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TITLE:
Tissue-Specific RNAi Tools to Identify Components for Systemic Stress Signaling

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SUMMARY:
Maintenance of organismal proteostasis requires the coordination of protein quality control responses such as chaperone expression from one tissue to another. Here, we provide tools used in *C. elegans* that allow monitoring of proteostasis capacity in specific tissues and determine intercellular signaling responses.

ABSTRACT:
Over the past decade, regulation of protein quality control processes went through a transformation that unveiled the importance of intercellular signaling processes to regulate cell-nonautonomous proteostasis. Recent studies are now beginning to uncover signaling components and pathways that coordinate protein quality control from one tissue to another. It is therefore important to identify mechanisms and components of the cell-nonautonomous proteostasis network (PN) and its relevance for aging, stress responses and protein misfolding diseases. In the laboratory, we use genetic knockdown by tissue-specific RNAi in combination with stress reporters and tissue-specific proteostasis sensors. We describe methodologies to examine and to identify components of the cell-nonautonomous PN that can act in tissues perceiving a stress condition and in responding cells to activate a protective response. We first describe how to generate hairpin RNAi constructs for constitutive genetic knockdown in specific tissues and how to perform tissue-specific genetic knockdown by feeding RNAi at different life stages. Stress reporters, and behavioral assays then function as a valuable readout, that allow for the fast screening of genes and conditions modifying systemic stress signaling processes. Finally, proteostasis sensors expressed in different tissues are utilized to determine changes in the tissue-specific capacity of the PN at different stages of development and aging. Thus, these tools should help clarify and allow monitoring the capacity of PN in specific tissues, while helping to identify components that function in different tissues to mediate cell-nonautonomous PN in an organism.

INTRODUCTION:

Cellular proteostasis is monitored by an intricate network of protein quality control components such as molecular chaperones, stress responses and degradation mechanisms including the ubiquitin proteasome system (UPS) and autophagy[1,2]. The activation of stress response pathways, such as the HSF-1 mediated heat shock response (HSR), the unfolded protein response of the endoplasmic reticulum (UPR^{ER}) and the mitochondria (UPR^{mito}) is vital for cellular adaptation to and survival during environmental challenges or protein misfolding disease that lead to toxic protein aggregation[1–6].

Cellular proteostasis is coordinated by an additional layer in multicellular organisms that requires the orchestration of cellular stress responses across different tissues to activate protective protein quality control components such as molecular chaperones[7]. In the past decade, cell nonautonomous activation of "cellular" stress response pathways has been observed for the heat shock response (HSR), the UPR^{ER} and the UPR^{mito}, as well as transcellular chaperone signaling (TCS)[3,4,7–10]. In each case, the nervous system as well as signaling from the intestine plays a crucial role in controlling the activation of chaperones across tissues, to protect against the toxic consequences of acute and chronic protein misfolding stresses[3,5,9,11]. This transmission from the neurons to the intestine and other cells in the periphery can be achieved by neurotransmitters as is the case for the UPR^{ER} and the HSR[6,8,11]. In one form of cell nonautonomous stress signaling, TCS, that is activated by the increased expression of HSP-90 in the neurons, secreted immune peptides play a role in the activation of *hsp-90* chaperone expression from the neurons to the muscle[5]. In another form of TCS, reducing the expression of the major molecular chaperone *hsp-90* in the intestine leads to an increased expression of heat-inducible *hsp-70* at permissive temperature in the body wall muscle[5,10]. In this particular case, the specific signaling molecules activated in the stress-perceiving intestine and the responding muscle cells are, however, unknown.

Thus, in order to identify how chaperone expression is activated from one tissue to another, an approach is required that allows to monitor the capacity of the proteostasis network (PN) and stress response activation at the tissue-specific level. To investigate which stress response pathway is activated in the individual tissues, an available selection of transcriptional chaperone reporters fused to fluorescent protein tags can be utilized (see also **Table 3**). These include fluorescently tagged *hsp-90, hsp-70* and *hsp-16.2* transcriptional reporters that indicate the induction of the HSR, *hsp-4* that indicates the activation of the UPR^{ER} and *hsp-6*, indicating the UPR^{mito}. Combination of these reporters with a tissue-specific stress condition then allows a powerful read-out that will pin-point individual tissues responding to an imbalance of the PN in a distal "sender" tissue perceiving the stress. To induce a stress condition or imbalance of the PN in a specific tissue, different approaches can be taken. For example, one such approach is by ectopic expression of the activated form of a stress transcription factor (e.g., *xbp-1s*) and another one is by reducing the expressing levels of an essential molecular chaperone (e.g., *hsp-90*) using tissue-specific promoters[8,10]. To deplete PN components in only one cell type, tissue-specific knockdown by RNAi is a useful tool.

In *C. elegans*, RNAi is however systemic; double stranded RNA in the environment can enter and spread throughout the animal to silence a targeted gene[12,13]. This systemic spread of ingested dsRNA is mediated by SID (systemic RNAi defective) proteins, such as SID-1 and SID-2 proteins that are dsRNA transporters, as well as SID-5, that colocalizes with late endosome proteins and is implicated in the export of ingested dsRNA[14–16]. SID-1 is a multi-pass transmembrane protein in all cells (except neurons) and required for dsRNA export as well as import into cells[17]. SID-2 expression is restricted to the intestine where it functions as an endocytic receptor for ingested dsRNA from the intestinal lumen into the cytoplasm of intestinal cells[16]. Neurons lack a response to systemic RNAi, and this correlates with reduced expression of the transmembrane protein SID-1 in neurons, that is essential for dsRNA to be imported[15,18]. Thus, for tissue-specific RNAi to be effective in only one cell-type, the systemic spread of dsRNA needs to be prevented. This can be achieved by utilizing the RNAi-resistant *sid-1(pk3321*) mutant that prevents the release and uptake of dsRNA across tissues[15]. Expression of a tissue-specific hairpin RNAi construct in this mutant or the ectopic expression of SID-1 in a specific tissue can then complement the function of mutant *sid-1* and will allow for tissue-specific RNAi [19].

So how is dsRNA ingested by the intestine in a *sid-1* loss of function mutant and how can it then reach neurons or muscle cells that ectopically express a SID-1 construct? In one current model explaining this mechanism, endocytosed dsRNA is taken up into the intestinal cytoplasm via SID-2 and then exported into the pseudocoelom by another SID-1 independent mechanism, involving SID-5 and transcytosis[17]. Thus because SID-1 is required for dsRNA import[17], only cells expressing wild type SID-1 will be able to take up the dsRNA released from the intestine into the pseudocoelom.

Here we demonstrate the use of a set of tools that allow for tissue-specific RNAi. We use the example of the molecular chaperone Hsp90 to describe the construction of hairpin RNAi that can be useful to constitutively knock down gene expression in a specific tissue[10]. The described approach could be used for any target gene of interest. The response of other tissues to the proteostasis imbalance caused by tissue-specific *hsp-90* RNAi can be probed by monitoring the expression of fluorescently tagged stress reporters in other tissues. As a second method for tissue-specific RNAi, we demonstrate how the *sid-1* mutant system can be adapted for feeding RNAi bacteria rather than expression of a hairpin RNAi construct. This can be useful when performing a candidate or genome-wide RNAi screen to identify components required for a tissue-specific response. Likewise, developmental defects associated with depletion of a vital PN component will require RNAi-mediated knockdown in specific tissues at later stages of development. We demonstrate how a SID-1 complementation system can be used on a candidate RNAi screen for tissue-specific TCS modifiers. In the example, we aim to identify signaling components that upon knockdown in the "stress-perceiving" sender tissue (intestine) and the stress effecting tissue (muscle) lead to the changed expression of a fluorescently tagged *hsp-70* reporter in muscle cells.

PROTOCOL:

1. Tissue-specific RNAi in two ways: Hairpin RNAi and tissue-specific SID-1

complementation

1.1. Generation of hairpin RNAi constructs for tissue-specific expression in *sid-1* mutants

1.1.1. Amplify the target gene sequence (e.g., *hsp-90* sequence isolated from the *hsp-90* RNAi clone from the Ahringer RNAi library[20]) by PCR. Place a nonpalindromic sequence at the 3' end of the *hsp-90* sequence, that is a SfiI site (ATCTA) [21].

NOTE: The primers used for cloning the *hsp-90* with the SfiI sequence (underlined) are:
as-hsp90-SfiI 5'-GGCCATCTAGGCCCTGGGTTGATTTCGAGATGCT-3'
as-hsp90 5' TCATGGAGAACTGCGAAGAGC-3'.

1.1.2. Subclone the amplified sequence into the commercial cloning kit (e.g., TOPO pCR BluntII).

1.1.3. Isolate the inverted *hsp-90* sequence from the *hsp-90* RNAi clone (Ahringer RNAi library)[20] by restriction digestion using XbaI and PstI restriction sites and place it downstream of the *hsp-90-SfiI* sequence in the vector (from step 1.1.1), resulting in an *hsp-90* hairpin construct (**Figure 1**).

1.1.4. Subclone the hairpin construct into a Gateway entry vector pDONR221 and fused with Gateway entry clones that contain tissue-specific promoters for either expression in neurons (*rgef-1p);* in the intestine *(vha-6p);* or the bodywall muscle *(unc-54p*) and the *unc-54 3'UTR* (or any other 3'UTR of choice) in a Gateway reaction as described in the protocol of the supplier.

1.1.5. Linearize the resulting hairpin RNAi constructs (**Figure 1**) using a unique restriction site outside the coding sequence and microinject as a complex array at a concentration of 1 ng/µL hairpin RNAi construct, mixed with 100 ng/µL N2 Bristol genomic DNA (digested with ScaI) into a *C. elegans* strain expressing the *hsp-70p::RFP* reporter (strain AM722) and crossed into the genetic background of *sid-1(pk3321)* mutants (strain NL3321). For a protocol on how to perform microinjection of complex arrays please follow[22].

1.1.6. As a negative control, use empty vector hairpin constructs expressing the nonpalindromic SfiI containing sequence (GGCCATCTAGGCC) under control of a tissue-specific promoter.

1.1.7. Use the increased *hsp-70p::RFP* expression of the reporter as a readout to score positive transformants expressing *hsp-90* hairpin RNAi (**Figure 3**). For a more general approach to verify the tissue-specific knock-down of any gene of interest, measure whole animal mRNA levels using qRT-PCR of the gene of interest.

1.1.8. Integrate the extrachromosomal array of the resulting strain expressing the intestine-specific *hsp-90* hairpin construct (PVH2; see Table 1) by gamma-irradiation. For integration of the extrachromosomal arrays into the genome, please see[22].

1.2. Tissue-specific SID-1 expression to allow for tissue-specific RNAi by feeding dsRNA-

expressing bacteria

1.2.1. Subclone the *sid-1* genomic DNA from vector TU867 (*unc-119p::SID-1*)[19] into the Gateway entry vector pDONR221. Primers for cloning of *sid-1* DNA can be found in[19]. Fuse the *sid-1* pDONR221 construct with Gateway entry clones containing muscle- (*myo-3p*) or intestine- (*vha-6p*) specific promoters and the *unc-54 3'UTR* (or any other 3'UTR of choice) in the Gateway reaction as described before in 1.1.4.

