

**Understanding the Role of the HSP70 Molecular Chaperone Family in Maintaining  
Protein Homeostasis**

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## 1. Abstract

The polyglutamine (polyQ) expansion disorders, such as Huntington's disease, are a class of neurodegenerative diseases marked by an accumulation of intracellular protein aggregates correlating with the length of the polyQ fragment and age. Cellular mechanisms to protect against protein misfolding and aggregation exist in the form of molecular chaperones, proteins which assist in the proper folding of their client proteins. One family of chaperones in particular, the HSP70s, are highly conserved proteins known to aid in protein folding. To study the link between chaperones and polyQ aggregation *in vivo*, we have established a transgenic model in the nematode, *Caenorhabditis elegans*, expressing a YFP fluorescent marker fused to polyQ tracts of different lengths in the body wall muscle cells. Using RNA interference (RNAi) in combinations and against specific chaperones within the family, I have successfully identified a network of HSP70 chaperones influencing polyQ aggregation, with key chaperones localized to different subcellular compartments and compensatory networks that modulate their expression in response to misfolded proteins. Overexpression of the highly conserved Hsc70 human homolog, HSP-1, results in suppression of the polyQ aggregation phenotype. While this suggests high chaperone levels can reduce the number of misfolded proteins in a cell, my data demonstrates that aggregation suppression comes at the expense of cellular function. Thus, new therapeutic strategies that consider chaperone expression levels will be needed before upregulation of these proteins is used as treatment for protein folding diseases.

## 2. Introduction and Literature Review

### A. Protein Folding and its Implications in Human Disease

Proteins are biological macromolecules composed of amino acids that catalyze a diverse set of kinetically unfavorable reactions within the cell. The linear sequence of amino acids, or primary structure of a protein, is faithfully stored in the DNA of an organism which, under appropriate cellular signals that drive gene expression, is transcribed into messenger RNA (mRNA). The mRNA product encoding the protein then exits the nucleus and is translated into a linear polypeptide at the ribosome.

While the primary structure of a protein is encoded directly in its corresponding gene, proteins must fold into unique and complex three-dimensional structures to obtain their catalytic activities. Initial experiments studying protein folding performed by Christian Anfinsen using ribonuclease A showed that under the appropriate conditions, proteins can spontaneously refold after denaturation and maintain their specific catalytic activity (Anfinsen 1973). Although this data provided insights into the reversibility of protein folding, it was still unclear how such a complex process could occur spontaneously.

To discover the molecular interactions occurring during protein folding, a variety of techniques have been developed to measure protein conformations in real time. These include spectroscopic approaches that measure either absorbance or fluorescence to detect folding intermediates on a millisecond timescale (Brockwell et al. 2000). Through the analysis of a large number of proteins and detection of a variety of folding intermediates, it has become clear that a number of different folding pathways can lead to a correctly folded protein. To complicate this process further, many factors dictate which

pathways are utilized, including amino acid composition of the protein, temperature, and solute concentration in solution (Radford 2000). To visualize the effect of all possible conditions affecting protein folding, the “energy landscape” model has been adopted to depict the variety of pathways and intermediates that can be traversed en route to a correctly folded product (Figure 2.1). One consequence of this theory is that a single protein can assume many different conformations before reaching its ground state and these conformations are somewhat dependent on environmental conditions. Thus, under certain circumstances, it is possible that particular folding intermediates can be stabilized, trapping polypeptides in a misfolded state, unable to achieve their native fold and function.

While it is possible to manipulate the external environment to optimize protein folding *in vitro*, the conditions in a cell are fine-tuned and, in some cases, present challenges for proper protein folding. For example, the high concentration of protein in the cytosol promotes intermolecular hydrophobic interactions that lead to protein aggregation (Ellis 2006). In addition, the linear production of proteins at the ribosome exit channel allows interactions between residues on the polypeptide before protein synthesis is complete (Agashe and Hartl 2000). How then, are these deterrents avoided so that protein folding is possible *in vivo*?

Throughout evolution, the cell has obtained various mechanisms to ensure proteins are folded correctly and misfolded proteins are quickly degraded, a process termed protein homeostasis (Figure 2.2). While many cellular processes influence protein homeostasis, two components, molecular chaperones and the proteasome, are essential in preventing protein aggregation (Voisine 2007). Molecular chaperones are a class of

proteins that bind to unfolded or misfolded polypeptide chains, sequestering them from the cellular environment, to allow the polypeptide to fold or re-fold into its native state (Hartl 2002). Therefore, chaperones are able to manipulate the microenvironment surrounding a protein to promote proper folding, similar to changing the external conditions to promote spontaneous folding in an *in vitro* experiment. When chaperones are unable to properly fold a polypeptide chain, the misfolded protein is targeted to the 26S proteasome for degradation (Goldberg 2003). Either refolding or degrading a misfolded protein is essential in maintaining protein homeostasis because misfolded proteins expose hydrophobic amino acids to the aqueous cellular environment (Gething and Sambrook 1992). These proteins can then self-associate or interact with other nonpolar molecules in the cell, leading to aggregation.

The appearance of protein inclusions or aggregates is a common feature in many human neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and the polyglutamine (polyQ) expansion diseases, suggesting a unified mechanism may contribute to these disease states (Soto 2003; Soto 2008). Although Alzheimer's and Parkinson's disease exhibit some genetic inheritance, the polyQ expansion diseases are a set of autosomal dominant disorders inherited with 100% penetrance, the most well-known being Huntington's disease (HD). The causative agent of the polyQ disorders is a trinucleotide CAG expansion within the coding region of a particular gene (Andrew et al. 1997). When translated into protein, the CAG expansion becomes a repeat of glutamine (Q) residues, hence the name polyQ diseases.

