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TITLE:

Using *Caenorhabditis elegans* to Screen for Tissue-Specific Chaperone Interactions

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SUMMARY:
To study chaperone-chaperone and chaperone-substrate interactions, we perform synthetic interaction screens in *Caenorhabditis elegans* using RNA interference in combination with mild mutations or over-expression of chaperones and monitor tissue-specific protein dysfunction at the organismal level.

ABSTRACT:
Correct folding and assembly of proteins and protein complexes are essential for cellular function. Cells employ quality control pathways that correct, sequester or eliminate damaged proteins to maintain a healthy proteome, thus ensuring cellular proteostasis and preventing further protein damage. Because of redundant functions within the proteostasis network, screening for detectable phenotypes using knockdown or mutations in chaperone-encoding genes in the multicellular organism *Caenorhabditis elegans* results in the detection of minor or no phenotypes in most cases. We have developed a targeted screening strategy to identify chaperones required for a specific function and thus bridge the gap between phenotype and function. Specifically, we monitor novel chaperone interactions using RNAi synthetic interaction screens, knocking-down chaperone expression, one chaperone at a time, in animals carrying a mutation in a chaperone-encoding gene or over-expressing a chaperone of interest. By disrupting two chaperones that individually present no gross phenotype, we can identify chaperones that aggravate or expose a specific phenotype when both perturbed. We demonstrate that this approach can identify specific sets of chaperones that function together to modulate the folding of a protein or protein complexes associated with a given phenotype.

INTRODUCTION:

Cells cope with protein damage by employing quality control machineries that repair, sequester or remove any damaged proteins[1,2]. Folding and assembly of protein complexes are supported by molecular chaperones, a diverse group of highly conserved proteins that can repair or sequester damaged proteins[3-7]. The removal of damaged proteins is mediated by the ubiquitin-proteasome system (UPS)[8] or by the autophagy machinery[9] in collaboration with chaperones[10-12]. Protein homeostasis (proteostasis) is, therefore, maintained by quality control networks composed of folding and degradation machineries[3,13]. However, understanding the interactions between the various components of the proteostasis network in vivo is a major challenge. While protein-protein interaction screens contribute important information on physical interactions and chaperone complexes[14,15], understanding the organization and compensatory mechanisms within tissue-specific chaperone networks in vivo is lacking.

Genetic interactions are often used as a powerful tool to examine relationship between pairs of genes that are involved in common or compensatory biological pathways[16-18]. Such relationships can be measured by combining pairs of mutations and quantifying the impact of a mutation in one gene on the phenotypic severity caused by a mutation in the second gene[16]. While most such combinations do not show any effect in terms of phenotype, some genetic interactions can either aggravate or alleviate the severity of the measured phenotype. Aggravating mutations are observed when the phenotype of the double deletion mutant is more severe than the expected phenotype seen upon combining the single deletion mutants, implying that the two genes function in parallel pathways, together affecting a given function. In contrast, alleviating mutations are observed when the phenotype of the double deletion mutant is less severe than the phenotype seen with the single deletion mutants, implying that the two genes act together as a complex or participate in the same pathway[16,18]. Accordingly, diverse phenotypes that can be quantified, including broad phenotypes, such as lethality, growth rates and brood size, as well as specific phenotypes, such as transcriptional reporters, have been used to identify genetic interactions. For example, Jonikas et al. relied on an ER stress reporter to examine interactions of the *Saccharomyces cerevisiae* ER unfolded protein response proteostasis network using pairwise gene deletion analyses[19].

Genetic interaction screens involve systematically crossing pairwise deletion mutations to generate a comprehensive set of double mutants[20]. However, in animal models, and specifically in *C. elegans*, this large-scale approach is not feasible. Instead, mutant strains can be tested for their genetic interaction patterns by down-regulating gene expression using RNA interference (RNAi)[21]. *C. elegans* is a powerful system for screens based on RNAi[22,23]. In *C. elegans*, double-stranded RNA (dsRNA) delivery is achieved by bacterial feeding, leading to the spread of dsRNA molecules to numerous tissues. In this manner, the introduced dsRNA molecules impact the animal via a rapid and simple procedure[21]. A genetic interaction screen using RNAi can, therefore, reveal the impact of down-regulating a set of genes or most *C. elegans* coding genes using RNAi libraries[24]. In such a screen, hits that impact the behavior of the mutant of interest but not the wild type strain are potential modifiers of the phenotype being monitored[25]. Here, we apply a combination of mutations and RNAi screening to systematically map tissue-specific chaperone interactions in *C. elegans*.

PROTOCOL:

1. Preparation of nematode growth media plates for RNAi

1.1 To a 1 L bottle, add 3 g of NaCl, 2.5 g of Bacto-Peptone, 17 g of agar and distilled water up to 1 L and autoclave.

1.2 Cool bottle to 55 °C.

1.3 Add 25 mL of 1 M KH_2PO_4, pH 6.0, 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, and 1 mL of cholesterol solution (**Table 1**) to make nematode growth media (NGM).

1.4 Add 1 mL of ampicillin (100 mg/mL) and 0.5 mL of 1 M IPTG (**Table 1**) to make NGM-RNAi solution.

NOTE: Commonly used HT115(DE3) *E. coli* bacteria contain an IPTG-inducible T7 DNA polymerase used for expressing the dsRNA-encoding plasmids. These plasmids also encode for ampicillin resistance.

1.5 Mix the warm solution by swirling the bottle.

1.6 In the hood or using sterile procedures, pour the NGM solution into plates or using a peristaltic pump, dispense the solution into plates. Use 6-well, 12-well, 40 mm or 60 mm plates for this screen. Agar should fill about 2/3 of the plate depth.

1.7 Let the plates dry overnight on the bench at room temperature, keeping the plates covered. NGM plates for RNAi can be stored at 4 °C for up to a month.

2. Growing RNAi bacteria and seeding the plates

2.1 In the hood or using sterile procedures, add 1 mL of ampicillin (100 mg/mL) and 2.5 mL of tetracycline (5 mg/mL) (**Table 1**) to a pre-autoclaved 1 L of LB solution and mix.

NOTE: Commonly used HT115(DE3) *E. coli* bacteria are tetracycline-resistant.

2.2 In the hood or using sterile procedures, add 600 µL of LB solution to each well in 2 mL-deep 96-well sterile plates. It is best to use a multichannel pipet for dispensing the media.

2.3 Using sterile procedures, inoculate wells with HT115(DE3) *E. coli* bacteria transformed with a dsRNA-encoding plasmid targeting a gene of interest or an empty plasmid, as a control. Cover the plates and incubate at 37 °C overnight. Libraries that consist of bacterial clones expressing dsRNA, corresponding to ~94% of predicted *C. elegans* genes were previously constructed[22,23] and are commercially available. The chaperone library used here was

constructed by Dr. Richard Morimoto laboratory[26].

2.4 Using sterile procedures, seed 75, 150 or 250 µL of bacteria onto the 12-well, 40 mm and 6-well or 60 mm NGM RNAi plates, respectively. Clearly mark the name of the target gene on the plate. Bacteria should cover 30-50% of the agar surface and should not touch the edges of the plate.