1.2.2. Microinject the resulting *vha-6p::SID-1::unc-54 3'UTR* or *myo-3p::SID-1::unc-54 3'UTR* constructs at a concentration of 30 ng/µL together with a red fluorescent pharyngeal co-injection marker (e.g., *myo-2p::RFP*; 5 ng/µL) into *sid-1(pk3321)* mutants.

1.2.3. Integrate the extrachromosomal intestine- or muscle-specific *sid-1* arrays into the genome as described in[22]. Here, this resulted in strains PVH5 [*myo-3p::SID-1; myo-2p::RFP];sid-1(pk3321) and* PVH65 [*vha-6p::SID-1; myo-2p::RFP];sid-1(pk3321).*

1.2.4. For neuron-specific expression of *sid-1* in the *sid-1(pk3321)* mutant, use strain TU3401 *uIs3401[unc-119p::SID-1; myo-2p::RFP];sid-1(pk3321)* that was generated previously by Calixto et al.[19].

1.2.5. As mentioned in 1.1.7, ensure tissue-specific knockdown of the gene of interest by measuring mRNA levels of the desired target gene by qRT-PCR. Alternatively, confirm tissue-specific RNAi sensitivity by using a fluorescent protein (e.g., GFP or RFP) expressed in the same tissue and treat worms with GFP or RFP RNAi. Expose nematodes to GFP/RFP RNAi as synchronized L1 stage larvae and grow on the RNAi bacteria until Day 1 of adulthood (**see Figure 2**). In our case, we used strains expressing SID-1 in the neurons, muscle or intestine and crossed into strains expressing HSP-90::RFP in neurons (AM987), in the intestine (AM986) and in the muscle (AM988).

2. Using stress reporters and proteostasis sensors to monitor cell autonomous and cell nonautonomous proteostasis

NOTE: To monitor PN capacity in specific tissues, use tissue-specific proteostasis sensors (such as strains expressing Q44 in the intestine or Q35 in the muscle – see **Table 3**) and stress reporters (such as the heat-inducible *hsp-70p::mCherry* reporter; **Table 3**).

2.1. Genetically crossing the *sid-1 (pk3321)* mutant allele into a proteostasis sensor strain and confirming the presence of *sid-1(pk3321)* by feeding RNAi

2.1.1. Genetically cross the proteostasis sensor/stress reporter strain into the genetic background of the *sid-1 (pk3321)* mutant strain. To establish genetic crosses between different transgenic strains, please follow [23] for a detailed protocol.

NOTE: *sid-1(pk3321*) mutants are resistant to feeding RNAi and hence treatment of embryos with

RNAi against an essential gene (such as *elt-2* or *hsp-90*) will only lead to developmental arrests or larval lethality in strains heterozygous or wildtype for the *sid-1* gene.

2.1.2. Let 10 gravid hermaphrodites lay eggs on RNAi plates against *elt-2* or *hsp-90* and control (empty vector; EV) RNAi plates at 20 °C. Remove the mothers after 1 - 2 h. Use N2 Bristol and the *sid-1(pk3321)* mutant as controls.

2.1.3. Observe development of the larvae on the RNAi plates over the next 2-3 days. *elt-2* RNAi will result in L1 larval arrest, while *hsp-90* RNAi results in L3 larval arrest in N2 Bristol. *sid-1* mutants will be unaffected by the RNAi treatment and will develop into gravid adults.

NOTE: *C. elegans* homozygote for *sid-1(pk3321)* will show a uniform population developing into adulthood. Heterozygotes will be indicated by mixed populations of some animals showing larval arrest, and some animals developing into adults.

2.2. Confirming the presence of *sid-1(pk3321)* by genotyping

2.2.1. Pick 15-20 worms of the selected candidate F2 strain into a PCR tube containing 15 µL of Worm Lysis Buffer (**Table 2**).

2.2.2. Place the tube at -80 °C for at least 10 min or overnight.

2.2.3. Incubate the tube in the PCR machine using the following program:
65 °C for 60 min (lyse worm); 95 °C for 15 min (inactivate Proteinase K); hold at 4 °C.

2.2.4. Use 2 µL of the worm lysate as a "template" to perform the PCR reaction for genotyping, using the following primers for *sid-1*: *sid-1 forw*: 5'-agctctgtacttgtattcg-3' and *sid-1 rev:* 5'-gcacagttatcagatttg-3'.

2.2.5. Use the following program for PCR genotyping: 1 cycle at 95 °C for 3 min; then 30 cycles of 95 °C for 10 , 55 °C for 30 s, and 72 °C for 30 s; 1 cycle at 72 °C for 10 min, hold at 4 °C.

2.2.6. Purify the ~650 bp PCR product using a PCR purification kit (**Table of Materials**) and sequence the *sid-1* PCR product to identify the G-to-A point mutation of the *sid-1 (pk3321)* allele. Alternatively, the G-to-A point mutation creates an ApoI restriction site, which can be used on the PCR product for genotyping as described in [24].

2.3. Using *iQ44::YFP* as a proteostasis sensor for the intestine

2.3.1. Synchronize *C. elegans* expressing Q44::YFP in the intestine (strain OG412) or crossed into the *sid-1(pk3321)* mutant background by bleaching, following the protocol described in [25]. Plate synchronized L1 larvae onto a 9 cm nematode growth media (NGM)-agar plate containing OP50 bacteria and grow until L4 stage at 20 °C.

2.3.2. Collect L4 animals by washing worms off the plate using 5 mL of M9 buffer. Transfer the M9 buffer containing L4 worms to a 15 mL tube using a glass pipette or a siliconized plastic pipette, and centrifuge at 1000 x *g* for 1 min at room temperature to gently pellet the worms. Remove the supernatant carefully, ensuring to leave the worm pellet undisturbed.

2.3.3. Critical Step: To transfer or plate out nematodes use a glass pipette or a plastic pipette tip that was treated with a siliconizing agent (e.g., SigmaCote) following the manufacturer's instruction. This prevents the sticking of worms to the plastic surface of a pipette tip.

2.3.4. Repeat step 2.3.2 three more times to wash off all OP50 bacteria from the worms.

2.3.5. Take up the worm pellet in 5 mL of M9 buffer and count the number of worms present in 10 µL.

2.3.6. Plate L4 animals on 6 cm NGM Agar plates containing empty vector control (EV) or *hsp-90* RNAi bacteria at a density of 10 worms per plate (prepare 5 plates per time point and biological replicate) and incubate for 24 -48 hours at 20 °C.

2.3.7. After 24 hours (=Day 1 adults) and 48 hours (=Day 2 adults) count the number of Q44 foci in the intestines of nematodes exhibiting aggregates. Score a total of at 30-50 nematodes per biological replicate.

3. Tissue-specific candidate RNAi screen for modifiers of cell nonautonomous proteostasis

NOTE: For the tissue-specific RNAi screen we used strain PVH172 allowing for intestine-specific RNAi by feeding RNAi bacteria and strain PVH171 allowing for muscle-specific RNAi (see Table 1 for genotype).

3.1. Preparation of the candidate RNAi plates

3.1.1. Prepare 6 cm NGM agar plates supplemented with 100 µg/mL ampicillin, 12.5 µg/mL tetracycline and 1 mM IPTG according to standard methods[25].

3.1.2. Use the Ahringer RNAi library to obtain the candidate RNAi clones for the RNAi screen[20].

3.1.3. Inoculate 3 mL of LB-amp media (50 µg/mL ampicillin in LB media) in at 15 mL tube with the desired RNAi clone using a plastic pipette tip. Grow at 37 °C overnight with agitation.

3.1.4. The next day add Isopropyl-b-D-thiogalactopyranosid (IPTG) (from a 1 M stock) to a final concentration of 1 mM in the bacterial overnight culture.

3.1.5. Agitate the cultures for a further 3 h at 37 °C.

3.1.6. Plate 300 µL of bacterial RNAi culture onto a 6 cm NGM agar plate supplemented with 100 µg/mL ampicillin, 12.5 µg/mL tetracycline and 1 mM IPTG. Let the plates dry on the bench for 2 days at room temperature, covered with aluminum foil to protect from light. Once dry, the RNAi plates can be stored in a box at 4 °C for several weeks.

3.2. Synchronization of *C. elegans* and treatment with RNAi bacteria

3.2.1. To synchronize worm strains, pick 15 gravid adults onto RNAi plates and allow to lay eggs for 1 h. Then remove the adults from the plate.

Crticial step: Synchronization by bleaching is avoided in this case, because it can induce the *hsp-70p::RFP* reporter, as it is a stressful condition for *C. elegans*.

3.2.2. Pick synchronized L4 stage larvae and transfer to a fresh RNAi plate.

3.2.3. Allow nematodes to grow on the relevant RNAi for two generations to ensure efficient uptake of dsRNA, making sure the temperature is kept at 20 °C.

3.2.4. For imaging and *hsp-70p::RFP* fluorescence quantification, use Day 1 adults.

3.3. Preparation of microscope slides

3.3.1. Prepare the microscope slides by placing ~250 µL of a 2% agarose solution (in M9 buffer) onto a glass microscope slide and a second slide place on top to create a flat disc.

3.3.2. Place 5 µL of 5 mM Levamisole solution (in M9 buffer) on the set agarose pad and transfer 5 Day 1 adult worms into the Levamisole drop. Leave the nematodes to paralyze for 5 min.

3.3.3. Once *C. elegans* are paralyzed, carefully align with a platinum wire pick and remove excess levamisole with a laboratory wipe before addition of a coverslip.

3.3.4. **Critical step:** Ensure to take images of the worms within 30 minutes after preparation of the microscope slides. Paralyzed nematodes on the microscope slide can dry out and burst, which can compromise the fluorescence measurements.

3.4. Microscope settings and image analysis

NOTE: Images are obtained using a confocal microscope equipped with an EM-CCD camera and a microscopy image automation & image analysis software.

3.4.1. Take images at 10x magnifications using a 561 nm laser for RFP fluorescence excitation. Ensure all images are taken using the same settings for laser power, pinhole size and fluorescence gain to enable comparisons.

3.4.2. Save all images as TIFF files.

3.4.3. Perform image analysis using ImageJ. Measure fluorescence intensity in each image as pixels per unit area, with background fluorescence subtracted. Normalize fluorescence intensity for each image to the image area as well as the length of the worms.

3.4.4. Measure the mean intensity using **Analyze | Measure** in ImageJ. Normalize the resulting intensity value to the image area by dividing the intensity by area.

3.4.5. To normalize the intensity to worm length, measure the worm by drawing a line along the length of the worm in ImageJ and using **Analyze | Measure**. The reason for normalizing fluorescence intensity to worm length, is that worms can vary in size, dependent on the gene that is knocked down by RNAi, and this could affect the mean intensity.

