Distinct Spatiotemporal Regulation of the Cytoprotective Heat Shock Response in Caenorhabditis elegans

Rosemary Nadine Plagens

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Distinct Spatiotemporal Regulation of the Cytoprotective Heat Shock Response in *Caenorhabditis elegans*

by

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A dissertation submitted to the College of Engineering and Science of Florida Institute of Technology in partial fulfillment of the requirements for the degree of

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Abstract

Distinct Spatiotemporal Regulation of the Cytoprotective Heat Shock Response in

*Caenorhabditis elegans*

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Every organism studied to-date utilizes the heat shock response (HSR) to maintain protein-folding homeostasis (proteostasis) during temperature or other protein-folding stress. The HSR has been well characterized using acute heat stress (HS) in single-celled models, but less is known about how distinct cell types and tissues respond to HS. Furthermore, how metazoans respond to prolonged HS at the molecular level remains relatively unexplored.

The model organism *C. elegans*, with its genetic tractability and distinct tissues and behaviors, has been used extensively in the field to characterize the acute HSR, but with considerable variability across labs regarding HS temperature and duration. Our objectives were to standardize HSR methodology and to examine spatiotemporal regulation of the HSR in this nematode by characterizing the effects of HS in different tissues and using extended timescales. These goals were met by combining genetic knockdowns, fluorescent protein reporters, qPCR, transcriptomic analyses, and behavioral assays. First, we demonstrated that the commonly used thermotolerance assay does not rely on the master regulator of the HSR, HSF-1, and instead proposed use of an alternate thermorecovery assay, which does rely on HSF-1, as standard practice for HSR studies. Next, we uncovered tissue-specific responses to proteostasis disruptions that are driven by the
balance between ubiquitously expressed HSR components and their tissue-specific substrates. Then, we identified a new cellular pathway affected by chronic HS—endocytosis—which was disrupted in oocytes, coelomocytes, and neurons at different points along a time course of chronic HS. Overexpression of molecular chaperones rescued endocytic defects and partially restored the associated phenotypes, suggesting chaperone titration during chronic HS as the driving mechanism. This mechanism is shared by neurodegenerative disease cell culture models but has not been shown in an intact, multicellular organism or with HS. Finally, we discovered that chronic HS induces activation of the hsf-1 promoter. This is particularly exciting because it has long been believed that HS does not affect hsf-1 transcription, since HSF-1 levels remain stable during a standard acute HS. Together, we have uncovered novel regulation of the HSR across space and time in C. elegans.
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Dedication

This is dedicated to my family—particularly to my husband, Derek, and the boys we lost, B-man, Jack-Jack, and Mickey. None of this would have been possible without you.
Chapter 1
Introduction

Overview
The primary role of cellular stress responses in living organisms is to maintain homeostasis across cells and tissues to sustain life. One of the most well-characterized stress responses is the heat shock response (HSR). This pathway is highly conserved and has been studied extensively a variety of organisms, including the multicellular eukaryote, Caenorhabditis elegans. These nematodes make excellent model organisms for studying the basic molecular mechanisms of stress responses due to their ease of culture, transparent bodies, and conserved genomes. Here, an overview is given for the HSR, protein-folding diseases, and the utility of using temperature to study general protein misfolding.

The heat shock response (HSR)
The observation by Ritossa et al. in the 1960s that Drosophila chromosomes responded to heat stress (HS) by visibly puffing provided the field of molecular biology with one of the first tools for studying rapidly induced gene expression changes (Ritossa 1962). While chromosomal puffs had been previously associated with RNA production, the discovery of unique and reproducible RNA banding patterns following HS paved the way for studies of transcriptional regulation. Soon after this discovery, scientists were able to link these heat-inducible changes with the expression of heat shock proteins (HSPs) and began to establish correlations between HS and genome-wide changes in gene expression levels (Snutch and Baillie 1983; Anckar and Sistonen 2011). The expression of these HSPs in response to protein misfolding caused by heat and other stressors became a widely studied pathway.
known as the heat shock response (HSR). Of the many proteins expressed in response to HS, the most prominent group belongs to the molecular chaperone protein families. These chaperones are recruited to misfolded proteins throughout the cell to either refold them or mark them for degeneration (Brunquell et al. 2016). This regulation of protein-folding homeostasis (proteostasis) plays a crucial role not only during stress-induced protein misfolding, but also during development and normal protein synthesis (Vihervaara and Sistonen 2014).

When cells are exposed to elevated temperatures, heavy metals, or other stressors that cause cytoplasmic proteins to become misfolded, the HSR is activated to restore proteostasis (Richter et al. 2010). The main regulator of the HSR is the transcription factor heat shock factor 1 (HSF1), which is also the only HSF found in invertebrates (whereas at least four HSFs have been described in vertebrates) (Anckar and Sistonen 2011). In vertebrates, HSF1 has been found to be expressed throughout all tissues and kept mostly inactive during periods of favorable conditions (Åkerfelt et al. 2010).

While there are several models to explain the general inhibition of HSF1 during basal conditions, the most widely accepted mechanism is through interactions with molecular chaperones known as heat shock protein 40 (HSP40), HSP70, and HSP90 along with the cytosolic chaperonin TCP1 ring complex TRiC/CCT (Neef et al. 2014; Gomez-Pastor et al. 2018). These proteins interact with HSF1 monomers to prevent them from trimerizing and becoming capable of transcriptional activation. Under conditions of protein-folding stress in the cytoplasm, the exposure of hydrophobic regions in misfolded proteins is sensed by chaperones, which are subsequently titrated away from HSF1 to stabilize the
partially unfolded proteins. HSF1 monomers are then released from their inhibitory complexes, form homotrimers, and accumulate in the nucleus while also undergoing extensive post-translational modifications, including acetylation and methylation (Budzyński et al. 2015). These activated HSF1 trimers bind to the promoters of HSP genes on a conserved, inverted repeat of the nGAAn pentamer known as the heat shock element (HSE) (Trinklein 2003). The regulon of genes whose expression is induced upon activation of the HSR is complex and includes not only members of the HSP family but also a large number of other genes, such as genes that function in cuticle structure, as will be discussed later. Groups of genes for many cellular pathways are also down-regulated during the HSR, including genes that encode for proteins with ATP- or ion-binding activity, kinase activity, or roles in transcription or reproductive development (Brunquell et al. 2016). The diverse functions of the genes regulated during the HSR indicate an intricate system that is designed to reduce some cellular activities while boosting overall protein-folding activity and structural integrity of the organism. The HSR is involved in a negative feedback loop wherein HSF1 becomes inactivated when HSP70/90 chaperone levels exceed the protein-refolding needs of the cell, thus allowing the chaperones to resume their role as HSF-1 suppressors and turn off the HSR when its job is done.

While the HSR is typically thought of as being a response to external stressors, it also plays pivotal roles during various stages of development. HSF-1 has been shown to interact with and regulate a distinct network of genes during C. elegans larval development, where it influences not only chaperones but also genes required for metabolism and gene expression (Li et al. 2016). Similarly, HSF1 has been linked to the regulation of another network of genes during carcinogenesis, where cancer cells can seemingly hijack the protective roles
of the HSR for their own gain (Mendillo et al. 2012). Therefore, the HSR is important for maintaining cellular proteostasis by combating external and internal stressors associated with high demands for de novo protein folding or protein refolding.

Proteostasis diseases

Based on the critical relationship between protein-folding states and cellular function, disruptions in proteostasis can lead to a range of severe clinical conditions, including Huntington’s disease (HD), Alzheimer’s disease (AD), and Parkinson’s disease (PD). Each of these diseases has been characterized by mutation or misfolding of distinct proteins with diverse clinical consequences. In HD, protein aggregation caused by polyglutamine expansions in the huntingtin (Htt) protein is associated with a particularly prominent loss of medium spiny neurons (MSNs), leading to degeneration in the striatum (Labbadia and Morimoto 2013; Jimenez-Sanchez et al. 2017). On the other hand, AD is associated with amyloid beta plaques and tau protein tangles in the hippocampus and cerebral cortex, whereas PD is associated with alpha synuclein in the substantia nigra and hypothalamus (Soto 2003).

While these diseases differ in the proteins and neurons affected, they share the pathological mechanism of protein misfolding and aggregation (Soto 2003; Labbadia and Morimoto 2013). Importantly, the HSR, which is the primary cytosolic response to disrupted proteostasis, has been linked with regulating cellular functions in various neurodegenerative disease cell culture models (Yu et al. 2014). These disease models have thus far provided a wealth of knowledge for understanding individual neurodegenerative diseases where specific mutations deleteriously affect proteostasis. Using a single variable
to disrupt protein folding can offer insight towards understanding general perturbations of proteostasis.

Using temperature to study proteostasis

In contrast to the single protein mutations associated with protein-folding diseases, temperature applies a uniform organismal stress and can be used as a powerful tool for studying the general effects of protein misfolding. Elevated temperatures cause proteins to denature due to the entropic costs associated with collapsing a very large number of unfolded conformations into a small number of conformations available for the native state (Dill et al. 2008). Moreover, many proteins have marginally stabilized native states, such that an increase of 4°C is predicted to destabilize the average protein by ~20% in E. coli (Ghosh and Dill 2010). Thus, temperature stress can have profound effects on the total proteome.

Since its discovery, the HSR has been found in every living organism investigated (Lindquist and Craig 1988). As yet, the acute HSR has been fairly well characterized using single-celled models and in vitro assays. However, the intricacies of how multicellular organisms respond to disruptions of proteostasis in the face of HS remain to be fully described. Also, determining whether organisms mount the same HSR during acute and chronic HS will be paramount in understanding the effects of prolonged proteostasis disruption. Thus, the ensuing chapters focus on characterizing the effects of temperature stress across space (cell and tissue types, such as intestine vs muscles) and time (duration of HS, such as 1-hour acute vs 48-hour chronic HS) in C. elegans.
Chapter 2
Standardized Methods for Measuring Induction of the Heat Shock Response in *Caenorhabditis elegans*


Abstract
The heat shock response (HSR) is a cellular stress response induced by cytosolic protein misfolding that functions to restore protein folding homeostasis, or proteostasis. *Caenorhabditis elegans* occupies a unique and powerful niche for HSR research because the HSR can be assessed at the molecular, cellular, and organismal levels. Therefore, changes at the molecular level can be visualized at the cellular level and their impacts on physiology can be quantitated at the organismal level. While assays for measuring the HSR are straightforward, variations in the timing, temperature, and methodology described in the literature make it challenging to compare results across studies. Furthermore, these issues act as a barrier for anyone seeking to incorporate HSR analysis into their research. Here, a series of protocols is presented for measuring induction of the HSR in a robust and reproducible manner with RT-qPCR, fluorescent reporters, and an organismal thermorecovery assay. Additionally, we show that a widely used thermotolerance assay is not dependent on the well-established master regulator of the HSR, HSF-1, and therefore should not be used for HSR research. Finally, variations in these assays found in the literature are discussed and best practices are proposed to help standardize results across the field, ultimately facilitating neurodegenerative disease, aging, and HSR research.
Introduction

The heat shock response (HSR) is a universal cellular stress response induced by cytosolic protein misfolding caused by temperature increases and other proteotoxic stresses. Activation of the HSR in Caenorhabditis elegans leads to transcriptional upregulation of heat shock genes such as \textit{hsp}-70 and \textit{hsp}-16.2. Many heat shock proteins (HSPs) function as molecular chaperones that restore protein folding homeostasis, or proteostasis, by directly interacting with misfolded or damaged proteins. The master regulator of the HSR is the transcription factor Heat Shock Factor 1 (HSF-1), whose activation is elegantly controlled via multiple mechanisms\(^1\). The role of HSF-1 is not restricted to stress. HSF-1 is required for normal growth and development, as deletion of \textit{hsf}-1 leads to larval arrest\(^2\). HSF-1 is also important during aging and age-related neurodegenerative diseases characterized by accumulation of protein aggregates and an inability to maintain proteostasis. Knockdown of \textit{hsf}-1 causes accumulation of protein aggregates and a shortened lifespan, while overexpression of \textit{hsf}-1 reduces protein aggregation and extends lifespan\(^3,4\). Therefore, regulation of HSF-1 at the molecular level has broad implications for organismal physiology and disease. \textit{C. elegans} is a powerful model organism for HSR research because the HSR can be measured at the molecular, cellular, and organismal levels\(^4,5,6\). Highlighting the power of this model, key advances in delineating the HSR pathway, such as tissue-specific differences in HSR regulation, have been discovered in \textit{C. elegans}\(^7,8\). Furthermore, \textit{C. elegans} is widely used for aging research and is an emerging system for modeling diseases linked to proteostasis disruption. Although heat shock experiments with \textit{C. elegans} can be quick and reproducible, there are several questions to consider before beginning. For example, which temperature should be used for induction
of the HSR and how long should the worms be exposed? Is it better to use a dry incubator or a water bath? Which developmental stage should be used? Unfortunately, the methodologies used to investigate the HSR vary widely from laboratory to laboratory, causing confusion when selecting the best methodologies and making it difficult to compare results across the field. We present robust and standardized protocols for using RTqPCR, fluorescent reporters, and thermorecovery to measure the HSR. While these three approaches are complementary, they each have unique advantages and disadvantages. For example, RT-qPCR is the most direct and quantitative measurement of the HSR, and this assay can be easily expanded to include many different heat shock-inducible genes. However, RT-qPCR is the most expensive, can be technically difficult, and requires the use of specialized equipment. In contrast, fluorescent reporters have the advantage of measuring tissue-specific differences in HSR induction. However, they are difficult to quantitate accurately, can only measure induction above a certain threshold, and require the use of a fluorescence microscope. Additionally, the reporter strains described here are developmentally delayed compared to the standard N2 strain. Although newer reporter strains containing single-copy transgenes are available, they have not been tested here\(^9\). The third assay, thermorecovery, has the advantage of providing a physiologically relevant readout at the organismal level. However, this assay is arguably the least sensitive and most indirect. Finally, we discuss some common variations found in these assays and propose a set of best practices to facilitate research in this field.
Protocol

1. Maintenance and synchronization of *C. elegans*

1. Maintain worms at 20°C on Nematode Growth Medium (NGM) plates seeded with *OP50 Escherichia coli* bacteria by transferring several adults to fresh plates approximately 2x per week\(^\text{10}\). Care should be taken to prevent worms from running out of food, because this can affect their physiology\(^\text{11}\).

1. Preparation of NGM plates.

   1. Mix 3 g of NaCl, 2.5 g of Bacto-peptone, 20 g of agar, and deionized (DI) H\(_2\)O up to 1 L in a flask.
   2. Autoclave the mixture for sterilization.
   3. Allow mixture to cool to \(\sim\)50°C.
   4. Add 25 mL of 1 M KH\(_2\)PO\(_4\) (pH = 6), 1 mL of 1 M CaCl\(_2\), 1 mL of 1 M MgSO\(_4\), and 1 mL of cholesterol (5 mg/mL in 100% ethanol).
   5. Using sterile technique, pour the mixture into 6 cm plates to yield approximately 100 plates. Pouring plates is easier if the mixture is first transferred to a 300 mL sterile beaker.
   6. Allow 1 day to solidify at room temperature (RT) before seeding with bacteria or storing at 4°C.

2. Seeding of OP50 bacteria onto NGM plates.

   1. Grow a saturated overnight OP50 bacterial culture in LB at 30°C or 37°C.
   2. Place approximately 300 μL of the culture onto the center of a 6 cm NGM plate.
3. Let plates dry at RT for 1-3 days as needed for the bacterial lawn to adhere to the plate. Plates can then be used or stored at 4°C.

2. Grow the worms synchronously either by isolating freshly laid eggs (described here) or alternatively by collecting eggs after dissolving worms with bleach.
   1. Transfer approximately 10 gravid adult worms to a fresh plate using a platinum wire pick. Egg-lay synchronization works best if the adults are in the first day of adulthood.
   2. After approximately 1 h, remove the worms from the plate. This should result in 40-60 eggs per plate, depending on the conditions and the strain.

2. Fluorescent imaging of HSR reporters
   1. Synchronize the worms (section 1.2) and maintain at 20°C until the desired developmental stage. For the AM446 (hsp-70p::gfp) and CL2070 (hsp-16.2p::gfp) fluorescent reporter strains, young adult worms that have not yet reached reproductive maturity are generated 64 h after the egg-laying synchronization. NOTE: The developmental timing varies with each strain and the temperature at which the worms are raised. Both HSR reporter strains exhibit a slight developmental delay relative to N2. Importantly, the magnitude of HSR induction declines approximately 2-4x after the onset of reproductive maturity (see Discussion).
   2. Heat shock the worms by wrapping plates with paraffin film and submerging in a circulating water bath at 33°C for 1 h. A thin strip of paraffin film should be wrapped 2x around the plate to seal the edges. Do not cover the bottom of the plate or it could interfere with heat transfer. Submerge the plates upside down using a test tube rack and a lead weight. Remember to include a negative control sample (no heat shock) if
necessary. NOTE: If the paraffin film is not secure, then water will enter the plate and the plate should not be used for data collection.

