Video Article

Using *Caenorhabditis elegans* as a Model System to Study Protein Homeostasis in a Multicellular Organism

Ido Karady¹, Anna Frumkin¹, Shiran Dror¹, Netta Shemesh¹, Nadav Shai¹, Anat Ben-Zvi¹

¹Department of Life Sciences, National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev

Correspondence to: Anat Ben-Zvi at anatbz@bgu.ac.il

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Abstract

The folding and assembly of proteins is essential for protein function, the long-term health of the cell, and longevity of the organism. Historically, the function and regulation of protein folding was studied *in vitro*, in isolated tissue culture cells and in unicellular organisms. Recent studies have uncovered links between protein homeostasis (proteostasis), metabolism, development, aging, and temperature-sensing. These findings have led to the development of new tools for monitoring protein folding in the model metazoan organism *Caenorhabditis elegans*. In our laboratory, we combine behavioral assays, imaging and biochemical approaches using temperature-sensitive or naturally occurring metastable proteins as sensors of the folding environment to monitor protein misfolding. Behavioral assays that are associated with the misfolding of a specific protein provide a simple and powerful readout for protein folding, allowing for the fast screening of genes and conditions that modulate folding. Likewise, such misfolding can be associated with protein mislocalization in the cell. Monitoring protein localization can, therefore, highlight changes in cellular folding capacity occurring in different tissues, at various stages of development and in the face of changing conditions. Finally, using biochemical tools *ex vivo*, we can directly monitor protein stability and conformation. Thus, by combining behavioral assays, imaging and biochemical techniques, we are able to monitor protein misfolding at the resolution of the organism, the cell, and the protein, respectively.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50840/>

Introduction

The interplay of the various steps of protein biosynthesis, such as mRNA transcription, processing, and translation, as well as protein folding, translocation, and assembly/disassembly, determines the load of metastable proteins that depend on the cellular protein quality control machineries for their function¹. The absence or malfunction of protein quality control machineries can, therefore, result in the functional decline of diverse types of cellular machineries and in the onset of protein misfolding diseases²⁻⁷. When the capacity of protein folding and clearance machineries is balanced with the load of metastable proteins, protein homeostasis (proteostasis) is achieved, a state that ultimately prevents the accumulation of misfolded proteins and aggregation within cells⁶. Accumulation of misfolded proteins is thought to be the signal that activates stress-inducible transcription factors, such as heat shock factor (HSF-1), and results in the activation of cyto-protective stress responses^{6,8}.

Our understanding of the functions of proteostasis networks in metazoans has mostly been derived from *in vitro* reconstitution studies and from observations made with tissue culture cells and unicellular organisms⁹. For example, research on molecular chaperones that prevent and resolve protein damage has focused on biochemical and cell biological studies of the mechanisms of chaperone-mediated protein folding, disaggregation and translocation¹⁰⁻¹³. In comparison, only limited information is available on the integrated function of various proteostasis components in the different cells and tissues of metazoans under "normal" growth conditions and in response to stress¹¹. The discovery that in *C. elegans*, cellular protein quality control can also be regulated cell nonautonomously, as reflected in experiments showing that mutations in the two neurons that perceive temperature can block the activation of the heat shock response and reduce thermotolerance, demonstrated the need to study proteostasis regulation in multicellular organism¹⁴⁻¹⁷. What is missing, however, is a cohesive picture of how proteostasis networks, such as the various molecular chaperone families, function in the tissues of an intact metazoan and how dynamic are these networks during development and aging. To meet this goal, reliable sensors for monitoring proteome maintenance in living animals are needed to determine the proteostatic capacity of different cells in a multicellular organism during the course of development and aging.

For a given protein to function as a sensor of cellular proteostasis, it must respond to changes in the cellular folding environment while only minimally interfering with the folding of unrelated proteins in the cell. To explore the maintenance and recovery of cellular proteostasis in a living organism, two complementary approaches that depend on folding sensors can be taken. The first relies on designed folding-sensors, based on experimentally identified metastable proteins that are known to depend on proteostasis machinery, such as firefly luciferase¹⁸⁻²⁰ or GFP tagged with a degen²¹⁻²⁴. In the second approach, endogenous metastable proteins, such as temperature-sensitive(ts) or age-dependent aggregating proteins that respond to incremental changes in the cellular environment, are traced²⁵⁻²⁷. Designed folding-sensors serve no essential biological function yet offer the advantage of being detectable by powerful reporting assays, such as GFP-tagged proteins, and can be employed with

many different cellular and animal models¹⁸. However, because introducing a single foreign protein can affect the folding environment²⁷, such polypeptides can overload the cellular proteostasis machinery. Alternatively, designed folding-sensors that are not native to the cell on which they report may not be affected by changes in the proteostasis capacity of the cell. For example, one GFP-tagged proteasome reporter substrate required ~90% of the proteasome to be inhibited before a phenotype could be detected²³. In contrast, endogenous metastable proteins that rely on the proteostasis machineries of the cell offer the advantage of being within the cellular sensitivity range. However, the loss-of-function associated with the misfolding of such proteins can also impact cellular function and organismal viability. Here, we will focus on the use of endogenous *C. elegans* folding-sensors.