3.4.6. Normalize the measured fluorescence intensities to untreated controls (i.e., transgenic *C. elegans* grown on control (EV) RNAi plates). Pool the normalized values to compare mean fluorescence intensities for each RNAi condition. Aim to image 20 worms per biological replicate and collect at least 3 biological replicate images.

3.4.7. Calculate P-values of the mean fluorescence intensity values using student's t test and perform a correction for multiple testing using the Benjamini-Hochberg method, using a false discovery rate of 0.01.

REPRESENTATIVE RESULTS:

Tissue-specific RNAi in two ways: Expression of hairpin constructs or tissue-specific SID-1 complementation

Expression of tissue-specific hairpin RNAi constructs allows for constitutive knockdown of a gene throughout development. However this can sometimes be impractical when the surveyed gene is required for organogenesis of that particular tissue, such as *elt-2* which is required for development of the intestine[26]. Tissue-specific SID-1 expression in the RNAi-resistant *sid-1* mutants has the particular advantage that tissue-specific gene knockdown can be timed at later stages of development. In both cases (for the expression of a hairpin construct or tissue-specific SID-1 complementation), the efficiency of the tissue-specific RNAi needs to be validated to confirm that only the targeted tissue is affected by RNAi. This is accomplished by co-expressing a fluorescently tagged protein such as HSP-90 fused to RFP (HSP-90::RFP) in different tissues.

We genetically crossed *sid-1(pk3321)* mutants alone or *sid-1* mutants expressing SID-1 in either neurons, intestine or bodywall muscle into *C. elegans* expressing HSP-90::RFP in the neurons (**Figure 2A**), intestine (**Figure 2B**) or the muscle (**Figure 2C**). The resulting strains were treated with *hsp-90* RNAi at L4 stage for 24 hours and HSP-90::RFP expression in specific tissues was examined by fluorescence microscopy.

HSP-90::RFP^{neuro} animals expressing SID-1 in the neurons (*unc-119p::SID-1*) exhibit reduced expression of HSP-90::RFP in neurons of the magnified tail region (**Figure 2A**). Likewise, *HSP-*

90::RFP^{int} animals expressing SID-1 in the intestine (*vha-6p::SID-1*) show reduced HSP-90::RFP expression in the intestine as expected (**Figure 2B**), whereas *sid-1(pk3321)* mutants expressing HSP-90::RFP in the intestine are unaffected by feeding *hsp-90* RNAi. Intestinal HSP-90::RFP expression also remains unaffected in animals expressing SID-1 in the neurons or the bodywall muscle, indicating that dsRNA is not spreading from the muscle or the neurons to the intestine (**Figure 2B**). Conversely, *HSP-90::RFP*^{muscle} animals expressing SID-1 in the muscle (*myo-3p::SID-1*) exhibit reduced HSP-90::RFP expression in the muscle during *hsp-90* RNAi, while HSP-90::RFP levels are unaffected in worms expressing SID-1 in the neurons or intestine (**Figure 2C**).

Using a stress reporter for a tissue-specific candidate RNAi screen

Reducing the function or expression of the major molecular chaperone HSP-90 induces the heat shock response and HSP-70 chaperone expression [27]. Tissue-specific *hsp-90* RNAi in the *C. elegans* intestine results in a strong upregulation of *hsp-70* in the muscle, as well as other tissues, as indicated by the stress-inducible *hsp-70* reporter (*hsp-70p::RFP*) (**Figure 3A**)[10]. *hsp-70* is a heat-inducible chaperone, and thus no expression can be observed in animals grown at permissive temperature (20 °C) (**Figure 3A**)[10,28]. Exposure to heat stress (35 °C) induces expression of *hsp-70*, as indicated by RFP expression of the reporter in the pharynx, spermatheca, intestine and bodywall muscle (**Figure 3A**). This can be quantified, by measuring RFP fluorescence intensity using Image J software (**Figure 3B**), as described in step 3.3 of the protocol section.

Constitutive knockdown of *hsp-90* in the intestine by *hsp-90* hairpin RNAi activates TCS [10] and results in a 20-fold upregulation of the *hsp-70p::RFP* reporter in primarily the bodywall muscle cells (but not detectably in other tissues) at permissive temperature (**Figure 3A,3B**).

To identify components that trigger the TCS-mediated induction of *hsp-70* expression from the intestine to the muscle, we performed a tissue-specific candidate RNAi screen of 58 candidate genes (see **Figure 4A** for an experimental flow chart). The candidate genes were identified in a preceding forward genetic screen and transcriptome analysis as potential modifiers of TCS in the *hsp-90*^{int} *hp-RNAi* strain (**Figure 4**); and consisted of components involved in cellular signaling processes, such as kinases, transcription factors and membrane proteins. We next wanted to determine in which tissue the candidate genes were acting as enhancers or suppressors of TCS-induced *hsp-70* expression in the muscle. This is achieved by measuring reduced (enhancer) or increased (suppressor) *hsp-70p::RFP* fluorescence intensity of the reporter.

To perform the tissue-specific RNAi screen we first genetically crossed the *hsp-90*^{int} *hp-RNAi* strain into *C. elegans* expressing SID-1 in either intestine or muscle. Intestinal SID-1 expression allows to screen for potential TCS signaling components acting in the intestine to mediate TCS, which is the tissue that perceives stress as reduced levels of *hsp-90*. Likewise, muscle specific SID-1 expression allows for screening TCS components required in the responding muscle tissue. The 58 candidate genes used for the tissue-specific RNAi screen were termed *txt (tcs-(x)cross-tissue)*. Animals were grown on RNAi plates for two generations until Day 1 of adulthood and *hsp-70p::RFP* fluorescence intensity in the muscle was measured by ImageJ software. As shown in **Figure 4**, RNAi-mediated knockdown of 58 candidate *txt* genes in the intestine (**Figure 4B**) or the muscle (**Figure 4C**) resulted in a range of modifiers that either suppress or enhance *hsp-70*

induction in the muscle. RNAi of candidates that result in a significant increase of *hsp-70p::RFP* fluorescence intensity indicate that the gene acts as a cell nonautonomous suppressor of TCS, whereas a reduction of RFP fluorescence intensity indicates that the candidate gene functions as an enhancer. The scored hits (enhancers/suppressors) can then be confirmed by measuring their effect on endogenous *hsp-70* mRNA levels by qRT-PCR and using proteostasis sensors.

Use of proteostasis sensors to monitor tissue-specific PN capacity.

TCS-mediated induction of *hsp-70* expression protects against protein misfolding and aggregation in a cell nonautonomous manner[5,10]. Proteostasis sensors can be used to survey the folding capacity in different tissues during stress conditions. These include endogenous, metastable proteins such as for example a conditional (temperature-sensitive; *ts*) mutant of myosin expressed exclusively in the bodywall muscle (*unc-54(ts)*)[29] or proteins containing expanded stretches of glutamine (PolyQ)[30–32] (See **Table 3** for a list of strains). Proteins within a length of 35-40 glutamines are particularly useful for this purpose, as they aggregate in an age- and stress-dependent manner and are thus highly suitable to report on the folding environment in specific tissues. These include strains expressing Q40::YFP in the neurons or Q35::YFP in the muscle and Q44::YFP in the intestine[30–32]. In addition to using PolyQ aggregation as a read-out, strains expressing Q40::YFP or Q35::YFP also exhibit an age-dependent motility defect[30], allowing quantification of motility by measuring thrashing rates in an automated manner (see [33] for a detailed example).

Here, we co-expressed intestinal Q44::YFP [32] in strains allowing for tissue-specific RNAi via SID-1 complementation. RNAi-mediated knockdown of *hsp-90* at L4 stage in the neurons, intestine or bodywall muscle, which induces TCS [10], resulted in a reduced accumulation of intestinal Q44 aggregates in Day 2 adults compared to control animals (**Figure 5**). Thus, this indicates that the TCS-mediated cell nonautonomous upregulation of *hsp-70* expression protects against age-associated protein misfolding in multiple tissues of *C. elegans*.

FIGURE AND TABLE LEGENDS:

Figure 1. Hairpin RNAi for constitutive gene knockdown in specific tissues. (**A**) The inverted repeats of *hsp-90* are generated by head-head ligation through a *SfiI* site (blue) introduced at one end of each repeat. The inverted repeats are under control of a tissue-specific promoter for either muscle- (*unc-54p*), neuron- (*rgef-1p*) or intestine- (*vha-6p*) specific expression. (**B**) The tissue-specific expression of the inverted *hsp-90* repeats will produce hairpin-loop RNA that induces tissue-specific RNAi in a strain with a *sid-1(pk3321)* mutant genetic background.

Figure 2. Tissue-specific expression of SID-1 to enhance tissue-selective RNAi-mediated knockdown. (**A**) Overexpression of HSP-90::RFP in the neurons of RNAi-resistant *sid-1(pk3321)* mutants. Expression of SID-1 in the neurons (*unc-119p::SID-1*) (strain PVH16); in the intestine (*vha-6p::SID-1*) (strain PVH17); and muscle (*myo-3p::SID-1*) (strainPVH18) enhances RNAi sensitivity in these specific tissues. Animals were exposed to *hsp-90* RNAi bacteria after L4 stage for 24 hours, leading to visibly reduced neuronal-specific HSP-90::RFP fluorescence intensity in the *unc-119p::SID-1* expressing animals only. Neurons in the tail region of the nematodes are magnified. (**B**) Overexpression of HSP-90::RFP in the intestine of RNAi-resistant *sid-1(pk3321)*

mutants. Expression of SID-1 in the neurons (*unc-119p::SID-1*) (strain PVH19); in the intestine (*vha-6p::SID-1*) (strain PVH20); and muscle (*myo-3p::SID-1*) (strain PVH21) enhances RNAi sensitivity in these specific tissues. Animals were exposed to *hsp-90* RNAi bacteria after L4 stage for 24 hours, leading to visibly reduced intestine-specific HSP-90::RFP fluorescence intensity in the intestine of *vha-6p::SID-1* expressing animals only. (**C**) Overexpression of HSP-90::RFP in the bodywall muscle of RNAi-resistant *sid-1(pk3321)* mutants. Expression of SID-1 in the neurons (*unc-119p::SID-1*) (strain PVH22); in the intestine (*vha-6p::SID-1*) (strain PVH23); and muscle (*myo-3p::SID-1*) (strain PVH24) enhances RNAi sensitivity in these specific tissues. This is indicated by a visibly reduced HSP-90::RFP fluorescence intensity in the muscle of *myo-3p::SID-1* expressing animals, but not in *unc-119p::SID-1* or *vha-6p::SID-1* expressing animals or control animals (*sid-1(pk3321)*) that are resistant to RNAi in all tissues. Yellow arrows indicate the red fluorescent pharyngeal co-injection marker (*myo-2p::RFP*). Scale bar = 50 mm.

