *hsp-70*^{9,41}. Like their UPR^{MT} and UPR^{ER} counterparts, *hsp-16.2p::GFP* and *hsp-70p::GFP* show minimal basal expression in the absence of stress. However, both reporters are robustly induced under conditions of heat stress, which can be easily visualized by microscopy or quantified using a large particle flow cytometer (**Figure 4**). Both reporters have large dynamic range, and induction is completely dependent on *hsf-1*, as RNAi-knockdown of *hsf-1* fully suppresses induction of *hsp-16.2p::GFP* and *hsp-70p::GFP*. While these reporters can be used interchangeably for most situations, there may be differences in expression levels and expression across tissues.

Physiological assays to measure stress sensitivity in *C. elegans*

C. elegans are a great model organism to measure stress sensitivity due to the low cost in maintenance and experimentation and ease of genome editing or genetic knockdown using RNAi, which provides the capacity to perform large-scale experiments in a whole organism. To assay stress tolerance to ER stress, we expose *C. elegans* to the chemical agent, tunicamycin, which causes accumulation of damaged proteins in the ER by blocking N-linked glycosylation¹⁰. Animals are exposed to tunicamycin post-development, as the drug causes developmental defects. When exposed to tunicamycin, adult worms exhibit a marked decline in lifespan. Moreover, knockdown of the gene, *xbp-1*, which encodes one of the primary transcription factors involved in UPR^{ER} induction, results in a significant increase in sensitivity to tunicamycin (**Figure 5A**)¹². Thus, this serves as a robust assay to measure ER stress sensitivity in adult worms.

To measure oxidative stress and mitochondrial stress, we expose animals to the chemical agent, paraquat. Paraquat causes mitochondrial stress by synthesis of ROS within the mitochondrial matrix, which can then be converted into hydrogen peroxide and diffuse out of the mitochondria to cause whole-cell oxidative damage¹³. Similar to ER stress assays, we expose animals to paraquat at adulthood. However, we perform paraquat assays in liquid to reduce cost and manual labor and agar plate-based assays would be difficult for most labs. Here, we show that animals exposed to paraquat in liquid show median survival of approximately 5 hours (**Figure 5B**). Moreover, knockdown of the insulin receptor, *daf-2*, results in an increased resistance to paraquat as activation of DAF-16/FOXO results in increased expression of involved in clearance of ROS, such as *sod-3*^{42,43}. Paraquat survival assays are short, lasting up to 14 hours, and thus serve as an efficient method to interrogate mitochondrial and oxidative stress responses.

Finally, survival at elevated temperatures is used to interrogate the physiological response to heat stress. These assays can be performed both in liquid or solid agar, and there exist numerous different protocols outlined²¹. It is recommended to standardize a single assay in the lab to decrease variability, which is exceptionally high in this assay. Thermotolerance should be performed in day 1 adult animals on standard agar plates, either at 34 °C or 37 °C. At 37 °C, a majority of death occurs between 7-11 hours, making this a simple single-day assay, whereas 12-16 hour experiments at 34 °C are most easily performed overnight (**Figure 5C-E**). Mutation in the gene, *ttx-3*, results in failure of specification of the AIY interneurons responsible for the thermosensory neural circuit, and causes a significant increase in thermosensitivity⁴⁴. While thermotolerance data can be plotted as a survival curve (**Figure 5C**), these assays should be performed at least 4-6 times and all replicates should be plotted against each other (**Figure 5D-E**), as thermotolerance shows incredibly high variability in comparison to other stress assays. This is due to the many caveats that exist in setting up these experiments, including variability in strains of interest, unequal cycling of air in incubators, uneven agar plates, etc.²¹. At 34 °C, median survival occurs at approximately 14 hours, and similar to 37 °C, *ttx-3* mutants exhibit decreased survival at 34 °C (**Figure 5E**).

FIGURE LEGENDS:

Figure 1. Using *hsp-4p::GFP* as a reporter for UPR^{ER} induction. (A) Representative fluorescent micrographs of *hsp-4p::GFP* expressing animals grown on control empty vector (EV) or *xbp-1* RNAi. Animals were grown on RNAi from hatch until L4 at 20 °C, then treated with 25 ng/μL tunicamycin or 1% DMSO floating in M9 at 20 °C for 4 hours, and recovered on an OP50 plate for 16 hours at 20 °C prior to imaging. Animals were paralyzed in 100 μM sodium azide on an NGM agar plate and imaged using a stereomicroscope. (B) Quantitative analysis of (A) using a large particle biosorter. Data is represented as integrated fluorescence intensity across the entire animal where each dot represents a single animal; DMSO control is in grey and tunicamycin treated animals are in red. Central line represents the median, and whiskers represent the interquartile range. n = 123-291 animals per strain. ****p* < 0.001 using non-parametric Mann-Whitney testing.

Figure 2. Using *hsp-6p::GFP* as a reporter for UPR^{MT} induction. (A) Representative fluorescent micrographs of *hsp-6p::GFP* expressing animals grown on control empty vector (EV), *cco-1*, *mrps-5*, or *nuo-4* RNAi. Animals were grown on RNAi from hatch and imaged on day 1 of adulthood at 20°C. Animals were paralyzed in 100 μM sodium azide on an NGM agar plate and imaged using a compound microscope. (B) Quantitative analysis of (A) using a large particle biosorter. Data is represented as integrated fluorescence intensity across the entire animal where each dot represents a single animal; EV control is in grey RNAi-treated animals are in red. Central line represents the median, and whiskers represent the interquartile range. n = 303-384 animals per strain. ****p* < 0.001 compared to EV control using non-parametric Mann-Whitney testing. (C) Representative images of *hsp-6p::GFP* animals treated with DMSO or Antimycin A. Animals were grown from hatch on 0.2% DMSO plates and transferred to plates containing 0.2% DMSO or 3 mM antimycin A for 16 hours prior to imaging on a Revolve ECHO R4 compound microscope. All growth was performed at 20°C. (D) Quantitative analysis of (C) using a large particle biosorter similar to (B). DMSO controls are in grey, and Antimycin A-treated animals are in red. n = 495 for DMSO and 219 for Antimycin A. ****p* < 0.001 compared to EV control using non-parametric Mann-Whitney testing.

Figure 3. Using *gst-4p::GFP* as a reporter for the OxSR. (A) Representative fluorescent micrographs of *gst-4p::GFP* expressing animals grown on control empty vector (EV), *skn-1*, or *wdr-23* RNAi. Animals were grown on RNAi from hatch until L4 stage at 20 °C. Animals were grown on RNAi from hatch until L4 at 20 °C, then treated with 2 mM TBHP in M9 or only M9 for “untreated” control at 20 °C for 4 hours, and recovered on an EV plate for 16 hours at 20 °C prior to imaging. Animals were paralyzed in 100 μM sodium azide on an NGM agar plate and imaged using a compound microscope. (B) Quantitative analysis of (A) using a large particle biosorter. Data is represented as integrated fluorescence intensity across the entire animal where each dot represents a single animal; untreated control is in grey and TBHP-treated animals are in red. Central line represents the median, and whiskers represent the interquartile range. n = 101-204 animals per strain. ****p* < 0.001 compared to respective EV control using non-parametric Mann-Whitney testing.

Figure 4. Using *hsp16.2p::GFP* and *hsp-70p::GFP* as reporters for the heat-shock response. (A) Representative fluorescent micrographs of *hsp16.2p::GFP* expressing animals grown on control empty vector (EV) or *hsf-1* RNAi. Animals were grown on RNAi from hatch at 20 °C until day 1. Day 1 animals were either left at 20 °C (untreated) or exposed to 2 hours of heat stress at 34 °C, then recovered for 2 hours at 20 °C. Animals were paralyzed in 100 μM sodium azide on an NGM agar plate and imaged using a stereomicroscope. (B) Quantitative analysis of (A) using a large particle biosorter. Data is represented as integrated fluorescence intensity across the entire animal where each dot represents a single animal; untreated control is in grey and heat-shocked animals are in red. Central line represents the median, and whiskers represent the interquartile range. n = 320-364 animals per strain. ****p* < 0.001 using non-parametric Mann-Whitney testing. (C) Representative fluorescent micrographs of *hsp-70p::GFP* expressing animals grown on control EV and *hsf-1* RNAi and treated as described in (A). (D) Quantitative analysis of (C) as described in (B). n = 773-941 animals per strain.

Figure 5. Physiological survival assays under stress in *C. elegans*. (A) Lifespans of nematodes grown on 1% DMSO containing 25 ng/μL tunicamycin (TM) plates. Animals were grown on 1% DMSO plates from hatch until day 1, and transferred to respective TM plates at day 1. Animals were kept on control empty vector (EV) or *xbp-1* RNAi from hatch until the end of the assay at 20 °C. Adult animals are manually moved away from progeny every day until ~ day 7-8 when progeny were no longer detected, then scored every 2 days until all animals were recorded as dead or censored. Animals with bagging, vulval protrusions/explosions, or those that crawled up the sides of plates were considered censored. (B) Survival curve of nematodes in 100 mM paraquat (PQ) dissolved in M9 solution. Animals were grown on EV or *daf-2* RNAi from hatch until day 1 of adulthood at 20 °C. Animals were placed into 50 μL of M9 + PQ solution in a 96 well-plate at 20 °C and visualized every 2 hours until all animals were motionless. (C) Survival curve of nematodes at 37 °C. Wild-type (N2), *ttx-3(KS5)*, and *sur-5p::hsf-1* animals were grown on EV plates from hatch until day 1 at 20 °C. At day 1, animals were moved to 37 °C and scored every 2 hours until all animals were scored as dead or censored. (D) Pooled data of all thermotolerance assays performed at 37 °C. Data are represented as percent alive at hour 9 of a thermotolerance assay, with each line representing a matched experiment performed on the same day. (E) Pooled data of all thermotolerance assays performed at 34 °C. Data are represented as percent alive at hour 14 of a thermotolerance assay, with each line representing a matched experiment performed on the same day. All statistics for A-C were performed using Log-Rank (Mantel-Cox) testing and can be found in **Table 5**.