3. Recover the worms by removing the plates from the water bath and drying with a paper towel. Remove the paraffin film and incubate the worms at 20°C for 6-24 h. This recovery period allows sufficient time for GFP synthesis and folding before imaging.

4. Prepare slides for imaging. Slides should be prepared fresh for each use.
   1. Make a 3% agarose solution in water and heat using a microwave until the agarose is dissolved.
   2. Place a microscope slide for imaging between two other microscope slides that have a strip of laboratory tape on them to create a spacer for the agarose pad.
   3. Using a 1,000 μL pipette, place a drop (~150 μL) of the heated 3% agarose in the center of the microscope slide.
   4. Immediately cover the microscope slide with a blank microscope slide perpendicular to the first slide so that the top slide rests on the laboratory tape on the adjacent slides. This spreads out the drop of agarose to create a pad of uniform width.
   5. Carefully remove the top slide.

5. Immobilize the worms by using a 200 μL pipette to add a small drop (~5 μL) of 1 mM levamisole in M9 buffer to the center of the agarose pad. Then transfer 10 worms into the drop of levamisole using a platinum wire pick. Cover with a coverslip. Sealing the coverslip is not necessary for an upright microscope. Optionally, the worms can be aligned when they become paralyzed by spreading the levamisole off, to the outside of the agarose pad, and aligning the worms with a platinum wire pick. Alternatively, the
levamisole can be soaked up using a laboratory wipe. NOTE: Image as soon as possible, because prolonged incubation in levamisole could alter fluorescence.

6. Image the worms using a fluorescence microscope. The details of image capture vary by microscope and software. NOTE: To directly compare image intensities, use identical microscope settings in one imaging session. Avoid oversaturating the image.

3. Measurement of HSR gene expression using RT-qPCR

1. Synchronize worms (section 1.2) and maintain at 20°C until the desired developmental stage. For N2 worms, young adult worms that have not yet reached reproductive maturity are generated 60 h after the egg-laying synchronization. NOTE: The developmental timing varies with each strain and the temperature at which the worms are raised. Importantly, the magnitude of HSR induction declines approximately 2-4x after the onset of reproductive maturity (see Discussion).

2. Heat shock worms as described in step 2.2.

3. Take the plates out of the water bath, remove the paraffin film, and immediately collect the worms. The worms can be collected by washing the plates gently with 1 mL of M9, collecting the liquid in a microcentrifuge tube, and then removing the M9 after centrifugation at 400 x g for 1 min.

4. Lyse the worms and purify the RNA using organic extraction.

   1. Add 250 μL of RNA isolation reagent (see Table of Materials).

   2. Vortex tubes by hand for 30 s.

   3. Vortex tubes for 20 min at 4°C using a microcentrifuge tube attachment (see Table of Materials).

   4. Add 50 μL of chloroform.
5. Vortex for 30 s.

6. Incubate the samples at RT for 3 min.

7. Centrifuge at ≥14,000 x g for 15 min at 4°C.

8. Transfer the aqueous layer (i.e., top layer, ~125 μL) to a new microcentrifuge tube. NOTE: Avoid the organic layer and the material in the interface.

9. Add 50 μL of chloroform.

10. Vortex for 30 s.

11. Incubate the samples at RT for 3 min.

12. Centrifuge at ≥14,000 x g for 5 min at 4°C.

13. Transfer the aqueous layer (~100 μL) to a new microcentrifuge tube. NOTE: Avoid the organic layer and the material in the interface.

14. Precipitate RNA with an equal volume (i.e., 100 μL) of isopropanol.

15. Incubate at -20°C for at least 30 min, but preferably overnight. NOTE: The experiment can be paused here and the RNA can be stored at -20°C.

16. Pellet the RNA by centrifugation at ≥14,000 x g for ≥30 min at 4°C.

17. Remove as much of the supernatant as possible without disturbing the pellet. NOTE: The pellet will be small and may not be visible. The pellet may not adhere tightly to the side of the tube, so caution is necessary to avoid dislodging it.

18. Wash the pellet with 250 μL of 70% ice-cold ethanol made with RNase-free H2O.

19. Centrifuge at ≥14,000 x g for ≥5 min at 4°C.

20. Remove as much supernatant as possible without disturbing the pellet.
21. Perform a quick spin at RT to remove any remaining 70% ethanol.

22. Dry the pellet by leaving the tubes open at RT as long as needed; typically at least 20 min. Tubes can be covered with a lint-free tissue or aluminum foil to prevent contamination.

23. Resuspend the pellet in 20 μL of RNase-free H2O.

24. Determine the RNA concentration using a small volume spectrophotometer (2 μL). NOTE: The experiment can be paused here and the RNA can be temporarily stored at or below -20°C.

5. Remove residual DNA by incubating with DNase I. It is recommended to use a commercially available kit (see Table of Materials) and to follow the manufacturer's instructions.

1. With this kit, prepare a 20 μL reaction with 500 ng of RNA and 1 μL of DNase I in a 37°C water bath for 30 min.

2. Add 2.5 μL of DNase inactivation reagent (included in the kit) to each sample and incubate at RT for 5 min with occasional flicking/vortexing.

3. Spin down at 14,000 x g for 2 min.

4. Without disturbing the white pellet, transfer 15 μL of supernatant to a fresh microtube for cDNA synthesis.

6. Conduct cDNA synthesis. It is recommended to use a commercially available kit (see Table of Materials) and to follow the manufacturer's instructions.

1. With the kit, prepare a 20 μL reaction with 15 μL of DNase I-treated RNA from the previous step and 1 μL of reverse transcriptase.
2. Use the following program for cDNA synthesis: 25°C for 5 min, 46°C for 20 min, 95°C for 1 min, 4°C hold.

3. Dilute cDNA by adding 80 μL of RNase-free H2O directly to the sample.

4. Briefly vortex, then spin down and store at -20°C until needed.

7. Perform qPCR. It is recommended to use a commercially available kit (see Table of Materials) and to follow the manufacturer's instructions.

   1. With the kit, prepare a 25 μL reaction containing 2 μL of cDNA and 200 nM (each) of forward and reverse primers in one well of a 96-well plate.

   2. Primer sequences for measuring the heat shock genes, *hsp*-70 and *hsp*-16.2, and *18S* rRNA (for a normalization control) are listed in the Table of Materials.

   Multiple normalization controls can be used as desired.

   3. Dilute cDNA samples 50x before measurement of *18S* to ensure that the assay is in the linear range. Appropriate qPCR conditions vary with the kit and primers used (see Representative Results).

   4. Use a real-time PCR detection system (see Table of Materials) for qPCR with 40 cycles of 95°C for 5 s denaturation, 58°C for 30 s annealing, and 72°C for 30 s extension. NOTE: Optimal annealing temperatures can vary by primers and conditions.

   5. Quantify using either the ΔΔCt or standard curve method.

4. Thermorecovery assay for measuring HSR at the organismal level

   1. Synchronize the worms (section 1.2) and maintain at 20°C until the desired developmental stage. For N2 worms, young adult worms that have not yet reached reproductive maturity are generated 60 h after the egg-laying synchronization. NOTE:
The developmental timing varies with each strain and the temperature at which the worms are raised. Importantly, the magnitude of HSR induction declines approximately 2-4x after the onset of reproductive maturity (see Discussion).

2. Heat shock the worms as described in step 2.2 for 6 h.

3. Remove the plates from the water bath, remove the paraffin film, and allow the worms to recover by incubation at 20°C for 48 h.

4. Count the number of worms that can immediately crawl away after mechanical stimulation without jerky movement or paralysis. NOTE: The 6 h incubation is optimal for examining conditions that reduce thermorecovery, but longer exposure times may be needed to look for conditions that enhance thermorecovery.

Representative results

Using the protocols described in this manuscript, HSR induction was measured using fluorescent reporters, RTqPCR, and thermorecovery assays. In each case, the procedure in section 1.2 was used to generate synchronized, young adult worms that had not reached reproductive maturity.

To visualize HSR induction at the cellular level, the AM446 (hsp-70p::gfp) and CL2070 (hsp-16.2p::gfp) fluorescent reporter strains were analyzed following section 2 of the protocol. In the negative control samples without heat shock, the hsp-16.2 reporter only showed normal autofluorescence, but the hsp-70 reporter had constitutive fluorescence in the anal depressor muscle as previously reported (Figure 1A). After 1 h of heat shock at 33°C, robust fluorescence was observed in both reporters; however, the pattern of expression was distinct depending on which reporter was used (Figure 1B). The hsp-70
reporter was brightest in the intestine and spermatheca, whereas the hsp-16.2 reporter was brightest in the pharynx. Additionally, the hsp-16.2 reporter had a high degree of worm-to-worm variability in the amount of induction as previously described, but the hsp-70 reporter did not.

A commonly used variation of section 2 is to perform the heat shock in a dry incubator instead of a circulating water bath. Therefore, the difference between the two methodologies was also tested. It was found that both protocols resulted in robust induction of the two fluorescent reporters using our conditions, although a circulating water bath is recommended as a best practice (see Discussion) (Figure 1B).

![Figure 2-1: (Fig. 1) HSR induction measured with fluorescent reporters.](image)

(A) The basal and (B) heat-inducible expression of hsp-70p::gfp and hsp-16.2p::gfp reporter strains after 1 h of heat shock at 33°C in a water bath or incubator. Worms were raised on OP50 bacteria for 64 h, heat shocked, and then recovered at 20°C for 8 h before imaging. For reference, the no heat-shock worms in (A) were renormalized in (B) to match the range and saturation of the heat-shocked worms. Representative images of two experimental replicates are shown. Scale bar = 250 μm.
To test the dependence of the reporters on the transcription factor HSF-1, feeding RNAi was used to knockdown hsf-1 before reporter induction was measured. It was found that fluorescence of both strains was severely reduced upon HSF-1 knockdown, indicating that these reporters are HSF-1-dependent as described in the literature⁴ (Figure 2). However, it was also observed that pharyngeal fluorescence persisted in both reporters upon hsf-1 knockdown, which is consistent with previous reports that the pharyngeal muscle is resistant to RNAi by feeding¹⁴.

![HSR induction measured with fluorescent reporters is dependent on HSF-1.](image)

**Figure 2-2: (Fig. 2) HSR induction measured with fluorescent reporters is dependent on HSF-1.**

Strains containing the hsp-70p::gfp and hsp-16.2p::gfp reporters were raised on control (L4440 empty vector) or hsf-1 RNAi plates for 64 h, exposed to a 1 h heat shock at 33°C in a water bath, and then recovered at 20°C for 8 h before imaging. Representative images of two experimental replicates are shown. Scale bar = 250 μm.

To quantitate whole worm induction of the HSR at the molecular level, two endogenous HSPs were measured with RT-qPCR using section 3 of the protocol. Samples were
measured in triplicate, a standard curve was generated for each of the primers, and a melt curve was analyzed for each sample for quality control. It was found that a 33°C heat shock for 1 h resulted in more than a 2,000x increase in relative expression for two heat shock genes, *hsp*-70 and *hsp*-16.2 (Figure 3). These results show that both endogenous genes are suitable for measuring HSR induction and that a 33°C heat shock for 1 h is sufficient to generate a substantial response. However, caution should be used in interpreting the absolute degree of heat shock induction, because the mRNA levels in the absence of heat shock are very low.

![Graph](image)

**Figure 2-3: (Fig. 3) HSR induction measured with RT-qPCR.**

N2 worms were raised on HT115 bacteria for 60 h and then heat shocked for 1 h in a 33°C water bath. The relative mRNA levels of *hsp*-70 (*C12C8.1*) and *hsp*-16.2 are shown normalized to the no heat-shock control.

Values plotted are the mean of four biological replicates and error bars represent ± SEM. Statistical significance was calculated using an unpaired Student's t-test. **p < 0.01.

To analyze a physiological response to heat shock, an organismal thermorecovery assay was tested using section 4 of the protocol. It was found that exposure of worms to a 6 h heat shock at 33°C led to a 20% decrease in worms with normal movement after a 48 h
recovery (Figure 4A). The dependence of this assay on the HSF-1 transcription factor was tested using feeding RNAi to knockdown *hsf-1* before exposing worms to the stress. It was found that knockdown of *hsf-1* caused a dramatic decrease in normal movement, with >95% of worms showing jerky movement or paralysis after being prodded with a platinum wire pick.

![Figure 2-4: (Fig. 4) Thermorecovery, but not thermotolerance, is dependent on HSF-1.](image)

N2 worms were raised on control (L4440) or *hsf-1* RNAi plates for 60 h and then shifted to either: (A) A 33°C water bath for 6 h and recovered at 20°C for 48 h before scoring for normal movement (thermorecovery), or (B) A 35°C dry incubator and removed every 2 h until dead (thermotolerance). Each assay was done with n ≥ 30 individuals on 2 independent days. The average is shown.

We compared this thermorecovery assay to a widely used alternative organismal assay commonly referred to as thermotolerance. In the thermotolerance assay, worms are exposed to a continuous 35°C temperature using a dry incubator, and the percentage of worms alive are measured at various timepoints. Using this assay, it was found that control worms continuously exposed to 35°C died after approximately 8 h of exposure (Figure 4B). However, when the dependence of this assay on HSF-1 was tested using RNAi
knockdown, it was found that inhibition of *hsf-1* did not cause a decrease in thermotolerance. Similar results have been previously shown using HSF-1 mutations (see Discussion). Therefore, the use of the thermotolerance assay to measure the HSR is not recommended, and thermorecovery is the preferred method for examining the HSR at the organismal level.

**Discussion**

In the literature a wide variety of temperatures, times, and equipment have been used to assay the HSR, which has introduced unnecessary caveats and led to difficulty in comparing results between laboratories. For example, temperatures ranging anywhere from 32-37°C and times from 15 min to several hours have been used to induce the HSR\textsuperscript{15}. However, it is reported that lethality occurs as early as 3 h at 37°C for all stages and 1.5 h for day 1 adults\textsuperscript{15}. Furthermore, we show that exposure of worms to 35°C causes lethality that is not HSF-1 dependent, making these conditions poorly suited for analysis of the HSR. In contrast, a heat shock of 33°C for 1 h is robust enough to elicit strong induction of heat shock genes, yet mild enough to not affect worm viability. Indeed, exposure to 33°C for as long as 6 h only causes 20% of worms to display abnormal movement. Therefore, we propose using a temperature of 33°C and a time of 1 h as a standardized condition for RT-qPCR and fluorescent reporter assays.

Recent experiments have revealed that developmental staging of worms for HSR experiments is particularly important. It was recently shown that in *C. elegans* the inducibility of the HSR declines (i.e., collapses) by >50% when hermaphrodites begin egg laying\textsuperscript{5}. Staging the worms correctly is critical because there are often differences in
developmental timing in strains carrying mutations. If temperature-sensitive mutants are used, this will also impact results if they are not synchronized by their reproductive age. Therefore, it is recommended to carefully measure the onset of egg laying for every strain to determine when the collapse occurs. The window of time after the L4 molt and before the initiation of reproductive maturity is narrow; therefore, care must be taken so that the HSR collapse does not inadvertently cause variability in results.

In addition to developmental timing, surprisingly small changes in temperature, as little as 1°C, can have substantial effects on the HSR. For example, thermosensory neurons in \textit{C. elegans} are sensitive to temperature changes as small as \(\pm 0.05^\circ\text{C}\)\textsuperscript{16}. Thus, it is imperative to use a thermometer that can accurately measure the temperature. Therefore, we propose as best practice the use of a calibrated device for temperature measurement that is precise enough to measure temperatures within \(\pm 0.1^\circ\text{C}\). Furthermore, a thermometer with a data-logging functionality should be used to measure temperature variations across time. Many incubators are specified to have thermal variations of more than 1°C in different parts of the incubator and across time, which can have significant effects on HSR experiments. As a best practice, we suggest using incubators that have sufficient insulation and circulation to minimize temperature fluctuations. For conducting heat shock experiments, we propose a best practice of a circulating water bath. The time it takes for an agar plate to reach a desired temperature is approximately 6-7 min in a water bath but much longer in a dry incubator\textsuperscript{15,17}. However, if a circulating water bath is not available, we have shown that robust HSR induction also occurs in a dry incubator using our conditions. If a dry incubator is used, opening of the incubator for the duration of the stress should be minimized.
It is well established that induction of heat shock genes is dependent on the master regulator of the HSR, HSF-1. Here, we present evidence that the two more indirect assays, fluorescent reporters and thermorecovery, are also dependent on HSF-1. Significantly, we found that a commonly used alternative organismal assay, thermotolerance, is not HSF-1 dependent using *hsf-1* RNAi (Figure 4). Similar results have been previously reported using an *hsf-1* mutant or a *ttx-3* mutant, which blocks the HSR\textsuperscript{18,19,20}. Together, these results indicate that the thermotolerance assay should not be used for HSR research. Furthermore, this suggests that a best practice is to test the HSF-1 dependence for any assay used to measure the HSR.