Figure 3. Expression of intestine-specific *hsp-90* hairpin RNAi induces the heat-inducible *hsp-70p::RFP* reporter at permissive temperature in the muscle. (**A**) Flow chart demonstrating the tissue-specific RNAi screening protocol using stress reporters and tissue-specific proteostasis sensors. (**B**) Fluorescent microscope images of animals expressing the *hsp-70* promoter fused to red fluorescent protein (RFP) and in the background of *sid-1(pk3321)* mutants (control) (strain AM994). Animals were either grown at 20°C (no HS) or treated with 1-hour heat shock at 35°C (HS) and allowed to recover for 6 hours post-HS. *hsp-90^{intestine} hp-RNAi* animals (strain PVH2) express an *hsp-90* hairpin RNAi construct under control of the intestine-specific promoter (*vha-6p*) in the genetic background of *hsp-70p::RFP;sid-1(pk3321)*. Scale bar = 100 mm (**C**) Quantification of RFP fluorescence intensity of control animals grown at 20°C (no HS), treated with a 1h HS at 35°C (HS) or expressing intestine-specific *hsp-90* hairpin RNAi at permissive temperature (20°C). Bar graphs represent the average of 3 biological replicates; error bars represent S.E.M. P-values were calculated using student's t test. *P < 0.05.

Figure 4. Tissue-specific RNAi screen to identify modifiers of transcellular chaperone signalling. (**A**) Intestine-specific RNAi screen by feeding dsRNA bacteria to *hsp-90^{intestine} hp-RNAi* animals expressing *vha-6p::SID*-1 (strain PVH172; see Table 1 for genotype). Shown are candidate genes that act as potential modifiers of TCS (***txt* for intestinal tcs-(x)cross-tissue**) by either suppressing or enhancing the *hsp-70p::RFP* fluorescence intensity in the bodywall muscle when knocked down in the intestine. (**B**) Muscle-specific RNAi screen by treating *hsp-90^{intestine} hp-RNAi* animals expressing *myo-3p::SID*-1 (strain PVH171; see **Table 1**) with *txt* candidate gene RNAi. (**A and B**) Fluorescence intensity of candidate genes are indicated as gray bars and are normalized to control (EV) RNAi which is indicated as a black bar. Error bars are S.E.M. of 5 biological replicates. The statistical significance of decreased or increased RFP fluorescence intensity between *txt* gene RNAi compared to empty vector (EV) control RNAi was calculated using student's t test, and correction for multiple testing was performed using the Benjamini-Hochberg method with a false discovery rate of 0.05. * P < 0.05.

Figure 5. Tissue-specific *hsp-90* RNAi reduces intestinal *Q44::YFP (iQ44)* aggregation. (**A & B**) Expression of intestinal Q44::YFP in the background of RNAi-resistant *sid-1(pk3321)* mutant allele

(strain PVH228) leads to accumulation of Q44 foci by Day 2 of adulthood. RNAi-mediated knockdown by feeding *E. coli* expressing *hsp-90* dsRNA from L4 stage onwards is ineffective compared to control RNAi (EV). (**C & D**) Neuron-specific (strain PVH229), (**E & F**) intestine-specific (strain PVH230) or (**G & H**) muscle-specific *hsp-90* RNAi (strain 231) leads to reduced accumulation of iQ44 foci at Day 2 of adulthood. (**B, D, F, H**) Quantification of the number of Q44 foci in worms exhibiting age-dependent Q44 aggregation at Day 1 and Day 2 of adulthood. Error bars are S.E.M of 3 biological replicates. Statistical significance between *hsp-90* and empty vector (EV) RNAi at Day 1 or Day 2 of adulthood was calculated using a student's t test. *n.s*. not significant; *P < 0.05; **P < 0.01.

Table 1. List of strains used in this work.

Table 2. Worm Lysis Buffer.

Table 3. List of proteostasis sensor- and stress reporter strains.

DISCUSSION:

The methods described here demonstrate the use of tools that allow for the tissue-specific knockdown of PN components in a constitutive and temporal manner. We have previously identified TCS, a cell nonautonomous stress response mechanism that is induced by tissue-specific alteration of Hsp90 expression levels[10]. Tissue-specific knockdown of *hsp-90* by expression of hairpin RNAi leads to cell nonautonomous upregulation of protective *hsp-70* chaperone expression in distal tissues, that increases organismal stress resistance[10]. We however do not know which signaling components in the stress-perceiving or responding tissue are activated to initiate this protective response. To identify signaling components mediating this process, tissue-specific reverse genetic screens are one of the important methods of choice.

Although tissue-specific knockdown by expression of a hairpin construct can be effective, this has disadvantages when a larger number of genes needs to be surveyed. Using RNAi-resistant *sid-1* mutants complemented by expression of SID-1 in intestine, neurons or muscle allows for tissue-specific gene knockdown by feeding RNAi and is thus an amenable tool for tissue-specific genetic screens. While we here described a small-scale RNAi screen of 58 candidate genes, the tissue-specific SID-1 system can be adapted for larger scale or genome-wide RNAi screens. For this, *C. elegans* growth in a 96-well plate format and automated scoring of fluorescence intensity by a plate reader will be required.

While the *sid-1* system can be effective for tissue-specific RNAi, an alternative method takes advantage of *rde-1*, an Argonaute protein that functions cell-nonautonomously to mediate systemic RNAi capacity[13]. Tissue-specific promoters driving *rde-1* rescue constructs also allow for RNAi to be effective in specific tissues, similar to the *sid-1* system [34]. However, *rde-1* mutations used in the *rde-1* system rely on a RDE-1 E411K missense mutation that may not completely abrogate RDE-1 function and so could lead to leakiness of RNAi activity in other tissues[34,35] . This issue however seems to be eliminated by the use of a newly generated *rde-1(mkc36)* indel mutation[35]. A particular current advantage of the *rde-1* system compared to the *sid-1* system is

the recent adaptation of the *rde-1* system for specific and effective RNAi in the germline[35]. This is important, as other currently existing germline-specific RNAi strains can also exhibit RNAi efficiency in the soma. Thus, the *rde-1* system allowing for germline RNAi could be a useful tool for researchers investigating the importance of the germline in various biological processes, such as for example aging research.

This method is based on multicopy expression of integrated tissue-specific SID-1 arrays. To achieve more physiological expression levels of SID-1 in the specific tissues, a CRISPR-Cas9 mediated single-copy knock-in method at defined genomic loci could be adapted for future use of the *sid-1* system and to express SID-1 under control of tissue-specific promoters[36].

To investigate stress pathway activation, one has the choice of a large selection of transcriptional chaperone reporters fused to green or red fluorescent proteins (**Table 3**). Tissue-specific (intracellular) stress as opposed to environmental stress, may also lead to a differential tissue expression profile of chaperone reporters, as shown by the results in **Figure 3**. For example, while heat stress leads to induction of the *hsp-70p::RFP* reporter in multiple tissues (muscle, spermatheca, pharynx, intestine), *hsp-90* hairpin RNAi in the intestine results in strong upregulation of *hsp-70* in the muscle (**Figure 3A**). This may indicate that muscle cells are more sensitive to changes in cell nonautonomous *hsp-90* levels, however it cannot be excluded that *hsp-70* is also induced in other tissues, albeit not visibly with the transcriptional reporter fused to a red fluorescent protein.

Therefore, proteostasis sensors are an important alternative, as they report on the actual folding environment or capacity of the PN in a specific tissue. The folding environment is not only dependent on chaperone expression, but also on PN components that regulate clearance of misfolded protein such as autophagy or UPS. For example, if enhanced folding capacity is indicated by one of the well-established folding sensors, but this does not overlap with increased chaperone expression, then this may suggest that other components of the PN are activated that increase proteostasis in a specific tissue. For example *hsp-4* is primarily induced in the intestine when the cell nonautonomous UPR is activated in the neurons, yet the accumulation of misfolded proteins expressed in muscle cells is also suppressed, possibly via lysosome activating signals from the intestine[3]. Likewise, the data shows that *hsp-90* RNAi in the intestine delays iQ44 aggregation in the intestine (**Figure 5**), even though expression of the *hsp-70* reporter was not detected in the same tissue (**Figure 3**). Thus in addition to folding sensors that report on the folding environment in a given tissue, reporters for autophagic flux such as *Cherry::GFP::LGG*-1[37] or reporters that indicate the activity of the UPS such as *UbG76V::Dendra2*[38] expressed in different tissues are just as crucial.

Taken together, we have described a tissue-specific RNAi system that allows for the examination of the PN capacity in different tissues in response to a cell nonautonomously activated stress response mechanism.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