In order to further understand the links between protein aggregation and disease, multiple *in vivo* approaches have been utilized, modeling the various neurodegenerative

diseases in well-established model organisms. One example is the R6 transgenic mouse, created by expressing the truncated Huntington gene (Htt), containing only the first exon, with varying polyQ tracts (Mangiarini et al. 1996). In lines with expanded polyQ (115Q or greater) an age dependent phenotype with physiological symptoms similar to HD are observed. In addition, nuclear Htt aggregates are detected at correlating time points with the disease symptoms. Furthermore, the ubiquitous expression of the Htt transgene confirms an essential feature of the disease, particularly that post-mitotic neurons are first to exhibit dysfunction. Using a similar strategy with the Htt exon-1 fragment, it was shown that expression of the transgene in *E. coli* also formed aggregates at higher polyQ lengths (Scherzinger et al. 1997). Additionally, a truncated protein, SCAJ/MJD, of another polyQ disorder, Spinocerebellar Ataxia 3, expressed in fruit fly, *Drosophila melanogaster*, leads to cell degeneration and the accumulation of nuclear aggregates when an expanded polyQ tract was present (Warrick et al. 1998). These systems demonstrate that the presence of a polyQ repeat at a particular length is necessary and sufficient to cause symptoms similar to those observed in the human disease.

Since the glutamine tract alone is sufficient to cause aggregation, newer models of the polyQ disorders have expressed polyQ fragments outside the context of their endogenous genes (Htt or SCAJ/MJD). Using the nematode *Caenorhabditis elegans*, aggregation *in vivo* is visualized by fusing a CAG repeat sequence to a gene encoding a yellow fluorescent protein (YFP) expressed in either the body wall muscle or neuronal cells (Morley et al. 2002; Brignull et al. 2006). These animals display a length dependent aggregation phenotype (Q35 and higher) correlating with diminished cellular function that can be observed and quantified in real-time using a fluorescent microscope over the

lifespan of the animal (Figure 2.3). The ease of genetic manipulation through RNA interference (RNAi) combined with the short life span of the organism have made this model a powerful tool for examining factors that influence protein aggregation (Nollen et al. 2004).

## B. Molecular Chaperones and the Heat Shock Response

Molecular chaperones are cellular proteins that assist in the folding of substrate proteins by allowing the polypeptide to undergo productive interactions en route to its native ground state (Bukau et al. 2006). Chaperones were first identified as proteins upregulated in response to elevated temperatures, earning the name heat shock proteins (HSPs). Subsequent studies showed that chaperone expression occurs in response to a variety of different stressors, including hypoxia, oxidative stress, chemical perturbations, and ethanol (Lindquist and Craig 1988). It is now understood that chaperones function as a general protective mechanism against conditions which promote protein misfolding and aggregation.

Although originally classified by size, chaperone proteins are now identified through homology searches because of high sequence conservation from *E. coli* to humans. For example, the HSP70 chaperone is an HSP of approximately 70 kilodalton (kDa) size, but also contains conserved ATPase and substrate binding domains that can be used to identify family members (Bukau et al. 2006). While prokaryotes lack organelles, eukaryotic cells have evolved many specialized compartments (i.e. mitochondria and endoplasmic reticulum) to carry out unique cellular tasks. To ensure proteins are correctly folded in these organelles, the eukaryotic cell contains chaperones specialized to subcellular compartments, which can be identified through leader sequence analysis and sequence homology to known localized chaperones (K. Orton and R.I. Morimoto, personal communication).

One major challenge in chaperone biology is understanding how a cell senses misfolded proteins and translates this information into a cellular response. A well

characterized stress response involves activation of the heat shock transcription factor-1 (HSF-1). This transcription factor is held in a monomeric and inactive form in the cytoplasm until appropriate cellular stressors promote trimerization of the protein, translocation into the nucleus, and binding to specific heat shock elements (HSEs) in the promoter region of certain genes, leading to increased transcription of its targets (Morimoto 1998). This process is known as the heat shock response (HSR) and is in place to minimize the number of misfolded proteins that accumulate under certain conditions, such as higher temperature. Not surprisingly, many of the proteins upregulated during the HSR are molecular chaperones which contain HSEs in their promoter regions, suggesting HSF-1 activation is a key messenger in gene activation during a stress response. While HSF-1 governs the cytoplasmic response to conditions that promote protein misfolding, recent data suggests transcription factor mediated responses to unfolded proteins also exist in the mitochondria and endoplasmic reticulum (ER). In these cases, compartment specific perturbation of either organelle activates transcription factors, upregulating chaperone expression within the compartment (Patil and Walter 2001; Haynes et al. 2007). This data suggests the eukaryotic cell has evolved cytoplasmic, mitochondrial, and ER specific stress responses to misfolded proteins. However, it remains unclear whether these responses to misfolded proteins act autonomously to misfolded proteins or coordinately to maintain protein homeostasis. In addition, it is unknown whether misfolded proteins in one organelle can cause global perturbations in the protein folding environment, effecting cellular processes throughout the cell.

### C. The HSP70 Family of Molecular Chaperones

The HSP70 chaperones are a well-studied class of cellular machines that function in a diverse set of cellular contexts. These include assisting in the folding of nascent polypeptides translated at the ribosome, translocating proteins across organellar membranes, disassembling protein aggregates, targeting misfolded and damaged proteins for degradation, and acting as regulatory molecules in stress response pathways (Bukau et al. 2006).