TABLE LEGENDS

Table 1. Recommended recipes for reagents used. All the exact recipes of the reagents used in this protocol are outlined here. Specific companies where reagents were purchased are also available in the **Table of Materials**. Many different sources of chemicals were tested, and those listed in the **Table of Materials** are those that exhibited the most robust and reproducible results.

Table 2. Recommended number of animals to plate post-synchronization to avoid starvation. To avoid starvation, we recommend plating a specific number of animals per condition. Since OP50 grows denser than HT115, more animals can be plated. All numbers listed here are the guidelines used in our lab, and numbers may be slightly different due to several variables and differences between laboratory conditions. Therefore, or recommended numbers are on the lower side in bold font. Max numbers are what could be plated in optimal conditions in our lab without reaching starvation, but it is not recommended to use these values without first titrating your conditions. All numbers are determined with the assumption that bacteria are seeded onto plates and allowed to grow for ~24 hours at ambient temperature (~22 °C) on plates prior to worms being placed on them. Although there is no major difference in starvation rates when plating eggs or L1s, we recommend plating ~10% higher numbers when plating eggs, since not all eggs will hatch post-bleaching.

Table 3. Transcriptional reporters for assessing activation of cellular stress responses. The strains listed here are all available through CGC or through special requests to laboratories for use in both qualitative and quantitative imaging methods described in this manuscript. These

strains are all derived from the Bristol N2 background. Recommended methods to apply stress to activate the reporters are also provided. All reporters, with the exception of *sod-3p::GFP⁴⁵* and *T24B8.5p::GFP⁴⁶* are described in the text.

Table 4. Recommended settings for fluorescent microscopy and quantification using a large particle biosorter. This table serves as guideline for recommended exposure times for fluorescent microscopy or PMT values for the large particle biosorter. These will serve as good starting points, but the exposure time and PMT value should be adjusted for every experiment to ensure that no saturation occurs and that fluorescent values are over the detection limit of background signal. If the sample with the brightest signal for an experiment is known (e.g., positive controls for stress induction), those samples can be used to determine the highest exposure time or PMT that can be used without saturating signal. If the brightest samples are not known, then the control can be used and an exposure time or PMT at the center of the dynamic range of your system can be used.

Table 5. Statistics for lifespans and stress survival assays. All sample sizes, statistics, and censorship rates for Figure 5 are available here.

Table 6. Recommended gene targets and primer pairs for measuring transcriptional upregulation of stress response genes.

DISCUSSION:

Here, methods to interrogate cellular stress responses in *C. elegans*, using fluorescent transcriptional reporters and physiological stress survival assays are described. The reporters all utilize GFP expression driven under the promoter of a downstream transcriptional target of the transcription factors involved in mounting cellular stress responses. The use of *hsp-4p::GFP* modulated by XBP-1s-mediated UPR^{ER}, *hsp-6p::GFP* controlled by ATFS-1-mediated UPR^{MT}, *gst-4p::GFP* under SKN-1-mediated OXSR, and *hsp16.2p::GFP* and *hsp-70p::GFP* under HSF-1-mediated heat-shock response are explained. Other standardized transcriptional reporters can be found in **Table 3**. All the transcriptional reporters presented here have a wide dynamic range and can be robustly activated by applying stress either through genetic perturbations or exposure to stress-inducing chemicals. Moreover, these reporters can all be suppressed by knockdown of the transcription factors upstream of the promoters employed. Finally, each transcriptional reporter is paired to a specific physiological stress survival assay to provide a physiological readout of the impact of either activating or repressing a specific stress response.

To successfully employ the use of transcriptional reporters, it is essential to determine the dynamic range of each reporter. Due to major lab-to-lab variability caused by differences in media, agar, ambient environment, etc., it is recommended to titrate each drug or stress induction paradigm for concentrations and timing using our recommendations as a baseline. Next, it is critical to ensure the animals are healthy and properly synchronized. Animals that have experienced some sort of stress (e.g., starvation, long-term exposure to light, exposure to elevated temperature, etc.) should be recovered for several generations prior to experimentation. Proper synchronization can be achieved by using the methods outlined in

section 2, which is essential as several stress responses have different levels of activation during the aging process. Finally, imaging protocols are essential to standardize, as there are various things that can affect image and data quality. For example, duration of animals in sodium azide should be minimized, as this causes stress to animals and can affect reporter signal. Moreover, microscope and biosorter specifications should be properly set to maximize signal-to-noise ratio and dynamic range, without causing saturation. Saturated pixels can cause major issues in quantitative analysis of samples, as maximum fluorescent signal can be severely underestimated.

While the transcriptional reporters described here provide a robust and efficient means to measure activation of stress responses, it is critical to understand that it is a single gene target of a known transcription factor. Therefore, while it serves as a reliable method for large-scale screens or first-pass tests of strains of interest, proper validations should be performed. We recommend performing qPCR to measure several canonical target genes activated upon induction of each stress response being assayed. A list of suggested gene targets can be found in **Table 6**. In addition, transcriptome profiling through RNA-seq is another alternative for a broader look at effects on multiple transcriptional targets at once. Of particular note, the imaging of fluorescent reporters provides spatial information regarding tissues that are affected by the perturbations. Such information cannot be obtained from qRT-PCR or RNA-seq, as it uses whole-worm extracts, except by scRNA-seq, FISH, and tissue-specific RNAseq protocols⁴⁷. Finally, these stress reporters are generally characterized as being specific to their stress response machinery. However, it is important to remember that not all stress response paradigms are unique and distinct. For example, ER stress can activate OxSR and vice versa⁴⁸, and overlaps between heat-shock response and ER stress responses have been commonly found⁴⁹. These are just a few of the many examples in the literature of cross-communication and overlap between stress responses, and thus it is important to understand that all assays should also be tested for specificity to derive final conclusions.

Another limitation of the methods described are that imaging and quantification through a biosorter has limited throughput. While biosorter quantification can be performed in 96-well plates for higher throughput, it is still limited by the necessity to transfer worms into solution, whereas imaging is limited by the investigator's capacity to prepare worms and perform microscopy. Therefore, large-scale screens will most likely involve only a visual screening of fluorescent reporter signal, with only hits being imaged and quantified.

An important caveat with using these fluorescent reporters is that activation or suppression of stress responses do not always contribute to physiologically meaningful phenotypes, or may reflect other global effects (e.g., decrease in protein synthesis). Therefore, every transcriptional reporter is paired to a method to assay stress survival. It is recommended to perform tunicamycin survival assays for the UPR^{ER}, paraquat survival assays for the UPR^{MT} and the OxSR, and survival in elevated temperatures for the heat-shock response. While these are generally robust, fast, and simple assays, they require intensive manual labor, and thus are severely limited in scalability. Moreover, almost all thermotolerance assays published to date have major variability, making a large number of replicates almost essential²¹. While the tunicamycin and paraquat survival assays do not suffer from this lack of reproducibility, they have their own challenges, including extensive

hands-on labor and duration of the protocol. As new technologies are born in automating lifespan and survival assays, it is likely that these stress survival assays can also become high-throughput⁵⁰. However, until these automated assays become standard, survival assays are currently limited to validation of physiological consequences in altering dynamics of stress response activity.

Beyond the stress survival assays described here, there are a number of other methods to measure physiology in animals. For example, a commercially available Seahorse XFp Analyzer can allow monitoring of cellular respiration, which provides additional mechanistic insight⁵¹. Another alternative to transcriptional reporters is the use of nuclear localization of fluorescently labelled transcription factors. There are numerous variants of this technique, but of particular interest to the methods described here include: HSF-1::GFP for the heat-shock response⁵², DVE-1::GFP for the UPR^{MT53}, and DAF-16::GFP and SKN-1::GFP for the OxSR^{54,55}. Finally, direct interrogation of morphology of specific organelles of interest can be performed. For example, mitochondrial morphology can be visualized by utilizing a fluorophore targeted to the mitochondrial matrix using a mitochondrial-localization sequence⁵⁶. ER morphology can be visualized by utilizing a fluorophore targeted to the ER through a signal-sequence fused to the N-terminus and HDEL fused to the C-terminus⁵⁷ or GFP fused to an ER membrane protein⁵⁸. Finally, actin cytoskeletal integrity can be used as a proxy for thermal stress sensitivity downstream of HSF-1. The actin cytoskeleton is regulated by transcriptional targets of HSF-1, specifically during aging and heat-stress, and thus actin organization can be visualized to determine functional readout of HSF-1 and heat-related stress^{59,60}.

All of the methods described here can be used independently or in combination with each other for a comprehensive analysis of genes or drugs of interest and their impact on stress response. Large-scale screens can be performed using high-throughput transcriptional reporter assays, and secondary screens can be performed using quantitative analyses of these reporters. Once more manageable candidate gene/drug lists are identified, physiological assays can be performed to identify those candidates that have direct impact on whole animal physiology. The other methods suggested above can also be used as validation or further investigation.

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DISCLOSURES:

The authors have nothing to disclose.