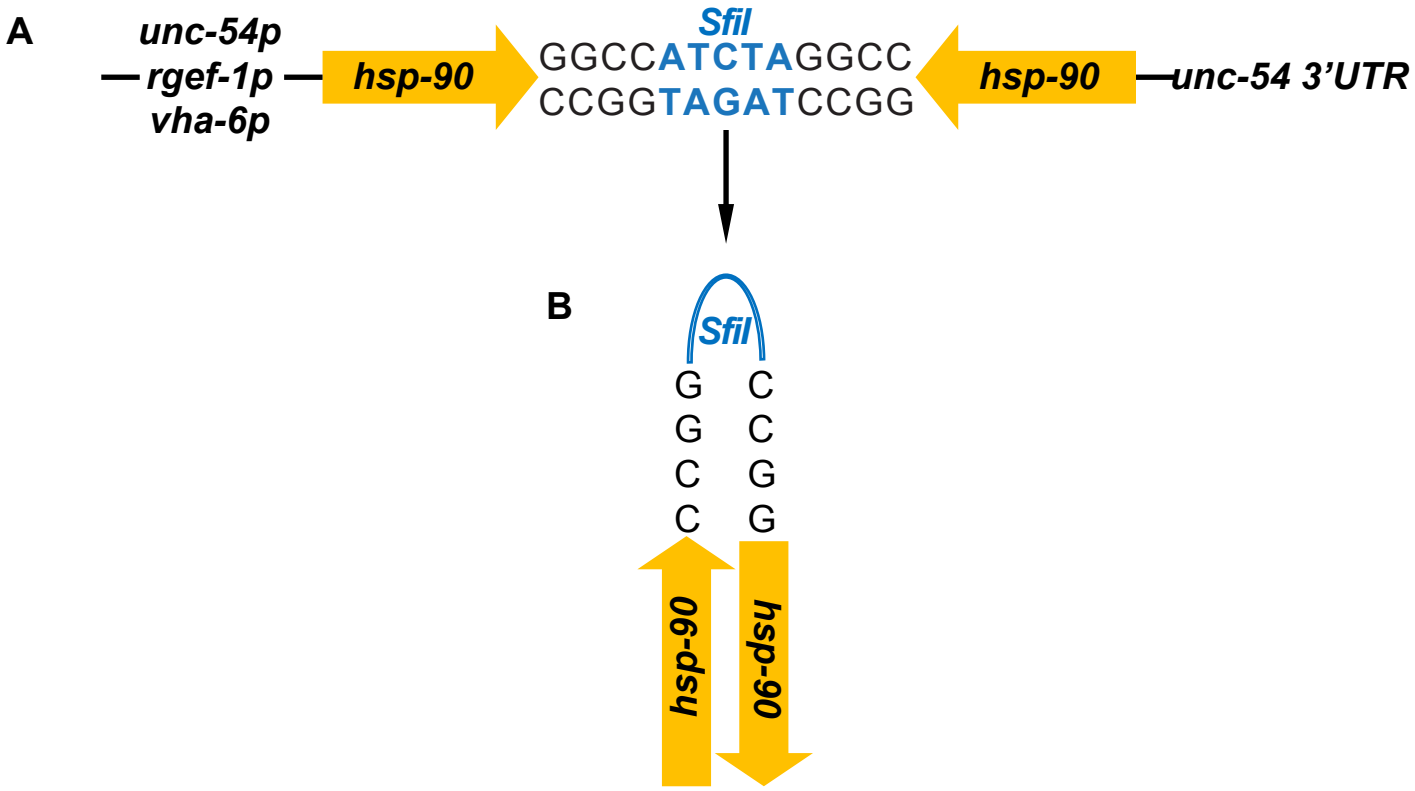

Figure 2

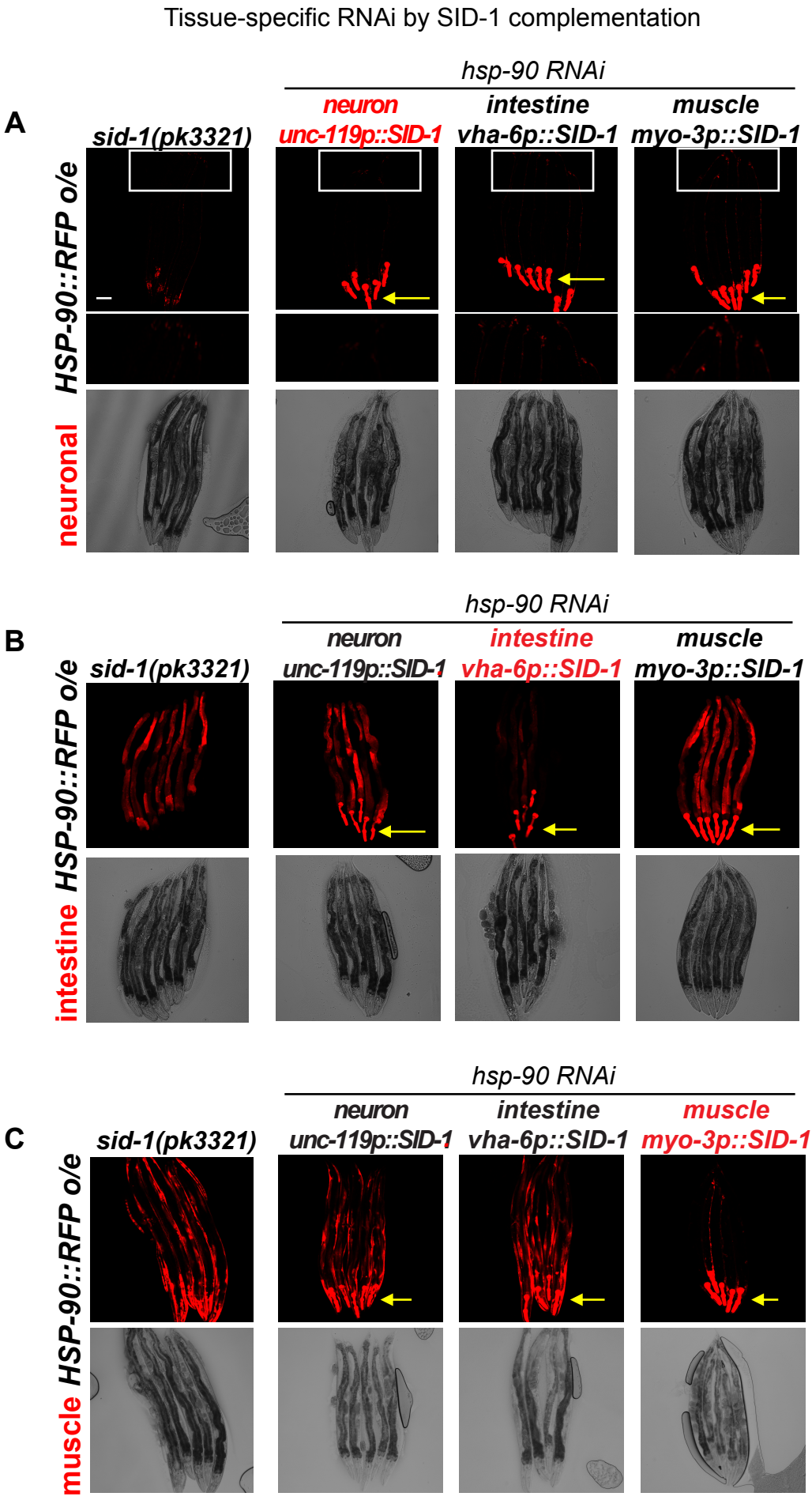

Figure 3

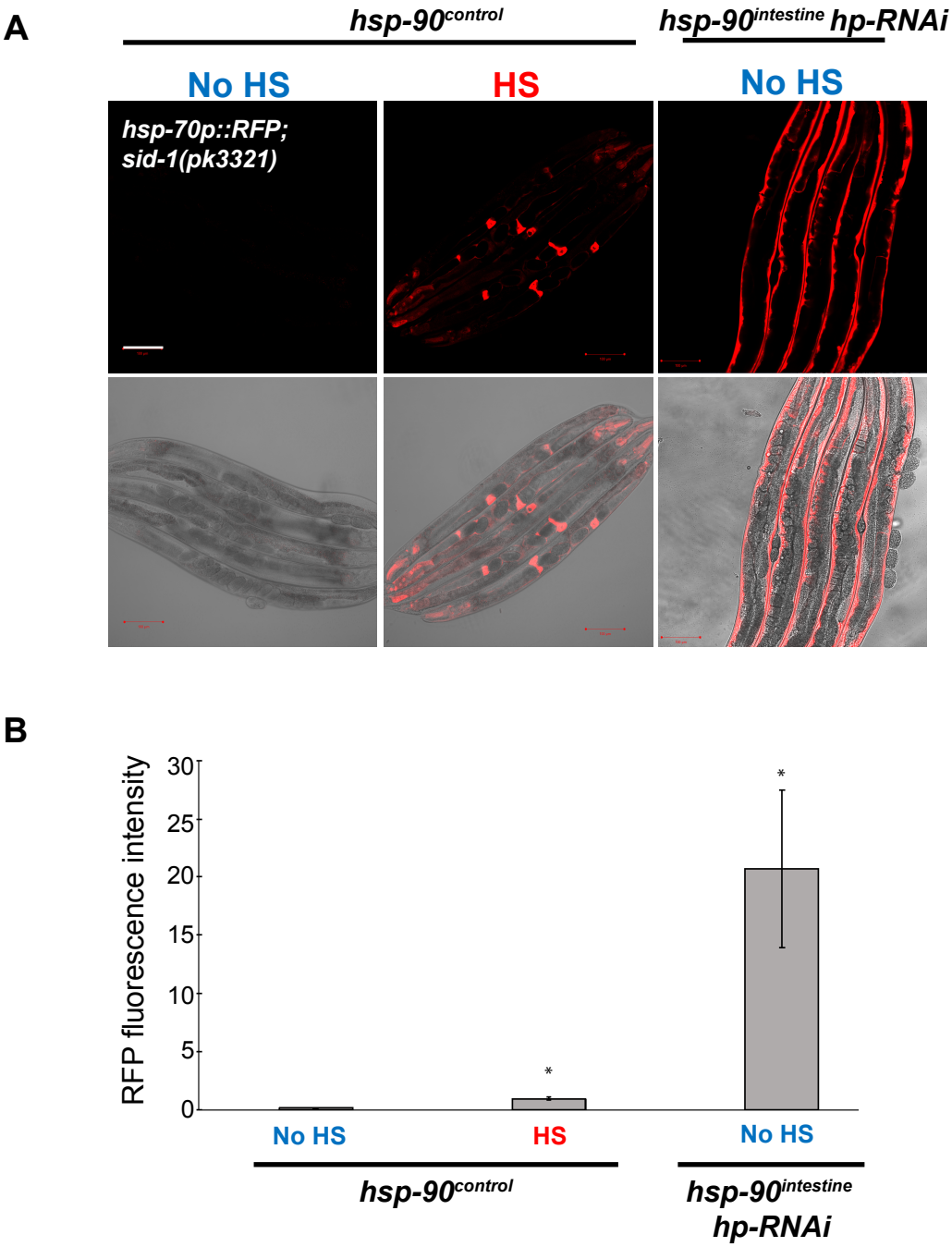

Figure 4

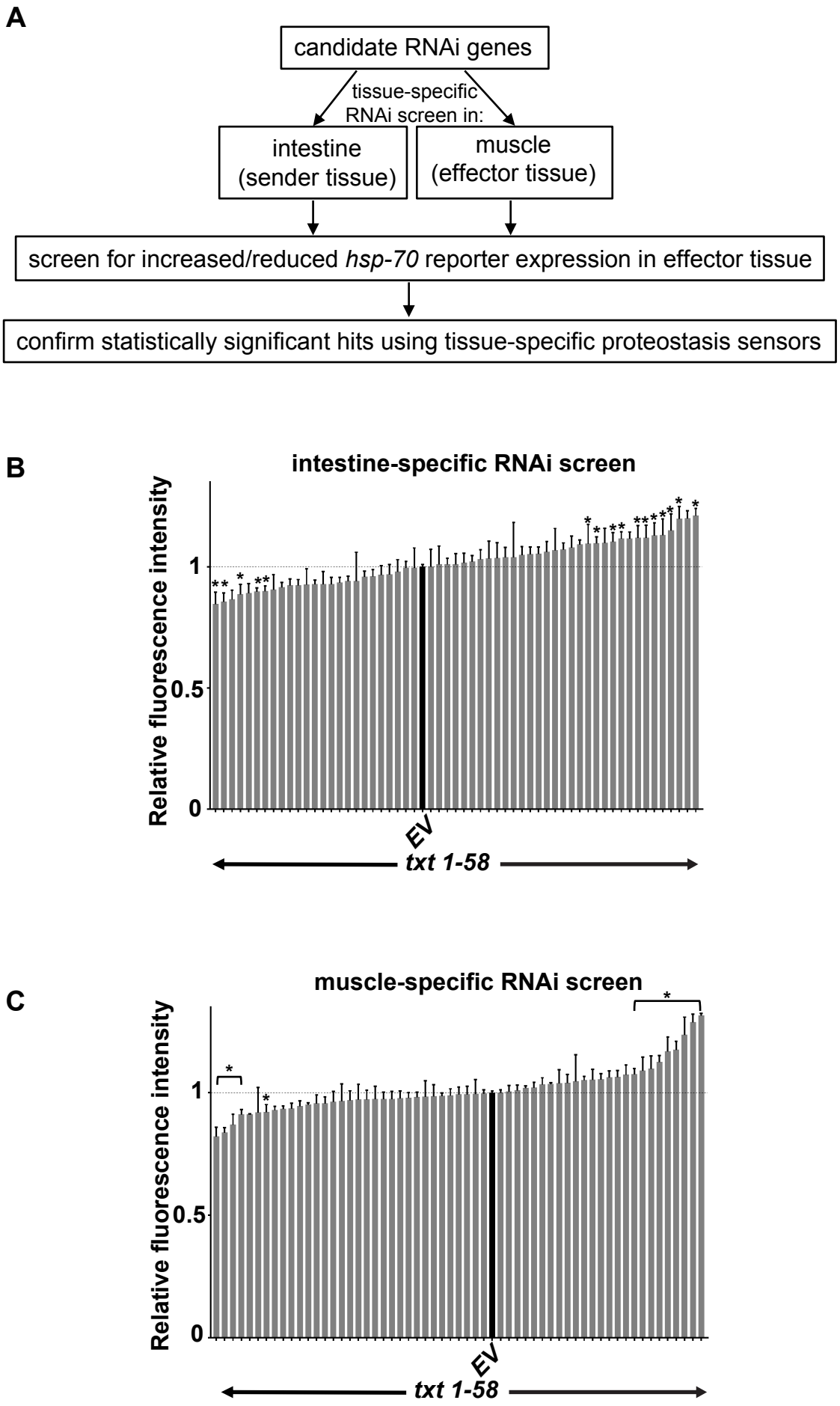

Figure 5

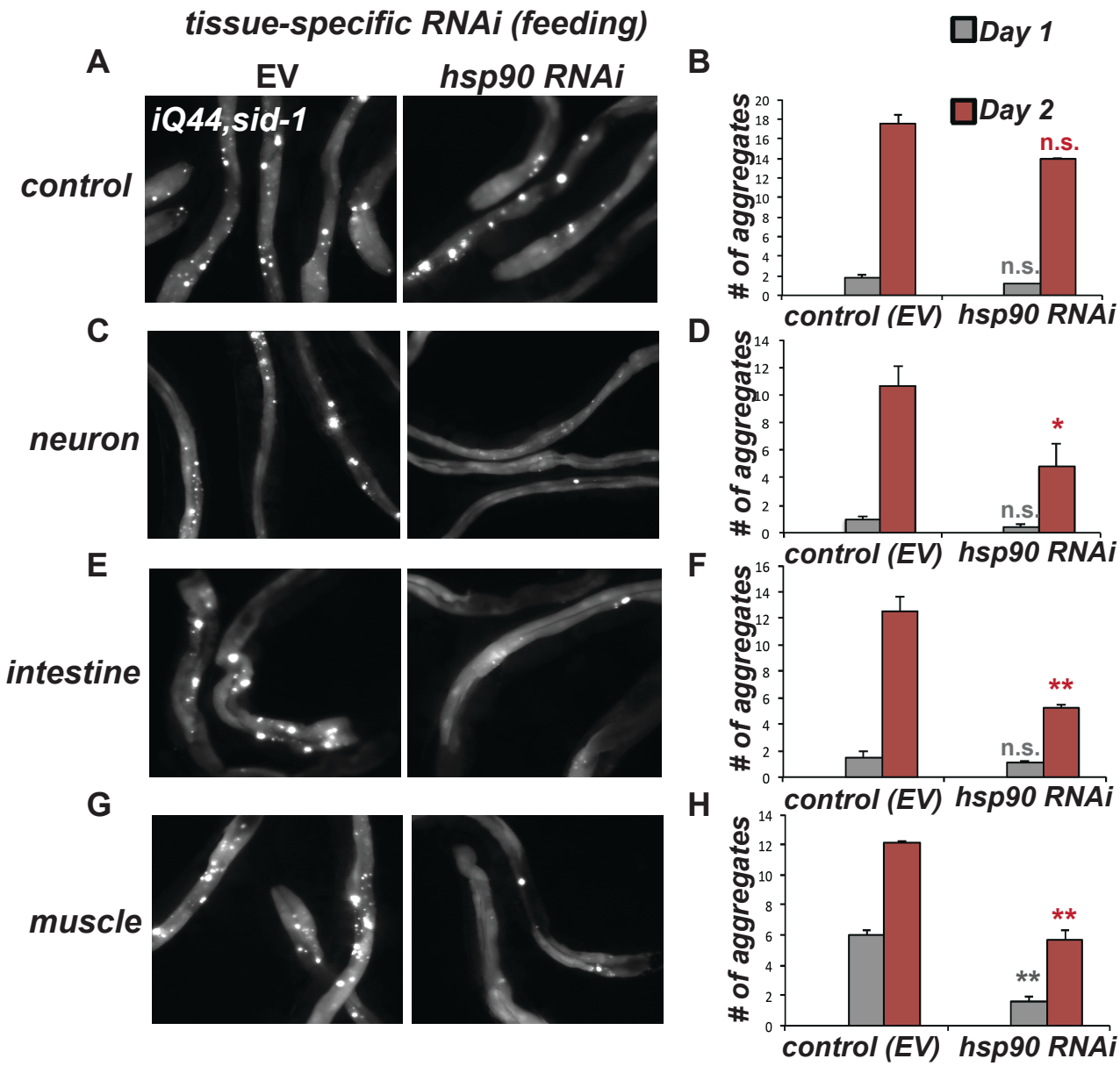

Table 1

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Strain name	Genotype	Source
N2	wild type	CGC
AM986	*rmIs346[vha-6p::HSP-90::RFP]*	PVO lab
AM987	*rmIs347[rgef-1p::HSP-90::RFP]*	PVO lab
AM988	*rmIs347[unc-54p::HSP-90::RFP]*	PVO lab
TU3401	*uIs69 [pCFJ90 (myo-2p::mCherry) + unc-119p::sid-1]; sid-1(pk3321)*	CGC
PVH2	*pccIs002[vha-6p::hsp-90 RNAi::unc-54 3'UTR]; rmIs288[hsp-70p::mCherry; myo-2p::CFP]; sid-1 (pk3321)*	PVO lab
PVH5	*pccIs005[myo-3p::SID-1::unc-54 3'UTR +myo-2p::RFP]; sid-1 (pk3321)*	PVO lab
PVH65	*pccIs004[vha-6p::SID-1::unc-54 3'UTR + myo-2p::RFP]; sid-1(pk3321)*	PVO lab
NL3321	*sid-1 (pk3321)*	CGC
AM722	*rmIs288[hsp-70p::mCherry; myo-2p::CFP]*	Morimoto lab
AM799	*hsp-90p::GFP*	Morimoto lab
OG412	*vha-6p::Q44::YFP*	CGC
PVH228	*vha-6p::Q44::YFP; sid-1(pk3321)*	PVO lab
PVH171	*sid-1 (pk3321); rmIs288 (myo-2p::CFP; C12C8.1p::mCherry); pccIs002 (vha-6p::hsp-90 RNAi::unc-54 3'-UTR); pccIs005 (myo-3p::SID-1::unc-54 3'UTR; myo-2p::RF*	PVO lab
PVH172	*sid-1 (pk3321); rmIs288 (myo-2p::CFP; C12C8.1p::mCherry); pccIs002 (vha-6p::hsp-90 RNAi::unc-54 3'-UTR); pccIs004 (vha-6p::SID-1::unc-54 3'UTR; myo-2p::RFP*	PVO lab
PVH26	*rmIs345[F25B3.3p::HSP-90::RFP]; rmIs317[hsp-90pr::GFP; pCeh361]; sid-1(pk3321)*	PVO lab
PVH16	*rmIs345[F25B3.3p::HSP-90::RFP]; rmIs317[hsp-90pr::GFP; pCeh361]; sid-1(pk3321); uIs69 [pCFJ90 (myo-2p::mCherry) + unc-119p::sid-1]*	PVO lab