C. elegans is a well-established metazoan model for the study of both development and aging that utilizes many conserved biological pathways and can be used to follow protein folding in the cell, using a combination of cell biology, biochemical and genetic approaches. We employed metastable proteins as probes of proteostatic capacity by monitoring changes in their phenotype, localization and stability. A variety of protein functions can, moreover, be studied by simple behavioral analysis. Likewise, substantial mislocalization of proteins occurs when cellular protein quality control networks fail to adjust to cellular demands. Proteins can be easily visualized in cells of living animals using fluorescently-tagged proteins or via immunostaining. Finally, using *ex vivo* methods, it is possible to monitor protein expression and stability. This allows for fast and simple screening of behavioral and physiological changes, coupled with in depth analysis of protein localization and stability, allowing for the monitoring of proteostasis modifiers. By combining these different methods, a broad view of the protein-folding environment of a cell can be obtained. Indeed, this strategy has been successfully used to monitor proteostasis perturbation in *C. elegans*, yeast, tissue culture and bacteria^{15,25-35}.

Protocol

Using behavioral assays to monitor protein folding in a living organism

1. Synchronizing Animals for Behavioral Assays

1. Follow standard methods for maintenance of *C. elegans*³⁶, including media and plates preparation, food preparation and animal growth conditions.

Carefully control cultivation conditions, including temperature (15-25 °C), population size and food availability. Discard any plate that shows bacterial or fungal contamination.

2. Pick 15-30 embryos, transfer them to new plates and grow at the desired temperature (15-25 °C) for the duration of the experiment. Alternatively, set 10-20 gravid adults on a plate, let them lay eggs for 30-60 min and then remove them from the plate. Both direct picking of embryos and setting gravid adults will give some variability in age (± 6 hr). To reduce this variability, either pick embryos at a specific stage, for example comma stage, or set 10-20 adults who are just starting to lay eggs (day 2 of adulthood). Avoid synchronization by bleaching whenever possible, given that this treatment is stressful and can affect proteostasis.
3. Pick synchronized animals, transfer to an assay plate for scoring and then discard.

2. Movement Assay

Pick synchronized animals and transfer to a clean plate at the desired temperature for movement scoring (15-25 °C). For each biological replicate, score >20 animals and repeat assay at least 3x per experimental condition. Use the Wilcoxon Mann-Whitney rank sum test to compare two independent experimental conditions.

1. To assay for mild movement impairment, set 5-7 animals together at the center of a clean bacterial lawn and allow the animals to move on the plate for 2 min. This time interval was determined based on the time it took 90% of wild type animals to clear a 1 cm circle.
2. Count the number of animals that did not clear a 1 cm circle (e.g. that remained within a 1 cm radius from the point at which they were set) within 2 min as uncoordinated.
3. To assay for severe movement impairment, set the animals on a clean bacterial lawn.
4. Photograph several animals at time zero (t=0) and again 5 min later (t=5). Score animals that did not move one body length after 5 min as paralyzed.

3. Thermo-resistance

1. Pick >20 synchronized animals and transfer to a 24-well plate containing 450 μ l Heat Shock (HS) buffer (**Table 1**). For each biological replicate, score >20 animals and repeat assay at least 4x per experimental condition.
2. Transfer 24-well plate into a heated bath. HS temperature and duration strongly depend on growth conditions, in particular, the cultivation temperature.
3. Supplement the HS buffer with 9 μ l SYTOX orange.
4. Score animal survival by monitoring dye uptake, using a fluorescent stereoscope with a TXR filter. Animals that took up the dye are dead. Use the Wilcoxon Mann-Whitney rank sum test to compare two independent experimental conditions.