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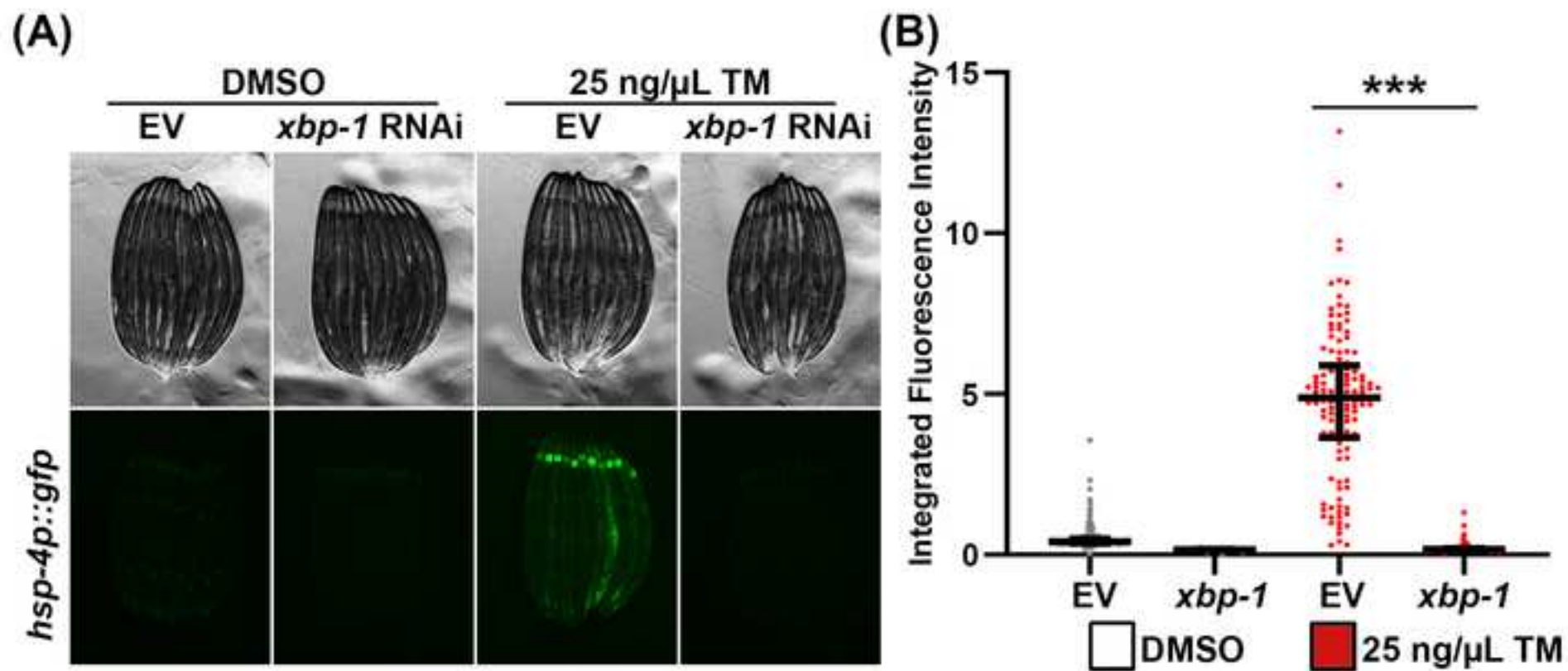
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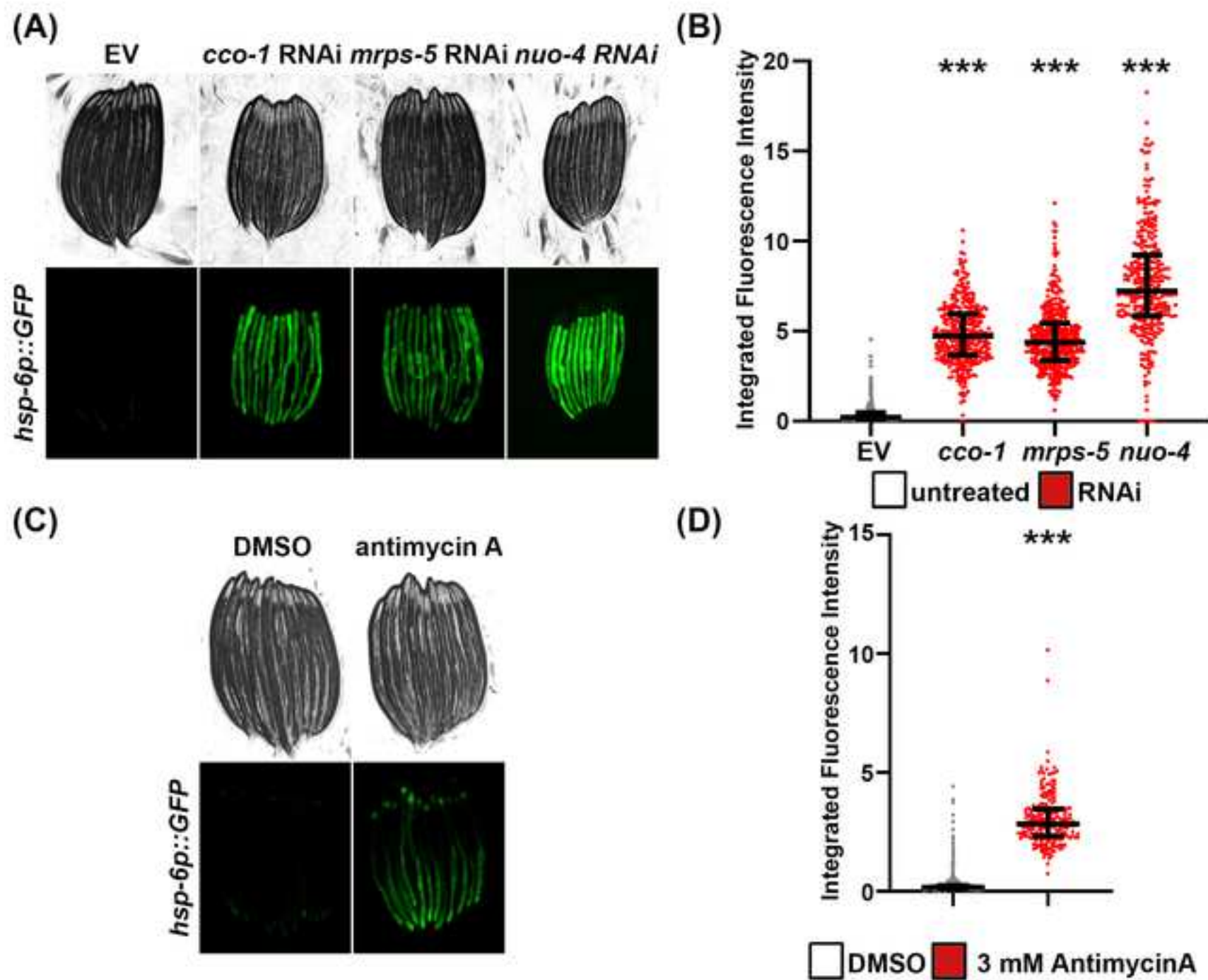
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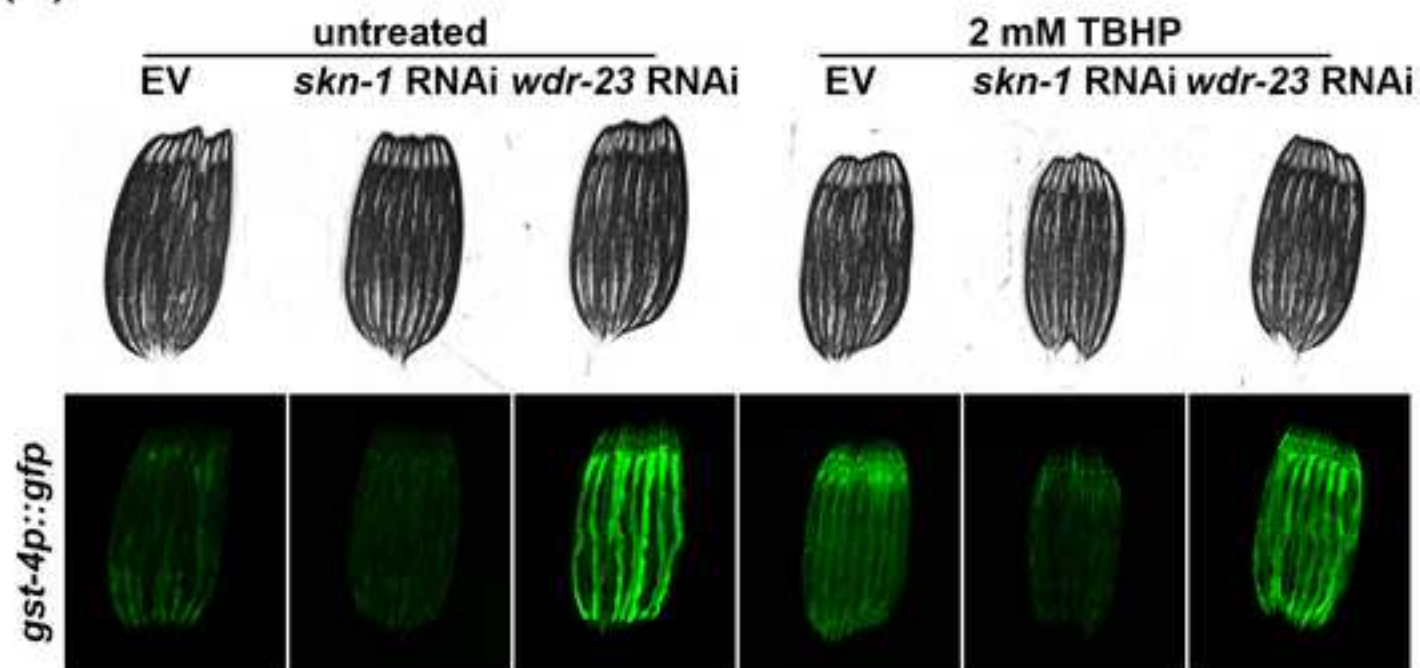
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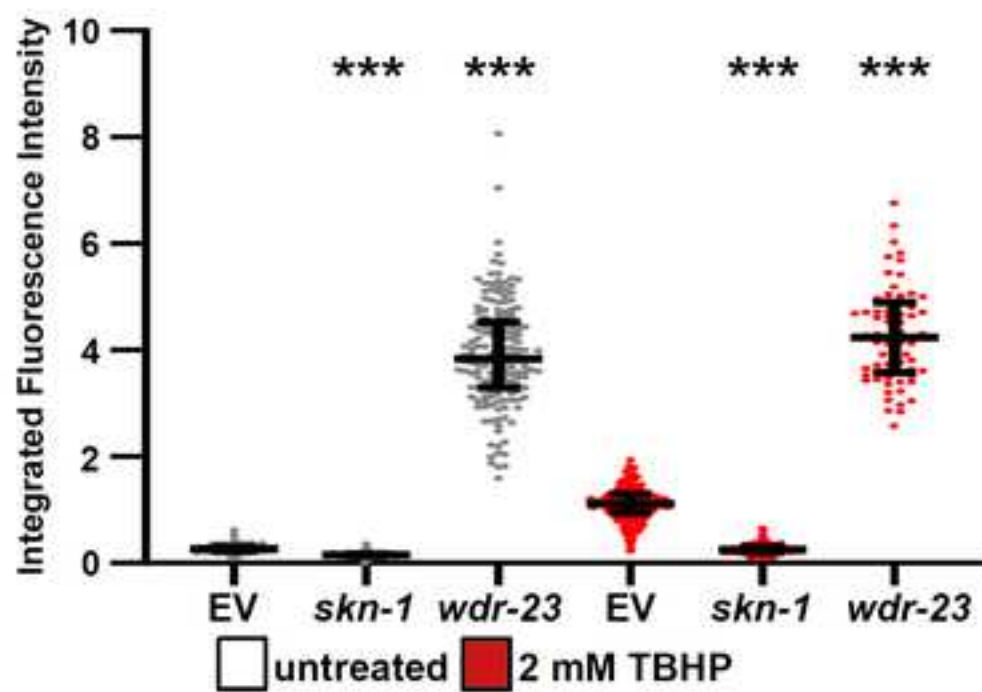