PVH17	*rmIs345[F25B3.3p::HSP-90::RFP]; rmIs317[hsp-90pr::GFP; pCeh361]; sid-1(pk3321); pccIs004[vha-6p::SID-1::unc-54 3'UTR + myo-2p::RFP]*	PVO lab
PVH18	*rmIs345[F25B3.3p::HSP-90::RFP]; rmIs317[hsp-90pr::GFP; pCeh361]; sid-1(pk3321); pccIs005[myo-3p::SID-1::unc-54 3'UTR +myo-2p::RFP;*	PVO lab
PVH14	*rmIs346[vha-6p::HSP-90::RFP]; rmIs317[hsp-90pr::GFP; pCeh361]; sid-1(pk3321)*	PVO lab
PVH19	*rmIs346[vha-6p::HSP-90::RFP]; rmIs317[hsp-90pr::GFP; pCeh361]; sid-1(pk3321); uIs69 [pCFJ90 (myo-2p::mCherry) + unc-119p::sid-1]*	PVO lab
PVH20	*rmIs346[vha-6p::HSP-90::RFP]; rmIs317[hsp-90pr::GFP; pCeh361]; sid-1(pk3321); pccIs004[vha-6p::SID-1::unc-54 3'UTR + myo-2p::RFP*	PVO lab

PVH21	*rmIs346[vha-6p::HSP-90::RFP]; rmIs317[hsp-90pr::GFP; pCeh361]; sid-1(pk3321); pccIs005[myo-3p::SID-1::unc-54 3'UTR +myo-2p::RFP*	PVO lab
PVH15	*rmIs347[unc-54p::HSP-90::RFP]; rmIs317[hsp-90pr::GFP; pCeh361]; sid-1(pk3321)*	PVO lab
PVH22	*mIs347[unc-54p::HSP-90::RFP]; rmIs317[hsp-90pr::GFP; pCeh361]; sid-1(pk3321) uIs69 [pCFJ90 (myo-2p::mCherry) + unc-119p::sid-1]*	PVO lab
PVH23	*rmIs347[unc-54p::HSP-90::RFP]; rmIs317[hsp-90pr::GFP; pCeh361]; sid-1(pk3321); pccIs004[vha-6p::SID-1::unc-54 3'UTR + myo-2p::RFP*	PVO lab
PVH24	*rmIs347[unc-54p::HSP-90::RFP]; rmIs317[hsp-90pr::GFP; pCeh361]; sid-1(pk3321); pccIs005[myo-3p::SID-1::unc-54 3'UTR +myo-2p::RFP*	PVO lab
AM994	*rmIs288[hsp-70p::mCherry; myo-2p::CFP]; sid-1 (pk3321)*	PVO lab
PVH229	*vha-6p::Q44::YFP; sid-1(pk3321);uIs69 [pCFJ90 (myo-2p::mCherry) + unc-119p::sid-1]*	PVO lab
PVH230	*vha-6p::Q44::YFP; sid-1(pk3321);pccIs004[vha-6p::SID-1::unc-54 3'UTR + myo-2p::RFP*	PVO lab
PVH231	*vha-6p::Q44::YFP; sid-1(pk3321);pccIs005[myo-3p::SID-1::unc-54 3'UTR +myo-2p::RFP*	PVO lab