Taken together, we present a series of standardized protocols and best practices for robust and reproducible measurement of HSR induction in *C. elegans*. We hope that these methodologies will decrease variability in HSR experiments and increase reproducibility. Facilitating direct comparisons of HSR research between laboratories will serve to accelerate research in the HSR field. Furthermore, standardization will benefit research into aging and neurodegenerative diseases with which the HSR is intimately associated.

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**Disclosures**

The authors have nothing to disclose.
Literature cited


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Chapter 3
Cellular Proteomes Drive Tissue-Specific Regulation of the Heat Shock Response

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Abstract

The heat shock response (HSR) is a cellular stress response that senses protein misfolding and restores protein folding homeostasis, or proteostasis. We previously identified an HSR regulatory network in Caenorhabditis elegans consisting of highly conserved genes that have important cellular roles in maintaining proteostasis. Unexpectedly, the effects of these genes on the HSR are distinctly tissue-specific. Here, we explore this apparent discrepancy and find that muscle-specific regulation of the HSR by the TRiC/CCT chaperonin is not driven by an enrichment of TRiC/CCT in muscle, but rather by the levels of one of its most abundant substrates, actin. Knockdown of actin subunits reduces induction of the HSR in muscle upon TRiC/CCT knockdown; conversely, overexpression of an actin subunit sensitizes the intestine so that it induces the HSR upon TRiC/CCT knockdown. Similarly, intestine-specific HSR regulation by the signal recognition particle (SRP), a component of the secretory pathway, is driven by the vitellogenins, some of the most abundant secretory proteins. Together, these data indicate that the specific protein folding requirements from the unique cellular proteomes sensitizes each tissue to disruption of distinct subsets of the proteostasis network. These findings are relevant for tissue-specific, HSR-associated human diseases such as cancer and neurodegenerative diseases. Additionally, we
characterize organismal phenotypes of actin overexpression including a shortened lifespan, supporting a recent hypothesis that maintenance of the actin cytoskeleton is an important factor for longevity.

**Introduction**

The HSR was first identified > 50 yr ago as a cellular, transcriptional response to increased temperature (Ritossa 1962). This response has subsequently been shown to be induced by protein misfolding, resulting in activation of the heat shock factor 1 (HSF1) transcription factor [reviewed in Guisbert and Morimoto (2013) and Guisbert et al. (2008)]. More recently, the HSR has been shown to have an important role in a variety of human diseases. In diseases of protein misfolding, such as Alzheimer’s disease, animal models have shown that activation of the HSR is beneficial [reviewed in Hipp et al. (2014)]. On the other hand, the HSR is constitutively active in several cancers, and inhibition of the HSR appears to be beneficial in cancer models [reviewed in Dai and Sampson (2016)]. While it remains to be seen whether manipulation of the HSR will prove to be a viable route for new disease therapeutics, a more complete understanding of HSR regulation will contribute to both basic biology and insight into human disease.

HSF1 senses protein folding through the HSP70 and HSP90 molecular chaperones, which bind to newly synthesized or misfolded proteins [reviewed in Hartl et al. (2011)]. Both HSP70 and HSP90 bind directly to HSF1 and inhibit its activity when the level of misfolded proteins is low (Shi et al. 1998, Zou et al. 1998). When misfolded proteins accumulate, chaperones are titrated away from HSF1, therefore liberating HSF1 to upregulate a set of genes called heat shock genes. Heat shock genes include many of the
molecular chaperones themselves, thus forming a negative feedback loop that elegantly links the total amount of chaperones in the cell to the cellular need for chaperones.

This molecular model for HSR regulation predicts a cell-autonomous response that couples chaperone expression to protein misfolding within each cell. Moreover, the central regulatory module of the HSR, consisting of HSF1, HSP70, and HSP90, is thought to be broadly expressed. Therefore, stress-sensing mechanisms were assumed to be conserved across all tissues in a multicellular organism. However, in a recent screen for HSR regulators, we discovered that regulators of the HSR display exquisite tissue specificity (Guisbert et al. 2013). We identified 52 negative regulators of the HSR, including HSP70 and HSP90. Unexpectedly, we found that each of the 50 new HSR regulators induced the HSR in only a subset of tissue types that were induced by HSP70 and HSP90. One set of new regulators that we identified contains the eight subunits of the TRiC/CCT chaperonin complex. Chaperonins are a class of ATP-dependent molecular chaperones that form large, barrel-shaped complexes that encapsulate protein substrates [reviewed in Lopez et al. (2015)]. The best characterized chaperonin is GroEL/GroES in prokaryotes. TRiC/CCT is the cytoplasmic chaperonin in eukaryotic cells that contains two hetero-oligomeric rings with eight subunits each. TRiC/CCT is essential for the proper folding of actin and tubulin and is estimated to participate in the folding of 10% of the proteome. We found that knockdown of each of the eight TRiC/CCT subunits causes induction of the HSR in muscle tissue, but not in the intestine or reproductive tissue. Importantly, regulation of the HSR by TRiC/CCT has been shown to be conserved in cultured human cell lines and involves direct regulation of HSF1 (Neef et al. 2014).
Another class of new HSR regulators includes subunits of the SRP and other components of the secretory pathway. The SRP is a well-conserved complex that contains both protein subunits and noncoding RNAs, which recognize signal sequences in nascent proteins, and targets them to the endoplasmic reticulum [reviewed in Elvekrog and Walter (2015)]. Knockdown of SRP subunits induces the HSR in the intestine but not in the muscle. Similar to TRiC/CCT, regulation of the HSR by SRP appears to be conserved across multiple species as components of the secretory pathway have been shown to directly regulate the HSR in *Escherichia coli* (Lim *et al.* 2013).

The tissue-specific regulation of the HSR highlights an import gap in our basic understanding of HSR regulation, which comes largely from unicellular organisms and cultured cells. Here, we investigate the molecular basis for tissue-specific HSR regulation in a multicellular organism for two distinct subsets of HSR regulators and show that the unique protein folding requirements of each tissue help to drive distinct patterns of HSR induction.

**Results**

**Expression of TRiC/CCT**

The HSR is regulated by highly conserved genes with well-established roles in cellular protein folding, yet these genes regulate the HSR primarily in a tissue-specific manner (Guisbert *et al.* 2013). To uncover mechanisms that drive tissue specificity, we first analyzed tissue-specific HSR regulation by the TRiC/CCT chaperonin complex. Knockdown of *cct-1*, a subunit of the complex, induces the HSR in muscle tissue, but not in the intestine or reproductive tissues (Figure 1 and Figure S1 in Supplemental Material,
As reported, this induction is quite distinct from induction by heat shock (Figure S2 in File S1).

**Figure 3-1:** (Fig. 1) Muscle-specific induction of the HSR by *cct-1* knockdown is dependent on *act-4.*

Fluorescent images are shown of strain AM446 containing a GFP-based HSR reporter. (A) Control, nonsilencing RNAi worms only display autofluorescence and constitutive reporter expression in the AD. (B) Knockdown of *cct-1* induces the HSR reporter in muscle tissue as indicated with the arrows labeled VM and BWM. (C) Knockdown of *act-4* prevents muscle-specific induction by *cct-1* knockdown. (D) Knockdown of *act-4* alone does not induce the HSR. (E) Knockdown of *unc-45* also induces the HSR in muscle tissue. (F) Knockdown of *act-4* does not affect HSR induction by *unc-45.* In these experiments, the single gene RNAi knockdowns were diluted with control, nonsilencing RNAi so that they had the same dosage as the double RNAi knockdowns. Quantitation of the results is given in Table 1. AD, anal depressor muscle; BWM, body wall muscle; GFP, green fluorescent protein; HSR, heat shock response; RNAi, RNA interference; VM, vulva muscle.

The simplest explanation for this muscle-specific induction might be that *cct-1* and other TRiC/CCT subunits are only expressed in muscle tissue. However, this possibility is unlikely given that TRiC/CCT is required for the folding of actin and tubulin, which are
essential components of the cytoskeleton. Supporting this, a functional role for the TRiC/CCT chaperonin in actin and tubulin folding in the *C. elegans* intestine has been characterized (Saegusa *et al.* 2014). Additionally, previous qualitative analyses of expression patterns of TRiC/CCT subunits have suggested a ubiquitous pattern of expression across tissues (Lundin *et al.* 2008). However, some fluorescent reporter constructs suggest that TRiC/CCT is enriched in muscle tissue (Leroux and Candido 1997). To resolve this discrepancy, we queried the expression of TRiC/CCT subunits from a genomic analysis of gene expression patterns in *C. elegans* as part of the modENCODE consortium (Spencer *et al.* 2011). These data were generated using tagged RNA-binding proteins expressed in a tissue-specific manner to examine the tissue-specific expression of endogenous mRNAs. In this dataset, the expression of TRiC/CCT subunits is relatively uniform across different cell types (Figure 2). Similarly, expression of the central regulatory module of the HSR, including HSP70, HSP90, and HSF1, also displays little or no tissue specificity (Figure S3 in File S1). This indicates that the muscle-specific effect of TRiC/CCT on the HSR cannot be explained by the expression pattern of TRiC/CCT or other major regulators of the HSR.
Figure 3-2: (Fig. 2) TRiC/CCT subunit expression is not strongly enriched in muscle tissue.

Mean normalized signal intensity for gene expression data are shown for each of the available TRiC/CCT subunits in various tissues from L2-staged larval worms relative to the L2 whole worm reference control. Data were taken from the modENCODE project (Spencer et al. 2011).

Requirement for actin in TRiC/CCT muscle-specific HSR regulation

If HSF1 and TRiC/CCT are both expressed across many tissues, then what could explain their specific genetic interaction in muscle tissue? For the well-established regulation of HSF1 by the HSP70 and HSP90 molecular chaperones, HSR regulation is mediated by the ratio between the chaperones and their substrates (Craig and Gross 1991). Therefore, we considered whether the substrates of TRiC/CCT might influence its tissue specificity. Actin is one of the major substrates of TRiC/CCT, (Lopez et al. 2015), therefore, we tested whether actin expression contributed to TRiC/CCT-mediated regulation of the HSR.

There are five actin isoforms in *C. elegans*, and four of these (*act-1, act-2, act-3, and act-4*) are 99% identical. The expression patterns of actin are not well-established, as reporter fusions suggest that the four similar isoforms are enriched in muscle tissues, whereas in
situ analysis reveals broader expression patterns (Stone and Shaw 1993, Macqueen et al. 2005). The remaining actin isoform, act-5, is 93% identical and expressed in the intestine where it has important roles in the formation of intestinal microvilli. Analysis of actin isoform expression patterns from the modENCODE dataset reveals that there is not a robust enrichment of actin expression in muscle tissue relative to the whole worm reference (Figure S2 in File S1). Nevertheless, there is enrichment for act-5 and a decrease in act-1, act-2, and act-4 expression in the intestine, resulting in an increased ratio of muscle to intestine expression. Supporting this, dominant mutants in actin isoforms preferentially affect muscle tissue, indicating a functional enrichment (Waterston et al. 1984).

We next tested whether the enrichment of actin in the muscle could influence the muscle-specific HSR induction from TRiC/CCT knockdown. We found that knockdown of the actin isoform act-4 resulted in inhibition of HSR induction caused by cct-1 knockdown (Figure 1). In contrast, act-4 knockdown did not affect HSR induction in the muscle by knockdown of unc-45, an HSR regulator unrelated to TRiC/CCT. Additionally, induction of the reporter by heat shock was not reduced upon act-4 knockdown (Figure S2 in File S1). Similar results were obtained by measuring induction of endogenous HSR genes using qPCR (Figure 3).
Figure 3-3: (Fig. 3) HSR induction of endogenous HSR genes by cct-1 knockdown

qRT-PCR analysis of two endogenous HSR genes, hsp-16.2 and C12C8.1 (hsp-70), reveals that HSR induction by cct-1 knockdown is decreased by knockdown of act-4. Both genes showed the same trend but the effect was only significant for hsp-16.2 due to the increased variability of C12C8.1 (p-value = 0.05 for hsp-16.2 and p-value = 0.09 for C12C8.1). In contrast, HSR induction by unc-45 knockdown was not decreased by knockdown of act-4 and is therefore marked as NS (n ≥ 6 for all samples except for C12C8.1 in the act-4 knockdown control where the signal was below the detection limit for many samples and is therefore marked as ND). HSR, heat shock response; mRNA, messenger RNA; ND, not determined; NS, not significant; qRT-PCR, quantitative real time polymerase chain reaction; RNAi, RNA interference.

Knocking down the three other broadly expressing actin isoforms also affects HSR induction by cct-1, indicating that this effect is not specific to any one actin isoform (Table 1). However, even though the RNAi constructs were designed to target specific actin isoforms, it has been previously shown that these constructs can knockdown multiple isoforms due to the high degree of sequence conservation among the isoforms (Velarde et al. 2007). Nevertheless, because the actin isoforms are thought to be largely redundant, this lack of specificity does not impact the conclusions.
Table 3-1: (Table 1) Dependence of muscle-specific HSR induction on actin isoforms.

<table>
<thead>
<tr>
<th></th>
<th>Induction (%)</th>
<th>Control (%)</th>
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<th>act-2 (%)</th>
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<td>90 ± 4</td>
<td>83 ± 3</td>
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Quantification of the percentage of worms with heat shock response (HSR) reporter induction from strain AM446 containing a green fluorescent protein (GFP)-based HSR reporter after double RNA interference (RNAi) knockdown of cct-1 or unc-45 with control, nonsilencing RNAi, act-1, act-2, act-3, and act-4 actin isoform knockdowns. Actin isoform knockdown leads to a decrease in the percentage of worms with HSR induction upon cct-1 knockdown (act-1 p-value = 2.8E-207, act-2 p-value = 2.8E-207, act-3 p-value = 2.4E-207, and act-4 p-value = 2.4E-205), but does not affect the percentage of worms with HSR induction upon unc-45 knockdown (act-1 p-value = 0.45, act-2 p-value = 0.33, act-3 p-value = 0.1, and act-4 p-value = 0.16). Data are from at least three trials and error represents SEM.

Sufficiency of actin for TRiC/CCT tissue-specific HSR regulation

Having established that the levels of actin are necessary for HSR induction by TRiC/CCT knockdown, we next asked whether actin was sufficient to drive induction of the HSR by TRiC/CCT in other tissues. Therefore, we generated a transgenic line containing the act-4 actin isoform expressed under the control of a broad, let-858 promoter. We found that overexpression of act-4 is not sufficient to induce the HSR on its own (Figure 4A). However, overexpression of act-4 combined with knockdown of cct-1 led to HSR induction in the intestine (Figure 4B). In contrast, act-4 overexpression did not sensitize the intestine to HSR induction by unc-45 knockdown (Figure 4C). These results indicate that actin expression is sufficient to sensitize the intestine to induce the HSR upon knockdown of TRiC/CCT. Therefore, HSR regulation by TRiC/CCT is not solely mediated by the levels of TRiC/CCT, but is instead determined by the ratio of TRiC/CCT to its substrates.
Figure 3-4: (Fig. 4) Actin overexpression expands the tissue-specific pattern of HSR induction by *cct-1* knockdown.

Fluorescent images are shown of strain EAG001, which overexpresses the actin isoform *act-4* and contains a GFP-based HSR reporter, after RNAi with nonsilencing control, *cct-1*, or *unc-45*. (A) Control, nonsilencing RNAi worms only display autofluorescence and constitutive reporter expression in the AD. (B) Knockdown of *cct-1* results in HSR induction in the intestine in addition to VM and BWM. Intestinal HSR induction occurs in 30 ± 9% of worms (n = 4 trials, error is SEM). (C) Knockdown of *unc-45* results in HSR induction in muscle tissue, but induction was not observed in the intestine. AD, anal depressor muscle; BWM, body wall muscle; GFP, green fluorescent protein; HSR, heat shock response; RNAi, RNA interference; VM, vulva muscle.