(A)



(B)



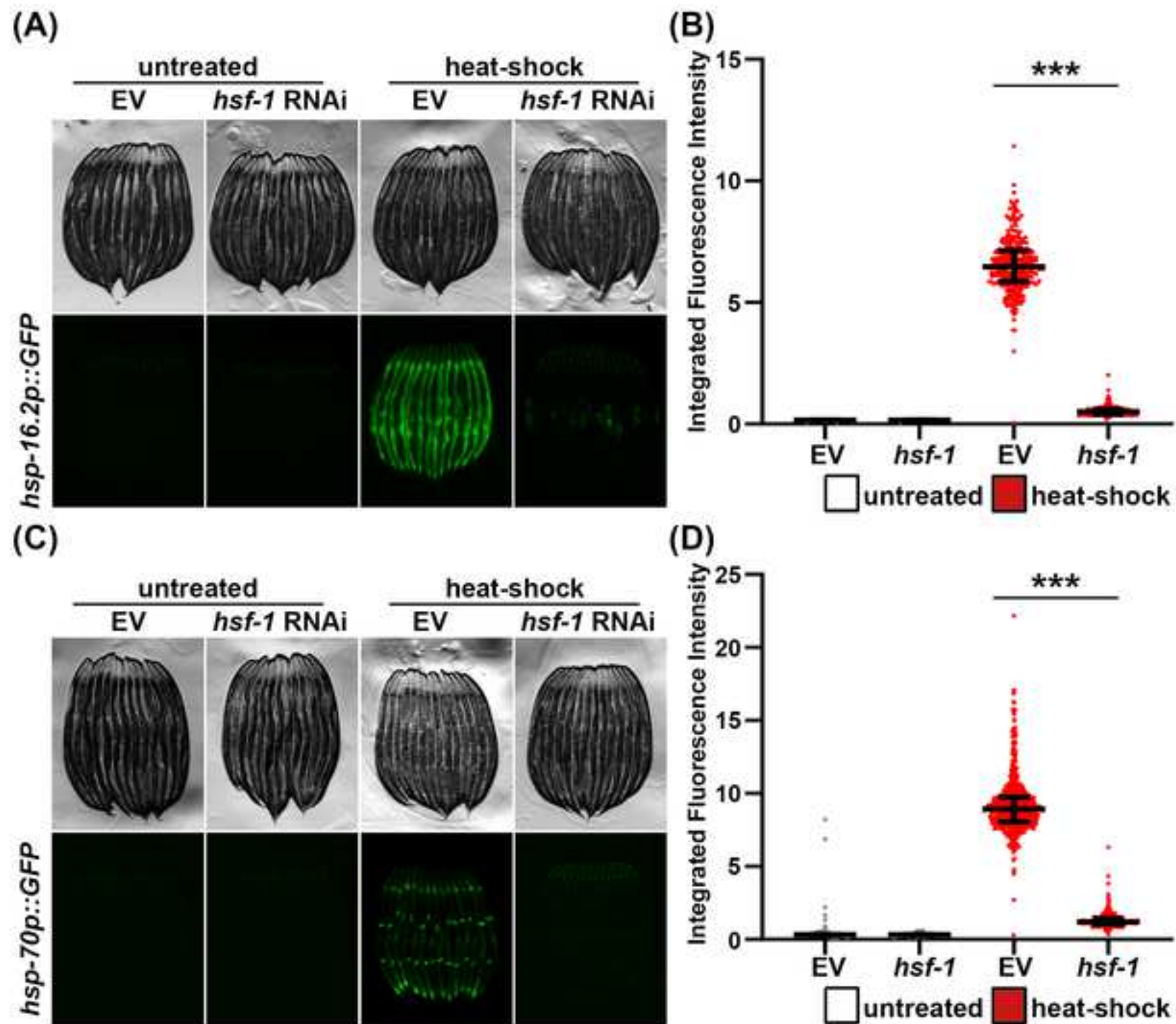
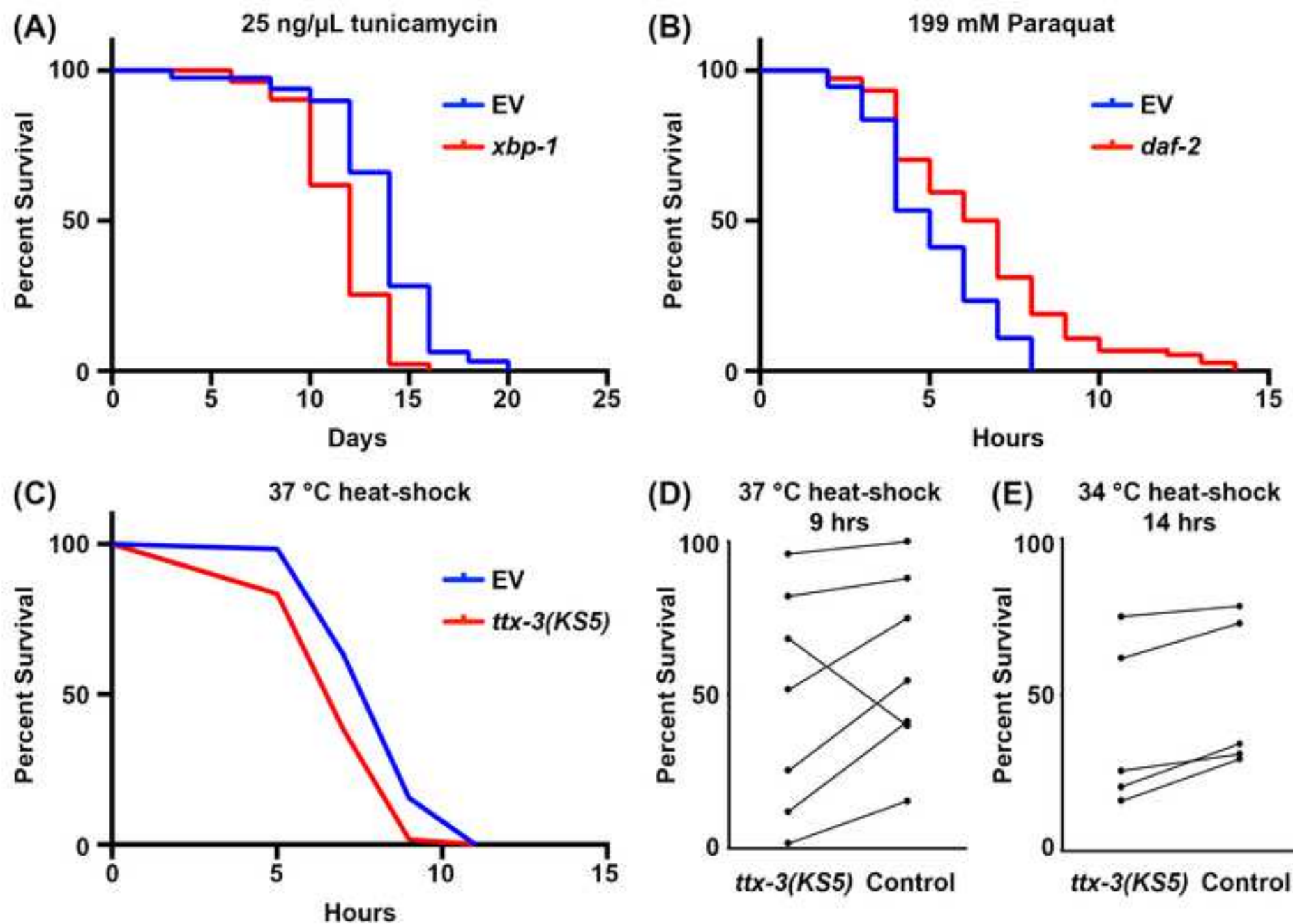


Figure 5



Reagent
Lysogeny Broth (LB)
Nematode Growth Media (NGM)
Bleach solution
M9 solution
NGM RNAi plates
Tetracycline
Carbenicillin
Sodium azide
Tunicamycin
Antimycin A
Paraquat
Tert-butyl hydroperoxide (TBHP)
NGM RNAi + DMSO0.2 (control for antimycin A)
NGM RNAi + antimycin A
NGM RNAi DMSO (control for tunicamycin)
NGM RNAi TM
IPTG

Recipe

In this protocol, commercial LB was used (see materials), but all standard LB home-made recipes using Bacto-tryptone, yeast extract, and NaCl are sufficient.