2.5 Allow plates to dry for at least 2 days on the bench at room temperature, keeping the plates covered.

NOTE: Plates can be incubated at 37 °C overnight. Make sure the inner wells are dry before using or storing the plates. For all long-term purposes (i.e., drying or storage) keep the plates in the dark. Dried, seeded plates can be stored at 4 °C for up to a month.

3. Non-stressful synchronization of embryos

3.1 Use a worm pick to move about 100 eggs from an unsynchronized worm plate to a newly seeded NGM plate.

3.2 Cultivate animals for 5 days at 15 °C, 3.5 days at 20 °C or 2.5 days at 25 °C. Animals should reach the first day of egg laying.

NOTE: Worms are commonly cultivated at 20 °C. However, different chaperone mutant strains may require specific cultivation temperatures. For example, many temperature-sensitive strains are cultivated at 15 °C but shifted to 25 °C to expose their phenotype.

3.3 Add 1 mL of M9 buffer (**Table 1**) slowly and away from the bacterial lawn. Rotate the plate so that the buffer completely covers the plate. Then tilt it to one side and remove the liquid from the plate and wash the animals off the plate.

NOTE: When using temperature-sensitive animals or chaperone mutants, it is best to maintain the buffers used in the protocol at the animals' cultivation temperature.

3.4 Repeat step 3.3 three times or until all the animals are washed off the plate.

3.5 Using a standard plastic tip, cut a square of agar from the washed plate where eggs are concentrated and place the piece of agar onto a newly seeded NGM plate.

NOTE: ~200 eggs are required to produce enough egg-laying animals; too many animals consume the bacteria too quickly. Low food levels can impact proteostasis[27].

3.6 Cultivate the animals for 5 days at 15 °C, 3.5 days at 20 °C or 2.5 days at 25 °C. At this point, the plates should be covered with synchronized eggs.

NOTE: Animals can be shifted to a new plate for a short duration for a more stringent synchronization. However, it is important to only use adults at the early stages of egg-laying as animals can retain eggs in their uterus impacting synchronization.

3.7 Add 1 mL of M9 buffer slowly and away from the bacterial lawn.

3.8 Rotate the plate so that the buffer completely covers the plate. Then tilt it to one side and remove the liquid from the plate and wash the animals off the plate.

3.9 Repeat step 3.7 three times or until all animals are washed off the plate.

3.10 Add 1 mL of M9 buffer and use a cell scraper to release the eggs from the plate.

3.11 Collect the M9 buffer containing the eggs from the plates.

3.12 Centrifuge the M9 buffer containing the eggs at 3,000 x *g* for 2 min.

3.13 Remove the supernatant and add M9 buffer to reach a volume of 1 mL.

3.14 Resuspend the eggs to disrupt any chunks of eggs and bacteria.

3.15 Repeat the washing procedure described in steps 3.11-3.13 five times. The egg pellet should appear white. If it is still yellow/brown, repeat the wash until a white pellet is attained.

NOTE: Bacteria that remain on the eggs can contaminate the dsRNA-expressing bacteria.

3.16 Remove most of the supernatant, leaving about 200 µL. Synchronized eggs can be used for RNAi screens.

4. Common phenotypic assays

4.1. Cultivation of animals during experiments

4.1.1 Place a drop of ~30 eggs close to the bacterial lawn in each RNAi-seeded plate. For reference, also place ~30 eggs on plates seeded with empty vector-containing (L4440) bacteria.

4.1.2 Cultivate age-synchronized animals on NGM RNAi-seeded plates. The duration of the experiment will depend on the stage at which the animals are to be monitored and the temperature of cultivation. Adjust the cultivation temperature when using temperature-sensitive mutant animals. Adjust the cultivation duration when using developmentally delayed mutant animals.

NOTE: While timing can vary, once animals reach adulthood, egg laying (and the resulting rapid food consumption), as well as age-dependent proteostsis collapse[28], could impact the results. It

is thus recommended to score animals before the onset of egg laying (day 1 of adulthood). Wild type animals reach this stage after 4 days at 15 °C, 3 days at 20 °C or 2 days at 25 °C.

4.1.3 The number of repeats used will depend on the size of the gene set examined. For the chaperone library (97 genes), repeat experiments at least four times. The size of the population depends on the assay used. In the behavioral assays discussed here, score >15 animals per experimental condition in each repeat. Data and statistical analyses also strongly depend on the type of assay used. Data in the assays discussed here can be presented as means ± SEM.

4.1.4 Compare the RNAi- and empty vector control-treated animals as independent populations. P values can be calculated using one-way or two-way ANOVA, depending on the changes examined, namely aggravating or alleviating alone or both. When examining a single RNAi treatment vs. control, P values can be calculated using one-way or two-way Mann-Whitney rank sum test. Other than statistical significance, consider a threshold for hits based on the degree of impact on the phenotype.

4.2. Developmental arrest/delay

4.2.1 Cultivate age-synchronized animals as in step 4.1 until animals grown on empty vector-containing control bacteria reach adulthood but before egg laying starts.

4.2.2 Monitor animals using a stereomicroscope and count the number of larvae and adults to score the percent of developmentally delayed animals. For reference, compare to mutant animals grown on empty vector-containing control bacteria. *hsp-1* or *hsp-90* RNAi treatment results in developmental arrest of wild type animals and can be used as a positive control.

4.2.3 To score the percent of developmentally delayed animals over time, repeat step 4.2.2.

NOTE: If there are egg-laying adults on the plate, transfer the developmentally delayed animals to a new NGM-RNAi plate labeled for the same target gene to avoid confusion with progeny.

4.3. Sterility or egg laying defects

4.3.1 Cultivate age-synchronized animals as in step 4.1 until animals grown on empty vector-control bacteria begin to lay eggs.

4.3.2 Monitor animals using a stereomicroscope and score the percent of animals with no visible eggs in their uterus. For reference, compare to mutant animals grown on empty vector-control bacteria.

4.3.3 Alternately, monitor animals using a stereomicroscope and score the percent of animals with a uterus full of eggs, defined as EGg Laying defective (Egl-d) phenotype[29].

4.4. Embryonic lethality

4.4.1 Cultivate age-synchronized animals as in step 4.1 until the animals begin to lay eggs.

4.4.2 Transfer ~100 eggs to an empty plate. Spread the eggs in rows to simplify counting.

4.4.3 Score the percent of unhatched eggs on the plate after 24-48 hours. For reference, compare to eggs of animals treated with empty vector-control bacteria.

4.5. Paralysis assay

4.5.1 Cultivate age-synchronized animals as in step 4.1 until animals grown on empty vector-control bacteria reach adulthood but before egg laying starts.

4.5.2 Draw a line on the back of a regular NGM agar plate using a fine marker.

4.5.3 Place 5-10 animals on the marked line.

4.5.4 Set a timer and wait for 10 min.

4.5.5 Score the percent of animals remaining on the line as paralyzed worms. For reference, compare to mutant animals grown on empty vector-control bacteria. Wild type animals treated with *unc-45* RNAi show severe paralysis phenotype and can be used as a positive control.