Organismal phenotypes for actin

We next characterized other phenotypes that arise from actin overexpression. We generated an integrated transgenic worm strain overexpressing *act-4* by irradiating the extrachromosomal *act-4* overexpression line and selecting for integration. The integrated line was validated to be similar to the extrachromosomal line with respect to induction of the HSR in the intestine upon *cct-1* knockdown (Figure S4 in File S1). Next, expression
levels were quantitated using qPCR, and act-4 was found to be 2.6-fold overexpressed relative to wild-type worms (Figure S5 in File S1). This level of overexpression is mild since it represents just one of the five actin isoforms.

Given the extensive post-translational regulation of actin localization and polymerization, it is unclear whether mild overexpression would cause serious functional consequences. However, as actin is one of the most abundant proteins in the cell, even mild overexpression could cause significant perturbations. To investigate muscle function, motility was measured using a thrashing assay and a 30% decrease in motility was observed (Figure 5A). Given the important role of actin in muscle tissue, this decrease is consistent with a mild perturbation of cellular function. Next, the role of actin in early development was investigated by measuring the brood size and the egg hatching rate (Figure 5, B and C). No decrease was observed in the number of eggs laid, but consistent with the established roles of actin in early development, less than half of the eggs laid were viable. Microscopic analysis of the eggs indicated a defect in gastrulation as unhatched eggs were observed that were arrested before and during the various embryonic stages associated with gastrulation, including the comma and the bean (Figure S5 in File S1). Of the eggs that hatched, they all reached adulthood even though there was a slight developmental delay (Figure 5D). Finally, the lifespan of adult worms was measured to test a recent hypothesis regarding the actin cytoskeleton and aging. Previously, it was shown that disruptions in muscle structure and function are associated with aging (Herndon et al. 2002). It was recently shown that overexpression of pat-10, a troponin-like protein that regulates the actin cytoskeleton, reverses age-dependent alterations to actin and extends lifespan, suggesting that the actin cytoskeleton has an important role in aging (Baird et al. 2002).
Supporting this hypothesis, we observed a significant decrease in lifespan upon mild actin overexpression (Figure 5E). This effect was validated by measuring lifespan in the extrachromosomal actin overexpression line where a similar decrease was observed (Figure S6 in File S1).

![Figure 3-5: (Fig. 5) Phenotypic effects of Act-4 OE.](image)

Various phenotypes were measured in strain EAG003, which contains an integrated Act-4 OE construct with an HSR reporter, and in the control strain AM446, which only contains the HSR reporter. All error bars represent SEM. (A) Actin OE causes a decrease in motility represented by a 32% decrease in thrashing rate per 30 sec (p-value = 0.00001, n = 30 worms). (B) Actin OE does not cause a decrease in brood size (n ≥ 35 worms). (C) Actin OE causes a decrease in hatching rate measured 24 hr after laying (p-value = 0.01, n ≥ 144 eggs). (D) Actin OE delays but does not impair postembryonic development (n ≥ 69 worms). (E) Actin OE causes a decrease in lifespan (p-value = 0.02, n ≥ 33 worms). Lifespan data were analyzed using a log-rank test in OASIS (Online Application for the Survival Analysis of Lifespan Assays Performed in Aging Research). HSR, heat shock response; OE, overexpression.
Requirement of vitellogenins for intestine-specific HSR regulation by SRP

To elucidate whether or not the ratio of HSR regulators to their substrates was a general feature of HSR regulation in *C. elegans*, we next asked whether the same principle applied to HSR regulators in the secretory pathway. Knockdown of the SRP subunit *F08D12.1* causes induction of the HSR in the intestine, but not in muscle tissue (Figure 6).

![Figure 3-6: (Fig. 6) Intestine-specific induction of the HSR by knockdown of the SRP subunit *F08D12.1* (*)srpa-72*) is dependent on the secreted vitellogenin *vit-3.*

Fluorescent images are shown of strain AM446 containing a GFP-based HSR reporter. (A) Control, nonsilencing RNAi worms only display autofluorescence and constitutive reporter expression in the AD. (B) Knockdown of *F08D12.1* induces the HSR reporter in the intestine. (C) Knockdown of *vit-3* prevents intestine-specific HSR induction by *F08D12.1*. (D) Knockdown of *vit-3* alone does not induce the reporter. (E) Knockdown of *hsp-6* also induces the HSR reporter in the intestine. (F) Knockdown of *vit-3* does not affect reporter induction upon *hsp-6* knockdown. Quantitation of these results is given in Table 2. AD, anal depressor muscle; GFP, green fluorescent protein; HSR, heat shock response; RNAi, RNA interference; SRP, signal recognition particle.
We hypothesized that intestinal cells may be particularly dependent on proper folding in
the secretion pathway due to the extremely high expression of the vitellogenin genes.

Vitellogenins, or yolk proteins, are synthesized in the intestine, secreted into the
pseudocoelomic space, and taken up in eggs through endocytosis (Kimble and Sharrock
1983). We knocked down the two vitellogenins from our RNAi library, vit-3 and vit-5, and
found that these genes prevented induction of the HSR upon knockdown of F08D12.1
(Figure 5 and Table 2). In contrast, they did not affect induction of the HSR in the intestine
by knockdown of hsp-6, a mitochondrial chaperone. Together, these data indicate that
regulation of the HSR by the secretory pathway also involves a balance between the
components of the secretory pathway and their substrates, and that this type of regulation is
a general feature of the HSR.

Table 3-2: (Table 2) Dependence of intestine-specific HSR induction on vitellogenins.

<table>
<thead>
<tr>
<th>Induction (%)</th>
<th>Control (%)</th>
<th>vit-3 (%)</th>
<th>vit-5 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F08D12.1</td>
<td>69 ± 10</td>
<td>3 ± 3</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>hsp-6</td>
<td>47 ± 12</td>
<td>60 ± 15</td>
<td>48 ± 20</td>
</tr>
</tbody>
</table>

Quantification of the percentage of worms with heat shock response (HSR) reporter induction from the
phsp70::gfp reporter strain for double RNA interference (RNAi) knockdown of F08D12.1 or hsp-6 with
control, nonsilencing RNAi, vit-3, and vit-5 vitellogenin genes. Vitellogenin knockdown leads to a decrease in
the percentage of worms with HSR induction upon F08D12.1 knockdown (vit-3 p-value = 0.0005 and vit-5 p-
value = 0.0005) but does not affect the percentage of worms with HSR induction upon hsp-6 knockdown (vit-3
p-value = 0.53 and vit-5 p-value = 0.97). Data are from at least three trials and error represents SEM.

Discussion

We have uncovered the mechanism of tissue-specific HSR regulation and found that it
reflects a balance between the unique cellular proteome and the more ubiquitous
proteostasis machinery. The simplest hypothesis explaining tissue-specific regulation of the HSR was tissue-specific expression of the regulators, but we found little evidence to support this hypothesis. Further, almost all HSR regulators have important functions in cellular protein synthesis, folding, trafficking, and degradation, and are therefore not likely to be expressed in a tissue-specific manner. Rather, we show that tissue-specific regulation is influenced by tissue-specific substrates. We demonstrate this principle with two orthogonal examples: an abundant TRiC/CCT substrate, actin, influences its HSR induction in the muscle and an abundant secretory protein, vitellogenin, influences HSR induction in the intestine by the secretory pathway (Figure 7).

![Figure 3-7](Fig. 7) Model figure.

Tissue-specific regulation represents the interplay between the proteostasis machinery (CCT/TRiC in A and SRP in B) and substrates (actin in A and yolk protein in B). (C) Interplay between organismal, tissue-specific, and core, cellular HSR regulation. HSR, heat shock response; SRP, signal recognition particle.

Together, these results indicate that the unique proteome of each tissue creates unique burdens upon the proteostasis network. Therefore, we predict that in the muscle, the large
amount of actin creates a higher burden upon the TRiC/CCT chaperone, sensitizing this tissue to its disruption. Similarly, the large amount of vitellogenin produced and secreted in the intestine sensitizes this tissue to disruptions in the secretory pathway. Our findings parallel the well-established regulation of the HSR by the HSP70 and HSP90 chaperone machines, where HSR regulation is thought to reflect a balance between the levels of the chaperones and the amount of their substrates, indicating that regulation of the HSR by components of the proteostasis network in relation to their substrates is a general feature of HSR regulation.

Recently, the HSR has been shown to have important connections to human diseases including cancer and neurodegenerative diseases, creating a renewed interest in studying the HSR pathway. Our results are particularly relevant given the exquisite tissue-specific nature of these diseases, and our data indicate that efforts to adapt the HSR as a therapeutic approach must include an analysis of the HSR in the relevant tissues. For example, a full analysis of HSR regulation in neurons is critical to generate foundational knowledge for the successful manipulation of the HSR in neurons to combat neurodegenerative diseases. Some research in this area has already been initiated, but with mixed results. Primary neuronal cultures from mice and rats have been shown to have impaired induction of the HSR due to decreased expression of HSF1 in some cases, and impaired HSF1 activation in others (Marcuccilli et al. 1996, Kaarniranta et al. 2002, Batulan et al. 2003). However, similar effects have not been observed in the mouse striatum in vivo (Carnemolla et al. 2015). Unfortunately, the technical limitations of RNAi in C. elegans neurons have thus far precluded an analysis of HSR regulation in this tissue, but our findings motivate a new investigation of HSR regulation in C. elegans neurons using sensitized RNAi strains.
Previous investigations into the organismal phenotypes of actin have used knockdowns and mutants to show that the actin cytoskeleton has important roles in motility and early development (Velarde et al. 2007, Waterston et al. 1984). Here, we have undertaken complementary approaches that support these earlier observations, showing that mild actin overexpression causes defects in motility and early development. Importantly, a recent study has suggested that maintenance of the actin cytoskeleton is an important factor in maintaining lifespan (Baird et al. 2014). Here, we validate a prediction of that model by showing that even slight disruption of the actin cytoskeleton by mild overexpression of a single actin isoform can shorten lifespan. Our findings on tissue-specific HSR regulation complement a growing body of literature exploring cell-autonomous and cell-nonautonomous regulation of the HSR. Cell-autonomous HSR regulation was robustly demonstrated using lasers to induce the HSR in individual cells in an intact C. elegans (Suzuki et al. 2013, Stringham and Candido 1993). We show that the mechanism of tissue-specific HSR regulation mirrors the core HSR regulation by HSP70 and HSP90, as they both act cell-autonomously to probe protein folding capacity relative to the protein folding requirements of the cell (Figure 7). This cell-autonomous regulation is quite distinct from other forms of HSR regulation at the organismal level (Prahlad et al. 2008, Van Oosten-Hawle et al. 2013, Douglas et al. 2015). For example, it was recently shown that mutations in neuronal genes can suppress the whole organismal response to temperature and that overexpression of HSP90 in a tissue-specific manner can affect HSR regulation in distinct tissues. These data indicate that the HSR can be coordinately regulated across tissues and at the level of the whole organism, at least under some conditions. Nonautonomous HSR regulation may be adaptive, enhancing the cellular stress response in anticipation of stress,
or alternatively, it may restrict cellular responses to maximize organismal performance. As the mechanisms behind nonautonomous regulation are not yet established, the coordination between nonautonomous and autonomous HSR regulation remain largely unexplored.

Advances in molecular biology have created an explosion in our understanding of the details and mechanisms behind many signal transduction pathways. The HSR has served as a foundational pathway for understanding signal transduction since its accidental discovery > 50 yr ago, due to its universal nature and the high degree of conservation among heat shock genes. Here, we use *C. elegans* to learn how the HSR is differentially adapted to each tissue in an organism and demonstrate how incorporating organismal and systems-level approaches to the study of classical signal transduction pathways can yield new insights into their functions.

**Materials and methods**

**Nematodes**

*Caenorhabditis elegans* were maintained at 20°C using standard procedures. Worms were synchronized by bleaching with hypochlorite and hatching overnight in M9 buffer. All nematode strains were derived from the N2 Bristol wild-type strain. The following strains were used: (1) AM446 rmls223[C12C8.1p::gfp;rol-6(su1006)] (Morley and Morimoto 2004); (2) EAG001 rmls223[C12C8.1p::gfp;rol-6(su1006)], eagEx1[let-858p::act-4;myo-2p::rfp]; and (3) EAG003 rmls223 [C12C8.1p::gfp;rol-6(su1006)], eagIs1[let-858p::act-4;myo-2p::rfp]. Plasmid pEAG58 was constructed using the Gateway system to combine an act-4 ORF (Reboul et al. 2003) with the let-858 promoter and the unc-54 392UTR. Strain EAG001 was generated by microinjection of plasmid pEAG58 along with a myo-
2p::rfp fluorescent marker into strain AM446. Strain EAG003 was generated by irradiation of strain EAG001, selection for integration, and backcrossing 4 times. The p-values represent raw p-values.

RNAi
RNAi was induced using a bacterial feeding approach with the Ahringer RNAi library (Kamath et al. 2003). Bacterial cultures were grown overnight in LB and induced with 1 mM IPTG for at least 2 hr in liquid cultures and on plates. RNAi was initiated at the L2/L3 stage of development by synchronizing worms at the L1 larval stage and then culturing them for 19 hr on OP50 plates. Nematodes were then grown to adulthood on RNAi plates ~48 hr prior to analysis.

Imaging
Worms were mounted on 3% agarose pads on a glass slide, immobilized in a drop of 1 mM levamisole, and imaged using a Nikon C1Si multi-spectral laser scanning confocal microscope and Nikon EZ-C1 software. Each individual worm was visually scored for induction of the fluorescent reporter. Unhatched eggs were collected in M9 after bleaching and directly visualized using a Zeiss Axioskop2 brightfield microscope and analyzed using Jenoptik’s ProgRes Capture Pro software.

qRT-PCR
RNA was isolated from whole animals using TRIzol (Invitrogen) according to standard protocols. DNA was removed using a DNA-free DNA Removal Kit (Ambion), cDNA synthesis was performed using an iScript cDNA Synthesis Kit (Bio-Rad), and qPCR was performed using iQ SYBR Green Supermix (Bio-Rad) using a CFX Connect Real Time
System (Bio-Rad). 18S RNA was used as a normalization control.

**Phenotypic assays**

Thrashing assays were conducted on day 1 of adulthood. Individual worms were picked into a drop of M9 buffer on a glass slide, acclimated for 1 min, and then the number of thrashing movements were visually counted in a 30 sec period using a dissecting microscope. Egg laying assays were conducted by counting the number of eggs laid in each 24 hr period from larva to cessation of egg laying. Total brood size was then calculated for each worm. Egg hatching was assayed by transferring young adult worms to a new plate, allowing egg laying to occur for 3 hr, and then removing the adult worms. The eggs were then incubated for 24 hr and the number of hatched larvae were counted. Development was assayed over time after synchronization with visual inspection of gonad morphology.

**Lifespan**

Lifespan experiments were performed at 20°C in the absence of FUDR. Worms were transferred to new plates at least every other day to separate adults from progeny. Lifespan data were analyzed using a log-rank test in OASIS (Online Application for the Survival Analysis of Lifespan Assays Performed in Aging Research).

**Data availability**

All strains and reagents are available upon request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.
Acknowledgments

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Supplemental files

Supplemental material is available online at

Supplemental figures

Figure 3-8: (Fig. S1) Muscle-specific induction of the HSR by cct-1 knockdown in strain AM446 containing a GFP-based HSR reporter.

(A) The image from figure 1B is shown with enlarged insets demonstrating: (B) muscle-specific induction in body wall muscle cells in the head, (C), constitutive expression in the anal depressor muscle, and (D) muscle-specific induction in the vulva muscle.
Figure 3-9: (Fig. S2) Heat shock induction of the HSR.

(A) No HSR induction is observed in the reporter strain in the absence of heat shock with control, non-silencing RNAi. (B) HSR induction in the reporter strain upon heat shock at 33°C for one hour with control, non-silencing RNAi. (C) HSR induction upon heat shock at 33°C for one hour is not affected by RNAi knockdown of act-4. (D) qPCR analysis of mRNA levels validate these same effects for endogenous HSR genes.
Figure 3-10: (Fig. S3) Expression patterns of genes across various tissues.

(A) HSF1, HSP70, and HSP90 expression levels do not indicate robust enrichment in specific tissues. Mean normalized signal intensity for gene expression data is shown for each of the three genes in various tissues from L2 worms relative to the L2 whole worm reference control. (B) Actin isoform expression reveals lower levels of act-1, act-2, and act-4 and higher levels of act-5 in the intestine relative to muscle. Data is from the modENCODE project.
Figure 3-11: (Fig. S4) Actin overexpression in an integrated line expands the tissue-specific pattern of HSR induction by *cct-1* knockdown.