1 mM CaCl₂, 5 µg/mL cholesterol, 25 mM KPO₄ pH 6.0, 1 mM MgSO₄, 2% (w/v) agar, 0.25% (w/v) Bacto-Peptone, 51.3 mM NaCl

1.8% (v/v) sodium hypochlorite, 0.375 M KOH

22 mM KH₂PO₄ monobasic, 42.3 mM Na₂HPO₄, 85.6 mM NaCl, 1 mM MgSO₄

1 mM CaCl₂, 5 µg/mL cholesterol, 25 mM KPO₄ pH 6.0, 1 mM MgSO₄, 2% (w/v) agar, 0.25% (w/v) Bacto-Peptone, 51.3 mM NaCl, 1 mM IPTG, 100 µg/mL carbenicillin/ampicillin. Store at 4 °C in dark for up to 3 months

10 mg/mL stock solution (500x) in 100% ethanol. Store at -20 °C

100 mg/mL stock solution (1000x) in water. Store at 4 °C for up to 6 months or -20 °C for long-term storage

1 M stock (~6.5%) stock solution of sodium azide in water. Store in the dark at 4 °C. This is a 10x solution and is diluted to a 100 mM working stock for most imaging experiments

2.5 mg/mL stock solution in 100% DMSO. Store at -80 °C for long-term storage. This is a 100x solution (25 ng/µL working solution)

15 mM antimycin A stock solution in 100% DMSO. Store at -20 °C. This is a 5000x solution (3 µM working stock)

50 µM solution in water – should be prepared fresh

7.7 M solution in water. This is a 3850x solution (2 mM working stock)

1 mM CaCl₂, 5 µg/mL cholesterol, 25 mM KPO₄ pH 6.0, 1 mM MgSO₄, 2% (w/v) agar, 0.25% (w/v) Bacto-Peptone, 51.3 mM NaCl,

1 mM CaCl₂, 5 µg/mL cholesterol, 25 mM KPO₄ pH 6.0, 1 mM MgSO₄, 2% (w/v) agar, 0.25% (w/v) Bacto-Peptone, 51.3 mM NaCl, 1 mM IPTG, 100 µg/mL carbenicillin/ampicillin, 0.2% DMSO, 3 mM antimycin A

1 mM CaCl₂, 5 µg/mL cholesterol, 25 mM KPO₄ pH 6.0, 1 mM MgSO₄, 2% (w/v) agar, 0.25% (w/v) Bacto-Peptone, 51.3 mM NaCl,

1 mM CaCl₂, 5 µg/mL cholesterol, 25 mM KPO₄ pH 6.0, 1 mM MgSO₄, 2% (w/v) agar, 0.25% (w/v) Bacto-Peptone, 51.3 mM NaCl, 1 mM IPTG, 100 µg/mL carbenicillin/ampicillin; 1% DMSO, 25 ng/µL tunicamycin

1 M solution in water.

Plate Size	Bacteria Type	# of animals to reach day 1 adulthood	# of animals to reach L4 stage
60 mm	OP50	100-150	150-300
60 mm	HT115	70-100	120-200
100 mm	OP50	600-1000	1500-2000
100 mm	HT115	350-600	700-1300

Strain name	Transgene	Purpose	Recommended application(s) of stress	Source
SJ4005	<i>hsp-4p::GFP</i>	UPR ^{ER}	25 µg/mL tunicamycin	CGC
SJ4100	<i>hsp-6p::GFP</i>	UPR ^{MT}	3 µM antimycin A; RNAi against ETC or mitochondrial ribosome	CGC
SJ4058	<i>hsp-60p::GFP</i>	UPR ^{MT}	3 µM antimycin A; RNAi against ETC or mitochondrial ribosome	CGC
CL2070	<i>hsp-16.2p::GFP</i>	heat-shock response	34 °C 2 hours	CGC
AM446	<i>hsp-70p::GFP</i>	heat-shock response	34 °C 2 hours	Morimoto Lab
CL2166	<i>gst-4p::GFP</i>	OxSR	50 mM paraquat; 2 mM tert-butyl hydroperoxide	CGC
CF1553	<i>sod-3p::GFP</i>	OxSR & insulin signaling	RNAi against <i>daf-2</i> (insulin receptor)	CGC
AU78	<i>T24B8.5p::GFP</i>	innate immune response	pathogen exposure (e.g. <i>P. aeruginosa</i>)	CGC

Transgene	Recommended application(s) of stress	Exposure time using a Leica M2250FA stereo microscope
<i>hsp-4p::GFP</i>	25 µg/mL tunicamycin (~4 hours with overnight recovery)	200 ms
<i>hsp-6p::GFP</i>	3 µM antimycin A (~16 hours); RNAi against ETC or mitochondrial ribosome (from hatch)	100ms
<i>hsp-60p::GFP</i>	3 µM antimycin A (~16 hours); RNAi against ETC or mitochondrial ribosome (from hatch)	200 ms
<i>hsp-16.2p::GFP</i>	34 °C 2 hours	400 ms
<i>hsp-70p::GFP</i>	34 °C 2 hours	400 ms
<i>gst-4p::GFP</i>	50 mM paraquat (~2 hours); 2 mM tert-butyl hydroperoxide (~4 hours with overnight recovery)	100 ms
<i>sod-3p::GFP</i>	RNAi against <i>daf-2</i> (insulin receptor)	300 ms
<i>T24B8.5p::GFP</i>	pathogen exposure (e.g. <i>P. aeruginosa</i>)	100 ms

Exposure time using a Revolve ECHO microscope	PMT values using a Union Biometrica COPAS biosorter
275 ms	450
50 ms	350
100 ms	450
200 ms	500
300 ms	500
50 ms	350
300 ms	475
50 ms	350

Corresponding Figure	Strain, Treatment	Median Lifespan	# Deaths/# Total	% change in median lifespan
5A	N2, vector RNAi, 1% DMSO	22 days	95/120	--
	N2, <i>xbp-1</i> RNAi, 1% DMSO	14 days	92/120	-36.4
	N2, vector RNAi, 25 ng/ μ L TM	14 days	97/120	-36.4
	N2, <i>xbp-1</i> RNAi, 25 ng/ μ L TM	12 days	98/120	-45.4
5B	N2, vector RNAi, 100 mM PQ	5 hours	73/73	--
	N2, <i>daf-2</i> RNAi, 100 mM PQ	6.5 hours	74/74	30
5C	N2, vector RNAi, 37 °C	8 hours	59/60	--
	<i>ttx-3(KS5)</i> , vector RNAi, 37 °C	7 hours	60/60	-12.5
	<i>sur-5p::hsf-1</i> , vector RNAi, 37 °C	9 hours	59/60	12.5

p-value (Log-Rank; Mantel-Cox)
--
< 0.001
< 0.001
< 0.001
--
< 0.001
--
0.002
0.012

Stress Response	Target Gene	Forward Primer	Reverse Primer
UPR ^{ER}	<i>hsp-3</i>	TCGCTGGATTGAACGTTGTTG	GTTGCGTTCTCCGTCCTTCTTG
UPR ^{ER}	<i>hsp-4</i>	GAACAACCTACTCGTGCGTTGG	GAACAACCTACTCGTGCGTTGG
UPR ^{ER}	<i>sel-11</i>	TTGATCTCCGGAACGCAACG	TTGATCTCCGGAACGCAACG
UPR ^{ER}	<i>ire-1</i>	TCCTCAACCGCTCCATCAACAT	TCCTCAACCGCTCCATCAACAT
UPR ^{ER}	<i>xbp-1</i>	GGACTTCTTCGGCTTCTGGAGT	GGACTTCTTCGGCTTCTGGAGT
UPR ^{ER}	<i>xbp-1 (spliced)</i>	GGTGGATGGAGGGAGAAGATT	GGTGGATGGAGGGAGAAGATT
UPR ^{ER}	<i>crt-1</i>	GAAGTAATAGCCGAGGGAAGC	GAAGTAATAGCCGAGGGAAGC
UPR ^{ER}	<i>T14G8.3</i>	CACCTCCATCAACAACAACAT	CACCTCCATCAACAACAACAT
HSR	<i>hsp-17</i>	TCGTTTCCACCATTCTCCCA	TGTTTGATCGGCCAGTATGGT
HSR	<i>hsp-70</i>	TGTTTGATCGGCCAGTATGGT	TTCGCAATGAGAAGGGACGACT
HSR	<i>F44E5.4</i>	TTCGCAATGAGAAGGGACGACT	CGTTGTGCTGCGTCTTCTTT
HSR	<i>hsp-16.2</i>	TCCATCTGAGTCTTCTGAGATTGTA	TGGTTAACTGTGAGACGTTGA
HSR	<i>hsf-1</i>	TTTGCATTTTCTCGTCTCTGTC	TCTATTTCCAGCACACCTCGT
UPR ^{MT}	<i>hsp-6</i>	GAGATCGTGGAACCGAAAGGA	CGGCATTCTTTTCGGCTTCCTT
UPR ^{MT}	<i>hsp-60</i>	CGGCATTCTTTTCGGCTTCCTT	CGTCGTTGCAGAGCTCAAGAAAG
UPR ^{MT}	<i>ymel-1</i>	CAAAACCTGATCTCGCTGGG	TTCTCAATGTCGGCTCCAGT
UPR ^{MT}	<i>clpp-1</i>	TGATAAGTGACCAAGTGCCA	TGATTCTGGAGTTCGGGAGA
UPR ^{MT}	<i>lonp-1</i>	CGATGATGGCCATTGTGCAG	CGCTTTGAAACATCAATTTTCATCCA
OxSR	<i>gst-4</i>	GATGCTCGTGCTCTTGCTG	CCGAATTGTTCTCCATCGAC
OxSR	<i>gst-6</i>	CCGAATTGTTCTCCATCGAC	TTTGGCAGTTGTTGAGGAG
OxSR	<i>gst-7</i>	TTTGGCAGTTGTTGAGGAG	TGGGTAATCTGGACGGTTTG
OxSR	<i>gcs-1</i>	TGGGTAATCTGGACGGTTTG	ATGTTTGCCTCGACAATGTT
OxSR	<i>skn-1</i>	GGACAACAGAATCCCAAAGG	TCAGGACGTCAACAGCAGAC
OxSR	<i>sod-3</i>	GTAACGGGCGATAGCATGAG	GCGAGAGCACATTGATGAC
OxSR	<i>ptps-1</i>	AATCGATTCTTTGGAGACC	CAATCTACTGCTCGCACTGCTCAAAGC
reference	<i>pmp-3</i>	TGGCCGGATGATGGTGTGCGC	ACGAACAATGCCAAAGGCCAGC
reference	<i>Y45F10.4</i>	CGAGAACCCGCGAAATGTCGGA	CGGTTGCCAGGGAAGATGAGGC
reference	<i>sap-49</i>	TGGCGGATCGTCGTGCTTCC	ACGAGTCTCCTCGTTCGTTCCA
reference	<i>tba-1</i>	TCAACACTGCCATCGCCGCC	TCCAAGCGAGACCAGGCTTCAG