Table 2

Solution	Reagent	Final Concentration
Worm Lysis buffer	1 M Tris pH 8.9	10 mM
	2 M KCl	50 mM
	50 mM $MgCl_2$	1 mM
	Tween 20	0.5%
	2% Gelatin	0.25%
	Proteinase K	0.1 mg/mL

Table 3

Strain name	Genotype	Source	Use
	Stress reporters		
AM722	*hsp-70p::mCherry; myo-2p::CFP*	Morimoto lab	**HSR**
AM446	*hsp-70p::GFP; rol-6*	Morimoto lab	**HSR**
AM799	*hsp-90p::GFP*	Morimoto lab	**HSR**
TJ375	*hsp-16.2p::GFP*	CGC	**HSR**
TJ3001	*hsp-16.2p::GFP;Cbr-unc-119p(+)*	CGC	**HSR**
CF1553	*sod-3p::GFP;rol-6*	CGC	**oxidative stress**
CL2166	*gst-4p::GFP::NLS*	CGC	**oxidative stress**
LD1	*skn-1p::SKN-1::GFP;rol-6*	CGC	**oxidative stress**
SJ4005	*hsp-4p::GFP*	CGC	**UPR^{ER}**
SJ4100	*hsp-6p::GFP*	CGC	**UPR^{mito}**
SJ4058	*hsp-60p::GFP*	CGC	**UPR^{mito}**
	Proteostasis reporters		
	Folding sensor (transgene)		
AM140	*unc-54p::Q35::YFP*	Morimoto lab	**muscle-specific polyQ expression**
AM141	*unc-54p::Q40::YFP*	Morimoto lab	**muscle-specific polyQ expression**
OG412	*vha-6p::Q44::YFP*	CGC	**intestine-specific polyQ expression**
FUH55	*unc-54p::FLUC::EGFP;rol-6*	Hartl lab	**muscle-specific of wt luciferase**
FUH134	*unc-54p::FLUCSM::EGFP;rol-6*	Hartl lab	**muscle-specific expression of R188Q mutant luciferase**
FUH135	*unc-54p::FLUCDM::EGFP;rol-6*	Hartl lab	**muscle-specific expression of R188Q,R261Q double mutant luciferase**
FUH48	*rgef-1::FLUC::EGFP;rol-6*	Hartl lab	**neuron-specific expression of wt luciferase**
FUH136	*rgef-1::FLUCSM::EGFP;rol-6*	Hartl lab	**neuron-specific expression of R188Q mutant luciferase**

FUH137	*rgef-1::FLUCDM::EGFP;rol-6*	Hartl lab	**neuron-specific expression of R188Q,R261Q double mutant luciferase**
	(endogenous) conditional folding sensors		
CB1301	*unc-54(e1301) I*	CGC	***myosin(ts)*** **, temperature sensitive mutant (muscle)**
CB1157	*unc-54(e1157) I*	CGC	***myosin(ts*** **), temperature sensitive mutant (muscle)**
CB1402	*unc-15(e1402)*	CGC	***paramyosin(ts)*** **, temperature sensitive mutant (muscle)**
HE250	*unc-52(e669su250) II*	CGC	**perlecan(ts), temperature sensitive mutant (muscle)**
SD551	*let-60(ga89)*	CGC	**Ras(ts), temperature sensitive mutant (multiple tissues)**
CX51	*dyn-1(ky51)*	CGC	**Dynamin(ts), temperature sensitive mutant (multiple tissues)**
CW152	*gas-1(fc21)*	CGC	**Gas-1(ts), temperature sensitive, EtOH sensitive**
	Autophagy reporter		
MAH215	*lgg-1p::mCherry::GFP::lgg-1;rol-6*	CGC	**tandem tagged autophagy reporter**
DA2123	*lgg-1p::LGG-1::GFP; rol-6*	CGC	**ubiquitious autophagy reporter**
DLM10	*myo-3p::CERULEAN-VENUS::lgg-1 + unc-119(+)*	CGC	**muscle-specific autophagy reporter**
DLM12	*rab-3p::CERULEAN-VENUS::lgg-1 + unc-119(+)*	CGC	**neuron-specific autophagy reporter**
DLM4	*vha-6p::CERULEAN-VENUS::lgg-1 + unc-119(+)*	CGC	**intestine-specific autophagy reporter**
DLM2	*eft-3p::CERULEAN-VENUS::lgg-1 + unc-119(+)*	CGC	**ubiquitous autophagy reporter**
	Ubiquitin Proteasome System (UPS) reporter		
	rgef-1p::UbG76V::Dendra2	Holmberg lab (Ref. 38)	neuron-specific UPS reporter
AGD1033	*unc-54p::UbG76::Dendra2*	CGC	muscle-specific UPS reporter
	dat-1p::UbG76::Dendra2	Holmberg lab (Ref. 38)	dopaminergic-neuron specific UPS reporter

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Ampicillin	Merck	A0166-5G	Protocol Section 3.1.
DNA Clean & Concentrator-500	Zymo Research	D4031	Protocol Section 2.2.
IPTG Isopropyl-β-D-thiogalactoside	Merck	367-93-1	Protocol Section 3.1.
Multisite Gateway Cloning Kit	Thermo Fisher	12537100	Protocol Section 1.2.
SigmaCote	Merck	SL2-25mL	Protocol Section 2.3.
Tetracycline	Merck	T7660-5G	Protocol Section 3.1.
Zero Blunt TOPO PCR Cloning Kit	Thermo Fisher	K280002	Protocol Section 1.1.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have proofread the manuscript.

2. Please include a table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

We have now included a Materials Table (Table 3).

3. Please revise the title for conciseness.

We have changed the title to: "Tissue-specific RNAi tools to identify components for systemic stress signalling.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We changed this accordingly.

5. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

We have removed any discussion points in the protocol section.

6. Please add more details to your protocol steps. 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.

We now added more detail throughout the protocol.

7. 2.2.5: How is purification done?
We used a PCR Purification kit (DNA Clean and Concentrator kit from ZymoResearch). This is now stated in 2.2.6.

8. 3.1.1: Please provide the details for the standard methods here so all the information to perform the experiment is contained in this article.

We have added a detailed description of the standard method of preparing RNAi plates (section 3.1).

9. 3.2.2: For how long?

5 minutes.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript from Miles J et al, describes a perfect tool to identify new players involved in the cell-non-autonomous proteostasis using C.elegans.
The article is very well written and already addressed and solved some of the potential problematic.
Indeed, this approach will be difficult if using a larger scale RNAi library. However, the authors already suggest solutions to bypass this flaw by using 96-well plate format.
The use of sid-1(pk3321) mutant is cleaver choice. Moreover, the steps in the protocol are very detailed.
This article is suitable for JoVE.

Minor Concerns:

I would be beneficial to mention that models of proteotoxicity based on expanded stretches of glutamine (Q44,Q35 and Q40) have motility impairment. Thus, by trashing assay it will be even faster to screen larger population of candidates.

We thank the reviewer for the kind words.
We have now added the suggested information to the results section and pinpoint to another paper that describes automated thrashing assays in a detailed manner.

Reviewer #2:
Manuscript Summary:
In this Methods paper, Miles and Oosten-Hawle describe the methods of modulating the levels of hsp-90 as a member of the proteostasis network using RNAi and how this combined with specific stress reporters can be used for screens to identify genes that are important for stress signaling.
This protocol is timely, important and of interest to the C. elegans research community. The article could benefit from some improvements.

Major Concerns:
1. The protocol needs to be expanded to include the RNAi screen and the proteostasis sensors. An experimental flow chart would be helpful.
To this end, it is not clear from the last paragraph of the introduction what will be described in the methods chapter and should be made more clear.

We thank the reviewer for this important point and have now added the flow chart to Figure 3A.
We also modified the last paragraph of the introduction to clarify what will be described in the methods chapter.

2. The introduction could be more specific with concrete examples.
- Line 107, compensatory activation of chaperone expression in other distal tissues"- it is unclear what these distal tissues are and it is also unclear whether just Hsp90 or other chaperones are also increased. Please clarify.

We have altered the sentence by clarifying what the distal tissues are and which chaperones are induced in these tissues.

- Line 108-111: First the authors list that the secretion of many a signaling molecules can activate the PN from one tissue to another and list many different types of molecules. Then in the next sentence they say that the specific molecules are unknown. I think this should be revised. First it would be helpful to list what tissues have been sown to release what kind of signaling molecule and whether that has been shown to act on a specific tissue. Lastly, the last sentence of what is unknown has to be more specific. As is this seems to be contradictory.

We agree that this paragraph was confusing and now specifically name the tissues responding to cell nonautonomous stress signalling as well as the identity of the signalling molecule(s) is involved in the response.

3. The section on the sid-1 system in the introduction should be expanded to explain how a sid-1 mutant with tissue-specific rescue of sid-1 in specific tissues (such as neurons or muscle), is able to transport the dsRNA from the site of uptake (intestine for RNAi by feeding and intestine or pseudocoelom for injections) to the site of sid-1 expression (neurons or muscle) if the transporter is missing. This is a frequent questions from students and would be helpful to be addressed, specifically in a methods paper.
We have now included a paragraph describing this mechanism in the introduction.

4. The authors could include a section into the introduction that introduces the different stress reporter strains that are available. It seemed a little late for it to come in the discussion. The discussion of the expression pattern of hsp-70 in the results is important and it would be good to include a general comment on the expression pattern already in the protocol section.

We now mention the different stress reporters in the introduction and also refer to Table 4 that provides a list of available stress reporters.
We also describe the expression pattern of hsp-70 in more detail in the results section.

5. Some of the sections of the protocol are written as a protocol(e.g. 1.1.1., 1.1.3 (where is 1.1.2??)), whereas other sections (e.g. 1.1.5., 1.1.6) are written in past tense and seem to be taken from the Methods section of the primary paper. This needs to be aligned.

We have corrected the numbering of the protocol section – 1.1.2 was indeed missing.
The methods section is now written in present tense.

6. In 1.1.4, the authors should specify which promoter is for which tissue.

We now specify which promoter drives expression in which tissue.

7. In 1.2.5. the strains expressing SID-1 in neuron is missing here. The promoters for tissue-specific expression of hsp-90 should be included. Why does Figure 2 not include the neuronal HSP-90::rfp reporter? This is important to demonstrate that the neuronal sid-1 strain is capable of knocking down hsp-90 in neurons. I understand that it may be difficult to demonstrate neuronal knockdown of hsp-90 in animals with a red pharyngeal marker, but higher magnification images should be able to show this.