NOTE: This assay highlights animals showing medium to severe paralysis. Such animals usually lie straight on the plate, rather than presenting the common curved shape. Moreover, a patch cleared of bacteria is visible around the heads of paralyzed worms.

4.6. Thrashing assay

4.6.1 Cultivate age-synchronized animals as in step 4.1 until animals grown on empty vector-control bacteria reach adulthood but before egg laying starts.

4.6.2 Pipet 100 µL of M9 buffer at the animals' cultivation temperature into a 96-well plate.

4.6.3 Place ~15 worms, one per well, into the M9 buffer-containing wells.

4.6.4 Let the animals adjust for 5 min.

4.6.5 Examine each animal under the stereomicroscope, start a timer counting down 15 s, and count the number of body bends each animal performs in that timespan. The values counted can be normalized to body bends per min. For reference, compare to mutant animals grown on empty vector-control bacteria.

NOTE: This motility assay is very sensitive and can detect very mild differences between

treatments. However, motility in liquid and motility on agar can differ.

5. Validation of protein knockdown

5.1. Place 250-300 synchronized eggs onto a 60 mm NGM-RNAi plate seeded with the relevant dsRNA-expressing or empty vector-containing (L4440) bacteria.

NOTE: RNAi knockdown could result in aberrant accumulation of embryos, in a lack of embryos or in developmental arrest that could impact gene expression. This should be considered when determining the age of the animals to be examined.

5.2. Cultivate animals for 4.5 days at 15 °C, 3 days at 20 °C or 2 days at 25 °C.

5.3. Pick and transfer a total of 200 young adult animals into the cap of a 1.5 mL tube fill with 200 µL of PBS-T.

NOTE: When using temperature-sensitive animals or chaperone mutants, it is best to maintain the buffers used in the protocol at the animals' cultivation temperature.

5.4. Close the cap carefully and centrifuge at 1,000 x *g* for 1 min.

5.5. Add 800 µL of PBS-T (**Table 1**) and centrifuge at 1,000 x *g* for 1 min.

5.6. Carefully remove the top 900 µL.

5.7. Repeat steps 5.4-5.6 three times.

5.8. Remove 900 µL, leaving 100 µL of solution containing the 200 worms.

5.9. Add 25 µL of 5x sample buffer (**Table 1**).

5.10. Heat the samples for 10 min at 92°C while shaking at 1000 rpm. Samples can then be frozen and kept at -20 °C.

5.11. Load 20 µL of each sample and run on an SDS-PAGE gel.

5.12. Perform western blot analysis using appropriate antibodies to determine the relative stability of the protein.

5.13. Determine the intensity of the bands using densitometric software, such as the freely available ImageJ gel module. Normalize all values to those measured in the control sample(s).

NOTE: The aim of RNAi knockdown in our screens is to lower protein levels of a specific chaperone/co-chaperone. Thus, the best way to assess the efficiency of the RNAi knockdown is

by western blot analysis. This requires specific antibodies. Alternately, qPCR can be used to quantify mRNA levels.

REPRESENTATIVE RESULTS:

Using temperature-sensitive mutations in UNC-45 to screen for aggravating or alleviating interactions under permissive or restrictive conditions, respectively

Muscle assembly and maintenance offer an effective system to study tissue-specific chaperone interactions. The functional unit of contractile muscles, the sarcomere, presents a crystalline-like arrangement of structural and regulatory proteins. The stability of the motor protein myosin and its incorporation into the thick filaments of contractile muscle sarcomeres depends on cooperation of chaperones and UPS components[30]. An example of one such chaperone is the conserved and specialized myosin chaperone UNC-45 that is mainly expressed in body wall muscle[31-35]. Mutations in UNC-45 have been shown to induce myosin disorganization and severe motility defects in *C. elegans*[31,36]. UNC-45 tandem modules assemble into a multi-site docking platform[37] that enforces collaboration between UNC-45, HSP-90 and HSP-1 and likely other chaperones and co-chaperones in myosin filament assembly[25,36-38]. To confirm known UNC-45 interactions and identify novel genetic interactions in muscle proteostasis, we established a strategy using *C. elegans* temperature-sensitive *unc-45* mutations as a sensitized genetic background for tissue-specific chaperone interaction screening[19,25].

Single amino acid substitutions in *C. elegans* UNC-45 (L822F and E781K, corresponding to the *e286* and *m94* alleles, respectively; *unc-45(ts)*) are responsible for temperature-dependent motility defects and myosin disorganization phenotypes when affected animals are grown under restrictive conditions (>22 °C). In contrast, these *unc-45(ts)* mutants show no movement or myosin organization defects at the permissive temperature (15 °C)[31]. In the proposed approach, age-synchronized *unc-45(e286)* animals at the first larval stage (L1) were depleted of different molecular chaperones by RNAi (97 genes) and then monitored for motility defects under permissive conditions (15 °C) (**Figure 1**). We confirmed the known interaction of UNC-45 and HSP-90 proteins in our genetic interactions screen and identified three *hsp-90* co-chaperones, *sti-1*, *ahsa-1,* and *daf-41*, as specifically causing a synthetic movement defect in *unc-45(ts)* mutant animals but not in wild-type animals[25]. We went on to examine whether *sti-1*-, *ahsa-1*- or *daf-41*-associated synthetic phenotypes were caused by myosin disorganization by monitoring the subcellular arrangement of myosin heavy chain A (MYO-3), using established immuno-staining techniques[39]. Whereas treatment of wild type animals with *sti-1*, *ahsa-1* or *daf-41* RNAi did not affect myofilament organization, depletion of these genes in *unc-45(e286)* mutant animals resulted in complete disruption of sarcomeric structures and MYO-3 mislocalization, even under permissive conditions (15 °C). This effect was comparable to what was seen with *unc-45(e286)* single mutants grown at the restrictive temperature (25 °C)[25]. These results were confirmed using another *unc-45(ts)*-allele, namely *unc-45(m94)* mutant animals[25].

To identify chaperones that destabilize UNC-45, we next screened for chaperones that improved the motility of *unc-45(286)* animals at 25 °C, relying on gene knockdown by RNAi. While *unc-45(e286)* mutant animals displayed severe movement defects at 25 °C, the motility of animals treated with RNAi against genes encoding four of the 97 chaperones screened was significantly

improved. Here too, the results were confirmed using *unc-45(m94)* mutant animals. Thus, the use of temperature-sensitive mutations allows for establishing both aggravating and alleviating screens, depending on the temperature at which the screen is conducted.