Early experiments with the *E. coli* HSP70 homolog, DnaK, suggested that HSP70 demonstrated greatest affinity to hydrophobic segments in extended conformations (Gragerov and Gottesman 1994). As discussed above, misfolded or aggregate prone proteins inappropriately display hydrophobic amino acids to the aqueous cellular environment. Thus, HSP70 is able to bind to general features of misfolded polypeptides, providing an explanation for the diverse functions of HSP70 within the cell. While this feature of the chaperone explains how it interacts with substrate polypeptides, kinetic studies of HSP70 *in vitro* revealed a folding mechanism dependent on an ATP hydrolysis cycle. During each round of hydrolysis, HSP70 binds and releases its substrate, transiently interacting with the polypeptide to encourage protein folding (Schmid 1994). Thus, HSP70 interacts with its substrate at exposed hydrophobic regions, preventing improper interactions leading to aggregation and allowing productive interactions between other areas of the polypeptide to allow refolding into a native state.

Later experiments, using HSP70 to refold firefly luciferase *in vitro*, have shown that the HSP70 reaction cycle is heavily influenced by the presence of co-chaperones, mainly members of the HSP40 (DnaJ in *E. coli*) chaperone family and the nucleotide

exchange factor GrpE (Szabo et al. 1994). These experiments led to the current model of the HSP70 reaction cycle (Figure 2.4). This mechanism begins with HSP40 co-chaperone delivery of a misfolded or unfolded substrate to an ATP bound HSP70 chaperone. ATP hydrolysis by the HSP70 is stimulated by HSP40, causing conformational changes that increase the affinity of HSP70 for its substrate. The nucleotide exchange factor, GrpE, then promotes dissociation of ADP from the HSP70, returning the HSP70/substrate complex to the low affinity state. An unfolded substrate may proceed through this cycle multiple times before reaching its native state. High resolution crystal structures of the HSP70 protein bound to a substrate peptide confirmed this reaction cycle and captured the conserved C-terminal substrate binding domain interacting with the unfolded polypeptide in two different, presumably the high and low affinity, conformations (Zhu et al. 1996).

Although assisted protein folding is necessary during protein translation, there are many other cellular processes that involve refolding polypeptides by the HSP70 chaperone. A well studied example involves the translocation of nuclear encoded proteins into organelles, such as the mitochondria. It was shown that one HSP70 chaperone in the budding yeast *Saccharomyces cerevisiae*, Ssc1p, is localized to the inner membrane of the mitochondria and guides linear polypeptides into the mitochondrial matrix (Kang et al. 1990). Ssc1p can then assist in the folding of the polypeptide using the reaction cycle described above, or deliver the chain to other chaperone machines. This discovery, namely that HSP70 could assist in translocation of proteins across organellar membranes, suggested the chaperone can participate in a broader range of functions within a cell.

While HSP70 and its co-chaperones attempt to fold their substrates, it was determined these machines can also act as intermediaries in targeting misfolded proteins for degradation to the proteasome. The 26S proteasome is a multisubunit ATP-dependent machine that recognizes ubiquitinated proteins and hydrolyzes polypeptides into individual amino acids that can be reused for protein synthesis (Goldberg 2003). It is believed HSP70 facilitates recognition of a misfolded protein by interactions with the ubiquitin E3 ligase (CHIP), polyubiquitinating the substrate and targeting it for degradation at the proteasome (Meacham et al. 2001). Downregulation of proteasomal subunits through RNAi, resulting in impairment of the machine, results in increased polyQ aggregation in *C. elegans* models, demonstrating the importance of this clearance mechanism in maintaining protein homeostasis (Nollen et al. 2004).

#### D. The Implications of Chaperones in Protein Folding Diseases

Because chaperones have the innate ability to promote the proper folding of proteins, more recent studies with chaperones have attempted to utilize their function to better understand protein folding disorders, particularly the neurodegenerative diseases discussed in section 2A. The basic approach in these studies has been to supply the cell with increased levels of chaperones, using overexpression, to provide added protection against aggregate prone proteins.

Initial experiments performed in yeast suggested overexpression of HSP70 could delay the aggregation phenotype independent of polyQ length (Krobitsch and Lindquist 2000). In addition, overexpression of other chaperones functioning in the HSP70 reaction cycle, namely the HSP40 co-chaperone, exhibited similar suppression phenotypes. Later, it was found that overexpression of HSP70 in a *Drosophila* polyQ model was able to compensate for deletions in an HSP40 gene, dHdj1, establishing a genetic relationship between the two chaperone genes (Chan et al. 2000). In addition, overexpression of both HSP70 and HSP40 produced a synergistic suppression of polyQ aggregation. These results suggest overexpression of a concert of molecular chaperones may be key to maintaining protein homeostasis in the presence of aggregate prone proteins.

As a corollary to chaperone overexpression, other experimenters have established a link between decreased chaperone levels and protein aggregation. More specifically, a progressive decline in HSP70 and HSP40 expression was measured in the brain tissue of the R6 HD mouse model over time, linking a lack in chaperone activity to disease progression (Hay et al. 2004). Using the *C. elegans* fluorescent Q35::YFP aggregation model, the entire genome of *C. elegans* was probed to find enhancers of polyQ

aggregation by RNAi (Fire et al. 1998; Nollen et al. 2004). Decreasing the levels of a diverse set of genes produced a polyQ aggregation phenotype earlier than the control Q35 strain, but only a small number of molecular chaperones were identified, including two members of the HSP70 family and one member of the HSP40 family. This result suggests compensatory networks of chaperones may have evolved in higher eukaryotes so that loss of function of a single chaperone does not negatively affect protein homeostasis.