Using immunostaining and tagged proteins to monitor protein folding in specific cells

4. Monitor Localization of Proteins by Immunostaining

1. At the required stage, transfer at least 30 animals into an Eppendorf tube containing M9 buffer³⁶.

2. Wash the animals a few times with M9 buffer by performing a short, low speed centrifugation step (3,000 rpm/900 x g, 2 min) and resuspending.
3. Place the tubes on ice for a few minutes to cool down.
4. Remove excess liquid and incubate with 500 μ l ice-cold 4% paraformaldehyde solution (**Table 2**). The time of incubation needs to be calibrated for each protein examined and should be around 5-30 min at room temperature. Prolonged incubation at 4 °C can also be performed but this may impair sample permeabilization.
5. Wash fixed animals 3x with PBS-Tween (pH 7.2) buffer by centrifugation (3,000 rpm, 1-2 min). **The animals can be stored at this stage at 4 °C for a few weeks.**
6. Collect the animals by centrifugation (3,000 rpm, 1-2 min).
7. In the hood, remove most of the supernatant and resuspend the pellet in 1 ml β -mercaptoethanol (β -ME) solution (**Table 2**).
8. Incubate at 37 °C overnight with gentle rocking on a nutator.
9. In the hood, wash animals 3x with PBS-Tween (pH 7.2) buffer.
10. Resuspend the animals in 50-100 μ l of PBS-Tween (pH 7.2). **The animals can be stored at this stage at 4 °C for a few weeks.**
11. Collect the animals by centrifugation (3,000 rpm, 1-2 min) and remove most of the PBS-Tween buffer.
12. Add 100-150 μ l of collagenase solution to each tube and incubate at 37 °C with strong agitation (using a Thermo-mixer or a shaking incubator).
13. Examine the animals frequently under a stereomicroscope, starting after 5 min of incubation. When 20% of the animals are broken, stop the reaction by placing the tube on ice. **This step is highly sensitive! The duration of treatment depends on fixation conditions and can vary widely between strains. A prolonged incubation could result in complete degradation of the animals. Conditions must be calibrated for each enzyme batch used and degradation should be monitored carefully.**
14. Wash the animals twice in PBS-Tween (pH 7.2) and then collect the animals by centrifugation (3,000 rpm, 1-2 min) and remove most of the PBS-Tween buffer.
15. Add 1 ml AbA solution (**Table 2**) and incubate with gentle rocking for 1 hr at room temperature. **The animals can be stored at this stage at 4 °C for a few weeks.**
16. Collect the animals by centrifugation (3,000 rpm, 1-2 min) and resuspend in 200 μ l AbA.
17. Add the primary antibody diluted to 1:100-1:1,000 in AbA solution and incubate with gentle rocking. The temperature and duration of incubation can vary between antibodies and should be calibrated for each protein examined. We begin with an overnight incubation at room temperature.
18. Wash the animals by centrifugation (3,000 rpm, 1-2 min).
19. Add AbA buffer (1 ml) and incubate with gentle mixing for 15-60 min at room temperature. **The animals can be stored at this stage at 4 °C for a few weeks.**
20. Optional: The sample can also be stained with fluorescent dyes. To do so, add the dye (e.g. DAPI (2 mg/ml) or Phalloidin (1:100)) to 200 μ l AbA and incubate with gentle rocking for up to 1 hr. This step can be performed during one of the steps for washing the secondary antibody.
21. Wash the animals 3x by repeating steps 4.18-4.19.
22. Add 100 μ l AbA with the appropriate fluorescently-tagged secondary antibody, diluted 1:100.
23. Maintain the samples covered (or in a dark tube) and incubate with rocking for several hours at room temperature.
24. Wash the animals 3x by incubating in AbA buffer (1 ml) with gentle mixing for 15-60 min at room temperature followed by centrifugation (3,000 rpm, 1-2 min). **The animals can be stored at this stage at 4 °C for a few weeks.**
25. To mount animals on slides, remove all liquid and resuspend in 10 μ l of the same buffer.
26. Cut the end of the pipette tip to avoid sheering of the animals and transfer 1-2 μ l of the sample onto a slide.
27. Add mounting medium in a 1:1 ratio.
28. Cover the slide **gently** with a coverslip and seal with nail polish. If the slide is not monitored immediately, the **slides can be stored at -20 °C for a prolonged period of time.**

5. Monitor Localization of Tagged Proteins

1. At the required stage, collect at least 15 animals into a 10 ml drop of M9 buffer on a slide.
2. Using a "worm pick", move the animals into a 10 ml drop of paraformaldehyde solution (4%) (**Table 2**) and wait until all animals stop moving (approximately 5 min).
3. Using a "worm pick", move the animals into a 2 ml drop of M9 buffer and mounting medium (1:1) (**Table 2**).
4. Gently cover the animals with a coverslip and seal with nail polish.