Using tissue-specific over-expression of a chaperone to screen for aggravating interactions

We next utilized tissue-specific over-expression of a single chaperone as a mild perturbation of the muscle chaperone network for screening purposes. Specifically, we utilized animals over-expressing wild type *dnj-24*, encoding the *C. elegans* homolog of the Hsp40 protein DNAJB6 in the *C. elegans* body wall muscle (DNJ-24M). As above, L1 DNJ-24M animals were treated with RNAi for different chaperones and monitored for motility defects (20 °C) (**Figure 1**). While DNJ-24M animals showed no notable motility defects, three genes (of the 48 chaperone-encoding genes examined; 6%), namely *hsp-1, rme-8*, and *dnj-8,* specifically affected the motility of DNJ-24M-expressing animals but not wild type animals. It is of note that testing the specificity of the hits using animals over-expressing a different chaperone, namely HSP-90, in muscle (HSP90M), showed no effect on HSP90M motility upon *hsp-1, rme-8*, or *dnj-8* RNAi treatment[40]. Taken together, the screening platform, employing mild perturbation to the chaperone network, such as the expression of metastable mutant proteins or tissue-specific over-expression, resulted in a highly specific hit rate (normally ~5%).

Using tissue-specific RNAi to screen for tissue-specific genetic interactions

Tissue-specific RNAi-sensitive strains allow for tissue-specific knockdown of genes while still using bacterial feeding for dsRNA delivery. These strains are mutant for the RDE-1 argonaute protein, a major component in the RNAi pathway required for effective gene silencing[21]. However, expressing wild type RDE-1 under the control of a tissue-specific promoter led to effective tissue-specific gene knockdown[41,42]. This tool thus allows for genetic interaction screens without using tissue-specific chaperones, such as UNC-45 or DNAJ-24M. For example, *hsp-6* (mortalin) knockdown in wild type animals during development resulted in a strong developmental arrest (96±1% of the RNAi-treated animals). At the same time, *hsp-6* knockdown in a strain expressing wild type RDE-1 in muscle did not cause developmental arrest, whereas expressing wild type RDE-1 in intestinal cells resulted in a strong developmental arrest phenotype (90±3%; **Figure 2**). Thus, HSP-6 function in intestinal cells is required for normal development. A mutation in *hsp-6(mg585)* that causes a mild growth delay can, therefore, be used to screen for aggravating or alleviating chaperone interactions by crossing that mutated gene into an intestinal-specific RNAi strain and screening the chaperone RNAi library.

Monitoring age-dependent changes in the folding environment using genetic interactions

Animals show an age-dependent decline in motility that is associated with sarcomeric disorganization[43-45]. Changes in protein folding capacity coincide with altered regulation and composition of the cellular proteostasis machinery[28], including altered levels of UNC-45, CHN-1, and UFD-2, proteins of the muscle quality control machinery[46]. In agreement, myosin folding and degradation are affected by alterations in proteostatic capacity at the transition to adulthood[47]. We, therefore, asked whether such changes could impact chaperone interactions. For example, we monitored the impact of *sti-1*, *ahsa-1* and *daf-41* knockdown on motility over time. We found that although *sti-1*-, *ahsa-1*- and *daf-41*-RNAi treated animals showed reduced motility during

larval development, motility strongly declined in *unc-45(ts)* adult worms. Moreover, MYO-3 organization in *unc-45(ts)* mutant animals treated with *sti-1*, *ahsa-1* or *daf-41* RNAi was similar to that of wild type worms at the fourth larval stage (L4), although the mutants exhibited disrupted sarcomeres after the animals reached adulthood (**Figure 3**). In contrast, both *unc-45(ts)* mutant animals treated with an empty vector control and wild type animals remained unaffected (**Figure 3**). Thus, proteostasis dynamics as a function of age or environmental conditions[27,45,46,48,49] could critically impact chaperone interactions.

FIGURE AND TABLE LEGENDS:

Figure 1: Setup of RNAi synthetic interaction screens using *C. elegans* carrying a mutation in a gene encoding a chaperone of interest. (**A**) Schematic representation of the basic setup of targeted chaperone interaction screens. (**B**) Hit validation requires confirmation of the genetic interaction using another chaperone mutant, as well as validating the specify of RNAi knockdown. (**C-D**) Simple readouts, such as motility defects, can be quantified to determine aggravating or alleviating interactions, using paralysis or thrashing assays, respectively.

Figure 2: **Tissue-specific RNAi of the mitochondrial chaperone *hsp-6* can be used to examine genetic interactions in one tissue.** Wild type intestine- and muscle-specific RNAi strains were treated with *hsp-6* RNAi and (**A**) developmental delay was scored or (**B**) images were taken on the first day of adulthood. Data are mean ± SEM, N=6. Scale bar is 1 mm.

Figure 3: Age-dependent effects of RNAi. (**A**) Motility with age. Wild type or *unc-45(e286)* embryos were placed on *sti-1*, *ahsa-1* or *daf-41* RNAi-seeded plates at 15 °C and scored for motility using a thrashing assay at each developmental stage, L1-L4, young adult and day 1 of adulthood. Data are mean ± SEM, N=15. (**B**) Confocal images of body wall muscle. Animals were treated as in A and fixed at the L4, young adult and day 1 of adulthood stages and immuno-stained with anti-MYO-3 antibodies. Scale bar is 10 µm.

Table 1: Solution Recipes

DISCUSSION:

An integrated picture of the proteostasis network reflecting how it is organized and functions in different metazoan cells and tissues remains lacking. To address this shortcoming, specific information on the interactions of various components of this network, such as molecular chaperones, in specific tissues during the course of development and aging is required. Here, we showed how the use of tissue-specific perturbations enabled us to examine the chaperone network in a given tissue. To explore tissue-specific chaperone genetic interactions, three different approaches were considered. In the first approach, UNC-45, a chaperone that is highly expressed in muscle cells, was used to screen for chaperone interactions via feeding-RNAi[25]. While the use of a specialized chaperone allows for discerning tissue-specificity, it can only report on that highly focused sub-network to which it contributes. Note that most *C. elegans* neurons are resistant to feeding-based RNAi delivery[50] and thus using this approach to identify genetic interactions in neuronal cells requires crossing the mutant chaperone examined with an RNAi-

enhanced strain[51]. In a second approach, a tissue-specific promoter was used to drive over-expression of a chaperone in muscle and thus specifically affect the muscle folding environment[40]. However, over-expressing a single chaperone could be generally beneficial to the folding environment and thus mask disruption caused by the two chaperones together. The third approach relied on tissue-specific RNAi knockdown to examine chaperone interactions in a given a tissue[41]. One advantage of this approach is that it allows for targeting neuronal cells that are resistant to RNAi delivery via feeding[42]. Still, this approach requires the use of a mild mutant (although not specialized), as well as that this mutant be crossed into a *rde-1* null mutant carrying a tissue-specific *rde-1* rescue gene. Importantly, these approaches can be combined so as to potentially modulate chaperone function in a single tissue or even a single cell.