E. The HSP70 Family in *C. elegans*: An *In vivo* Approach to Identify Chaperone Networks Influencing Protein Homeostasis

To understand the role of molecular chaperones in maintaining protein homeostasis in higher eukaryotes, I have investigated the functional role of the HSP70 family. In *C. elegans*, the HSP70 family is comprised of twelve members with predicted expression in specific cellular compartments (Figure 2.5). Two of these chaperones, HSP-1 and HSP-6, have been previously shown to enhance polyQ aggregation in the *C. elegans* Q35::YFP model upon RNAi knockdown, however, no phenotype for the remaining has been observed. Therefore, it is likely that redundancies in the HSP70 chaperone network have evolved to produce chaperones with overlapping functions. To test this hypothesis, I performed comprehensive RNAi experiments to knockdown expression of specific HSP70 members individually and in combinations, using enhancement of polyQ aggregation and upregulation of other chaperones as a readout for a disruption in protein homeostasis. My studies demonstrate that four additional HSP70 chaperones, C12C8.1, HSP-3, HSP-4, and T14G8.3 are modulators of protein homeostasis. In addition, my data suggests that distinct nodes of an HSP70 chaperone network are localized to three cellular compartments: the cytoplasm, mitochondria, and ER, supporting recent data that specific responses to unfolded responses are localized to these areas (Patil and Walter 2001; Yoneda et al. 2004). Although focused only on the HSP70 family, this data could be extrapolated to other chaperone families and models to provide a dissection of the relationships between chaperone genes in higher organisms.

As a corollary to chaperone knockdown studies, I also overexpressed the highly conserved cytoplasmic *C. elegans* Hsc70 homolog, HSP-1, as a tool to study the effect of

chaperone overexpression in the background of chronic challenges to the protein folding environment *in vivo*. These experiments both test if HSP70 overexpression can suppress polyQ aggregation, as shown in other models, and also provide insight into the effect of chaperone overexpression on cellular function. My data shows that while HSP-1 overexpression suppresses polyQ aggregation, this comes at the expense of cellular function, suggesting chaperone levels are critical when designing strategies for preventing neurodegenerative disease. Furthermore, HSP-1 overexpression globally protects against cytoplasmic polyQ aggregation, as it suppresses RNAi aggregation phenotypes of chaperones localized to different areas in the cell. Thus, although perturbation of specific cellular compartments activates localized upregulation of chaperones, misfolded proteins may impart global cellular challenges that require chaperone upregulation throughout the cell.

### 3. Materials and Methods

#### A. DNA Cloning

To generate the overexpression construct for HSP-1 in the body wall muscle of *C. elegans*, PCR amplification of an *unc-54p::cfp* construct as well as the *unc-54 3'UTR* was performed from *C. elegans* vectors (a gift from Susana Garcia, primer sequences can be found in Table 3.1). Genomic DNA was isolated (3x freeze thaw in liquid nitrogen, 1 µg/µL Proteinase K treatment at 65 °C for 1hr, followed by 5 min. boil to heat inactivate) from wildtype (N2) worms as template for PCR amplification of the *hsp-1* gene. The Gateway cloning system and protocols (Invitrogen) were used for all subsequent cloning. The *unc-54* promoter element along with the *cfp* gene were cloned into p4-p1 vector, the *hsp-1* genomic sequence into p221, and the *unc-54 3'UTR* into p2-p3 using the BP Clonase enzyme which recognizes recombination sites designed into the PCR primers. The three clones were assembled into the final expression vector used for microinjection through an LR clonase recombination reaction to yield an in-frame *unc-54p::cfp::hsp-1::unc54 3'UTR* sequence. The integrity of all DNA sequences was confirmed through DNA sequencing (Seqwright, Coralville, IA).

#### B. *C. elegans* Methods

*C. elegans* were handled using standard methods (Brenner 1974). For experiments requiring a synchronized population of animals, gravid hermaphrodites were washed into a 7.75 mL solution of M9 buffer (KH<sub>2</sub>PO<sub>4</sub> 3 g/L, Na<sub>2</sub>HPO<sub>4</sub> 6 g/L, NaCl 5 g/L, 1 mM final concentration MgSO<sub>4</sub>) and then shaken with 2 mL commercial bleach and 250 µL of 10N NaOH for 4 minutes. The resulting eggs obtained through centrifugation were washed twice with M9 buffer and allowed to hatch overnight at 20 °C to arrest at the first larval

stage (L1). The next day, arrested L1 worms were plated on *C. elegans* agar media with appropriate bacterial lawn (this time point is referred to as day 0).

For creation of transgenic lines, digested *unc-54p::cfp::hsp-1::unc54* (1 ng/uL) was coinjected with *PvuII* digested genomic DNA (100 ng/uL) into the gonad of a hermaphrodite adult animal. F1 progeny containing the transgene were singled based on CFP fluorescence. Transgenic lines were then established based off transmission of the transgene to the F2 progeny (>70%). To create integrated transgenic animals, adult hermaphrodites were gamma irradiated and allowed to reproduce for 10 days at room temperature. This ensured that non integrated arrays would be lost over multiple generations. The starved progeny were transferred to fresh plates containing food for two days and approximately 500 CFP positive worms were singled onto individual plates to select for independent integrated lines. Twelve strains displaying 100% transmission were obtained, but only three, displaying different levels of CFP expression, were selected for further experiments (HSP-1L, HSP-1M, HSP-1H, named for low, medium, and high levels of CFP fluorescence, respectively). These lines were then backcrossed (4X) to wildtype worms to eliminate background mutations from irradiation and crossed to the Q35::YFP transgenic model. Only homozygous strains for both the Q35::YFP and CFP::HSP-1 were used in experiments.

### C. Aggregation Quantification

All quantification was performed on fluorescent microscopes. As reported previously, aggregates were defined as structures with distinguishable boundaries from the surrounding fluorescence on all sides (Morley et al. 2002). One tail unpaired student

t-tests (Microsoft Excel) were performed to determine statistical significance between samples.