Fluorescent images are shown of strain EAG003, an integrated line that overexpresses the actin isoform *act-4* and contains a GFP-based HSR reporter, after RNAi with non-silencing control and *cct-1* knockdown. Non-silencing control RNAi does not demonstrate HSR induction, but knockdown of *cct-1* results in HSR induction in both muscle tissue and intestine.
Figure 3-12: (Fig. S5) Expression levels of Act-4 and its effects on embryonic development.

(A) Quantification of relative *act-4* mRNA levels in the EAG003 *act-4* overexpression strain worms using qRT-PCR. Values were normalized to 18S RNA and error bars represent SEM (n=6, p-value = 0.01). (B-D) Images of embryos reveal arrests in various embryonic stages including comma, bean, and cleavage stages. This indicates that Actin overexpression causes a range of defects before and during gastrulation.
Figure 3-13: (Fig. S6) Extrachromosomal Actin overexpression causes a decrease in lifespan.

The lifespan of EAG001 act-4 overexpression from an extrachromosomal element was compared to the AM446 control strain. Data was analyzed using a log-rank test in OASIS (Online Application for the Survival Analysis of Lifespan Assays Performed in Aging Research). A decrease in lifespan was observed (N = 90 worms, p-value=0.0002).
Literature cited


Chapter 4
Chronic Temperature Stress Inhibits Reproduction and Disrupts Endocytosis via Chaperone Titration in *Caenorhabditis elegans*


Abstract

**Background:** Temperature influences biology at all levels, from altering rates of biochemical reactions to determining sustainability of entire ecosystems. Although extended exposure to elevated temperatures influences organismal phenotypes important for human health, agriculture, and ecology, the molecular mechanisms that drive these responses remain largely unexplored. Prolonged, mild temperature stress (48 h at 28 °C) has been shown to inhibit reproduction in *Caenorhabditis elegans* without significantly impacting motility or viability.

**Results:** Analysis of molecular responses to chronic stress using RNA-seq uncovers dramatic effects on the transcriptome that are fundamentally distinct from the well-characterized, acute heat shock response (HSR). While a large portion of the genome is differentially expressed ≥ 4-fold after 48 h at 28 °C, the only major class of oogenesis-associated genes affected is the vitellogenin gene family that encodes for yolk proteins (YPs). Whereas YP mRNAs decrease, the proteins accumulate and mislocalize in the pseudocoelomic space as early as 6 h, well before reproduction declines. A trafficking defect in a second, unrelated fluorescent reporter and a decrease in pre-synaptic neuronal...
signaling indicate that the YP mislocalization is caused by a generalized defect in endocytosis. Molecular chaperones are involved in both endocytosis and refolding damaged proteins. Decreasing levels of the major HSP70 chaperone, HSP-1, causes similar YP trafficking defects in the absence of stress. Conversely, increasing chaperone levels through overexpression of the transcription factor HSF-1 rescues YP trafficking and restores neuronal signaling.

Conclusions: These data implicate chaperone titration during chronic stress as a molecular mechanism contributing to endocytic defects that influence multiple aspects of organismal physiology. Notably, HSF-1 overexpression improves recovery of viable offspring after exposure to stress. These findings provide important molecular insights into understanding organismal responses to temperature stress as well as phenotypes associated with chronic protein misfolding.

Background
The fate of an organism can rest on its ability to accurately sense and respond to stress. A ubiquitous stress that often disrupts homeostasis is temperature fluctuation [1]. Consequently, biological systems have a variety of mechanisms to re-establish homeostasis when faced with elevated temperatures. These adaptations to temperature can be both evolutionary and organismal. Evolutionary adaptations are driven by selection of heritable genetic changes. Organismal responses are driven by cellular and molecular mechanisms.

At the organismal level, animals exhibit changes in behavior, autonomic reflexes (e.g., sweating), cardiovascular function, and neuroendocrine signaling when exposed to elevated temperatures [2]. For livestock, temperature is one of the largest stressors in
animal production, and organismal response mechanisms are typically detrimental to performance due to their high metabolic costs [3, 4]. Heat stress decreases growth, reproduction, milk production, and meat production, while increasing the occurrence of disease. Furthermore, organisms have evolved to survive specific ranges of temperatures, and an increase of only a few degrees is predicted to result in the extinction of as many as 16% of all species [5].

At the molecular level, elevated temperatures disrupt protein-folding homeostasis, or proteostasis [6]. Proteostasis is normally maintained by an extensive network of factors collectively known as the proteostasis network, which includes pathways that regulate the synthesis, folding, trafficking, and degradation of proteins. However, many proteins adopt native states that are only marginally stabilized, such that an increase of only 4 °C destabilizes the average protein by ~ 20% in *Escherichia coli* [7]. To deal with the massive accumulation of misfolded proteins during temperature stress, all organisms utilize an adaptative response known as the heat shock response (HSR) [8]. This highly conserved pathway is mediated by the transcription factor heat shock factor 1 (HSF1). Mutations in HSF1 cause thermosensitivity, whereas overexpression of HSF1 enhances thermotolerance [9, 10].

An important class of HSF1-regulated genes is the molecular chaperone family that assists in protein folding [11, 12]. In the absence of stress, chaperones participate in *de novo* folding of newly synthesized proteins and in cellular processes that require assembly and disassembly of macromolecular complexes. For example, chaperones are required for several steps of endocytosis [13–15]. The initial invaginations of coated pits, dissociation
of clathrin during vesicle uncoating, and the stabilization of clathrin after dissociation are regulated by the chaperone HSC70. Another chaperone, RME-8, is important for downstream steps of clathrin-mediated endocytosis and is a shared regulator of both receptor-mediated endocytosis (RME) in oocytes and fluid-phase endocytosis in coelomocytes in Caenorhabditis elegans [16–19]. In the presence of stress, chaperones help to repair misfolded or damaged proteins [20]. This serves to titrate specific chaperones away from an inhibitory complex with HSF1. Therefore, the activity of HSF1 is intimately linked to the protein-folding state of the cell. Conditions other than temperature stress similarly lead to chaperone titration. During aging, a substantial decline in the capacity of the proteostasis network is attributed to age-associated accumulation of misfolded proteins [21]. Numerous neurodegenerative diseases are also associated with extended chaperone titration [22].

The consequences of stress are fundamentally distinct depending on the degree and duration of the stress. The HSR has been extensively studied at the molecular level using a variety of model systems exposed to acute, severe temperature stress. However, physiological responses to temperature stress in metazoans are typically observed during mild stress on much longer timescales. The connection between the molecular events that take place during acute stress and the organismal-level responses to chronic stress remains to be fully characterized. Recently, correlations have been made between molecular and organismal responses to temperature stress. For example, we have shown that a chronic, 4-week exposure to thermal stress in Hippocampus erectus seahorses results in altered feeding frequency and overall ventilation rates, along with increased expression of HSR genes and genes involved in regulating reproduction [23]. Additionally, the advent of next-
generation sequencing and genomic information has enabled a multitude of studies to examine transcriptomic responses to temperature stress in a variety of metazoans [24–26]. While these studies generate a treasure trove of data, this methodology is limited to providing descriptive correlations.

The multicellular, transparent nematode *C. elegans* is an ideal candidate for examining the effects of temperature stress at the molecular, cellular, tissue-specific, and organismal levels. An acute, nearly lethal temperature stress (1 h at 33–35 °C) that elicits the HSR has no reported ill effects on organismal fecundity in *C. elegans* [27–29]. In contrast, a chronic, mild temperature stress (48 h at 28 °C—a few degrees above the normal growth temperatures of 15–25 °C) completely inhibits reproduction while only mildly affecting phenotypes such as viability and motility [30, 31]. Here, we combine genetics and transcriptomic analyses with cellular and organismal phenotypes to identify molecular mechanisms driving the dramatic differences in fate between acute and chronic stress.

**Results**

**Chronic heat stress inhibits egg laying and elicits distinct transcriptomic responses**

Previous studies have shown that worms display a reproductive defect after prolonged exposure to mild heat stress (HS) [30, 31]. The temperature shifts in those studies were initiated prior to the onset of egg laying. However, more recent work showed that multiple stress responses attenuate at the onset of egg laying, such that stress-inducible gene expression in reproductive adults is half that of pre-reproductive worms [32]. Therefore, we first established organismal responses to chronic temperature stress after this final stage
of development. Egg-laying adults were evaluated for reproduction and motility over a 2-day HS time course that reflects ~ 10% of the *C. elegans* lifespan. Worms exposed to 28 °C HS laid similar numbers of eggs as the control worms (20 °C) during the first 12 h, but only ~ 40% as many eggs between the 12- and 24-h HS timepoints (Fig. 1a). After the 24-h timepoint, the HS worms had essentially ceased reproduction. Despite the shutdown of reproduction, worms displayed no other obvious defects. This suggests that reproduction was not inhibited as a result of a catastrophic decline in worm health.

**Figure 4-1**: (Fig. 1) Chronic heat stress inhibits reproduction and affects global gene expression.

Chronic exposure of day 1 adult N2 wild-type (WT) worms to 28 °C heat stress (HS) inhibits the number of eggs laid (a), mildly affects motility (b), and alters global gene expression (c-e) compared to 20 °C control worms. Data in a and b represent mean ± SD of n = 30 worms, collected across three independent trials. **p < 0.01, ****p < 0.0001 (Student’s t test versus 20 °C at the same timepoint). Data in c–e represent densities of genes at each fold-change (log2) value relative to the 0-h (20 °C) controls following 1-h (c), 24-h (d), or 48-h (e) 28 °C HS as determined by RNA-seq analysis. Dashed vertical lines denote upper and lower expression
limits of the genes that were up- or down-regulated, with the total number of differentially expressed genes in each category in parentheses. Arrows in e represent the fold-change expression of the six vit genes.

To quantitatively assess whether chronic HS impacted motility, we used a thrashing assay. Exposure to 28 °C HS did not affect motility after 24 h and only had a slight effect (< 15%) after 48 h (Fig. 1b). Therefore, a dramatic loss of reproductive capacity but not motility is evident during chronic temperature stress after the onset of egg laying. To identify molecular changes in these chronic stress conditions, the transcriptome was characterized using RNA-sequencing from three biological replicates at four timepoints: 0 h (20 °C control), 1 h, 24 h, and 48 h at 28 °C HS. These timepoints were selected to compare the effects of acute (1-h) and chronic (24- and 48-h) exposures to 28 °C HS, based on the distinct phenotypic changes observed in reproduction and motility. Each sample generated between 39 and 59 million clean reads, with 5.91 to 8.98 G of clean bases. The samples each had a Q20 score > 97.4% and the Pearson correlation coefficients between the biological replicates ranged between 0.973 and 0.994, indicating high sequencing quality and reproducibility. Differentially expressed genes were identified using the DESeq R package comparing each HS timepoint to the 0-h 20 °C controls (see Additional file 1) [33]. Genes with an adjusted (Benjamini-Hochberg) p value < 0.05 were considered differentially expressed [34]. Analysis of the RNA-seq data revealed that 1 h at 28 °C caused differential expression of only a few genes (10 genes down-regulated; 12 genes up-regulated) (Fig. 1c). The down-regulated genes are largely uncharacterized, but include three genes with predicted solute carrier activity, two with predicted roles in coenzyme A processes, and several with predicted catalytic activity. The up-regulated genes include six heat shock proteins (HSPs) as expected, as well as four genes of unknown function and
two genes with diverse roles (ckb-2 and cebp-1). This dataset, while using a duration commonly used for acute HS (1 h), is distinct from standard acute HS protocols that employ higher temperatures and observe larger changes in gene expression. In contrast, 24 h of 28 °C HS led to downregulation of 4755 genes and up-regulation of 4775 genes (Fig. 1d). Approximately 28% of the differentially expressed genes (697 genes down; 1934 genes up) experienced a ≥ 4-fold change in expression. The 48-h 28 °C HS led to down-regulation of 4365 genes and up-regulation of 4770 genes (Fig. 1e), ~ 33% of which (749 genes down; 2303 genes up) were altered by ≥ 4-fold. Venn diagrams showed that all of the genes differentially expressed during 1-h 28 °C HS were shared with at least one of the longer HS conditions, whereas 30% of the 24-h and 27% of the 48-h 28 °C HS genes were uniquely regulated by those timepoints (see Additional file 2: Fig. S1 and Additional file 3). Together, these data reveal extensive, duration-specific remodeling of the transcriptome in response to chronic stress. Furthermore, the molecular responses to chronic, mild temperature stress extend beyond the well-characterized heat shock response (HSR). Only 8% of the genes regulated during 48-h 28 °C HS were shared with a previously published gene set representing a classical acute HSR [35] (see Additional file 2: Fig. S2 and Additional file 3). To determine which cellular pathways were affected during chronic HS, gene ontology (GO) enrichment analysis was used (see Additional file 2: Fig. S3 and Additional file 3) [36, 37]. The 24-h HS gene enrichments showed an up-regulation in stress responses, such as defense and immune system responses, along with down-regulation of metabolic processes and nucleotide binding. Interestingly, both the 24-h and the 48-h HS timepoints elicited down-regulation of genes associated with the structural constituent of the cuticle, with the 48-h HS also up-regulating many cuticle-associated
genes, suggesting a cuticle restructuring event in response to chronic HS. The most dramatic phenotypic effect of chronic stress is inhibition of egg laying. If the reproductive cessation during chronic HS was being driven by transcriptional shutdown of oogenesis, we would expect to see a general repression of these genes. However, GO analyses did not reveal enrichment for reproduction-associated processes. To investigate this further, the genes involved in reproduction were specifically queried by examining the expression levels of oogenesis-enriched genes, as described by Reinke et al. [38]. Together, the 920 oogenesis-enriched genes identified in our RNA-seq data showed no change in expression at the 1-h timepoint, slight repression at 24 h, and a smaller degree of repression at the 48-h HS timepoint (see Additional file 2: Figs. S4A-C). Among these genes, ~3.8% were affected at least 4-fold by exposure to 28 °C HS for 24 or 48 h. Therefore, our data indicate that the cessation of reproduction during chronic HS does not involve an overall shutdown of oogenesis-enriched gene expression.

### Chronic heat stress disrupts endocytosis of yolk by oocytes

Among the most strongly repressed genes at the 48-h HS timepoint was the six-member vitellogenin gene family (*vit*-1 through *vit*-6) that encodes the yolk proteins (YPs) (Fig. 1e, arrows), with levels ranging from a ~3.9-fold to 216.8-fold decrease in expression compared to controls. The *vit* genes and their YP products play important roles in providing nutrients to developing oocytes [39–41]. In *C. elegans*, the six *vit* genes produce three major YP species—YP170, YP115, and YP88—that are named according to their molecular weights (see schematic in Fig. 2a). The YP170 species is composed of two protein products: YP170A, encoded by *vit*-3, *vit*-4, and *vit*-5; and YP170B, encoded by *vit*-
and vit-2. The other two protein species—YP115 and YP88—are cleaved in the pseudocoelom (body cavity) from a 180-kDa precursor protein produced by vit-6 [42, 43].

**Figure 4-2:** (Fig. 2) Chronic heat stress causes yolk protein accumulation and mislocalization.

a) A schematic showing the relationship between the six vit genes, their initial protein products, and the three major yolk protein (YP) bands. b) Coomassie-stained SDS-PAGE reveals accumulation of all three major YP species following chronic exposure to 28 °C HS. Arrows indicate YP170, YP115, and YP88 bands. The rme-4(b1001) mutant YP170::GFP strain (RT362) was included for YP band identification. Shown is a representative image from three independent trials. MW: molecular weight marker; WT: wild-type. c–h) Chronic exposure to 28 °C HS causes accumulation and mislocalization of the YP170::GFP (RT130) fluorescent reporter. Arrows in c, e, and g demonstrate proper localization of YP170::GFP in embryos at the control temperature. Shown are representative images from three independent trials with n ≥ 10 worms in each trial. Scale bar: 150 μm.
Since *vit* expression was decreased during chronic HS, YP levels were analyzed by SDS-PAGE to determine whether they also decreased [44, 45]. In contrast to the repression of YP mRNA levels, chronic 24- and 48-h HS led to accumulation of all three major YP species when compared to age-matched controls (Fig. 2b). YP identities were validated by comparison with a mutant known to accumulate YPs (compare YP170::GFP; *rme-4* mutants to YP170::GFP;WT in Fig. 2b) [46].