The quality control machinery can impact the function of many gene products by perturbing proteostasis. This is a major challenge when using genetic interactions to explore the quality control network[18]. For example, chronic expression of aggregation-prone proteins or proteostasis collapse in aging resulted in phenotypic aggravation of many unrelated metastable proteins in *C. elegans* and yeast[45,52,53]. Moreover, clathrin-mediated endocytosis in mammalian cells was inhibited upon functional sequestration of Hsc70 to protein aggregates. Yet, it was shown that endocytosis could be rescued by Hsc70 over-expression, while aggregation could not[54,55]. Likewise, a genetic screen in *Drosophila* designed to uncover regulators of the heat shock response identified a missense mutation in flight muscle actin that constitutively activated the heat shock response[56]. Taken together, perturbation of the proteostasis network can expose metastable proteins or induce stress that can impact result specificity. Nonetheless, analyzing genetic interactions can yield highly specific and functional insight. For example, epistatic analyses of yeast genes required for folding in the endoplasmic reticulum identified specific genetic interactions between molecular chaperones that were subsequently validated[19]. Likewise, an aggravating screen for chaperones that enhance the toxicity of two aggregation-prone models (as measured by motility) identified a specific subset of 18 chaperones, orthologs of which impacted Huntingtin aggregation in human cells[26]. Here, we showed that various perturbations of the proteostasis network can uncover specific and functional chaperone interactions.

The main advantage of using RNAi feeding-based genetic interaction screens is the relative simplicity of the method. Even employing a general behavioral output, such as motility, can reveal novel genetic interactions (**Figure 3**). However, variability and partial effects of expression knockdown can limit the robustness and specificity of the results[57]. Moreover, genetic interactions are not indicative of physical interactions and the relationship between two genes could thus be indirect. Exploring the nature of the interactions and discarding non-specific interactions can be time consuming[57-59]. This is a concern that needs to be addressed in the screen setup and validation. For example, using null alleles in genetic screening allows for determining whether these genes function in the same or distinct pathways in a given biological process. However, using partial loss of gene function, hypomorphic alleles, such as temperature-sensitive alleles, or RNAi-dependent down-regulation of expression, results in residual activities that can yield aggravating or alleviating phenotypes, regardless of whether the genes act in the same or in parallel pathways[59]. Thus, the nature of any interaction requires further analysis.

However, using hypomorphic alleles and RNAi could identify a broad range of interactors, including genes in the same protein complex or pathway or in a redundant pathway[59]. While this larger scope of possible interactions can yield more hits in a genetic screen, it can also lead to non-specific interactions, such as, for example, the non-specific exposure of temperature-sensitive alleles in proteostasis collapse[45,53].

Hit validation and non-specific interactions can be examined in several ways. The number of hits should be low. For example, down-regulation of chaperone expression in an *unc-45* mutant background resulted in a small percentage of hits (4%), with most chaperone gene down-regulation not showing any effect on motility. A similar rate was observed when DNJ-24M was over-expressed (6%). As noted above, a screen for chaperone interactions with aggregation-prone proteins identified 18 chaperones of 219 screened (8%).

Several mutant alleles, over-expression lines or disease models should be used. The use of different mutant alleles that have different impacts on chaperone function, as well as different strains with different genetic backgrounds, can support the specificity of any screen hits. Alternatively, using mutation or over-expression of a different chaperone that does not lead to similar perturbation can serve as a negative control. For example, both *unc-45(e286)* and *unc-45(m94)* showed aggravating behavior when *sti-1*, *ahsa-1* or *daf-41* were down-regulated. Moreover, a similar interaction was observed when animals carrying the *ahsa-1(ok3501)* deletion mutant were treated with *hsp-90* RNAi[25].

The identity of the chaperone hits and their known interactions should be examined. For example, chaperones identified in a *unc-45* aggravating screen are a highly specific set of chaperones required for the HSP-90 ATPase cycle, including those encoding a client recruiter (STI-1), a remodeling co-chaperone (AHSA-1), and a client maturation co-chaperone (DAF-41). In fact, this set of co-chaperones forms a complete HSP-90 folding cycle[60]. Likewise, a DNJ-24M screen identified HSP-1, the main chaperone partner of Hsp40s[40].

Changes in interactions over the lifespan of the animal should also be examined. For example, chaperones identified in *unc-45* aggravating screen strongly impacted motility and myosin organization in adulthood but had a milder effect during development. This could be due to changes in the proteostasis network in adulthood[28] or to changes in myosin folding requirements between myo-fiber folding and maintenance.

Use complementary biochemical approaches to directly examine interactions between the proteins, as well as their localization in the cell. For example, the tHSP-90 co-chaperones, STI-1, AHSA-1 and DAF-41, are localized to the sarcomere where they interact with myosin[25].

C. elegans is a well-established metazoan model for monitoring quality control. It is often used to monitor cellular and organismal proteostasis using a variable toolkit of cell biology, biochemical and genetic approaches. Here, we employed genetic screening approaches and available tools[57-59], such as a mutant bank, available RNAi libraries[22,23] and tissue-specific RNAi strains[41,42], to monitor chaperone interactions in a living animal during development and aging.

The use of simple behavioral assays, such as motility, simplify the screen of many possible gene pairs to explore novel genetic interactions. This can then serve as a platform to further explore chaperone localization and physical interactions using biochemical tools to mechanistically study their potential interactions in vivo and in vitro. The protocol described here has been successfully used to identify novel chaperone interactions in *C. elegans* body wall muscle[25,40].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