#### D. RNAi Experiments

RNAi experiments were performed with a previously established RNAi library (Kamath et al. 2003). The *E. coli* strain HT115(DE3) was transformed with either an empty control vector or with a plasmid targeted to the indicated chaperone gene. For RNAi experiments, bacteria cultures were inoculated at 37 °C for twelve hours and then treated with IPTG (1 mM final concentration) for another four hours. The cultures were then plated on *C. elegans* agar media containing additional IPTG (1.429 g/L), ampicillin (.075 g/L), and tetracycline (.0125 g/L) and kept under aluminum foil to avoid exposure to light. After the bacterial lawn had dried, synchronized animals were plated and allowed to grow normally at 20 °C.

#### E. DNA Isolation

Isolation of *C. elegans* genomic DNA for microinjection follows the protocol for RNA isolation in section 3F. However, following ethanol precipitation, the nucleic acid pellet was resuspended in water and treated with RNase A to digest RNA. The resulting DNA was then quantified (Amersham Biosciences: Ultraspec 2100 *pro*) and digested with the *PvuII* restriction enzyme (New England Biolabs: cat#151, using suggested protocol) for microinjection.

#### F. RNA Isolation and RT-PCR

RNA was harvested from synchronized animals on the indicated day or stage of development. Animals were washed (2x) in M9 buffer, pelleted, and flash frozen in liquid nitrogen for storage until use. The pellet was then treated with 500 uL of triazol reagent

(Invitrogen: cat#15596-026), vortexed for 1 minute, and subjected to 3-5 rounds of freeze/thaws to break open the worm cuticle. An additional 250 uL of triazol was added to the mixture and vortexed for 30 seconds before one final freeze/thaw. Nucleic acids were isolated by treatment with 500 uL of chloroform, incubation for 5 min. at room temperature, and a 15 min. centrifugation to separate the solution into two layers. The aqueous top layer was extracted and added to 500 uL of isopropanol to precipitate RNA and DNA. A nucleotide pellet was obtained after centrifugation and washed with 75% ethanol to remove salts. Another centrifugation yielded a purified pellet and, after removal of ethanol solvent, the pellet resuspended in nuclease free water.

DNA was removed from the pellet through treatment with DNase I following a described protocol (Ambion: cat# 1906). The concentration of RNA in each sample was determined by measuring the absorbance on a spectrophotometer (Amersham Biosciences: Ultraspec 2100 *pro*) in TE buffer (pH 8). Samples exhibiting a 260/280 ratio between 1.8 and 2.2 were selected for RT-PCR experiments. 1 ug of purified RNA was synthesized into cDNA using a described protocol and kit (BioRad: cat# 10864A) in a reverse transcriptase reaction.

Gel based RT-PCR was performed on cDNA samples with varying input concentrations as indicated for each experiment and visualized on an agarose gel containing ethidium bromide. For quantitative RT-PCR (qRT-PCR) experiments, DNA was amplified from a SYBR green PCR master mix (BioRad: cat#170-8882) in fluorescence PCR plate reader (BioRad: cat#170-8740) reading absorbance at 490 nM. Optical plates (Biorad: cat#2239441) and tape (Biorad: cat#2239444) were used in all trials. Either linear standard curves were created to determine exact transcript numbers

for quantification or relative amounts were reported based on an actin loading control. The reliable linear range for absorbance was determined to be approximately between 50 and 500,000 transcripts, with slight variations observed based on the size of the template. All reported qRT-PCR data fell within the linear region of the curve.

#### G. Protein Isolation and Western Blots

Protein was isolated from synchronized worms after washing (3x) in M9 buffer and once in PELE buffer (20 mM Tris pH 7.4, 10% glycerol, 5 mM MgCl<sub>2</sub>, 0.5% Triton X-100, 0.2 mM PMSF, 1ug/uL leukopeptin, 1 tablet protease inhibitor cocktail (Roche Diagnostics: cat#11-836-170-001)). For protein extractions involving polyQ western blots, 5% SDS was added to the PELE buffer to prevent aggregation. Pelleted animals were subjected to four rounds of freeze/thaw in liquid nitrogen to fragment the *C. elegans* cuticle. An additional 1:1 volume of PELE buffer was added based on the volume of the pellet. The lysates were then sonicated (Branson 1510 water sonicator) at a low water level until all cuticle remains dissolved. In samples without 5% SDS in the PELE buffer, quantification of protein was performed using a standard Bradford assay (BioRad: cat#500-0006), measuring absorbance at 595 nM. Readings were compared to a standard BSA curve to obtain exact concentrations.

SDS-PAGE was performed with both stacking and resolving sections according to standard protocol. Protein was transferred from the polyacrylamide gel to a nitrocellulose membrane in a semi-dry transfer apparatus (BioRad: Transferblot SD®) at a constant 400 mA. Blots were then blocked (5% milk, 0.1% Tween in PBS buffer, for at least 12 Hrs) at 4 °C. For treatment with antibodies, blots were washed (at least 3X) using PBS buffer with 0.1% TWEEN-100 and incubated for 1 hour with 1:4,000 and 1:10,000

dilutions for primary and secondary antibodies respectively. Visualization of the protein bands was performed with the Odyssey infrared imaging system (LI-COR Biosciences) and all quantification was calculated in Adobe photoshop.

The antibodies used for these experiments include a conjugated GFP antibody (Conjugated Rockland Immunochemicals: cat#600-132-215) with fluorescence at 800 nM, a primary mouse tubulin antibody (Sigma: cat#T5168) visualized with an anti-mouse secondary (Molecular Probes, Eugene, Oregon: cat#A-21057) with fluorescence at 680 nM, and an HSP-1 antibody (Openbiosystems, See table 3.1) visualized with an anti-rabbit secondary (Conjugated Rockland Immunochemicals: cat#611-732-127) with fluorescence at 800 nM.