We have added confocal images of strains expressing HSP-90::RFP in the neurons – please see Figure 2A.

8. In 2.1.2. What is strain NL3321? It should just say sid-1(pk3321) mutant. Overall the authors need to be more consistent in using genotypes and strain names. Best would be full genotypes. The strain names and full genotypes should be included in the figure legends at least.

We corrected this by providing the genotype in 2.3.1. We added strain names in the Figure legends. The strain names and full genotypes are given in Table 1.

9. In 2.2.4. The PCR protocol should be included here. Annealing temperature and time. Maybe even a picture of the gel and sequencing reaction.

We have included the PCR programme for genotyping (2.2.5).

10. Line 387 onward: The screen setup has to be described more thoroughly. A schematic would help. The nature of the candidate genes needs to be elaborated on. The expected outcomes of the possible RNAi hits should be elaborated on. Are you expecting increase in hsp-70 fluorescence or decrease? What does that mean? Where are the statistics in Figure 4? Why is this not included in the protocol? The strain (PVH2?) used for the screen should be listed with name and full genotype in the figure legend (Figure 4).

We have now expanded on this section to answer the reviewer's questions. We briefly elaborate on the nature of the candidate genes (kinases, transcription factors and membrane proteins) but do not want to disclose the identity of the genes as these are to be published elsewhere (manuscript in preparation).
We have included the strain names in the figure legend as well as the statistics and also indicated in Figures 4B and 4C which RNAi condition results in a significant increase/decrease of RFP fluorescence intensity, so that it becomes clear which genes were identified as enhancers or suppressors.

11. The use of proteostasis sensors should also be included in the protocol. Stats should be added to Figure 5.

The iQ44::YFP proteostasis sensor is now included in the protocol section 2.3
Stats are added to Figure 5.

Minor Concerns:
12. Line 114: The abbreviation PN should be spelled out here as it is the first time to be used in the introduction.

We have now added the full spelling of the abbreviation PN.

13. In Figure 2, The label "tissue-specific hsp-90 RNAi" should be changed to simply "hsp-90 RNAi"

We have changed the label to hsp-90 RNAi.

14. In Figure 1, an illustration of what the hairpin will look like would be a nice addition.

We have included an illustration of the hairpin structure.

15. In 2.2.3. Not all labs will have a program called "Single Worm Lysis": Just the protocol is necessary here.

This has been changed into "….using the following programme".

16. The authors should indicate that daf-21 is the c. elegans name for hsp-90 in the table.
We have changed daf-21 into hsp-90 to be conform with the current nomenclature for C. elegans chaperone genes.

Reviewer #3:
Manuscript Summary:
This manuscripts describes methods for performing genetic screens via tissue-specific gene knockdown by RNAi in C. elegans. The authors are primarily interested in genes involved in proteostasis. Specifically, they used their system to identify genes/proteins that are required for normal transcellular chaperone signaling. They knocked down the expression of 58 genes in the intestine or body wall muscle cells and identified those that had the greatest effect on transcellular chaperone signaling.

Major Concerns:
My main concern is that the method described herein is so specific—the use of tissue specific RNAi to identify genes involved in transcellular chaperone signaling—that I am not convinced that it will garner broad interest. Further adding to this concern is the fact that it is seemingly written for members of the van Oosten-Hawle

lab rather than for a general audience. For example, PCR program names are given, such as "Single Worm Lysis" (step 2.24), but this is lab-specific.

We think that the described method to achieve tissue-specific RNAi by either expression of a hairpin construct or by feeding RNAi bacteria, will garner broad interest. It is here described on the example of tissue-specific *hsp-90* RNAi, but the approach can be used for any target gene of interest. Moreover, conducting a tissue-specific RNAi screen is an approach that allows to investigate the responses between different organs and tissues within an entire organism. This is relevant for our specific interest in transcellular chaperone signalling, but also for investigators looking into intertissue communication in general, as our understanding of proteostasis has now transcended from being regulated in a cell autonomous to a cell nonautonomous manner. We have now made this clear in the last paragraph of the introduction and tried to give examples for a more general approach throughout the manuscript.

Minor Concerns:
The manuscript would benefit from copy-editing. In addition, some aspects of the protocol would benefit from additional clarification:
- In step 2.2.4, it says to use 2uL as "template." While one can figure out that the authors are referring to worm lysate, this should be stated explicitly.

Worm lysate is now stated explicitly.

- In step 2.2.5, the authors say that PCR product should be purified, but they don't say how they do this. Then they say to "send for sequencing" but all that matters is that it is sequenced. Whether readers will choose to do sequencing in-house or to outsource sequencing is irrelevant. This is another example of where the protocol seems to be written for one specific lab rather than for a broader audience.

We changed this into: "Purify the ~650 bp PCR product using a PCR purification kit (we used DNA Clean and Concentrator from ZymoResearch) and sequence the PCR product to identify G-to-A point mutation". Section 2.2.6.

- In step 3.3.1, the authors should remove the word "equal" as results should be comparable, but not necessarily equal.

"equal" has been removed.

- Step 3.1.3. At what larval stage are the animals transferred to a fresh plate?

We added "L4 stage larvae". Section 3.2.2.

Reviewer #4:

Manuscript Summary:

Miles and van Oosten-Hawle describe two methods to perform tissue-specific RNAi in C. elegans. Firstly, they describe a protocol for the generation of hairpin RNAi constructs that can be expressed under tissue-specific promoters. Secondly, they provide detailed methods for tissue-specific RNAi in sid-1 mutants, which have sid-1 rescued in the tissue of interest. These methods enable the study of cell non-autonomous stress responses, by analysing tissue-specific changes in proteostasis sensors, such as polyQ, and fluorescent stress reporters, such as hsp70p::RFP.

These protocols are useful not only for the study of cell non-autonomous processes, but also more generally whenever tissue-specific RNAi would be informative. I therefore believe the community would benefit from the publication of these methods. However, in parts of the protocol, the information is currently very specific to the authors' established system using hsp-90 knockdown. In order to make this method broadly useful, some additional comments regarding controls and validation processes are needed. Please see the following points for details.

Major Concerns:

1. In sections 1.1.5 and 1.2.5, the method for validation of successful generation of tissue-specific RNAi strains, involves the authors' genes of interest, hsp-90 and hsp-70. What are some other ways in which readers can validate their strains, e.g. qPCR? Can you make some general points or suggestions?

We have added information on alternative ways to validate successful generation of transgenic strains in both sections.

2. Although the authors do mention other proteostasis reporters in the discussion, it would be useful to have this information summarised in a table, listing the different sensors and fluorescent reporters for different stress responses. This would help make the protocol more broadly useful.

We now provide a Table (Table 4) listing different proteostasis and stress reporter strains.

3. More experimental details are required in the following sections:

Section 1.1.3: Please add some extra detail regarding the cloning procedure. Is the hsp-90 sequence from the library clone ligated into the TOPO vector? This is a little unclear.

Current section 1.1.2: we have added the extra detail to clarify the cloning procedure.

Section 1.1.5: What RNAi controls can be used? For example, is it recommended to use non-specific or scrambled RNAi? This is usually done in other model systems, so it would be important to address this. It is also unclear what "control" means in Figure 3.

Current section 1.1.5: we have added information on empty vector hairpin RNAi constructs that can be used as negative controls.
Control in Figure 3 means that no hairpin RNAi construct is expressed in this strain.

Section 1.2.5: Please add details of the RNAi procedure or refer to previous publications that have the procedure outlined in detail.

We have added more detailed information regarding this point. We now also include an entire section (3.1) describing the preparation of RNAi plates.

Section 3.2.1: How much agarose is added to the slide?

we have added "250 ul".

Section 3.3.1: Please add laser power to the list of parameters that need to be kept constant.

We have added "laser power".

Section 3.3.2: Please go into detail regarding the image analysis process; this section is currently very light. How can intensity be measured as pixels per unit area without first applying a threshold and making the image binary? How was background fluorescence determined and subtracted? Please also explain more why and how fluorescence intensity is normalised to worm body length.

The following information was added to 3.3.3:
Image analysis can be performed using Image J software. Fluorescence intensity in each image is measured as pixels per unit area, with background fluorescence subtracted. Fluorescence intensity for each image is normalized to the image area as well as the length of the worms. For this the mean intensity is measured using the "Analyze" -> "Measure" function in ImageJ. The resulting intensity value is then normalised to the image area by dividing the intensity by area.
To normalise the intensity to worm length, measure the worm by drawing a line along the length of the worm in ImageJ and using the "Analyze" -> "Measure" function. The reason for normalising fluorescence intensity to worm length, is that worms can vary in size, dependent on the gene that is knocked down by RNAi, and this could affect the mean intensity.

4. Please highlight the critical steps in the procedure and also highlight any common technical issues you have identified, with troubleshooting suggestions where possible.

Critical steps are highlighted in sections 2.3.3; 3.2.1; and 3.2.4.

5. The materials and equipment list is missing some information, including (but not

limited to) the kits used for cloning and the RNAi reagents.

We have now added the materials and equipment list used.

Minor Concerns:
1. The title is very general and does not reflect the main subject of the protocol, i.e. the tissue-specific RNAi systems.

We have modified the title into: "Tissue-specific RNAi tools to identify components for systemic stress signalling"

2. The authors mention the rde-1 system in the discussion, but it should be raised more explicitly because this is a common alternative to the sid-1 system. Perhaps address any advantages/disadvantages of the sid-1 system and when the rde-1 system could be used instead (e.g. when there is a chromosome conflict for crossing). Please point the readers to some key rde-1 papers.

We have now expanded on this section in the discussion to address advantages and disadvantages. Key rde-1 papers are cited in this section.

3. Figure 5 is missing panel lettering and stats.

We have added panel lettering as well as stats.

Reviewer #5:
This manuscript introduced tools that can be used to test proteostatic responses in specific tissues of C. elegans. The topic is very interesting and the manuscript is well prepared. I have only a few minor suggestions to make the paper more readable.

Line 100: Ref "78" should be "7,8".
It should actually be reference 7 only, and we changed this accordingly.

Lines 71, 74 and 114: The "PN" in Line 71 is correct, but the one in Line 74 is not. Also, full name of "PN" in Line 114 should be given as it occurs for the first time in the main text.

We have removed the parenthesis from PN in line 74 and provided the full name of PN in line 114.

Lines 178-181: It would be better for a non-specialist to read if a reference is provided for "Ahringer RNAi library" rather than only Ref 21 although people working on C. elegans are supposed to know who Julie Ahringer is.

We have added the appropriate reference to Ahringer RNAi library.

Lines 242-245: It will be easier for readers to follow the paper if examples of proteostasis sensors and/or stress reporters are given here, e.g. those from representative results can be briefly mentioned.

We have added examples for both proteostasis sensors and stress reporters accordingly.

Line 259: Provide the full name here for "EV" control.

We added the full name "empty vector" for EV.