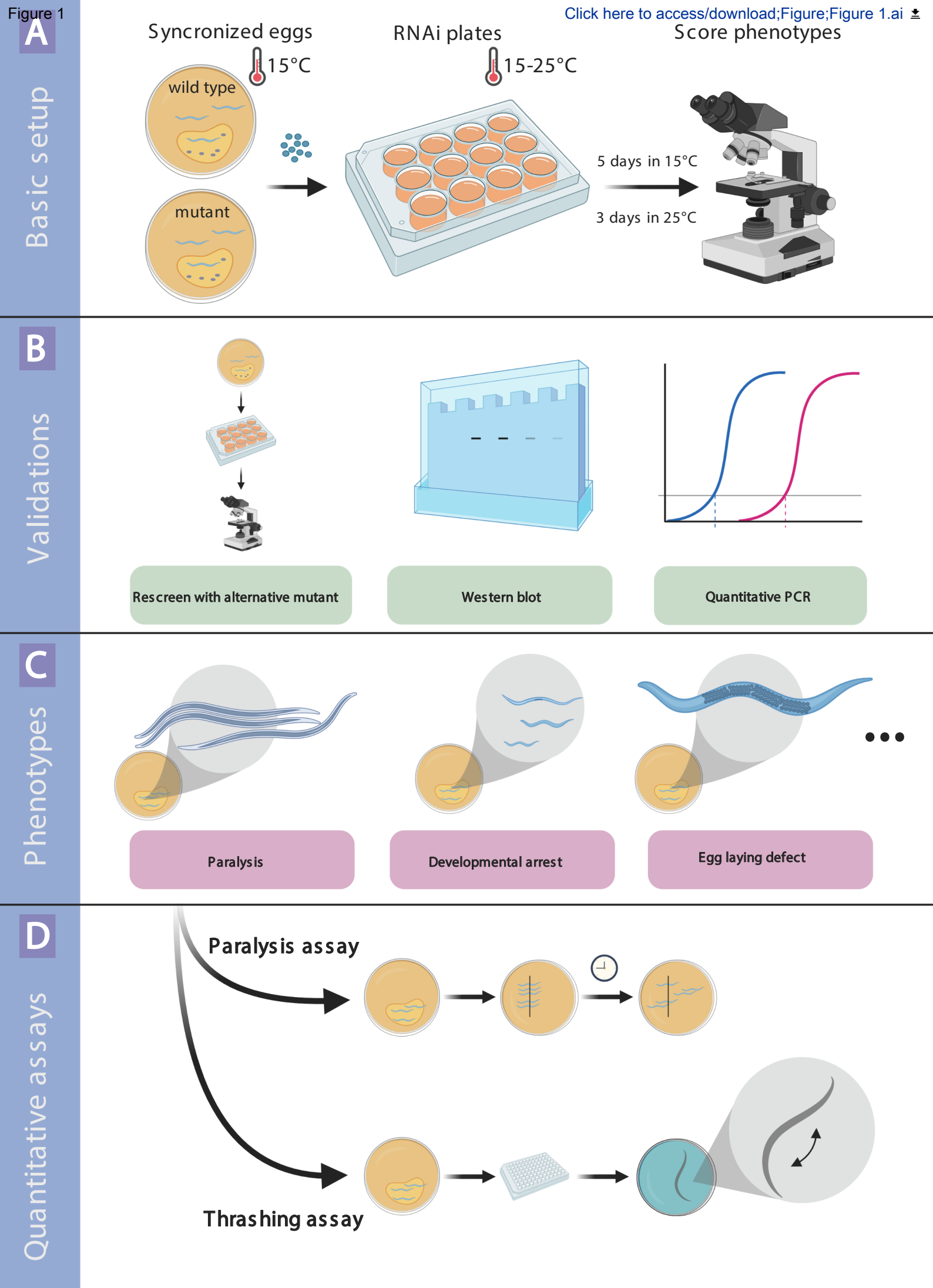

Figure 2

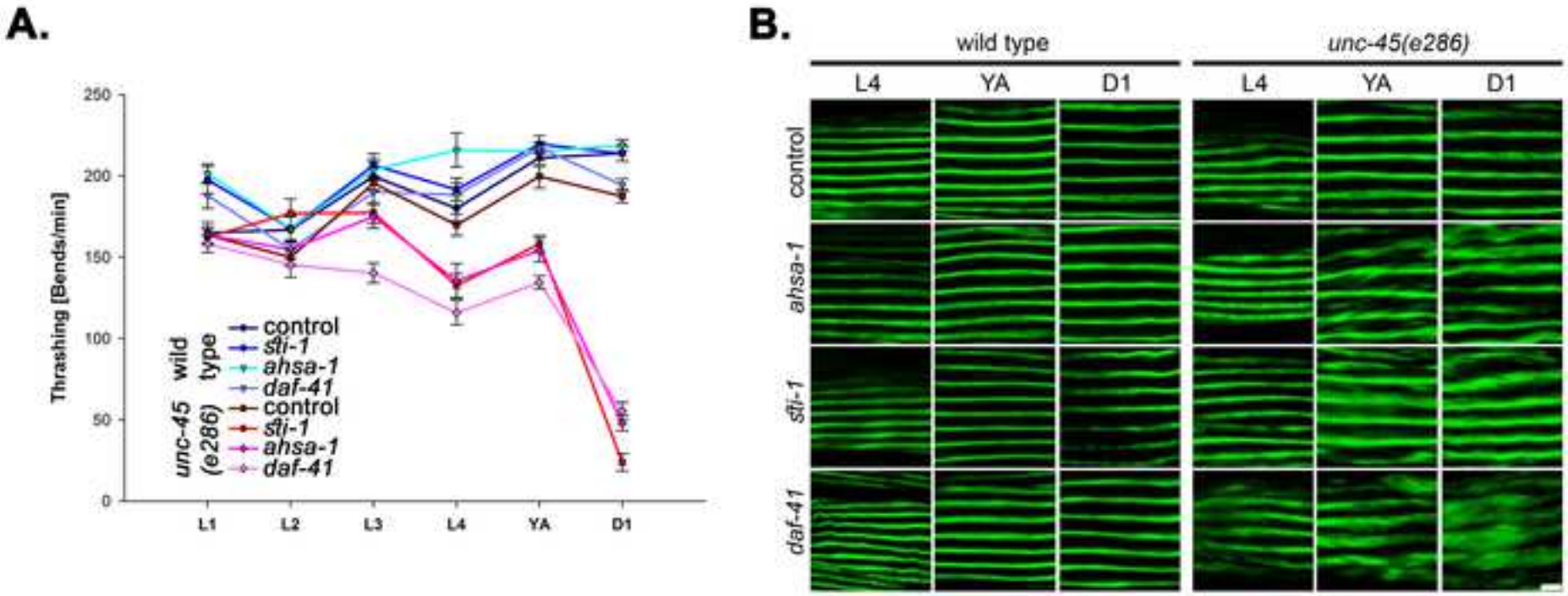

Figure 3

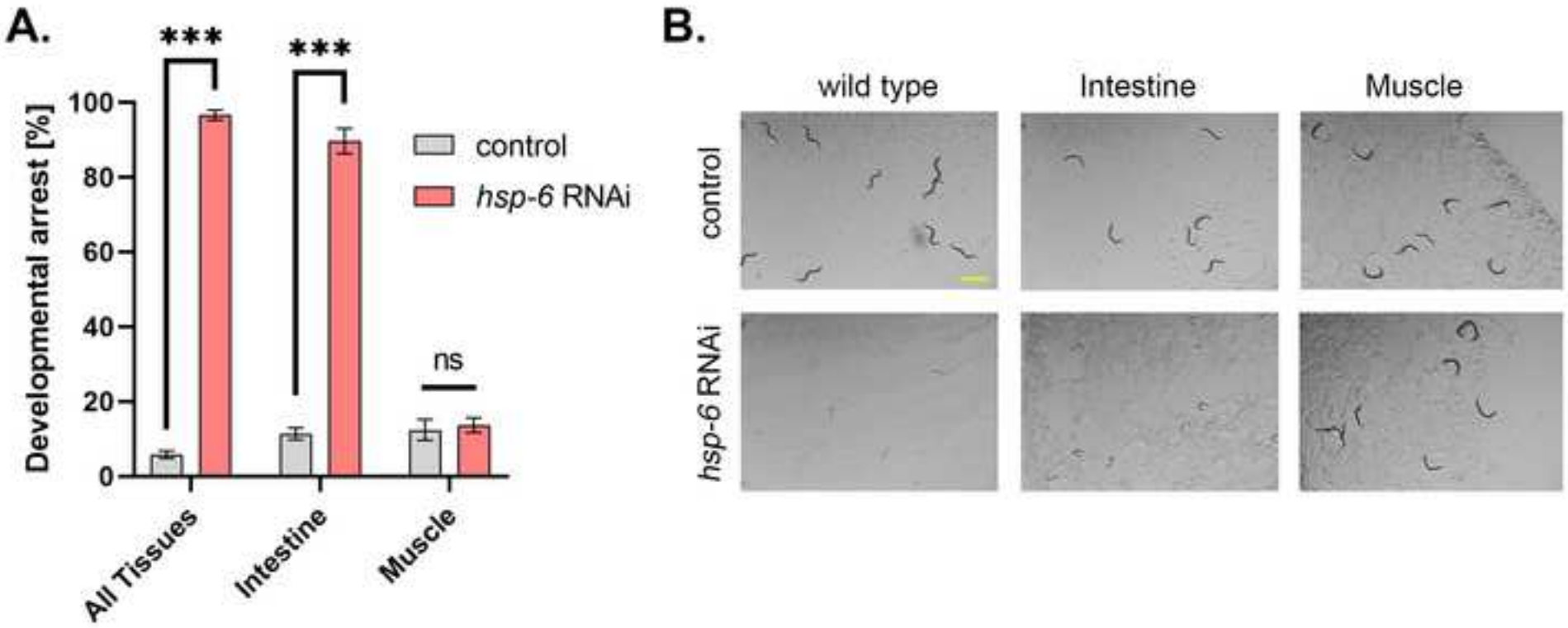

Table 1 Click here to access/download;Table;Table 1 .xlsx

Solution	Preparation instructions
1 M $CaCl_2$ (1 L)	Add 147.01 g $CaCl_2 \cdot 2H_2O$ Add dH_2O to 1 L Autoclave or filter (0.22 µm)
1 M KH_2PO_4, pH 6.0 (1 L)	Add 136.09 g KH_2PO_4 Add 800 mL dH_2O Mix using magnetic stirrer until dissolved Titrate pH using KOH Add dH_2O to 1 L Autoclave or filter (0.22 µm)
1 M $MgSO_4$ (1 L)	Add 248.58 g $MgSO_4 \cdot 7H_2O$ Add dH_2O to 1 L Autoclave or filter (0.22 µm)
Cholesterol solution (50 mL)	Add 250 mg cholesterol to a 50 mL Falcon tube Completely dissolve in 40 mL ethanol Add ethanol to 50 mL
1 M IPTG (50 mL)	Add 11.9 g IPTG (isopropyl-β-D-thiogalactopyranoside) to a 50 mL Falcon tube Completely dissolve in 40 mL dH_2O Add dH_2O to 50 mL Filter (0.22 µm), aliquot 1mL tubes
Ampicillin stock (50 mL)	Add 5 g ampicillin to a 50 mL Falcon tube Completely dissolve in 40 mL dH_2O Add dH_2O to 50 mL Filter (0.22 µm), distribute 1 mL aliquot into Eppendorf tubes
Tetracycline stock (50 mL)	Add 250 mg tetracycline to a 50 mL Falcon tube Completely dissolve in 40 mL ethanol Add ethanol to 50 mL Aliquot 1 mL tubes
M9 buffer (1 L)	Add 5.8 g $Na_2HPO_4 \cdot 7H_2O$ Add 3 g KH_2PO_4 Add 5 g NaCl Add 0.25 g $MgSO_4 \cdot 7H_2O$ Add dH_2O to 1 L Filter (0.22 µm)
1X PBS-T (pH 7.4) (1 L)	Add 8 g of NaCl Add 200 mg of KCl Add 1.44 g $Na_2HPO_4 \cdot 7H_2O$ Add 240 mg KH_2PO_4

1X PBS-T (pH 7.4) (1 L)	Completely dissolve in 800 mL dH_2O Titrate pH using KOH tp pH 7.4 Add 500 µL Tween-20 Add dH_2O to 1 L
5x sample buffer	Add 6.8 mL dH_2O Add 2 mL 0.5M Tris pH 6.8 Add 3.2 mL Glycerol Add 1.6 mL 20% SDS Add 0.8 mL ß-mercaptoethanol 1% bromophenol blue

Storage
Store at RT
Store at RT
Store at RT
Store at -20 °C
Store in the dark at -20 °C
Store at -20 °C
Store at -20 °C
Store at RT
Store at RT

Store at -20 °C

Name of Material/ Equipment	Company
12-well-plates	SPL
40 mm plates	Greiner Bio-one
60 mm plates	Greiner Bio-one
6-well plates	Thermo Scientific
96 well 2 mL 128.0/85mm	Greiner Bio-one
Agar	Formedium
Ampicillin	Formedium
bromophenol blue	Sigma
$CaCl_2$	Merck
Camera	Qimaging
Cholesterol	Amresco
Confocal	Leica
Filter (0.22 µm)	Sigma
Fluorescent stereomicroscope	Leica
Glycerol	Frutarom
IPTG	Formedium
KCl	Merck
KH_2PO_4	Merck
KOH	Bio-Lab
$MgSO_4$	Fisher
Myosin MHC A (MYO-3) antibody	Hybridoma Bank

$Na_2HPO_4 \cdot 7H_2O$	Sigma
NaCl	Bio-Lab
Peptone	Merck
Plate pouring pump	Integra