#### H. Motility Assays

For thrashing experiments, single animals were placed in a drop of M9 buffer and each bend was scored for 1 minute. A bend was defined as a definitive body movement to one side; twitches and head movements were not scored. Alternatively, motility was measured by placing worms at the center of a lawn of food and scoring the percentage able to move outside a circle boundary for a set time. Boundaries were set so that control worms escaped approximately 50% of the time. This ensured motility phenotypes above or below the control could be measured. Comparison of the two types of assays yields similar results. For statistical methods, a one-tail unpaired student t-test was used to determine significance between strains in thrashing assays and the Fischer exact method was used for agar plate motility experiments.

## 4. Results

### A. Establishing an HSP70 Chaperone Network in *C. elegans*

#### i. An RNAi Approach to Identify HSP70s Responsible for Maintaining Protein Homeostasis

As mentioned above, a genome-wide RNAi screen in *C. elegans* revealed two HSP70 chaperones, the cytoplasmic HSP-1 and mitochondrial HSP-6, which enhance the Q35 aggregation phenotype upon knockdown (Nollen et al. 2004). However, the screen did not identify any role for the other ten HSP70 members, suggesting cooperative or redundant relationships might exist amongst the remaining HSP70s. In addition, no quantification of the aggregates was included in the analysis, raising the possibility smaller effects may have been missed in the genome-wide screen.

To confirm the results of the screen, synchronized Q35 animals were fed bacteria containing a control vector (L4440) or expressing double stranded RNA (dsRNA) of a particular HSP70 gene. Confirming the previously published data, knockdown of both HSP-1 and HSP-6 significantly enhanced the polyQ aggregation phenotype (Figure 4.1A). RNAi against the other HSP70s did not affect the number of aggregates compared to control.

We hypothesized the inability to identify other HSP70 members influencing polyQ aggregation may be the result of chaperone networks that modulate their expression to compensate for loss of a particular chaperone. To answer this question, combinatorial RNAi (cRNAi) experiments were performed between pairs of HSP70s to concurrently reduce their expression. We found cRNAi knockdown of two HSP70s localized to the ER, HSP-3 + HSP-4, displayed an enhanced aggregation phenotype

(Figure 4.1B). In addition, cRNAi against the cytoplasmic inducible HSP70 chaperone, C12C8.1, and HSP-1 increases the number of aggregates compared to HSP-1 RNAi alone. Thus, C12C8.1 appears to protect the cytoplasmic folding environment only under stress conditions that activate its expression.

The results of the singlet and cRNAi experiments implicate the function of HSP70 chaperones in multiple cellular compartments: the cytoplasm, mitochondria, and ER. To investigate if these components define different nodes in maintaining the protein folding environment, cRNAi knockdown was performed between the HSP70 chaperones of different subcellular localization. The aggregation phenotype of HSP-1 RNAi is enhanced by cRNAi with HSP-6 or HSP-3/HSP-4 (Figure 4.1B). Because the mitochondrial and ER HSP70 chaperones are able to enhance the cytoplasmic polyQ aggregation phenotype, it is likely these organelles maintain homeostasis in their respective compartments, separate from the machinery present in the cytoplasm.

#### ii. Identifying Compensatory Chaperone Networks through Gene Expression

The HSP70 compartmentalization model can explain the aggregation enhancement observed in the HSP-1 cRNAi experiments with HSP-6 and HSP-3/HSP-4. However, in the case where cRNAi pairs enhance aggregation from within the same compartment, such as HSP-1 + C12C8.1 in the cytoplasm and HSP-3 + HSP-4 in the ER, we hypothesized compensatory networks that induce expression of particular chaperones may explain the observed aggregation enhancement. Previous publications support this hypothesis, as HSP-4 has been shown to increase in expression upon HSP-3 knockdown (Kapulkin et al. 2005). In addition, HSP-6, the single HSP70 in the mitochondria, is expressed at higher levels when the function of other mitochondrial chaperones is

compromised (Yoneda et al. 2004). With these findings in mind, and knowing C12C8.1 is a highly inducible HSP70 chaperone, we reasoned it might play a role in maintaining the cytoplasmic folding environment after loss of HSP-1 expression. To test this hypothesis, gel based RT-PCR experiments were performed on animals treated with HSP-1 RNAi to measure changes in C12C8.1 expression. After knockdown of endogenous HSP-1 on day three, the same day on which aggregates were quantified, a significant increase in C12C8.1 transcript was measured, suggesting this chaperone has the role of restoring protein homeostasis when the cytoplasmic HSP-1 chaperone machinery is impaired (Figure 4.2A). The inducible character of this chaperone also explains why an RNAi aggregation phenotype is observed only in combination with HSP-1 RNAi, as basal levels of C12C8.1 are low. The normally low levels of C12C8.1 expression also illustrate why the chaperone does not exhibit an RNAi aggregation phenotype on its own.

Because other HSP70 members may function in these compensatory networks, quantitative RT-PCR experiments measuring the expression of all 12 HSP70 members were performed on HSP-6 and HSP-3/HSP-4 RNAi lysates to determine if knockdown of compartmental HSP70s would have effects on other members of the family. We found C12C8.1 expression increases in HSP-6 and HSP-3/HSP-4 RNAi samples and another HSP70 localized to the ER, T14G8.3, is upregulated in response to HSP-3/HSP-4 cRNAi (Figure 4.2B). While we believe T14G8.3 upregulation represents another intracompartamental compensatory network, the induction of C12C8.1 by local perturbation of the ER and mitochondria suggests a deficiency in the protein homeostasis in specific compartments can have global consequences on the folding environment of a

cell. This result correlates with the enhanced cytoplasmic polyQ aggregation observed upon HSP-6 or HSP-3 + HSP-4 knockdown. While RNAi knockdown of these chaperones compromise the folding machinery in their respective organelles, indirect effects likely perturb the cytoplasmic folding environment, leading to an accumulation of misfolded proteins and induction of cytoplasmic chaperones.