The striking accumulation of YPs seen in adults could occur in one of the three locations involved in the YP trafficking pathway: the intestine (site of synthesis), the embryos (site of uptake), or the pseudocoelomic space that separates the intestine from the gonad [44, 47, 48]. To determine the location of YP accumulation, a fluorescent reporter containing YP170 fused with GFP (YP170::GFP) was used (Fig. 2c–h) [47]. Compared to 20 °C controls, where YP170::GFP was localized in the oocytes (see arrows in Fig. 2c, e, g), exposure to 28 °C HS for 24 h (Fig. 2f) or 48 h (Fig. 2h) resulted in increased levels of YP170::GFP in the pseudocoelom but not in the intestine or the oocytes (see Additional file 2: Fig. S5 for higher magnification at the 24-h timepoint). As oocytes normally take in yolk using receptor-mediated endocytosis, the pseudocoelomic accumulation of YPs during HS phenocopies mutations in endocytosis [47]. Importantly, this phenotype can be observed after a 6-h HS exposure, with prominent YP170::GFP puncta around the embryos and reduced YP170::GFP intensity inside the embryos compared to the 20 °C controls (Fig. 2c vs 2d). This finding revealed that YP accumulation preceded, and was therefore not a consequence of, the egg-laying defect that developed between 12 and 24 h of HS.
Chronic heat stress disrupts endocytosis in coelomocytes and affects neuronal signaling
To determine whether disruption of YP trafficking is indicative of a generalized defect in trafficking, an independent protein trafficking reporter was used. This reporter contains a muscle-specific myosin promoter that drives a GFP transgene containing a signal sequence (myo-3p:ssGFP). The ssGFP is secreted from the muscles into the pseudocoelom and then taken up via fluid-phase endocytosis into the six macrophage-like coelomocytes, where its fluorescence is readily observable [19]. Whereas fluorescence was localized in the coelomocytes at the control temperature (Fig. 3a, c), exposure of this reporter to 24- or 48-h 28 °C HS resulted in diffuse fluorescence across the pseudocoelom (Fig. 3b, d). Thus, this protein trafficking reporter with a distinct endocytic process revealed a second trafficking defect in a different target tissue.
Figure 4-3: (Fig. 3) Chronic heat stress inhibits endocytosis in coelomocytes and disrupts neuronal signaling.

a–d) Chronic exposure to 28 °C HS (b and d) disrupts coelomocyte endocytosis compared to 20 °C controls (a and c) as observed in myo-3p::ssGFP (GS1912) adults. Shown are representative images from three independent trials with n ≥ 10 worms. Arrows in a and c demonstrate proper localization of ssGFP in coelomocytes at the control temperature. Scale bar: 150 μm. e) A schematic showing the cycle of synaptic vesicle formation and neurotransmitter release at a neuromuscular junction. Synaptic vesicle components are normally recycled through endocytosis (dashed arrows). f) Exposure of WT (N2) adults to 48-h 28 °C HS increases resistance to aldicarb, but not to levamisole, compared to 20 °C controls as measured using paralysis assays. Data represent the mean ± SD of n = 3 independent trials. * p < 0.05 (Student’s t test).
However, trafficking of the myo-3p::ssGFP reporter could be indirectly affected by the high levels of accumulated YP in the pseudocoelomic space during chronic stress. To test this, we knocked down YP in the myo-3p::ssGFP reporter strain using RNA interference (RNAi) against ceh-60, a regulator of vitellogenin expression [49, 50]. While ceh-60 RNAi effectively knocked down YP in the YP170::GFP reporter, which showed little to no GFP fluorescence (see Additional file 2: Figs. S6E-H), it did not affect the coelomocyte trafficking defect in the myo-3p::ssGFP reporter during exposure to 24-h 28 °C HS (Additional file 2: Figs. S6A-D). These results indicate that yolk accumulation in the pseudocoelom does not inhibit coelomocyte uptake and that a general defect in endocytosis occurs during chronic stress.

If endocytosis is globally disrupted during chronic stress, then neuronal signaling, which is heavily dependent on endocytosis, would also be affected. Such a defect would localize to the pre-synaptic junctions that rely on clathrin-mediated endocytosis to recycle synaptic vesicle components [51–53] (see schematic in Fig. 3e). Disruption of pre-synaptic versus post-synaptic neuronal signaling can be distinguished by sensitivity to the paralysis-inducing drugs aldicarb, an acetylcholinesterase inhibitor that acts on pre-synaptic signaling, and levamisole, a nicotinic acetylcholine receptor agonist that acts on post-synaptic signaling [54, 55]. Exposure to 48-h 28 °C HS caused a dramatic reduction in sensitivity to aldicarb, with ~ 36% of the stressed worms showing aldicarb resistance (not paralyzed) compared to only ~ 19% of the control worms remaining motile (Fig. 3f). In contrast, no difference was observed in the sensitivity to levamisole between the unstressed controls and the heat-stressed worms. These data suggest a pre-synaptic signaling defect
during chronic stress, which supports the hypothesis that chronic stress globally disrupts endocytosis.

Heat shock factor 1 (HSF-1) overexpression rescues chronic stress-induced endocytic disruptions
Several steps in endocytosis are dependent on molecular chaperones for assembly and disassembly of macromolecular complexes [14, 15]. These molecular chaperones are also important for protein folding during temperature stress and are responsible for negative regulation of the HSR in the absence of stress [11]. Activation of the HSR in our transcriptomic data indicates that chaperones are titrated by misfolded proteins during chronic stress. Therefore, we hypothesized that titration of chaperones away from their constitutive roles in endocytosis could be the mechanism causing disruption of protein trafficking and subsequent organismal phenotypes during chronic stress. If chaperone titration disrupts endocytosis, then reducing or increasing chaperone levels would exacerbate or suppress endocytic defects, respectively. To test this, we first inhibited the predominant cellular HSP70 chaperone, hsp-1, using RNAi knockdown. At the control temperature of 20 °C, knockdown of hsp-1 in the YP170::GFP reporter resulted in pseudocoelomic YP accumulation that mirrored chronic HS (Fig. 4). Therefore, depletion of HSP70 disrupts YP trafficking, consistent with previous reports demonstrating the role of HSP70 in endocytosis in other systems [13–15].
hsp-1 knockdown causes yolk to accumulate in the pseudocoelomic space, as shown in YP170::GFP (RT130) worms that were raised to the L4 stage on OP50 and then exposed to either a) control (L4440, empty vector) or b) hsp-1 RNAi for 48 h at 20 °C. Shown are representative images from three independent trials with n ≥ 10 worms. Scale bar: 150 μm.

Next, we tested the effects of increased HSP levels by using worms overexpressing the transcription factor heat shock factor 1 (HSF-1) [56]. To determine whether YP levels in the embryos were affected by chaperone levels, we collected equal numbers of eggs from wild-type and HSF-1 overexpression adults with and without HS. Consistent with a defect in endocytosis, isolated wild-type embryos contained lower levels of YPs per egg upon chronic stress (Fig. 5a). Quantitation of this effect showed that wild-type embryos had a 50% reduction in YP170 uptake capacity during HS (Fig. 5b). Remarkably, HSF-1 overexpression effectively restored embryonic YP accumulation, with these embryos maintaining ~ 98% of their YP170 uptake capacity. YP115 and YP88 also showed similar trends of rescue. However, HSF-1 overexpression was not sufficient to rescue YP accumulation inside adults during stress (Fig. 5a). Together, these results indicate that overexpression of chaperones is sufficient to rescue the YP endocytosis defect in oocytes during chronic stress.
Figure 4-5: (Fig. 5) HSF-1 overexpression partially rescues yolk endocytosis, neuronal signaling, and recovery of offspring following chronic HS.

a, b) Coomassie-stained SDS-PAGE reveals that exposing adults to 24-h 28 °C HS results in a loss of YP170 in WT (N2) embryos that is rescued in HSF-1 overexpression (HSF-1 O/E; EQ140) embryos. a) Representative image from one of three independent trials. Arrows indicate YP170, YP115, and YP88 bands. The rme-4(b1001) mutant YP170::GFP strain was included for YP band identification. MW: molecular weight marker; WT: wild-type. b) Quantification of a shows the relative change in each YP level upon HS. Data represent the mean ± SD of n = 3 independent trials.

c) HSF-1 O/E worms show restored sensitivity to aldicarb compared to WT worms that show resistance to aldicarb following exposure to 48-h 28 °C HS. Neither strain shows significant changes in drug response after 24-h 28 °C HS compared to 20 °C controls. Data represent the mean ± SD of n = 3 independent trials. d–f) HSF-1 O/E worms show improved reproductive function compared to WT during a 20 °C recovery period after a 24-h 28 °C HS, as shown by the percent of adults that recovered viable offspring (d) and the average number of eggs laid (e) and viable offspring produced (f) by each worm. Data in d represent the mean ± SD of n = 3 independent trials. Data in e and f represent the mean ± SD of n = 30 worms collected across three trials. * p<0.05, ** p<0.01 (Student’s t test).
We next tested whether chaperone titration represents a mechanism that generally affects cellular endocytosis by examining the effects of HSF-1 overexpression on neuronal signaling. HSF-1 overexpression reversed the aldicarb resistance induced by 48 h at 28 °C (Fig. 5c). Therefore, overexpression of HSF-1 can rescue multiple endocytosis defects and is not specific to YPs. The effects of HSF-1 overexpression are presumably through upregulation of chaperones; however, these data do not exclude other pathways regulated by HSF-1. Interestingly, no significant differences in aldicarb sensitivity were observed in wild-type or HSF-1 overexpression worms after 24-h 28 °C HS. This indicates that neuronal signaling is more resistant to stress-induced changes than the other pathways that showed endocytic defects at this timepoint.

Given that HSF-1 overexpression restored the endocytic defect in oocytes and the neuronal phenotype in adults, we next examined whether HSF-1 overexpression could protect reproductive output during chronic HS. Although HSF-1 overexpression was not sufficient to restore egg laying during continuous 28 °C HS, it did enhance recovery of viable offspring after a 24-h exposure to 28 °C HS (Fig. 5d). In these conditions, 100% of adults from both the wild-type and HSF-1 overexpression strains laid eggs during a 20 °C recovery period, but the HSF-1 overexpression worms laid significantly more eggs (Fig. 5e) and produced more larvae (Fig. 5f). Notably, only HSF-1 overexpression worms were capable of producing viable offspring in the first 24 h of recovery, whereas wild-type worms did not produce viable offspring until the second day of recovery (see Additional file 2: Fig. S7). These data suggest that increased chaperone levels can provide a stronger recovery of viable offspring following a period of chronic HS. Together, the recovery of YP endocytosis, neuronal signaling, and viable offspring indicate that a persistent
disruption of proteostasis during chronic stress titrates chaperones and that this is a common molecular mechanism that contributes to multiple physiological processes.

**Discussion**

We have identified chaperone titration as a molecular mechanism that contributes to a range of cellular and organismal phenotypes during exposure to chronic temperature stress (28 °C) (Fig. 6). The organismal phenotypes include cessation of egg laying and altered neuronal signaling. The cellular phenotypes include inhibition of YP uptake by oocytes and fluid-phase endocytosis by coelomocytes. A common feature of these phenotypes is their dependence on endocytosis. Our data indicate that chronic but mild temperature stress titrates chaperones away from their constitutive roles in endocytosis. Validating this model, induction of chaperones through HSF-1 overexpression partially rescues these phenotypes. Together, this work uncovers a unifying mechanism that connects molecular, cellular, tissue-specific, and organismal responses to chronic temperature stress.
Figure 4-6: (Fig. 6) Chaperone titration and disrupted endocytosis link chronic heat stress with organismal phenotypes.

Chronic protein-folding stress causes a titration of chaperones and subsequent disruption in endocytosis across cell types, ultimately leading to diverse physiological defects. Here, we provide evidence of this process in endogenous yolk levels and a fluorescent yolk reporter for embryos, a GFP reporter for coelomocytes, and functional assays for neurons coupled with HSF-1-mediated rescue of endogenous yolk uptake and neuronal signaling.

Recent experiments have suggested that inhibition of endocytosis via chaperone titration plays an important role in multiple neurodegenerative diseases [57]. It was shown that ectopic expression of disease-associated, aggregation-prone proteins in cultured cells leads to endocytic disruptions that can be rescued by chaperone overexpression. Here, we demonstrate that a general temperature stress can have the same effects on chaperones and endocytosis as those seen with specific aggregation-prone proteins. Therefore, our work extends the model of chaperone titration-induced endocytic defects to an intact organism.
Furthermore, the discovery that chaperone titration is a shared mechanism may help to explain why neurodegenerative diseases have such a large environmental component.

Our investigation has also uncovered critical spatiotemporal aspects of the responses to chronic temperature stress. A uniform, mild stress affects several processes central to organismal health, but at different timescales and with varying degrees of severity. The first observed phenotype in response to HS is a disruption of yolk trafficking into embryos seen in the first 6 h of 28 °C HS. By 24 h of HS, egg laying is terminated and fluid-phase endocytosis in coelomocytes is compromised. After 48 h of HS, neuronal signaling in response to aldicarb is altered and motility is slightly decreased. Thus, different tissues display unique temporal sensitivities to a stress applied uniformly to the organism. Distinct tissue-specific responses have also been observed through genetic disruption of protein folding, as tissues maintain unique balances between components of the proteostasis network and their tissue-specific substrates [58]. In addition, protein-misfolding mutations in a broadly expressed protein can elicit a “bystander” effect whereby the folding and trafficking of a specific but unrelated protein can be disrupted, possibly through titration of a selective, shared chaperone [59]. This effect was shown to produce cell-specific phenotypes despite the ubiquitous expression of the mutated protein. Recent work has also suggested cell-specific requirements for broad-specificity chaperones [60]. Therefore, the chronic stress-mediated phenotypes uncovered here contribute to the growing recognition of a range of cellular proteostasis capacities existing within an organism.

The earliest observed chronic stress phenotype, YP mislocalization at 6 h, precedes the reproductive defect. However, the reproductive defect is not likely to be caused by YP
mislocalization alone, since YPs are not required for oogenesis, fertilization, or egg laying. Several mutations that block YP production do not affect egg laying, and mutations in the yolk receptor (RME-2) do not prevent production of viable offspring [47, 49, 61]. The observation that HSF-1 overexpression partially rescues reproduction after 24 h of stress indicates that chaperone titration contributes to the reproductive defect independently of yolk trafficking. However, since the rescue is not complete, there must be additional mechanisms behind this reproductive cessation. As temperature stress also disrupts embryonic development [62], these other mechanisms could include signaling pathways that coordinate development of the embryos with egg-laying behavior in the adult.

Notably, the repression of vit genes occurs after the YP trafficking defect. However, there is no visible accumulation of YP in the intestine, indicating that this effect is cell non-autonomous. Together, these data suggest a signaling pathway between the site of YP accumulation in the pseudocoelom and the site of synthesis in the intestine.

The framework we have established here provides a solid foundation for further investigations into the molecular and organismal responses to elevated temperature. The impact of this research is not limited to reproduction, as we have uncovered a wealth of gene expression changes induced by chronic stress. These gene expression changes are strongly enriched for specific categories, such as collagen genes, that are associated with specific organismal biology, such as cuticle structure. Our analysis connecting organismal responses to temperature with tissue-specific, cellular, and molecular pathways has wide-ranging implications for agricultural, ecological, and evolutionary studies. Furthermore,
understanding the effects of extended but mild temperature stress grows increasingly critical in the face of elevated temperatures due to climate change.

Conclusions
We have established C. elegans as a model organism for the systematic analysis of the effects of chronic stress. We have identified chaperone titration as a molecular mechanism that contributes to defects in protein trafficking, disruption of neuronal signaling, and cessation of reproduction during exposure to extended temperature stress.

Methods
Worm strains and maintenance
The following Caenorhabditis elegans strains were used in this study: Bristol strain N2, RT130(pwIs23[YP170::GFP]), RT362(rme-4(b1001);pwIs23[YP170::GFP]), GS1912(arIs37[myo-3p::ssGFP + dpy-20(+)] I), and EQ140(iqIs37[-pAH76(hsf-1p::myc-hsf-1)+pRF4(rol-6p::rol-6(su1006))]). Unless otherwise stated, worms were maintained at 20 °C. Worm populations were synchronized for experiments by allowing day 2 adult hermaphrodites to lay eggs for 1–1.5 h. The synchronized egg populations were then raised at 20 °C until day one of adulthood, after the onset of egg laying (~ 72 h post-synchronization for the N2 strain). For all heat stress (HS) experiments, plates of day 1 adults were transferred to a 28 °C dry incubator (HS worms) for the specified time course.

Egg-counting experiments
Synchronized wild-type (N2) worms at the late L4/young adult (YA) stage (~ 58 h after synchronization) were singled onto 35mm NGM plates seeded with 100 μL OP50 and kept at 20 °C for 12 h to reach egg-laying adulthood. The individuals were then transferred to
fresh 35mm plates and shifted to 28 °C for HS or maintained at 20 °C for control conditions (this represents the 0-h timepoint) and the eggs laid on the first plate were counted. Worms were transferred to fresh, pre-warmed plates every 12 h with minimal exposure (< 5 min) to ambient temperatures.