RNAi Chaperone library	NA
SDS	VWR Life Science
ß-mercaptoethanol	Bio world
stereomicroscope	Leica
Tetracycline	Duchefa Biochemie
Tris	Bio-Lab
Tween-20	Fisher

Catalog Number	Comments/Description
BA3D16B	
627160	
628102	
140675	
780278	
AGA03	
69-52-3	
BO126-25G	
1.02382.0500	
q30548	
0433-250G	
DM5500	
SCGPUO2RE	
MZ165FC	
2355519000024	
367-93-1	
104936	
1.04873.1000	
001649029100	
22189-08-8	Gift from the Morimoto laboratory
5-6	

s-0751

001903029100

61930705001730

does it p920

NA

0837-500

41300000-1

MZ6

64-75-5

002009239100

BP337-500

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 re-edited the manuscript.

2. Please revise the 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.

>> The table was revised and all the missing information is now included.

3. Figure 2B: Please provide scale bars.

>> Scale bar were added to Figures 2B and 3B.

4. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

>> The table was removed from the text and will be uploaded as a .xlsx file named Table 1.

5. 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.

>> Protocol steps were revised, as suggested. Specifically, section 4 sub-steps are now detailed.

6. What RNAi bacteria is used and how much?

>> We used the *E. coli* HT115(DE3) strain. We have included more details on the bacteria used.

7. 3.1: Where did these eggs come from? What NGM plate? The same as from step 1.1? Please specify its preparation.

>> We have modified the text to clarify the missing details and abbreviations.

8. Please include a space between each protocol step.

>> We have added a space between each protocol step.

9. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) 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.

10. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

>> We have marked sections 4-5 of the protocol as essential steps for the video.

11. Please do not abbreviate journal titles.

>> We have used JoVE style in Endnote with the Full journal titles option to format the reference list.

Reviewers' comments:
Reviewer #1:
Major Concerns:
-Line 70: what is the meaning of ´RNA knockdown of the entire genome´?
-Lines 74-78: Could this part be better explained?

>> We have modified the text in lines 86-93 of the Introduction for clarity.

-Line 90: Plates are open or close during drying? Does it have to be done in a hood?

-Line 95: Sterilized plates?

>> We have included more detail on the sterile procedures required in sections 1-2.

-Line 97: Please, explain or give reference to ´RNAi bacteria´.