To complete our analysis of the HSP70 chaperone family in *C. elegans*, current experiments include obtaining comprehensive HSP70 expression data after knockdown of each individual HSP70 member. As discovered above, we hope to identify additional compensatory networks and relationships between the cytoplasmic, mitochondrial, and ER chaperones. In addition, we are also completing cRNAi aggregation experiments with HSP-3/HSP-4 + HSP-6, HSP-6 + C12C8.1, and HSP-3/HSP-4 + C12C8.1 to confirm our conclusion that three autonomous folding environments are maintained by HSP70 chaperones.

## B. HSP70 Overexpression and its Effect on Protein Homeostasis in *C. elegans*

### i. Generation of Transgenic HSP-1 Overexpression Strains

Our initial studies with the HSP70 family in *C. elegans* have implicated one chaperone in particular, the cytoplasmic HSP-1, to be essential for protecting against polyQ aggregation and a possible target for understanding chaperone networks in the cell. While these experiments have shown the deleterious consequences of chaperone knockdown and the mechanisms in place to compensate for loss of chaperone function, we are also interested in understanding how chaperone overexpression may provide protection against aggregate prone proteins such as polyQ. In addition, we asked if overexpression can globally compensate for the loss in protein homeostasis or if this effect is limited to specific compartments within the cell. To test this, *C. elegans* transgenic lines were created overexpressing a CFP tagged HSP-1 protein under the *unc-54* muscle specific promoter. Out of twelve integrated lines, three were chosen for further experimentation, exhibiting variable CFP expression levels under a fluorescent microscope (Figure 4.3A). To confirm the expression of the 90 kDa CFP::HSP-1 protein, we created an antibody against HSP-1 (Figure 4.3B). Western blots probed with the anti-HSP-1 antibody detected the presence of the CFP::HSP-1 protein and quantification confirmed the variable expression levels apparent from the fluorescence (Figure 4.3C). Thus, we have named these strains HSP-1L, HSP-1M, and HSP-1H for their low, medium, and high transgene expression.

### ii. HSP70 Overexpression and PolyQ Aggregation

It has been previously reported in other model organisms that HSP70 overexpression is able to suppress polyQ aggregation (Chan et al. 2000; Krobitsch and

Lindquist 2000). Thus, to test the functionality of CFP::*HSP-1* in *HSP-1L*, *HSP-1M*, and *HSP-1H*, the lines were crossed to the *Q35::YFP* model and aggregates were quantified over a period of eight days. We found the aggregation phenotype of *Q35* was reduced in a manner dependent on the levels of *HSP-1* overexpression, with the highest levels of suppression observed in *Q35;HSP-1H* (Figure 4.4A). While the decrease in aggregation was slight in *Q35;HSP-1L* and *Q35;HSP-1M*, it was determined to be statistically significant (Figure 4.5A inset).

To determine if the reduction in aggregation was due to a decrease in *Q35::YFP* transgene levels, quantitative RT-PCR experiments were performed to measure the level of *Q35::YFP* transcript on day three. We measured no detectable difference between *Q35::YFP* transcript levels of *Q35;HSP-1L* and *Q35;HSP-1H* and the *Q35* wildtype strain (Figure 4.5B). However, experiments with *Q35;HSP-1M* were more variable, indicating the *Q35::YFP* expression in this strain may be affected by the CFP::*HSP-1* transgene. To determine if the reduction in aggregation was due to a decrease in *Q35::YFP* protein, the relative levels of *Q35::YFP* protein was calculated using western blots probed with a GFP antibody capable of recognizing YFP. Using gamma tubulin as a normalization control, no differences were again observed in *Q35;HSP-1L* and *Q35;HSP-1H* (Figure 4.5C). *Q35;HSP-1M*, however, again showed a decrease in *Q35::YFP* transgene levels. Also of note, the GFP antibody confirmed the same relative levels of CFP::*HSP-1* transgene as the *HSP-1* antibody. Based on these results, all further experiments, both in wildtype and *Q35* backgrounds, were performed with either *HSP-1L* and *HSP-1H*.

### iii. The Effects of Chaperone Overexpression on Cellular Function

After confirming CFP::HSP-1 overexpression suppressed Q35 aggregation in *C. elegans* models, we next asked if chaperone overexpression affected cellular function. With CFP::HSP-1 overexpression sequestered to the muscle tissue under the *unc54* promoter, we tested this question by using organismal motility as an indicator of muscle cell function. While HSP-1L showed movement similar to wildtype levels, HSP-1H exhibited a marked decrease with two different types of motility assays (Figure 4.5A and 4.5B). This phenotype was also confirmed on day two and day six after synchronization (data not shown). Thus, even though aggregation is markedly suppressed in HSP-1H, this comes at the expense of cellular function, suggesting high levels of chaperone overexpression might interfere with normal cellular processes.

### iv. HSP-1 Overexpression Globally Rebalances Protein Homeostasis

Our combinatorial RNAi (cRNAi) experiments propose HSP70 chaperones function in three chaperone networks, localized to the cytoplasm, mitochondria, and ER (Figure 4.1B). However, it is still unclear how perturbation of the mitochondria or ER could effect cytoplasmic polyQ aggregation. Using the HSP-1 overexpression model, we hypothesized that disruption of the local protein folding environment in these organelles by RNAi indirectly taxes the protein folding machinery in the cytoplasm. If this were the case, overexpression of the HSP-1 chaperone should suppress the polyQ aggregation phenotypes of other chaperones. As before, RNAi specific to chaperones known to enhance polyQ aggregation were fed to synchronized Q35;HSP-1L animals, and aggregates were quantified on day three. We found Q35;HSP-1L was able to suppress the aggregation phenotype of different chaperones families localized to different cellular

compartments (Figure 4.6). This suppression was also observed for the Q35;HSP-1H strain (data not shown). Consistent with our hypothesis, HSP-1 overexpression can rebalance the cytoplasmic folding environment, even if the RNAi insult reduces chaperone function in a separate cellular compartment.