**Motility assays**
Thrashing was scored at room temperature (RT) in a 10-μL drop of M9 on NGM agar. Individual worms were placed into the droplet and allowed to acclimate for 30 s, then each body bend was counted for 30 s.

**RNA-sequencing**
Synchronized day 1 adult wild-type (N2) worms were either collected immediately (0-h 20 °C control), or shifted to 28 °C HS for 1, 24, or 48 h before collection. RNA was collected as described previously [63]. Library preparation, sequencing, and initial data analyses were performed by Novogene. Total RNA was poly-A selected and then subjected to 150-bp paired-end Illumina HiSeq sequencing using three biological replicates for each condition. The raw reads were filtered to remove reads with adaptor contamination, reads consisting of > 10% uncertain nucleotides, and reads where > 50% of the bases were low quality (base quality < 20). The remaining clean reads (~ 97% for each sample) were mapped to the WBcel235 *C. elegans* reference genome. Approximately 92–93% of the clean reads were mapped per sample. Genes that had no mapped reads for control or HS conditions were excluded from the differential expression analysis. Differentially expressed genes were identified using DESeq (v.1.18.0) by normalizing reads based on the negative binomial distribution method and comparing each HS timepoint to the 0-h control.
Fluorescence microscopy

Worms were anesthetized in 0.5–1.0mM levamisole on a 3% agarose pad for imaging, as described previously [63]. Images were analyzed using EZ-C1 software, Gold Version 3.90 build 869 (Nikon Corporation, Tokyo, Japan). The range and saturation levels of each image were normalized across conditions at each timepoint to allow for direct comparisons. Each trial scored n ≥ 10 worms per condition.

SDS-PAGE and Coomassie staining

For adult protein lysates, equal numbers of adults (50–60) from each condition were picked into microcentrifuge tubes containing 33 μL Laemmli sample buffer. Samples were then boiled for 10 min, centrifuged for 10 min at 14000 x RPM at RT, and the supernatants were transferred to fresh tubes. Equal volumes were loaded per lane. For embryo lysates, ~100–200 adults from each condition were collected, and fresh bleach solution (20% sodium hypochlorite + 0.25M NaOH in ultrapure H₂O) was added to each tube to dissolve the adults. After ~8 min with gentle mixing (or until adults were dissolved), embryos were washed once with M9, resuspended in 15 μL M9, and then the number of eggs was counted using a dissecting scope. Samples were then mixed with 2x Laemmli sample buffer, boiled, and centrifuged before loading. Equal numbers of eggs (~800) were loaded per lane. Lysates were loaded into a freshly prepared 8% SDS-PAGE. Gels were stained using Coomassie brilliant blue and imaged using a ChemiDoc™ XRS System (Bio-Rad Laboratories Inc., Hercules, CA).
Yolk protein quantification
YP bands were identified based on comparisons to literature and validated by comparing banding patterns with RME mutants known to accumulate YPs [44–46]. Using ImageLab 6.0.0 (Bio-Rad), each gel was quantified by normalizing background levels and then calculating each adjusted YP170, YP115, and YP88 band volume relative to the total lane volume for that sample. The relative accumulation or loss of each YP was calculated by dividing the adjusted YP volume in the HS lane by the adjusted YP volume in the control lane for that timepoint.

RNAi experiments
RNA interference (RNAi) was performed against the indicated genes, with L4440 as the vector control, using feeding RNAi in *E. coli* HT115(DE3) [64]. RNAi clones were sequence-verified before use. RNAi against *ceh-60* was initiated from egg-lay synchronization. RNAi against *hsp-1* was initiated in L4 worms to avoid developmental defects and was carried out for 48 h at 20 °C before imaging.

Neuronal transmission assays
Paralysis plates were made using NGM containing final concentrations of 1 mM aldicarb (Adipogen Corporation, San Diego, CA) or 0.5 mM levamisole (Acros Organics, Thermo Scientific, Waltham, MA). For each paralysis assay, drug plates were freshly seeded with OP50 and dried at RT for 24 h. Then, 25 adult worms that had been exposed to 20 °C (control) or 28 °C (HS) for 24 or 48 h (as described for HS conditions) were picked to each drug plate and placed in a 20 °C incubator. Levamisole sensitivity was scored 2 h after plating and aldicarb sensitivity was scored 8 h after plating. Worms were scored as
paralyzed if they did not move after being gently prodded 3x on the head and tail with a platinum wire pick. Worms that ruptured or crawled up the sides of the drug plate were censored. The percentage of worms not paralyzed in each trial was calculated from the total number of non-censored worms for that plate.

Reproduction recovery experiments
Synchronized day 1 adult wild-type (N2) or HSF-1 overexpression (EQ140) worms were singled onto 35 mm OP50 plates, shifted to 28 °C HS for 24 h, and then shifted back down to 20 °C for recovery. During the recovery period of up to 7 days, adults were transferred to fresh OP50 plates daily and the eggs on each plate were counted. The same plates were scored again the following day for appearance of larvae (viable offspring).

Statistics and figures
Graphs were prepared with accompanying statistics using R version 4.0.4 (R Foundation for Statistical Computing, Vienna, Austria). A p value < 0.05 was considered statistically significant. Model figures (Figs. 3e and 6) were created with BioRender.com.

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Authors’ contributions
RNP performed experiments, analyzed data, and drafted and edited the manuscript. IM collected and analyzed data. RNP, KSKG, and EG designed the experiments, analyzed the data, and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets supporting the conclusions of this article are included within the article and its additional files. Complete sequence reads are available in the Sequence Read Archive (SRA) at NCBI under project number PRJNA705210 [65].

Declarations
Ethics approval and consent to participate: Not applicable. Consent for publication: Not applicable. Competing interests: The authors declare that they have no competing interests.
Supplementary information

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Additional file descriptions

**Additional file 1. DEGs.** Worksheet containing the DESeq output file containing statistically differentially expressed genes from each timepoint with read counts, log2 fold-change, and $p$-values. **Additional file 2: Figure S1-S7.** File containing all supplemental figures. **FigS1.** Venn diagram comparing Acute (1-h), Mid (24-h), and Chronic (48-h) 28 °C HS differentially expressed genes. **FigS2.** Venn diagram comparing Chronic (48-h) 28 °C HS and Classical Acute HSR differentially expressed genes. **FigS3.** YP170::GFP reporter ±24-h 28 °C HS at 20x and 40x magnification. **FigS4:** GO term enrichment analyses highlight dynamic stress-induced transcriptomes. **FigS5.** Oogenesis-enriched genes show temporal response to stress. **FigS6.** Knockdown of YP with ceh-60 RNAi does not restore coelomocyte endocytosis during 24-h 28 °C HS. **FigS7.** HSF-1 overexpression provides faster recovery of offspring after 24 h of 28 °C HS. **Additional file 3: Gene lists.** Worksheet with separate tabs for: **FigS1**, all differentially expressed genes shared between Acute (1-h), Mid (24-h), and Chronic (48-h) 28 °C HS; **FigS2**, all differentially expressed genes shared between Chronic (48-h) 28 °C HS (this work) and Classical Acute HSR genes (33 °C HS for 30 min, from Brunquell et al. [35]); **FigS3**, GO analysis results including all significantly enriched GO terms and the up- and down-regulated genes from each term for each 28 °C HS timepoint (listed by WormBase ID).
Supplemental figures

Figure 4-7: (Fig. S1) Venn diagram comparing Acute (1-hr), Mid (24-hr), and Chronic (48-hr) 28°C HS differentially expressed genes.

All differentially expressed genes compared across the chronic 28°C HS time course. Lists of genes in each overlapping (shared) region can be found in AdditionalFile3_GeneLists.xlsx.
Figure 4-8: (Fig. S2) Venn diagram comparing Chronic (48-hr) 28°C HS and Classical Acute HSR differentially expressed genes.

All differentially expressed genes from chronic, 48-hour 28°C HS (this work) compared with genes differentially expressed by a classical acute HS (30-minute 33°C HS) from Brunquell et al. (ref [35]). Lists of all genes and of the overlapping (shared) genes can be found in AdditionalFile3_GeneLists.xlsx.
Figure 4-9: (Fig. S3) GO term enrichment analyses highlight dynamic stress-induced transcriptomes.

GO term enrichment was analyzed using RNA-seq data collected from WT (N2) hermaphrodites following exposure to 1-hour, 24-hour, or 48-hour 28°C HS initiated on day 1 of adulthood. The top 10 enriched GO terms among the genes that were up- or down-regulated ≥ 4-fold relative to 0-hour (20°C) controls are shown as the significance of the GO enrichment (-log10 of the Q value). The number of up- or down-regulated genes in each term are indicated in each bar. See AdditionalFile3_GeneLists.xlsx.
Figure 4-10: (Fig. S4) Oogenesis-enriched genes show temporal response to stress.

Analysis of oogenesis-enriched genes reveals time-dependent responses to 28°C HS in adult WT (N2) worms. Curves represent the densities of all genes (gray) or oogenesis-enriched genes (color) at each expression fold-change (log2) value relative to the 0-hour (20°C) controls following 1-hour (A), 24-hour (B), or 48-hour (C) 28°C HS as determined by RNA-seq analysis.
Day 1 adult YP170::GFP (RT130) hermaphrodites were exposed to 24 hours of 20°C (control) or 28°C HS before imaging. Scale bar: 50 µm.
Figure 4.12: (Fig. S6) Knockdown of YP with ceh-60 RNAi does not restore coelomocyte endocytosis during 24-hour 28°C HS.

The effects of yolk knockdown during chronic stress were assessed using hermaphrodites that were raised from egg to day 1 adults on either control (L4440, empty vector) or ceh-60 RNAi before being exposed to a 24-hour 28°C HS. A-D) Yolk knockdown has no effect on coelomocyte endocytosis at control (20°C) or HS temperatures, as observed in myo-3p::ssGFP (GS1912) worms. E-H) Yolk knockdown efficiency is demonstrated in YP170::GFP (RT130) worms. Shown are representative images from three independent trials with n ≥ 10 worms. Scale bar: 150 μm.
Figure 4-13: (Fig. S7) HSF-1 overexpression provides faster recovery of offspring after 24 hours of 28°C HS.

HSF-1 overexpression (HSF-1 O/E; EQ140) worms show faster recovery of viable offspring compared to WT (N2) during a 20°C recovery period after a 24-hour 28°C HS, as shown by the number of larvae produced daily by each worm. Data represent n = 30 worms collected across three trials, where the larval counts for individual worms are pooled from recovery days 4-7.
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Chapter 5
Potential Autoregulation of Heat Shock Factor 1 (HSF-1) in *C. elegans*

Abstract
One of the most prominent cellular responses to protein misfolding during stress is the heat shock response (HSR), regulated by the transcription factor Heat Shock Factor 1 (HSF1). HSF1 coordinates expression of molecular chaperones called heat shock proteins (HSPs) that help to refold proteins or target them for degradation. The HSR maintains protein-folding homeostasis not only upon exposure to stress, but also during development and *de novo* protein synthesis. While many studies have focused on the activation and downstream effects of the HSF1 protein, little is known about how its gene, *HSF1*, is regulated. The *HSF1* gene is generally believed to be stably expressed in cells, but the mechanics dictating its expression are not well understood. We have preliminary evidence that HSF1 levels may play a direct role in the regulation of *HSF1* gene expression. Using a transgenic *C. elegans* strain with a red fluorescent protein fused to the *hsf-1* promoter, we have found that RNAi directed against *hsf-1* induces activation of the *hsf-1* promoter. Moreover, this effect is seen both in the absence of stress as well as during chronic but mild heat stress (HS). This suggests that HSF-1 is involved in an autoregulatory feedback loop, and we hypothesize that HSF-1 levels are sensed at the *hsf-1* promoter in a manner that is enhanced upon exposure to prolonged protein-folding stress. This autoregulatory feedback loop may be mediated through other proteins or through non-canonical binding by HSF-1.
These findings offer a new regulatory pathway to explore that may have clinical relevance for various protein-folding diseases.

**Background**

In vertebrates, at least four transcription factors associated with the heat shock response (HSR) have been found and are named heat shock factors 1-4 (HSF1-4) (Nover et al. 2001). The primary regulator of the HSR in vertebrates is HSF1, which is also the only HSF found in invertebrates (Anckar and Sistonen 2011). HSF4 seems to function primarily during development, specifically during development of eye lenses, and is not known to have any stress-inducible roles at this time (Dayalan Naidu and Dinkova-Kostova 2017). HSF3 was originally thought to be specific to avian species but has been found in mouse cells more recently, although its function is still undetermined (Akerfelt et al. 2007; Anckar and Sistonen 2011). HSF2, unlike the other HSFs, is localized in specific tissues and cell types, and can form heterotrimers with HSF1. HSF2 has been studied mostly in terms of developmental roles, but may be linked to neurodegenerative disease progression (Dayalan Naidu and Dinkova-Kostova 2017). HSF2 also plays a role in stimulating the HSR, but seems to depend on HSF1 for that effect (Östling et al. 2007). HSF1 is by far the most-studied member of the HSF protein family, and is crucial for mounting a cellular response to stress as evidenced in studies of Hsf1-/- knockout mice that cannot stimulate stress-induced transcription of genes encoding HSPs (Östling et al. 2007; Anckar and Sistonen 2011).

While the structure of HSF1 is highly dynamic and has not yet been fully solved, five functional domains of HSF1 have been described in some detail (Fig. 1) (Anckar and
Sistonen 2011; Neudegger et al. 2016). The N-terminal region contains the DNA-binding domain (DBD) spanning from amino acid positions 15-110. This is followed by the first heptad repeat, a leucine-zipper-like domain known as HR-A/B (or LZ1-3). The HR-A/B domain is responsible for forming trimers with other HSFs, but is negatively regulated by the HR-C (LZ4) domain to prevent sporadic trimerization. The regulatory domain (RD) falls in the middle of the protein and functions to repress the C-terminal trans-activation domain (TAD) from spurious transcriptional activation of target genes. The RD contains 15 of the 22 known phosphorylation sites in HSF1. Hyperphosphorylation of HSF1 through the RD was thought to be necessary for trans-activation activity, although recent research has suggested hyperphosphorylation to be an independent event playing a larger role in repressing the TAD instead of activating it (Budzyński et al. 2015).

Figure 5-1: (Fig.1) The five functional domains of human HSF1.

The 529-amino acid protein with known domains labeled. DBD = DNA-Binding Domain, LZ = Leucine Zipper, HR = Heptad Repeat, RD = Regulatory Domain, TAD = Trans-Activation Domain.

While many studies have focused on the activation of HSF1 and its downstream effectors, very little information is available regarding the regulation of the HSF1 gene. A previous study examined the promoter region of the mouse Hsf1 gene and found potential binding sites for specific protein 1 (Sp1) and CCAAT-box-binding protein transcription factors (CTFs) (Zhang et al. 1998). The authors made mutations in the Hsf1 promoter region to delete those binding sites and found that the constitutive expression of the Hsf1 gene appears to be under the control of those two transcription factors. More recently,
researchers have seen that the mRNA levels of HSF1 can be variable in human cells if stimulated by chemicals or disease, potentially through the CCAAT enhancer-binding protein (C/EBP) (Xue et al. 2012), a newer name used to describe the CTF above, but these results have not yet been supported with in vivo studies.

As a principal function of HSF1 is to protect cells during proteotoxic stress by upregulating molecular chaperones, diseases that negatively impact the ability of cells to properly fold proteins or to successfully break-down misfolded proteins would benefit from increased activation of HSF1 and the HSR. For example, experiments with the polyglutamine (polyQ) expansion of the huntingtin protein (Htt) in Huntington’s Disease (HD) model organisms have shown that certain compounds can limit the toxicity of the expanded Htt, possibly through activation of the HSR (Wang et al. 2005; Fujikake et al. 2008). Other studies have shown that the activity of Purkinje cells, the primary neuronal components of the cerebellum, can be recovered in Alzheimer’s disease (AD) models by overexpressing HSF1 (Jiang et al. 2013). Conversely, the levels of HSF1 were seen to be slightly down-regulated in the disease state, which could account for some of the toxicity seen in the disease. The recovery of Purkinje cells upon HSF1 overexpression is believed to be caused by the subsequent upregulation of chaperones. Additionally, while studying the protective role of the HSR in the brain of AD patients may be obvious since decreased mental capacities make up the primary symptoms of AD, many other tissues suffer from dysfunction in patients with this disease. Cardiovascular health has been shown to decline in AD patients, and a recent study explored the role of amyloid-β (which accumulates in plaques in the brain during AD progression) in cardiomyocytes to search for mechanisms that may lead to the secondary effects of AD (Zhang et al. 2015). Researchers found that
accumulation of amyloid-β in cardiomyocytes led to increased apoptosis, an effect that could be abrogated through forced overexpression of HSF1. Taken together, increasing HSF1 activity would be beneficial to help clear out misfolded proteins in neurodegenerative diseases, and increasing the expression levels of HSF1 would be a way to ensure higher levels of molecular chaperone expression.