Name of Material/ Equipment	Company
Antimycin A	Sigma-Aldrich
Bacto Peptone	Fisher Scientific
BD Difco granulated agar	VWR
Calcium chloride dihydrate	VWR
Carbenicillin	BioPioneer
Cholesterol	Sigma-Aldrich
COPAS Biosorter	Union Biometrica
COPAS Cleaning Solution	Union Biometrica
COPAS Sheath Solution	Union Biometrica
DMSO	Sigma-Aldrich
IPTG dioxane free	Denville Scientific
LB Broth Miller	Fisher Scientific
M205FA stereoscope	Leica
Magnesium sulfate heptahydrate	VWR
Paraquat	Sigma-Aldrich
Potassium Chloride	Fisher
Potassium phosphate dibasic	VWR
Potassium phosphate monobasic	VWR
Revolve	ECHO
Sodium Azide	Sigma-Aldrich
Sodium Chloride	EMD Millipore

Sodium phosphate dibasic	VWR
Tert-butyl hydroperoxide	Sigma-Aldrich
Tetracycline hydrochloride	Sigma-Aldrich
Tunicamycin	Sigma-Aldrich

Catalog Number	Comments/Description
A8674	for mitochondrial stress
DF0118072	for NGM plates
90000-782	for NGM plates
97061-904	for NGM plates
C0051-25	for RNAi
57-88-5	for NGM plates
350-5000-000	equipped with a 488 nm light source.
300-5072-000	to use with COPAS
300-5070-100	to use with COPAS
472301	solvent for drugs
CI8280-4	for RNAi
BP1426500	for LB
10450040	equipped with a Leica DFC3000G
EM-MX0070-3	for NGM plates, M9
36541	for oxidative/mitochondrial
P217-500	for bleach soluton
EM-PX1570-2	for NGM plates
EM-PX1565-5	for M9
75990-514	equipped with an Olympus 4x Plan Fluorite
71289-50G	for imaging
SX0420-5	for NGM plates, M9

71003-472	for M9
458139	for oxidative stress
T7660-5G	for RNAi
T7765-50MG	for ER stress

We thank the editorial team and all the reviewers for an exceptionally comprehensive review of the manuscript. We have put in considerable efforts to address all the points brought up by the editorial team and the reviewers, and believe this has significantly strengthened the manuscript. We believe that some points brought up by reviewer 3 were beyond the scope of this manuscript and reflected more what would be appropriate for a literature review, but have addressed the concerns to the best of our abilities for what would be appropriate for a protocol/methods manuscript.

Editorial comments:**General:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done.

2. Please ensure that the manuscript is formatted according to JoVE guidelines—letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Done.

3. Please avoid the use of personal pronouns (you, your, we).

Done.

4. Please include email addresses for all authors in the manuscript itself.

Done.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Sigma-Aldrich, Biometrica, BioSorter, etc.

Done – microscope names were still included. Should these be removed as well?

Protocol:

1. There are several long, non-instructional, paragraphs in the protocol; these should be moved to the Introduction, Results, or Discussion, as appropriate.

Done.

2. The 'Materials' sections are not necessary—all information there should be in the Table of Materials, in numbered protocol steps as instructions in the imperative, or possibly in separate tables.

All materials have been moved to Table 1, and all commercial product names have been removed within the manuscript and is available only in the table of materials.

3. The content 'Notes and Troubleshooting' should either be notes immediately after the relevant steps or be in the Discussion section.

All notes have been moved to their relevant sections.

4. There is a (roughly) 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing; see formatting guidelines above) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have attempted to minimize the protocol and highlighted sections as much as possible. Upon re-review, we will work directly with the editor (Phillip Steindel) to further decrease the length.

5. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Done.

Tables:

1. Please remove the embedded Tables from the manuscript. Please instead upload as .xls/.xlsx-formatted files.

All tables have been converted to xls format.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

This is confirmed.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this manuscript Bar-Ziv et al. present protocols for examining stress response and stress resistance in *C. elegans*. They describe how to use fluorescent reporter strains to quantify the mitochondrial unfolded protein response, the heat shock response and the ER unfolded protein response. They also provide protocols for examining resistance to mitochondrial/ROS, heat and ER stress. Overall, this is a well written, meticulously prepared manuscript that will be useful for those examining stress in *C. elegans*.

Major Concerns:

No major concerns.

Minor Concerns:

1. It would be important for the authors to note that it is possible for these reporters to be activated by

other pathways aside from the stress pathways being tested. For example *gst-4* can be activated by EGF signaling (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5190145/>).

A statement has been added to the discussion reviewing examples of where stress response reporters are not specific to each individual paradigm as a caution to readers.

2. The authors describe the use of the COPAS biosorter to quantify fluorescence in the fluorescent reporter strains. However, most laboratories do not have access to this equipment. It would be useful to provide a protocol for quantifying fluorescence from images of the worms. This can be done using free software such as ImageJ.

We have added a section for quantification using ImageJ.

3. The authors mention an increased sensitivity to stress for worms grown on the standard OP50 bacteria. It would be useful for the authors to provide a reference. If unpublished, perhaps the authors could add a figure comparing stress resistance in worms grown on OP50 versus HT115 as this would be useful information for the *C. elegans* community.

A reference is provided in the following sentence, which describes the increased stress sensitivity is due to vitamin B12 deficiency (Revtovich, A.V., Lee, R., Kirienko, N.V. Interplay between mitochondria and diet mediates pathogen and stress resistance in *Caenorhabditis elegans*. *PLOS Genetics*. **15** (3), e1008011, doi: 10.1371/journal.pgen.1008011 (2019).)

4. Line 135. Why is it recommended to transfer young animals? In a sick strain these could become sterile and fail to produce progeny.

A sentence has been added to chunk animals when dealing with sterility or sickness issues.

5. Line 252 and multiple other places. 3-4 days seems long for worms to reach adulthood. On OP50, WT worms typically develop from hatch to adult in just under 2 days. Is this slower development because of the RNAi plate? Is it because of the RNAi treatment? This would be worth mentioning.

In the WormAtlas, it is described that animals take about 65-96 hours to make it to Day 1 of adulthood, which we describe as “gravid adults” or “egg-laying maximal.” We have made it more clear in the protocol that this is what we describe as a Day 1 adult.

DEVELOPMENT AT DIFFERENT TEMPERATURES

	"16°C" (16.0 ± 0.3°C)	"20°C" (19.5 ± 0.5°C)	"25°C" (25.0 ± 0.2°C)
Egg laid	0 hr	0 hr	0 hr
Egg hatches	16-18 hr	10-12 hr	8-9 hr
First-molt lethargus	36.5 hr	26 hr	18.0 hr
Second-molt lethargus	48 hr	34.5 hr	25.5 hr
Third-molt lethargus	60.0 hr	43.5 hr	31 hr
Fourth-molt lethargus	75 hr	56 hr	39 hr
Egg-laying begins	~90 hr	~65 hr	~47 hr
Egg-laying maximal	~140 hr	~96 hr	~62 hr
Egg-laying ends	~180 hr	~128 hr	~88 hr
Length at first molt	360 µm	370 µm	380 µm
Length at second molt	490 µm	480 µm	510 µm
Length at third molt	650 µm	640 µm	620 µm
Length at fourth molt	900 µm	850 µm	940 µm
Length at egg- laying onset	1150 µm	1060 µm	1110 µm
Maximal egg- laying rate	5.4/hr	9.1/hr	8.1/hr
Total eggs laid	275	280	170

6. It was not always clear whether the superscript number referred to a note or a reference.

All notes were moved directly into their respective sections, and now all superscripts are references.

7. Line 272. "translocates" This is a bit misleading. ATFS-1 is in cytoplasm and MTS allows for import into mitochondria. Under stress, this import is blocked leaving ATFS-1 in cytoplasm. This allows NLS to bring ATFS-1 to nucleus.

The wording has been fixed.

8. Section 2.2.2.1 is essentially the same as 2.2.1.1 except the gene being knocked down is different. Perhaps the authors could just mention that the procedure is the same except for the RNAi clone.

Done.

9. Line 293-298 It was unclear why there is a specific materials section for this subsection but not the others. Could these materials be included in the list at the start of section 2?

All materials sections have been removed and have been turned into Table 1.

10. Line 343, 355 Why is there a 16-24 hour recovery for TBHP exposure, and 2 hour recovery for PQ exposure?

These are the times that we saw the most robust induction of *gst-4p::GFP*. It is possible that because TBHP is much more stable than PQ, there is a longer-term effect.

11. Line 351 "THBP" should be "PQ"

Done.

12. Line 357 It might be worth putting RNAi before compound exposure for consistency with previous sections.

Done.

13. Line 397 Is 100 mM correct? We use 10 mM which is sufficient to immobilize long enough to image.

Yes – we had a note that stated that sodium azide can be brought down to 10 mM and now this has been moved directly to the section.

14. Line 657 The time points for 34 degrees are noted in the main protocol while the critical time points for 37 degrees are found in the note. It might be worth having them in the same place.

Done.

15. Note 23 and note 33 are identical. Please check.

All notes have been removed.

16. Line 847 5 days should be 5 hours

Done.

17. Table 2. It would be useful to include a reference for each strain.

A reference for each strain is included both in the introduction and the references section.

18. Table 3. The duration of exposure is only included for heat stress. It would be useful to include this information for all.

Done.