>> We have included more detail and references on the bacteria used for RNAi experiments.

-Line 106: It will be for the best if explain what ´NGM´ stands for.

>> We have introduced this abbreviation in the text, as the reviewer suggested.

-Page 3, items 3.3 to 3.7: What is the temperature for the M9 that is added?
-Line 171: Temperature for the PBST buffer?
-Line 172: Temperature for the centrifugation procedure?

>> We have included more information on the recommended temperatures for the solutions and procedures used in the protocol.

-Line 144: Please, explain or give reference to ´egl phenotype´.

>> We have included an explanation and reference for the ´egl phenotype´.

-Please, include at least a comment (a more detailed method section will be better) on statistics.

>> We have included information on the experimental set up and use of statistics in section 4.1.

-When possible, refer to alpha and/or beta Hsp90.

>> *C. elegans* only have one HSP90 gene; we now use the worm name for HSP-90, for clarity.

-Include a section on ups and downs of the technique and, when possible, alternatives.
>> We have included advantages and disadvantages of the technique in the Discussion section.

Minor Concerns:
-Review Introduction writing to make it more interesting.
-Review writing: lines 137, 151, 152, 161 and 141.
-Line 165: Please, check ´10 mm´
>> We have corrected the mistakes in the text and re-edited the manuscript.

Reviewer #2:
Major concern.
…The authors must include 1-2 paragraphs in their discussion that focus on proper inference of genetic interactions (or weave into the existing discussion), and all three of the aforementioned reviews should be added to the references.

>> We have included a more detailed discussion on how to interpret genetic interaction results using RNAi and have included all the references suggested by the reviewer.

Minor concerns.
1. No mention of biological and/or technical replication as necessary in the protocol.

>> We have included information on the experimental set up and use of statistics in section 4.1.

2. Protocol section 4.4: Review the sentences on lines 155-156- if a 96-well plate is used, it seems unrealistic be able to observe body bends of an individual animal when there are 15 animals in a single well.

>> The reviewer is correct, animals were set at one per well. This is now stated clearly in the text.

3. The short abstract is focused on the representative results, and not the method.

>> We have modified the short abstract, as the reviewer suggested.

4. Beginning of introduction- sequestration of damaged proteins is another protective strategy involving chaperones- in addition to repair and breakdown- to maintain proteostasis and should be referenced (as overviewed in Walther, Dirk M., et al. "Widespread proteome remodeling and aggregation in aging C. elegans." Cell (2015).)

>> We have modified the Introduction (lines 53-56) and included a reference to a current review discussing sequestration of damaged proteins, as the reviewer suggested.

5. The last paragraph of the introduction should be revised for clarity: the brief description of RNAi (lines 74 - 77) seems to be out of order; the mention of specificity when not using combinations of null mutants is more of a point for the discussion.

>> We have revised the Introduction (lines 85-93) for clarity and moved the mention of specificity to the Discussion section, as the reviewer suggested.

6. Were 10 cm plates intended instead of 10 mm plates? (lines 88, 165)

>> We have corrected the mistakes in the text.

7. Provide a guideline for the number of plates necessary for a typical experiment, accounting for replication (section 1).

>> We have included information on the experimental set up and use of statistics in section 4.1.

8. 2.4 (lines 99-100)- Provide typical volumes for bacterial culture to seed, as this likely depends on the plate type and may not be the entire 300 μL culture.

>> We have included information on typical volumes for seeding.

9. 2.5 (lines 101-102)- What temperature should plates be allowed to dry at, presumably room temperature?

>> We have included more information on the recommended temperatures for the solutions and procedures used in the protocol.

10. 3.1 (line 106)- what size plate?

>> We have included more information on the size of plates to be used in the protocol.

11. 3.2 (line 107)- why 15c? Presumably this is due to working with ts-mutant backgrounds where 25c would be a non-permissive temperature, but adding a note about this would improve clarity.

>> We have included more information on the recommended temperatures for the experimental set up.

12. There are many mentions of raising animals for experiments at temperatures between 15 - 25 C, however there is little discussion of selecting an appropriate temperature outside of the context of temperature-sensitive mutants.

>> We have added a sub-section on “Cultivation of animals during experiments” in which we have noted the impact of temperature on experimental design.

13. 3.5 (lines 115-117)- how would this avoid collecting adults?

>> We have included the wash steps (revised 3.7-3.8) originally missing from the protocol.

14. 3.11 note (lines 132-134)- cite reference for age-dependent proteostasis collapse; additional mention of time point selection in the discussion could be helpful to some readers.

>> We have added a reference for proteostasis collapse in the revised protocol, as the reviewer suggested. We have also noted the impact of age on monitoring genetic interactions in the Discussion.

15. Protocol section 4- each of these sub-steps gives only a very brief outline of the relevant assays- consider breaking them down further in more detail, or otherwise adding references where more detailed protocols may be found.

>> We followed the reviewer’s suggestion and divided the sub-heading into sub-steps, as well as adding references to section 4.

16. 4.2- It should be noted here that different strains may require different developmental time periods to reach the egg-laying stage; this point may be relevant to other aspects of the method as well, but is critical here so as to avoid calling a case of developmental delay as sterile.

>> As noted above, we have added a sub-section on “Cultivation of animals during experiments” in which we noted the impact of developmental delay on experimental design.

17. 4.3, 4.4- What age animals would be typically used for these assays?

>> We now address the recommended age for different experiments in section 4.

18. Protocol section 5- choose a more informative heading to reflect that the steps are for validating knockdown/mutation at the protein level, and not validation of phenotype or gene expression.

>> We have modified the title of section 5, as the reviewer suggested.

19. Lines 295-6: The authors state: "UNC-45, a chaperone that is highly expressed in one tissue was used to screen for chaperone interactions.". UNC-45 is also expressed in muscle, the authors should make this clear.

UNC-45 is highly expressed in different muscle cells. We modified the text in the discussion line 1575 to note this.

20. Inefficiency of RNAi in neurons in non-RNAi-enhanced strains is not addressed as a caveat in the discussion.

>> We have addressed the inefficiency of RNAi in neurons in the Discussion section.

21. Revise sentences 324-326- while specificity of the interaction is important to address, the issue is not clearly introduced.

>> We have revised the text in the Discussion section, as the reviewer suggested.

22. Some materials mentioned in the protocol are not included in the materials table:
a. M9 buffer
b. RNAi bacterial stocks
c. PBST
d. 5x sample buffer

>> We have included preparation instructions for M9, PBST and 5x sample buffer in Table 1 and for the RNAi library and solution components in the Material Table.

23. There are some typographical errors, and some cases where alternative terms might be clearer including:
a. "Using a worm pick to move…" (line 106)
b. "stereomicroscope" instead of "binocular" (lines 125, 157)
c. "…at the at 15-…" (lines 137, 141)
d. "…medium that severs paralysis" (line 152)
e. "…sti-1-, ahsa-1-, or daf-41 on…" (line 278)
f. "…caused by the wo chaperones…" (line 301)
g. "…calthrin-mediated endocytosis…" (line 312)

>> We have corrected the mistakes in the text and re-edited the manuscript.