Using ex vivo assays to monitor protein folding and stability

6. Partial Digestion

1. Wash animals from 15 plates (60 mm) using 1-2 ml M9 buffer in a microfuge tube. Please note that animals should not be crowded and sufficient food should be available throughout the duration of the experiment.
2. Wash the animals a few times with M9 buffer by centrifuging (3,000 rpm, 2 min) and resuspending animals.
3. After the final spin, resuspend animals in 100 μ l M9 buffer.
4. Flash-freeze in liquid N₂. **Samples can be stored at -80 °C.**
5. Using a chilled pestle and motorized drill (a hand held tissue culture drill can also used), grind the sample. **Keep sample on ice and avoid over-heating.**
6. Examine the animals frequently under a stereomicroscope to determine if grinding is complete. If intact animals remain, repeat the procedure.
7. Estimate the volume by pipetting the worm extract.
8. Add 1:1 ratio of (2x) worm lysis solution (**Table 3**). **Do not use protease inhibitors of any kind.**
9. Incubate on ice for 15 min.
10. Remove debris by centrifugation (1,000 x g for 2 min) in a precooled centrifuge (4 °C).
11. Transfer the supernatant to a clean microfuge tube. **Samples can be used directly or stored at -80 °C.**

12. Prepare chymotrypsin solution (**Table 3**) and maintain on ice until use.
13. Determine total protein concentration using the Bradford assay.
14. Dilute the sample if necessary such that the total protein concentration is 3-3.5 mg/ml.
15. Add chymotrypsin solution to the total protein lysate (1:1,200).
16. To determine time of incubation, incubate the sample with chymotrypsin for 60 min. Remove 20 μ l aliquots from the sample at different time points (e.g. 0, 5, 20, 40, and 60 min), mix immediately with SDS sample buffer and boil for 5 min at 96 °C. *Alternatively, incubate the sample with different concentrations of chymotrypsin and stop the reaction after a specific time by adding SDS sample buffer and boiling for 5 min at 96 °C.
17. Run samples on a SDS-PAGE gel.
18. Perform western blot analysis using appropriate antibodies to determine the relative stability of the protein.
19. Determine the optimal conditions (chymotrypsin concentration and digestion time) that show significant change between samples and use these conditions for future experiments.
20. Determine the intensity of different digest products (lower molecular weight fragments) using densitometry software, such as the NIH image gel module.

Representative Results

A compromised folding environment can lead to protein misfolding and aggregation. Protein misfolding is associated with altered conformation and results in a loss of protein function. We use complementary approaches to monitor protein folding and function in intact animals, specific cells and protein extracts. An intelligent choice of folding-sensor, based on strong behavioral and cellular phenotypes that are associated with a known destabilizing mutation, can then be used to monitor changes in the protein-folding environment. An example of an endogenous temperature-sensitive mutant protein is the L799F mutation in the thick muscle filament protein, paramyosin (*C. elegans* UNC-15)³⁷. The missense mutation affects coiled-coil interactions such that paramyosin(ts) misfolds and mislocalizes to paracrystalline assemblies instead of to muscle sarcomeres at the restrictive temperature. Such mislocalization disrupts thick filament formation and leads to temperature-dependent embryonic and early larval lethality and to movement defects in adults²⁷. Likewise, animals expressing the P70S mutation in the endocytosis protein dynamin (*C. elegans* DYN-1) display temperature-dependent endocytosis defects in various cell types associated with misfolding of the mutant protein^{28,38}. Thus, we can monitor changes in proteostasis regulation by monitoring the function, localization, and conformation of metastable proteins.

Using behavioral assays to monitor protein folding in a living organism

Protein loss-of-function can be monitored in an intact organism by following a detectable phenotype associated with that function. For example, an E781K ts mutation in the muscle chaperone *unc-45(m94)*, *unc-45(ts)*, strongly affects myosin folding and assembly and, therefore, animal motility at the restrictive temperature. When *unc-45(ts)* mutant animals were grown at temperatures above 20 °C, they were completely paralyzed, with less than 4% of the animals moving a distance of one body length after 5 min (**Figures 1A and 1B**). In contrast, *unc-45(ts)* mutant animals were fully motile when grown at 15 °C (97.8 $\pm$ 2.2%). Thus, loss of UNC-45 function offers a robust and easy way to score by monitoring motility.