19. In table 2 and table 3, *sod-3* is indicated as an output for the insulin-IGF1 signaling pathway. In table 5, *sod-3* is indicated as an output measure for OxSR mediated by SKN-1. This could be confusing to readers and should be clarified.

In Table 2, it has been put in as an output for both OxSR and insulin-IGF signaling. In table 3, the parentheses is to explain that *daf-2* is the gene encoding the insulin receptor. It is not describing what the *sod-3* reporter measures.

20. Figure 5. It would be helpful to label the stress on the figure e.g. paraquat, heat, TM.

Done.

Reviewer #2:

The authors have done a terrific job and a service to the field in assembling these assays and protocols to evaluate organelle-specific stress responses in nematodes. The protocols are beautifully described and will be of use to scientists with or without expertise in *C. elegans* techniques. The manuscript is beautifully written and comprehensive. Thus, I only have several minor suggestions.

Page 6. Please include a sentence indicating that the mechanism by which induction of *hsp-4::gfp* during ER stress is regulated (IRE-1 and XBP-1). The other stress response reporters indicate the regulators (SKN-1, ATFS-1, etc). filleted

We have described a more detailed mechanism of how each of the stress responses are activated in their respective sections of the introduction & results.

What is tag-335?

All RNAi recommendations have now been paired to a description of what the gene encodes and what the function of the protein is.

Line 368. Please use either HSF1 or HSF-1.

Everything has been standardized to HSF-1 (the *C. elegans* nomenclature).

Line 748. As mentioned, tunicamycin is expensive. DTT can be used as a less expensive alternative as it also induces the *hsp-4::gfp* reporter due to ER stress.

We have had very little success with DTT in inducing *hsp-4p::GFP* and thus have not included this method in our protocols.

The word "filleted" is used throughout and may be confusing as it is not clear to me what it means in context of the manuscript? A Google search definition indicates it means "to remove bones from "a fish."

This is a colloquial *C. elegans* term for lifespans and was originally defined during both of its uses in the manuscript. However, this word has been removed.

Reviewer #3:

The authors describe assays to study various stress responses in *C. elegans*, focusing on transcriptional readouts studied by means of GFP reporter assays and functional assays using population survival. The methods are fairly standard in the field and there aren't any major issues with them (see below for some specific questions/comments). The protocols provided herein are well described and clear, and should

be therefore be useful to a lab not familiar with them.

However, I'd like to authors to more specifically consider some of the general points discussed below; as is, the manuscript has adopted a fairly proteostasis centric view, and could better explain the non-linearity and exclusivity of transcription factors in gene regulation.

We understand that this protocol has taken a fairly proteostatic viewpoint, but this was intentional as describing all the functions of transcription factors in gene regulation is beyond the scope of this protocol.

- General (this is noted below in specific instances): The authors should clarify that their chosen reporters merely report on a fraction of the underlying response pathways and in no way reflect the organisms' complete response to a stress. I.e., hsp-4 is not equal to the UPR, gst-4 is not equal to the OSR, etc. Highlighting the organisms' response complexity and redundancy is an important point that needs to be cleared up. Otherwise, an uninformed reader may adopt an overly simplistic view of these stress responses. Further below, line 1010, text such as "can all be suppressed by knockdown of the primary transcription factors upstream" should be revised for that reason - the view of the "primary/master" regulator is outdated and a factor being genetically required to induced a gene in stress buy no means makes it "primary/master". Indeed, it's quite likely that many factors are required to induce the 4 reporter genes described here.

In the discussion, we have dedicated an entire section to describing the limitations of the reporters and alternative methods that can be used to supplement all reporter assays have been provided. We have adjusted all references to "primary" transcription factors to state either "one of the primary transcription factors" or "an important transcription factor."

- General; Throughout the manuscript, the authors appear to equate "stress responses" with "protein quality control" (e.g. p2 line 29, p2 line 44 and below, etc). The two terms should not be conflated. Please, state up front that proteostasis is merely a part of any one stress response, and may not be of equal importance in all stresses. Further, please clarify that stress response goes well beyond ensuring protein folding, modification, and degradation. More extensive referencing in the intro would help.

We have edited the introduction to reflect that this protocol is dedicated to protein quality control.

- Some statements are poorly supported by references. For example P2L53 and below, "Some cellular stress responses are not dedicated to a specific cellular compartment, but rather focus on dealing with a particular type of stress. For example, the OxSR serves to alleviate the toxic effects of reactive oxygen species (ROS)." Please provide citations that provide evidence that the OxSR (which is what, exactly, anyway?) is mounted in all cellular compartments. If not available, please temper this statement; the two possibilities are not mutually exclusive. Indeed, the whole paragraph is supported by only one citation; more extensive referencing would be appropriate.

This statement has been removed. OxSR has been described as oxidative stress response in its first mention, as have all the other stress responses.

- P3L69 revise "Additionally, the relatively quick physiological response to stress (between hours and a few weeks)" - weeks?

The word "weeks" has been removed.

- P4L1128: "1. Grow a culture of OP50 in LB or equivalent media of choice for 24-48 hours in ambient temperature (~22-25 °C)." Why? We always do this overnight at 37. Much easier. Is there is reason not to? Then please state so. Otherwise, suggest both, as preferred by the experimenter.

The reason is stated in the notes, which have now been included directly in the text.

- P6L158: "7. Invert the bleach and worm mixture for ~5 minutes (do not exceed 10 minutes). Vigorous shaking will help to dissolve worm carcasses faster and is recommended for optimal preservation of eggs." We find that many mutants, especially stress sensitive mutants (of interest here), are sensitive to bleaching. I'd suggest to the authors to suggest to readers that bleaching time may be shorter for mutants/transgenic strains.

Again, this was already in the notes, which have now been added directly into the text.

- P6L195, recipe/source for IPTG missing

The IPTG was in the table of materials, but we have also now included this in our new Table 1.

- P7L233 and below: the view described here of the UPR as solely a proteostasis response is outdated. It is now clear that the UPR also monitors ER membrane status. The authors should clarify this and reference pertinent papers, especially because used the same *hsp-4* reporter. By comparison, the authors' description of the UPR-mito below is more comprehensive in its inclusivity. Indeed, the authors observation of *hsp-4::gfp* expression fluctuating at various temperatures (L713) may be an indirect consequence of temperature induced lipid alterations (saturation).

We have modified our description of UPR^{ER} to include its role in ER membrane homeostasis (line 707). However, to keep within the scope of this JOVE collection, our manuscript focuses on the role of UPR^{ER} in protein homeostasis, as all of our assays utilize tunicamycin.

Our recent paper has shown that *hsp-4* is a direct readout for protein homeostasis as a downstream target of UPR^{ER}, and that other genes are involved in ER membrane status. Moreover, these two phenomenon can be distinctly separated (<https://www.biorxiv.org/content/10.1101/471177v1>). However, we focused this protocol on protein homeostasis and thus we believe addressing this is outside the scope of the manuscript.

- P7L239 and below: it is my understanding that *hsp-4* is a specific readout of the *ire-1/xbp-1* branch of the UPR, but isn't under control of *pek-1/atf-4* or *atf-6*. The authors should clarify this; it's important to delineate specificities of transcriptional reporters.

It had been described both in the introduction and the results section that *hsp-4* is a direct target of XBP-1.

- P7L255 and below, "2.2.2.2 Inducing ER stress using a chemical agent". The authors surprisingly describe a protocol using tunicamycin in liquid culture; a reason for this is not described, and other chemicals below are described as being used on solid media. The authors should clarify that solid/liquid worm culture transition may cause gene expression changes that may affect genes under study. They

should further clarify that many labs have used Tunicamycin in solid culture, reference pertinent papers, and describe the corresponding protocol. In addition, it is not clear to me what the 15-20hr or even o/n recovery step does - are animals that don't recover eliminated? If so that should be stated. Otherwise, what's the point of the recovery step?

We do not believe that it is within the scope of this manuscript to describe all the available methods to performing tunicamycin assays. We have provided both a liquid treatment assay and a solid agar survival assay. Moreover, we have provided a solid agar RNAi method. Finally, we have added an extra line within the protocol to describe that the tunicamycin reporter assay can also be performed on solid agar.

We use a 16-20 hour recovery because this is necessary to accumulate enough GFP to be visible. This has been explicitly stated in the text.

- P9L314 and below. The description of oxidative stress response is incomplete. It is especially surprising that the authors propose using TBHP as an inducer of the "SKN-1 response". Recent papers show that a substantial fraction of TBHP regulated genes are SKN-1 independent (and that *gst-4* is regulated by other TFs in oxidative stress); these papers should be cited and this clarified. Further, TBHP and paraquat are non-equivalent, which should be clarified.

We understand that TBHP and paraquat are not equivalent, and that is why both assays have been provided. Moreover, we had originally cited a paper that shows a much more comprehensive review of the protocols available for oxidative stress (Senchuk, M.M., Dues, D.J., Van Raamsdonk, J.M. Measuring Oxidative Stress in *Caenorhabditis elegans*: Paraquat and Juglone Sensitivity Assays. *Bio-protocol*. **7** (1), doi: 10.21769/BioProtoc.2086 (2017)). This reference has been moved to the introduction for ease of finding.

- P15L575: It's not clear to me why paraquat was chosen for oxidative stress assays when both PQ and TBHP were used to induce *gst-4*? TBHP is just as fine and has been used quite a bit for OSR studies. Please, again, clarify that PQ is merely one way of causing oxidative stress. A brief attempt to do this is made in L583, but please expand.

Again, a comprehensive overview of oxidative stress response assays have been provided in the introduction. It is out of the scope of this manuscript to provide this.

- P15L602 and below. I don't think the experiment described here is an appropriate readout of ER status. The concentration of 25 ng/μL tunicamycin seems very low. Does it really have an effect (life span effect in the figure could easily just represent short life span of *xbp-1* mutant)? In our hands, Tunicamycin wears off after a few days and any effects after that likely have nothing to do with ER stress, but rather simply reflect life span phenotypes, which may or may not be connected to stress response phenotypes. To truly monitor ER stress responses, higher concentrations should be used in my opinion. The fact that the other chemicals in 4.2 are used in a more classical, short-term stress survival assays would agree with this view. Also, P16L621 "5. At day 1, lifespans are "filleted". These are not (should not be, anyway) lifespans, but stress assays. Also, remove "filleted".