The mechanisms dictating how HSF1 expression levels may be altered by diseases is unclear, and further studies are needed to elucidate this pathway. The significance of altered HSF1 levels on disease outcomes has been most strongly demonstrated in carcinogenesis. Researchers have shown that Hsf1-null (Hsf1−/−) mice, when exposed to carcinogens, are far more resistant to tumor formation than mice with wild-type Hsf1 levels, especially when combined with a defective tumor suppressor p53 background (Dai et al. 2007). It was later shown that the network of gene expression regulated by HSF1 in cancer differs from the genes regulated by HSF1 during heat shock, implicating HSF1 in a previously unknown transcriptional program distinct from the HSR (Mendillo et al. 2012). Moreover, higher levels of HSF1 expression are correlated with poorer prognosis and larger tumor size in estrogen receptor (ER)-positive breast cancers (Santagata et al. 2011). Several other cancers have been studied with their relation to HSF1 activity and levels, and some researchers have even linked HSF1 overexpression with chemotherapeutic drug resistance (Hoang et al. 2000; Vydra et al. 2013; Vilaboa et al. 2017). These findings stress the importance of understanding the regulatory mechanisms driving gene expression of HSF1, seeing as the cytoprotective role of HSF1 seems to have been hijacked by cancer cells.
As the regulation of the HSF1 gene is poorly understood, it is crucial to elucidate the details of the mechanisms involved. We have preliminary data demonstrating a change in hsf-1 gene promoter activity in C. elegans when worms are exposed to chronic mild HS and/or depleted of endogenous hsf-1 levels using RNA interference (RNAi). These findings suggest previously undescribed regulation of hsf-1 during HS and a potential autoregulatory feedback loop of HSF-1.

Results

Chronic HS induces the hsf-1 promoter
Exposure to a mild but chronic 24- or 48-hour HS at 28°C strongly induced fluorescence in C. elegans adults carrying red fluorescent protein fused to the hsf-1 promoter (hsf-1p::his-24::mCherry) (Fig. 2). Induction appeared to be localized in the intestinal cells and hypodermal nuclei in the soma, but prominent induction also appeared in the embryos contained within the uterus.
Figure 5-2: (Fig. 2) Chronic 28°C HS induces the $hsf-1$ promoter.

Day 1 adult RW10438 ($hsf-1p::his-24::mCherry$) worms raised on OP50 were exposed to 24 or 48 hours of 28°C HS. Shown are fluorescent images with corresponding white light images, which are representative of $n=3$ independent trials using 10 worms each. Scale bar: 100 µm.
hsf-1 RNAi induces the hsf-1 promoter

While examining potential regulators of hsf-1, we found that RNAi against hsf-1 itself induced the promoter reporter in the absence of stress (Fig. 3). This effect was variable, however, likely owing to the inherent variability of RNAi efficiency. This induction appeared most often in the first two intestinal cells, but some worms showed additional induction in several pairs of intestinal cells. Induction in all worms was enhanced upon exposure to chronic 28°C HS.

Figure 5-3: (Fig. 3) hsf-1 RNAi induces the hsf-1 promoter.

Day 1 adult RW10438 (hsf-1::his-24::mCherry) worms raised on L4440 (empty vector control) or hsf-1 RNAi were exposed to 24 or 48 hours of 28°C HS. Shown are fluorescent images with corresponding white light images, which are representative of n=3 independent trials using 10 worms each. Scale bar: 100 µm.
Chronic HS induces endogenous *hsf-1* mRNA levels in qPCR but not RNA-seq

Endogenous *hsf-1* mRNA levels increased ~3-fold following 48-hr 28°C HS relative to non-stressed control worms as measured by reverse-transcriptase quantitative PCR (RT-qPCR) (Fig. 4). This effect was not seen, however, in RNA-sequencing analyses, even though the same RNA samples were used (Plagens et al. 2021). This discrepancy remains to be resolved, but could be caused by the differences in RNA processing or normalization methods that differ between these two techniques.

![Figure 4](image)

**Figure 4:** Chronic HS induces endogenous *hsf-1* mRNA levels in qPCR.

Wild-type (N2) day 1 adult hermaphrodites were subjected to a chronic HS time course at 28°C, with RNA collected at each time point. Data are normalized to the 0-hour controls and represent the mean ± SEM from n=4 biological replicates. *p* < 0.05 (Student’s *t* test).

**Discussion**

Despite being long-believed to remain stably expressed during HS, we have discovered that chronic but mild 28°C HS induces the *hsf-1* promoter and possibly increases
endogenous hsf-1 mRNA levels. The induction of hsf-1 may have been missed in previous HS experiments because most HS studies utilize acute stress durations to induce the HSR. Normal levels of HSF-1 may be sufficient to endure an acute HS, but the continuous need for transcription of molecular chaperones during prolonged HS may require increased levels of HSF-1. Similarly, reducing HSF-1 levels through hsf-1-directed RNAi induces the hsf-1 promoter in the absence of stress, suggesting that the level of HSF-1 is being sensed at its own promoter.

To better study this new regulation, the first step is to verify that increased and decreased protein levels of HSF-1 affect the hsf-1 promoter activity in opposing manners. If our initial findings are correct, then overexpressing HSF-1 should decrease promoter induction following chronic HS. Such experiments are currently ongoing, using the EQ140 (hsf-1p::myc-hsf-1) HSF-1 overexpression transgenic strain crossed with the hsf-1p::his-24::mCherry promoter reporter strain.

The next question to address is whether the hsf-1 promoter is sensing HSF-1 levels or activity. To make this distinction, the hsf-1p::his-24::mCherry strain will be crossed with the PS3351 strain (hsf-1(sy441)), which carries a point mutation in hsf-1 that does not affect HSF-1 levels but renders HSF-1 incapable of activating in response to stress (Hajdu-Cronin et al. 2004). If the hsf-1 promoter is sensing reduced HSF-1 levels, then this new strain should only induce the promoter reporter during chronic HS or with hsf-1 RNAi, matching induction of the reporter alone. However, if the promoter is sensing reduced HSF-1 activity, then this new strain may induce the promoter reporter constitutively to correspond with reduced HSF-1 activity during development and normal conditions. These
results will guide future studies to determine whether the effects of HSF-1 levels/activity act directly or indirectly on the hsf-1 promoter.

The dramatic induction of the hsf-1 promoter in embryos during chronic HS is particularly exciting, as we have recently demonstrated that chronic HS disrupts endocytosis of yolk proteins in embryos through chaperone titration (Plagens et al. 2021). This new finding indicates that, although eggs laid at 28°C are not viable and 48-hour 28°C HS prevents adults from laying eggs, these embryos may still be transcriptionally active and sensing HS.

If the levels or activity of the HSF-1 protein are being directly sensed at the hsf-1 promoter, whether in somatic or germline cells, this would reveal novel mechanisms for the regulation of the hsf-1 gene. Although the promoter region of HSF1 has not yet been reported to contain HSEs in other organisms, we have found a potential HSE located 511 bp upstream of hsf-1 in the C. elegans genome (data not shown). This could serve as an HSF-1 binding site. Alternatively, the regulation by HSF-1 may be indirect or through non-canonical binding with DNA. It is possible that this regulatory mechanism has evaded discovery in vertebrates if similar sites exist at enhancer elements located at great distances from the HSF1 locus. Otherwise, C. elegans may utilize unique hsf-1 regulation to compensate for only having one heat shock factor. To the best of our knowledge, neither the chronic stress-induced regulation nor the autoregulatory feedback loop has yet been explored. These mechanisms could provide answers regarding the functional roles of HSF-1 in development, induction of the HSR, and diseases such as AD and cancer. Given the
results of our preliminary data, we hypothesize that the promoter of hsf-1 is regulated, at least in part, by feedback with its own product.

Methods

Worm strains and maintenance
These trials used the strains N2 (Bristol strain) and RW10348 [hsf-1p::his-24::mCherry]. In-progress trials are crossing the RW10438 strain with EQ140(iqls37[pAH76(hsf-1p::myc-hsf-1)+pRF4(rol-6p::rol-6(su1006)))] to overexpress HSF-1 simultaneously with the promoter reporter. Unless otherwise stated, worms were maintained in a 20°C (control temperature) dry incubator on nematode growth medium (NGM) plates seeded with OP50 E. coli. Experimental worms were synchronized by allowing day 2 adult hermaphrodites to lay eggs on OP50 plates for 1 hour; these eggs were placed in 20°C for ~72 hours to develop to day 1 (egg-laying) adults prior to shifting to 28°C HS.

RNAi knockdown
Unless otherwise stated, synchronized larvae were picked to RNAi plates ~31 hours post egg-lay synchronization (~L2 stage larvae), where they remained at 20°C for an additional ~41 hours to reach day 1 of adulthood (egg-laying adults). RNAi constructs used were L4440 (empty vector control) and hsf-1. RNAi plates and cultures contained ampicillin, tetracycline, and IPTG as described previously, where the RNAi cultures were induced 2-3 hours with 1 mM IPTG prior to seeding 300 µL onto prepared plates (Golden et al. 2020).
Heat stress (HS) conditions
Synchronized day 1 adult hermaphrodites were either maintained at 20°C (controls) or shifted to 28°C heat stress (HS) for 24 or 48 hours prior to imaging. Both conditions utilized dry incubators.

Fluorescence microscopy
At each timepoint, 10 adult worms from each condition were mounted onto 3% agarose on glass slides using 1 mM levamisole prior to imaging using a Nikon confocal microscope, as described previously (Golden et al. 2020). Images at each timepoint have normalized range and saturation levels to allow for direct comparison of fluorescence intensity across conditions.

qPCR
Day 1 adult wild-type (N2) worms were either collected immediately (20°C controls), or shifted to 28°C (HS) for 1, 24, 48, or 72 hours. Total RNA was collected using Trizol as described previously (3 of the 4 biological replicates were subjected to RNA-seq in my chronic HS manuscript, but all 4 original replicates were used for qPCR here) (Plagens et al. 2021). Next, 250 ng RNA were treated with DNase I before being subjected to cDNA synthesis and qPCR using technical duplicates. Ct values were normalized to 18S rRNA using the standard curve method and data are shown relative to the 0-hour, day 1 controls.

Literature cited


Chapter 6
Discussion

While the mechanics of the acute HSR have been well characterized in single-celled organisms, the complexities of how multicellular organisms respond to varying durations of HS remain to be fully characterized. Here, we have discovered that a uniformly applied stress such as HS affects an organism in a highly tissue-specific manner and at vastly different timescales. Specifically, we have shown that oocytes are highly sensitive to HS, as demonstrated by a reduction in yolk uptake that is evident in as little as 6 hours into a 48-hour 28°C HS time course, whereas neurons were the last examined cells to display a defect, which was only evident at the 48-hour time point. These findings are consistent with our data showing that the balance between the components of the proteostasis machinery and their tissue-specific substrates drives tissue-specific responses to protein misfolding in an organism. Together, these data reveal insight into how proteostasis diseases can have profound tissue-specific consequences, and emphasize the importance of examining stress responses across tissues using extended timescales.

The discovery that molecular chaperones are titrated away from their endocytic roles in C. elegans during chronic HS has substantial implications for organismal responses to prolonged temperature stress. However, with the vast array of environmental toxins and conditions that negatively impact proteostasis, it is likely that chronic exposure to stressors other than temperature would have similar outcomes on cellular function. As the molecular mechanism of chaperone titration inhibiting endocytosis is shared with neurodegenerative disease models, our findings implicate a variety of environmental stressors in sporadic
protein-folding diseases such as Alzheimer’s disease. Thus, this work motivates future studies probing the relationship between different chronic environmental stressors, proteostasis, and neuronal function.

Moreover, this system has allowed us to identify novel regulation of HSF-1 levels, which were previously believed to be unaffected by HS. Currently, little is known about the regulation of the HSF1 promoter across organisms. The finding that chronic HS activates the hsf-1 promoter in not only the somatic cells but also the embryos in C. elegans presents a new regulatory mechanism to explore. Knowing that different tissues respond uniquely to disruptions of proteostasis and that HSF-1 regulates unique networks during stress compared to development, it is likely that hsf-1 is regulated differently in somatic cells compared to embryos. This is supported by the finding that depleting hsf-1 through RNAi induced the hsf-1 promoter at basal temperatures only in somatic cells but not in the embryos. This also indicates that the potential autoregulatory feedback loop with HSF-1 levels is distinct from the induction seen with chronic HS. These results could be explained by a unique combination of canonical and non-canonical binding of DNA by HSF-1, recruitment of novel cofactors, and/or various post-translational modifications of HSF-1 during these different conditions in these distinct cell types. Future work determining the mechanisms driving promoter activation and gene expression of the master regulator of cytosolic proteostasis could have substantial implications for protein-folding diseases and general organismal health.

An ecologically relevant finding revealed by transcriptomic analyses of C. elegans exposed to chronic 28°C HS was a potential cuticle restructuring event. Previously, cuticle collagen
genes were shown to be up-regulated in *C. elegans* larvae after an acute, 30-minute 33°C HS (Brunquell et al. 2016). Here, adult worms exposed to a milder 28°C HS showed enrichment for both up-regulation and down-regulation of cuticle-associated genes at the 24 and 48-hour timepoints, but no changes at the 1-hour timepoint. Thus, the molecular signatures in our RNA-seq dataset provide a resource for exploring new phenotypes that are uniquely sensitive to mild but persistent protein-folding stress and could be of great ecological interest in understanding the impacts of organismal responses to climate change.

The most striking phenotype in *C. elegans* during chronic HS is the cessation of egg laying. One factor contributing to this phenotype appears to be chaperone titration, as HSF-1 overexpression could improve reproduction after HS but could not restore reproduction during continuous HS. Thus, other mechanisms contributing to reproductive cessation remain to be identified. Our RNA-seq data shows thousands of transcriptomic changes taking place during chronic HS that could drive these mechanisms. To determine which gene expression changes are most important for protection against chronic HS, we have designed a forward genetic screen that will select for worms that can reproduce during prolonged exposure to 28°C. Following the most common method of random mutagenesis in *C. elegans*, we will expose adult hermaphrodite worms (generation P0) to the alkylating agent ethyl methanesulfonate (EMS) (Jorgensen and Mango 2002; Kutscher 2014) to produce worms that are heterozygous for random mutations (generation F1). After allowing the F1 generation to self-fertilize and produce an F2 generation of worms homozygous for random mutations, we will then shift the F2 worms to 28°C HS for 48 hours. Under these conditions, only F2 mutants with superior reproductive thermotolerance will be able to produce viable eggs, and the resulting progeny (F3) will be shifted back
down to 20°C to recover at normal temperatures. The F3 larvae will then be cloned by picking single worms to self-fertilize and create large populations of the selected mutations for downstream genetic and functional characterization.

Based on our findings that chronic HS titrates HSPs away from their endocytic roles, we expect the parameters of this genetic screen to uncover regulators of HSP expression levels or other components of endocytosis. Alternatively, this screen will reveal other modulators of the chronic HSR. For example, this screen may unmask novel regulators of hsf-1 gene expression, as we have demonstrated that hsf-1 is induced through currently unknown mechanisms during chronic HS. Some recent work has shown that individual worms with higher stress resistance tend to have lower reproductive fitness, but this was assessed using short-term exposures to strong HS at 35°C (Casanueva et al. 2012). Based on the mild nature of our HS, and the evidence that the damages incurred at 28°C are not permanent, our screen has high chances of successfully identifying mutations that offer reproductive thermotolerance. This method will select genetic mutations that confer thermotolerance and unique reproductive resistance to chronic HS. Moreover, as successful reproduction relies on many basic cellular processes such as endocytosis, using this method to select for thermotolerance will have wide-reaching implications.

Together, the key findings in this body of work provide a deeper understanding of how an organism responds to proteotoxic conditions across space and time. Additionally, these findings highlight the utility of using multicellular organisms with genetic tractability to explore the nuances of the HSR and other stress responses. Given the vital role that the HSR plays in maintaining organismal health, particularly in the face of protein-folding
stress during disease or temperature shifts, this work paves the way for future research spanning various biological fields of study.
References