One can then ask if a specific treatment affects muscle proteostasis by monitoring the induced change in animal motility under permissive or restrictive conditions. Treatments that cause paralysis at permissive temperatures or rescue motility at the restrictive temperature are potential modulators of proteostasis. For example, expression of aggregation-prone poly-glutamine proteins²⁷ or mutant super-oxide dismutase³³ in the *C. elegans* body wall muscle resulted in a severe loss of coordination. Likewise, modifiers of protein aggregation and toxicity identified in a whole genome RNAi screen were shown to improve UNC-45 egg-laying and motility defects³⁴. This approach, involving different temperature-sensitive mutant proteins, has been used in *C. elegans* to monitor changes in the folding environment associated with the expression of unrelated aggregation-prone proteins, genetic and chemical modifiers and age-related changes^{15,27-31,33,34}. While not as sensitive as monitoring the misfolded protein directly, this approach allows for the fast and simple screening of modifiers that can then be further examined.

The ability of animals to cope with stress can be monitored as readout of changes in protein-folding homeostasis at the organismal level^{28,39,40}. Many different treatments that affect protein-folding homeostasis were shown to affect stress resistance, in general, and the response to heat shock, specifically. Indeed, thermo-resistance is a well-established method to monitor proteostasis^{6,41-43}. For example, cultivation temperature has strong impact on *C. elegans* lifespan⁴⁴ and was shown to alter protein expression, in particular chaperone levels⁴⁵. In agreement, we find that raising the cultivation temperature affects *C. elegans* thermo-resistance. When wild-type animals raised at 25 °C were subjected to a prolonged HS, 72.3 $\pm$ 8.2% survived, while only ~30% survived if raised at 15-20 °C. To monitor the effect of modifiers on the HS response in different temperatures, we adjusted the stress conditions to reach a ~70% survival rate for wild type animals under control conditions. We, therefore, subject animals grown at 15 °C to a 34 °C HS for 4 hr, while we subject animals grown at 25 °C to a 37 °C HS for 6 hr. It is important when calibrating this assay to examine several temperatures and follow survival over time so as to optimize conditions. These data suggest that protein-folding capacity varies as a function of cultivation temperature (**Figure 2**) and support the use of stress survival as a tool to monitor proteostatic capacity.

Using immunostaining and tagged proteins to monitor protein folding in specific cells

Protein misfolding can be associated with protein mislocalization. The localization of several metastable proteins was shown to shift using specific antibodies^{15,27,28}. For example, paramyosin(ts) [*unc-15(e1402)*] mislocalizes to paracrystalline assemblies instead of to muscle sarcomeres under restrictive conditions. Likewise, myosin(ts) [*unc-54(e1301)*] forms disorganized filaments instead of the highly ordered filamentous structures observed by immunostaining assays at the restrictive temperature (**Figures 3A and 3B**).

A complementary assay to monitor protein localization employs a fluorescently tagged partner that can serve as a proxy for a protein complex. For example, monitoring different temperature-sensitive mutations in muscle proteins can be realized by employing myosin heavy chain A tagged with green fluorescence protein (GFP) (MYO-3::GFP) as a reporter of muscle integrity. Animals expressing various ts-mutant proteins coexpressed with MYO-3::GFP showed a rapid deterioration in myofilament organization, detected by monitoring MYO-3::GFP localization

(Figure 3C). While myofilament organization differed between different strains, all strains examined showed MYO-3::GFP mislocalization under restrictive conditions. This assay was also used to monitor age-dependent changes in muscle proteostasis^{28,46}.

Using ex vivo assays to monitor protein folding and stability

Protein misfolding can be directly examined using protease sensitivity as a tool to monitor conformational changes²⁷. Proteolytic fragmentation patterns of paramyosin(ts), for example, were shown to be affected by coexpression of poly-glutamine proteins, as well as by age^{27,28}. Similar to MYO-3::GFP localization reporting on muscle proteostasis, changes in myosin conformation can also reflect changes in the folding capacity of muscle cells. For example, the proteolytic fragmentation pattern of myosin heavy chain A (MYO-3) coexpressed with unc-45(ts) shows distinct characteristics compared with the digestion profile of myosin from wild-type animals (Figure 4), supporting the claim that myosin folding is affected by UNC-45 loss of function^{47,48}.

Lists of strains and antibodies used in this section are included in Tables 4 and 5.

Table 1. HS buffer.

Solution	Reagent	Final Concentration	Comment
HS buffer (100 ml)	83.9 ml Tris-HCl, pH 7.4 (100 mM)	83.9 mM	
	4 ml NaCl (1 M)	40 mM	
	1 ml cholesterol	5 mg/ml	Dissolve in ethanol
	11.1 ml OP50-1 in LB + streptomycin (100 mg/ml)		Bacteria O.D = 0.3

Table 2. Immunostaining solutions.