In our assay, the animals are always on tunicamycin plates. Therefore, they are always in the presence of the ER stress and the effect does not wear off. We have shown in previous work that *xbp-1* RNAi does not decrease lifespan in basal conditions. Thus, sensitivity of these *xbp-1* RNAi animals to tunicamycin (shorter survival compared to N2) is not due to them having an inherently short lifespan. Our data

presented here is not novel, but merely representative (e.g. Taylor, R.C., Dillin, A. XBP-1 is a cell-nonautonomous regulator of stress resistance and longevity. *Cell*. **153** (7), 1435–1447, doi: 10.1016/j.cell.2013.05.042 (2013).).

Due to the solubility of tunicamycin, we have found 25 ng/μL to be a high enough level without causing toxicity from DMSO concentration. We have gone as low as 5 ng/μL of tunicamycin and see a dose-dependent effect on lifespan and *hsp-4p::GFP* induction, and have only included the most robust concentration in this protocol for clarity and ease.

In addition, we have tested tunicamycin plates that were greater than 2 months old (stored in the dark at 4C), and found that these can induce *hsp-4p::GFP* and cause L1 arrest of larvae, giving us confidence that the plates we are using have long-term functionality – at least enough for a 15-day assay as we describe. We unfortunately have not been able to adapt a “short-term” tunicamycin protocol that is robust, although that would certainly be an interesting area of investigation for a future study.

- P16L629: Why is paraquat assay in liquid media? No justification is given. I see no reason not to do it on solid media.

We have attempted to do a paraquat assay on solid agar and had very inconsistent results with lower concentrations and extremely high censorship in higher concentrations. To avoid discouraging this assay, we have provided a reference that covers many of the available oxidative stress response assays.

- P17L716: The authors observation on starvation inducing ER stress is not novel; they should cite pertinent literature, particularly Jo H et al., *Cell Metab* 9: 440-448, 2009.

We have removed this statement from the text during revisions.

- P20L813: "chemical oxidant, TBHP, results in a milder, but still significant, activation of *gst-4p::GFP* (Figure 3)." The Blackwell lab has shown that arsenite produces a much higher induction of *gst-4*

Again, a reference for a full list of oxidative stress response assays has been provided.

- P25 table 2: "and bolded strains are those where representative data are provided in this manuscript." No strains were bolded. Further, the authors should provide citations for the *sod-3::gfp* and *T24B8.5p::GFP* strains.

This statement has been removed. Citations have been added.

- P30L1040: "Such information cannot be obtained from qRT-PCR or RNA-seq, as it uses whole worm extracts". Inaccurate. Please consider mentioning scRNA-seq and several methods for tissue-specific gene expression profiling (from Waterston, Moerman, Riddle, Kim, Murphy labs, most recently Kaletsky et al. *PLoS Genetics* 2018).

Citation has been added.

Reviewer #4:

Manuscript Summary:

Here, the authors describe a series of assays and tools for analyzing different QC systems using reporters in *C. elegans*. In addition to the detailed protocols for each assays, the authors are making extra effort to guide readers so that even non-nematodologists should be able to use these tools. Overall, this is an excellent and thorough resource for the community.

Major Concerns:

None

Minor Concerns:

There are part of the manuscript that are in highlighted yellow.

Apologies, this is solely for the editorial staff.

In Figure 5B, author should highlight that RNAi against *daf-2* is not necessarily as effective to show phenotypes as long-lived alleles of *daf-2*.

In our hands, the *daf-2* RNAi was sufficient to show major differences in phenotypes, and thus it was not essential to use the *daf-2* mutant.

It would be useful for authors to highlight the details of the protocol associated with lining up worms for imaging. This is a relatively challenging technique for beginners, but this method allows readers to clearly observe the reproducibility phenotypes (a variation across a cohort), which is quite valuable. This should hopefully become standard in the field and authors could highlight the benefits of this approach.

This is a very good point, and we have full intentions to capture the importance of this section in the video – unfortunately, this is a difficult thing to do in writing.

Reviewer #5:

Manuscript Summary:

In their manuscript „Measurements of physiological stress responses in *C. elegans*“, Bar-Ziv et al. describe routine protocols for examining proteotoxic stress responses in the nematode *C. elegans* in response to important environmental stressors, such as heat and oxidative stress. The authors are „key opinion leaders“ in the field of research into proteotoxic stress and aging in *C. elegans*, such that I expect great interest of new and established *C. elegans* labs in their precise protocols, which they normally do not/cannot share in their original publications. The level of detail is such that no extensive pre-existing knowledge in *C. elegans* methods/techniques is necessary to perform these protocols such that they will be extremely helpful for new undergraduate/graduate students, in particular when considering that a video will be provided in addition. Lastly, in their discussion, the authors point to complementary assays (e.g. qPCR) and highlight particular strength/limitations of the assays they describe, such that researchers can make an „informed decision“ on which assay to use. Accordingly, I only have very few specific comments/questions on the manuscript which I suggest to address before publication in JOVE (which I definitely recommend), with the intention to make the manuscript even more helpful to researchers new to *C. elegans*.

Major Concerns:

none

Minor Concerns:

Section 2.1: specify the number and stage of worms to be transferred on RNAi-plates and give a

recommendation at which stage/age worms should be examined; double-check step (5): do you intend to point the reader to section 2.2.1?

In Table 2, we have specified the number of eggs that can be transferred onto RNAi plates. Moreover, we have more accurately described the stages at which experiments should be performed – we describe “Day 1” adults as 65-96 hours of development, which is a “gravid” adult described by WormAtlas as an “egg-lay maximal” worm where the entire egg sac is full of eggs. We hope this would give some more direct guidance.

Section 2.2.1.1. The time window provided to give the reader a rough idea how long it takes for worms cultured at 20 °C to reach day 1 of adulthood is larger than 24 h. Why? I guess this is because some RNAi-constructs may slow down development. Please explain why you provide such a broad time window. Provide instructions on how to set up the experiment if worms develop at different speed (e.g. staggered plating or staggered analysis).

A section has been added to incorporate all the information for this.

Section 2.2.3. Ptypo: TBH should read TBHP

Done.

Section [2.2.2.2](#): The protocol provided assesses the ability to recover from ER-stress, rather than the activation of the ER-stress response, as „promised“ in the introduction to this section. Clarify.

We have added additional information that this specific assay requires recovery in order to detect the induction of *hsp-4*. We also offered an alternative method to induce *hsp-4*, which is a chronic tunicamycin exposure.

Section 2.2.3.1. and 2.2.4. Similar to comment on [2.2.2.2](#): recovery, rather than activation?

Again, the recovery time is required for GFP accumulation to detectable levels. All these notes have been added into the protocols in their respective sections.

Section 3: The COPAS-BioSort is not a standard-device in many labs. Provide a clear recommendation of how to quantitatively analyze images if such a device is not available, even though these alternatives are much lower throughput (e.g. manual outlining of worms in ImageJ)

We have added a section on quantification using ImageJ.

Section 4.1. Provide an idea of how long it takes for wildtype worms to die under the conditions described. For plate assays, why not use FUDR to avoid the additional stress to worms during frequent manual transfer? Also for plate assays, specify the number of worms per plate and the number of plates per condition, similar to how you specify the number of wells per condition and number of worms per well for the paraquat assay in liquid. For the assay in liquid (which my lab does not perform because of the particular concern that I want to rise here): are there cases (mutants/RNAi-treatments) where survival is that long that one has to fear that worms may experience starvation in addition to oxidative stress? Can this risk be reduced by on purpose transferring lots of bacteria into the well, together with the worms?

The average expected survival was added into the section. While FUDR might minimize stresses from manual transfer, it comes with its own set of issues. For all survival assays, suggested numbers of worms have been recommended. We are uncertain of whether starvation or the actual oxidative stress itself has a greater impact, but we have provided a reference that gives a comprehensive overview of all the pros and cons of oxidative stress response protocols in the introduction.

Comment on Table 2: In some studies it may be of interest to assess the effect of an RNAi-treatment of interest on multiple reporters. In this case, the reporter constructs need to be crossed into a common wildtype background.

All of the reporters are on the Bristol N2 background. This information has been added to Table 2 (now Table 3 in the revised version).

Comment on Table 5. For a qPCR-experiment, one also needs reference genes; thus, for reasons of completeness, also recommend specific reference genes and primers.

We apologize for this oversight and reference genes have been added.

Reviewer #6:

Manuscript Summary:

The manuscript by Bar-Ziv, Frakes et al. describe protocols related to the assessment of stress responses using the model organism *C. elegans*, including the ER UPR, mitochondrial UPR, cytoplasmic heat shock response and the oxidative stress response. A thorough set of methods have been provided including growth conditions of *C. elegans* and bacterial cultures, imaging techniques, conditions to induce each stress response, quantification of stress response reporter strength using either small sample sizes (microscopy) or larger quantifications using a COPAS biosorter. The authors also provide physiological assays to compliment the readouts obtained using the stress reporters. Sufficient resources are also provided for the listed protocols including transgenic *C. elegans* strain IDs, microscopy settings, qPCR gene primer sets, and manufacturer information. The discussion also offers sufficient considerations to be made when using stress reporters as readouts of these cellular pathways as well as suggestions to further validate findings derived from these type of experiments. Overall, this is a very well written and thorough overview describing this method.

Major Concerns:

None.

Minor Concerns:

Two minor changes are suggested:

1. Line 568: I would suggest changing "...tunicamycin, which blocks N-linked glycosylation and subsequent accumulation of misfolded proteins in the ER" to "...tunicamycin, which blocks N-linked glycosylation causing accumulation of misfolded proteins in the ER."

Done.

2. Line 738 and 766 seem to be identical statements which can be condensed into one.

All the notes have been removed and placed into their appropriate sections, thus resolving this issue.