Solution	Reagent	Final Concentration	Comment
4% paraformaldehyde solution (25 ml)	Dissolve 1 g paraformaldehyde into 22.5 ml dH ₂ O	4%	
Heat to 55-60 °C in a water bath and add 2-4 drops of 1 M NaOH		Heat until the solution clears	
Cool solution on ice and add 2.5 ml 10x PBS (pH 7.4)		Store at -20 °C until needed	
20x PBS (2 L)	320 g NaCl	2.74 M	
8 KCl	54 mM		
57.6 g Na ₂ HPO ₄	200 mM		
9.6 g KH ₂ PO ₄	36 mM		
Add dH ₂ O to 2 L			
PBS-Tween (pH 7.2) (500 ml)	25 ml 20x PBS (pH 7.4)		
475 ml dH ₂ O			
250 ml Tween-20 (100%)	0.5%	Use cut tip	
Collagenase solution (~600 ml)	480 ml dH ₂ O		
120 ml Tris-HCl 0.5 M (pH 7.4)	100 mM		
0.6 ml CaCl ₂ (1 M)	1 mM		
12 ml collagenase (stored at 4 °C)	2 g/L	Dissolve 100 mg Collagenase in 1 ml of 0.1 M Tris-HCl, pH 7.4 NOTES: Collagenase activity varies from stock to stock. The first three ingredients can be combined and stored as a stock solution at room temperature. Add the collagenase immediately before use.	
B-ME solution (~1.5 ml)	1 ml dH ₂ O		
400 ml 0.5 M Tris-HCl (pH 6.8)	133 mM		
15 ml Triton X-100 (100%)	1%	Use cut tip	
76 ml β-ME	5%	Add β-ME in the hood!	

M9 buffer (1 L)	5.8 g Na ₂ HPO ₄ ·7H ₂ O	21 mM	Filter (0.22 mm) and bottle
3.0 g KH ₂ PO ₄	22 mM		
5.0 g NaCl	86 mM		
0.25 g MgSO ₄ ·7H ₂ O	1 mM		
Add dH ₂ O to 1 L			
Mounting medium (100 ml)	10 ml 1x PBS (pH 7.2)		
DABCO	0.1 mg/ml	Add DABCO to PBS first	
90 ml glycerol (100%)	90%	Use cut tip	
AbA (~40 ml)	38 ml dH ₂ O		
2 ml 20x PBS			
200 ml Triton X-100	0.5%		
0.4 g BSA	10 g/L		

Table 3. Partial digest solutions.

Solution	Reagent	Final Concentration	Comment
Worm lysis solution (50 ml)	2.5 ml Tris-HCl, pH 7.4 (1 M)	50 mM	
250 ml MgCl ₂ (1 M)	5 mM		
250 ml Triton-X (100%)	0.5%		
add dH ₂ O to 50 ml			
Chymotrypsin solution (1 ml)	1 ml HCl (1 M)	1 mM	Solution can be stored at -20 °C for one week.
2 ml CaCl ₂ (1 M)	2 mM		
100 mg chymotrypsin	100 mg/ml		
add dH ₂ O to 1 ml			

Table 4. List of strains and abbreviations used in this work.

Strain (ref)	Abbreviation	Genotype	Source
N2	<i>wt</i>	---	CGC
CB286	<i>unc-45(ts)</i>	<i>unc-45(e286)</i>	CGC
HE250	<i>unc-52(ts)</i>	<i>unc-52(e669su250)</i>	CGC
CB1402	<i>unc-15(ts)</i>	<i>unc-15(ts)</i>	CGC
CB1301	<i>unc-54(ts)</i>	<i>unc-54(e1301)</i>	CGC

Table 5. List of antibodies used in this work.

Antibody Name	Protein detected	Source
5-6	Myosin MHC A (MYO-3)	Hybridoma Bank
28.2	Myosin MHC B (UNC-54)	Gift from Prof. Epstein
5-23	Paramyosin (UNC-15)	Hybridoma Bank

Figure 1. Typical temperature-dependent sensitivity of metastable folding-sensors. Age-synchronized animals were grown at the indicated temperature (15-25 °C) on OP50-1 bacteria until the first day of adulthood. We set the first day of adulthood (day 1) for each cultivation temperature as beginning after the L4 molt and before the onset of egg-laying (90 hr at 1 °C, 65 hr at 20 °C, and 50 hr at 25 °C). **(A)** The percentage of moving animals was scored on the first day of adulthood at the indicate temperature. **(B)** To score motility, images taken at the start (t=0) and following a 5 min (t=5) interval were compared for animals grown at 15 °C or 25 °C. Arrows indicate a representative animal location at t=0. The data represent the means $\pm$ SEM of >3 independent experiments. [Click here to view larger image.](#)

Figure 2. Stress tolerance is an indicator of proteotaxis modulation. Age-synchronized wild type (wt) animals were grown at the indicated temperature (15-25 °C) on OP 50-1 bacteria (90 hr at 15 °C, 65 hr at 20 °C, and 50 hr at 25 °C). **(A)** Animals were exposed to a 37 °C heat shock for 6 hr on the first day of adulthood and survival was assayed using the Sytox orange uptake assay. Animals with cells that were permeable to Sytox orange are counted as dead. **(B)** Bright field (left) and fluorescent (right) representative images of animals exposed to Sytox orange following heat shock treatment. White arrows indicate dead animals (Sytox permeable) while yellow arrows indicate live animals (Sytox impermeable). The data represent the means $\pm$ SEM of >3 independent experiments. [Click here to view larger image.](#)

Figure 3. The localization of folding-sensors can report on cell-specific proteostasis. (A,B) Confocal images of muscle cells stained with antiparamyosin [5-23] or antimyosin heavy chain B [28.2] (green) and rhodamine-phalloidin (red) of age-synchronized wild type or temperature-sensitive animals grown at 25 °C on the first day of adulthood (50 hr at 25 °C). Scale bar is 5 μm. (C) Confocal images of age-synchronized animals (day 4-5 of adulthood) grown at 25 °C expressing MYO-3::GFP in a wild type (control) or temperature-sensitive paramyosin(ts) [*unc-15(e1402)*], myosin [*unc-54(e1301)*] and perlecan [*unc-52(e669su250)*] mutant background. Scale bar is 10 μm. [Click here to view larger image.](#)

Figure 4. Protease sensitivity as a tool to monitor proteostasis. Protein extracts were produced from wild type or *unc-45(ts)* animals. (A) Extract were incubated with chymotrypsin for the indicated times and the digestion of myosin heavy chain A was monitored by western blot using antimyosin antibodies [5-6]. (B) To quantify digestion, the appearance of a low molecular weight band was determined using ImageJ software. [Click here to view larger image.](#)

Discussion

Probes of the cellular proteostasis capacity must be highly sensitive to changes in the folding environment and be easily monitored so as to provide real-time assessment of protein folding quality control capabilities. Using metastable proteins as probes of proteostasis capacity, we monitored changes in phenotype, subcellular protein localization, and protein conformation. The protocols presented here focus on muscle protein-associated phenotypes, including motility, muscle filament organization and myosin stability. However, these protocols can be modified for monitoring other folding sensors. For behavioral phenotypes, it is important to design a simple and robust assay to determine protein function at the organismal level. For example, a ts mutation in *dyn-1* leads to the rapid loss of endocytosis and synaptic vesicle recycling at the restrictive condition. Consequently, dynamin function in coelomocytes and neurons can be monitored by the uptake of GFP secreted into the body cavity fluid or by localization of the synaptic protein, synaptobrevin-1, tagged with GFP (SNB-1::GFP), respectively²⁸. Likewise, the function of mitochondrial complex I protein GAS-1 can be examined by monitoring animals sensitivity to ethanol²⁸. Many genes in *C. elegans* are classified by their phenotype and are associated with simple behavioral readouts that can be easily monitored. However, it is important to identify when and where a gene is expressed since its expression pattern will directly impact its ability to function as a folding sensor.

Thermo-resistance is an accepted method to monitor proteostasis^{6,41-43}. For *C. elegans*, survival is typically monitored by manually scoring a large population of worms (>70 for each condition tested) every 1-2 hr until no animals survive (typically >15 hr). To render the thermo-resistance assay less laborious, faster and more reproducible, we adapted uptake of Sytox orange⁴⁹, a membrane impermeable dye, as a marker of dead cells in the animal and, thus, as a visual readout of animal mortality. Under our conditions, the two thermo-resistance assays gave comparable results, although the use of sytox allows for fast and reproducible screening. We avoid using prolonged stress treatments because the time resolution of a 15 hr survival assay can be limiting when monitoring changes in HS survival. For example, upon transition to adulthood, there is a fast change in the ability of *C. elegans* to activate the HS response, an event that is shorter than the typical kinetic thermo-resistance assay⁵². Moreover, by monitoring survival rates when ~70% of wild type animals survive under control conditions, we can easily detect modifiers that enhance or reduce stress survival by monitoring only a single time point, simplifying the assay. It is possible to modify the use of Sytox orange to monitor survival under other stress conditions, extending the possible application of this technique.

For cellular localization, the immunostaining protocol presented here is robust and was employed to study a variety of protein targets. When adjusting the protocol to a specific protein sensor, it is best to first adjust the duration of the fixation treatment (step 4.4) because fixation can affect epitope detection. The duration of the fixation step, however, can also affect the sensitivity of the animals to the collagenase treatment (step 4.13) that is required for permeabilization. It is, therefore, important to also adjust the duration of the collagenase treatment. In general, prolonged fixation will require prolonged incubation with collagenase. This step is crucial because insufficient permeabilization will result in poor antibody staining and samples will be lost if permeabilization is not stopped in time. It is, therefore, recommended to calibrate the protocol for each antibody tested before applying it to your samples.

For protein conformation, the limited proteolysis assay presented here is a sensitive tool to probe changes in protein conformation since such changes can be associated with altered accessibility to proteolytic enzyme. Generally, treatments that destabilize protein conformation can expose new proteolytic sites that are normally buried and inaccessible to proteases. However, aggregation can also result in reduced accessibility to proteolytic enzymes. Thus, this assay can only be used to probe for conformational changes and not to report on the protein folding state. It is, therefore, recommended to employ a general protease, such as trypsin or chymotrypsin, that has many predicted proteolysis sites for most protein sequences. An alternative approach can be to employ a specific protease. For example, DYN-1 has a unique thrombin proteolysis site on the loop, where the ts mutation is located. The fold of this loop is thought to be destabilized at the restricted temperature³⁸ and proteolysis with thrombin can be employed to monitor changes in DYN-1 conformation. Before selecting a protease for this analysis it is thus important to examine the protein sequence and identify if there are predicted proteolysis sites and where they are located.

The ability to monitor protein folding at the level of the organism, cell and protein allows for diverse application of these probes. For example, use of these folding-sensors in genetic or chemical screens can be highly effective in mapping pathways that regulate cellular proteostasis, using a simple behavioral readout. While this approach offers a rapid and easy tool for screening, it is not direct and, hence, other assays to monitor proteins misfolding are required. Likewise, we can assess the functional capacities of different tissues by comparing protein localization under different treatments and conditions. This approach requires imaging and is more laborious but can provide information on the folding environment of specific cells during the lifespan of the organism. Combining the different readouts provides a powerful set of tools to study proteostasis in depth.

Environmental signals, such as temperature and nutrient availability, can modulate cellular proteostasis^{6,9,14,16,17,28,35,46}. These findings highlight the importance of carefully controlling experimental conditions when examining proteostasis. For example, cultivation temperature can affect the folding of metastable proteins (**Figures 1, 3, and 4**), as well as the induction of protective stress responses (**Figure 2**). It is, therefore, imperative to use controlled cultivation conditions. Specifically, it is important to maintain cultivation temperature by reliable incubators, keep the population size constant between experiments, control food availability and avoid bacterial and fungal contaminations by discarding contaminated plates. It is also important to note that strains expressing folding sensors or metastable proteins can acquire genetic modifiers under normal cultivation conditions. This trend can be accelerated by the introduction of stress, as, for example, during synchronization animals by bleaching. We, therefore, recommend periodically replacing the strains used by either crossing to wild type animals (N2) or thawing new frozen stocks. Using these guidelines regulates the environmental contribution and reduces the effect of nonspecific effectors on proteostasis.

Proteins used as folding-sensors interact with the folding machineries^{18,28} and can thus change the folding environment on which they report. This outcome strongly depends on the folding capacity of the cell and its load of misfolded proteins. For example, overloading the cell with misfolded protein upon expression of aggregation-prone polypeptides was shown to affect the folding of many unrelated proteins, while over-expression of proteostasis components was shown to rescue their folding^{27,33,50,51}. As such, it is good practice to use more than one sensor to monitor protein folding *in vivo* and best to use wild type proteins that do not harbor any mutations yet are naturally metastable and responsive to the cellular folding environment. In *C. elegans*, a recent study used mass spectrometry to identify proteins that undergo age-dependent aggregation³¹, similar to either endogenous or designed folding-sensors^{18,28}. Such proteins are good candidates for use as folding-sensors and can be tagged with a fluorescent protein³¹. These folding-sensors composed of a stable fluorescent protein fused to a metastable protein can be

used to monitor changes in the folding environment by monitoring both function and localization. However, such sensors are highly specific to the cell or organism on which they report.

In summary, we have described a set of complementary molecular tools using diverse metastable proteins and fluorescent reporters for the real-time assessment of protein folding homeostasis in *C. elegans*. Although we now have versatile and powerful tools to study protein folding, there is still a need to further develop our toolbox of folding sensors to report on proteostasis in the cells and tissues of live organisms.

Disclosures

The authors declare that they have no competing financial interests.

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