## 5. Discussion

### A. Cellular Compartmentalization and Compensatory Networks Characterize Chaperone Function in Eukaryotes

Taking advantage of genetic manipulation by RNAi in *C. elegans*, we have identified HSP70 chaperones that maintain protein homeostasis in three cellular compartments: HSP-1 in the cytoplasm, HSP-6 in the mitochondria, and HSP-3/HSP-4 in the ER (Figure 4.1A, 4.1B). Because combinatorial RNAi (cRNAi) across compartments further enhances individual polyQ aggregation phenotypes (Figure 4.1B), it is likely these chaperones exhibit specialized functions within their organelle independent of chaperone activity elsewhere in the cell. Thus, we conclude three HSP70 nodes modulate protein homeostasis in *C. elegans*. In addition to defining subcellular chaperone nodes, our data suggests activation of compensatory chaperones, namely C12C8.1 in the cytoplasm and T14G8.3 in the ER, is a cellular response to protect against locally misfolded proteins (Figure 4.1B, 4.2A, 4.2B). All of this data, summarized in a *C. elegans* HSP70 model (Figure 5.1), demonstrates eukaryotes utilize compartment specialization and local compensatory regulation of chaperones to maintain protein homeostasis. This model correlates with recent publications suggesting in addition to the cytoplasmic HSR regulated by HSF-1, stress responses also exist in the ER and mitochondria and are modulated by organelle specific transcription factors (Haynes et al. 2007), providing an explanation for how a cell is able to upregulate certain chaperones to respond to localized protein misfolding, while others remain repressed.

While the three HSP70 nodes maintain protein homeostasis locally, it is likely a compartment specific disruption in protein homeostasis can have global cellular

consequences. My finding that the cytoplasmic compensatory HSP70, C12C8.1, is upregulated in response to HSP-6 and HSP-3 + HSP-4 RNAi illustrates this hypothesis. While knockdown of HSP-6 is likely to increase the number of misfolded proteins inside the mitochondria, it may also hinder other processes, such as synthesis of ATP. Since ATP is required for HSP70 function throughout the cell, a decrease in ATP levels may explain how mitochondrial insult could affect the protein folding environment in the cytoplasm, leading to activation of C12C8.1. Alternatively, HSP-6 translocates nuclear encoded mitochondrial proteins across the mitochondrial membrane and into the organelle (Kang et al. 1990). If HSP-6 was disrupted by RNAi, unfolded mitochondrial proteins, unable to enter the organelle, could self-associate in the cytoplasm, leading to aggregation and upregulation of cytoplasmic chaperones. This same rationale can be used to explain the HSP-3 + HSP-4 cRNAi phenotype, as these ER chaperones are also involved in the translocation of proteins into the ER lumen (Brodsky et al. 1995).

Further support that a compartment specific insult can present challenges to protein homeostasis throughout the cell stems from HSP-1 overexpression experiments demonstrating constitutive upregulation of this cytoplasmic chaperone can suppress RNAi aggregation phenotypes of mitochondrial chaperones (Figure 4.6). In these cases, it is possible added levels of HSP-1 are able to compensate for an increase in cytoplasmic misfolded proteins resulting from inefficient translocation into the mitochondria. However, the possibility that diminished ATP levels may also contribute to protein misfolding still remains. To differentiate these mechanisms, RNAi against specific subunits of the electron transport chain would reduce ATP levels while minimally effecting the mitochondrial protein folding environment. If an enhancement in

aggregation is measured in these experiments, it is likely ATP levels are important for maintaining protein homeostasis in the cytoplasm.

While I have only studied the HSP70 family in one model organism, the increased number of chaperones in eukaryotes compared to bacteria suggests compartment specialization and inducible compensatory chaperone networks could define chaperone relationships in other eukaryotic organisms as well. Further chaperone studies in *C. elegans* and other model systems will continue to reveal the importance of these proteins in maintaining protein homeostasis on a local and global cellular level.

## B. Insights into Chaperones as Therapeutics for Preventing Protein Misfolding Diseases

It is now well established in many organisms that chaperone overexpression can prevent aggregation in protein misfolding disease models (Glover and Lindquist 1998; Chan et al. 2000), and we have shown, in *C. elegans*, HSP-1 overexpression follows this trend (Figure 4.4A). However, our data also suggests high chaperone levels, while able to protect against misfolded proteins, also impairs muscle cell function (Figures, 4.5A, 4.5B). One would expect if high chaperones levels led to increased fitness, organisms would have obtained this benefit through evolution. Instead, chaperone expression appears to be fine-tuned and regulated via stress responses which are activated depending on the amount of misfolded proteins present in a particular cellular compartment.

In order for chaperone therapeutics to be a treatment option for protein misfolding diseases, it is likely other approaches, and not overexpression of a single chaperone, need to be taken to avoid negative consequences on cellular function. For example, previous studies have shown HSP70 and HSP40 co-overexpression synergistically suppresses polyQ aggregation phenotypes (Chan et al. 2000), raising the possibility that the overexpression of a concert of chaperones is needed to obtain the beneficial effects of increased chaperone levels. Further experiments, upregulating HSP40 or GrpE chaperones in HSP-1L and HSP-1H, may yield a similar result as co-chaperone availability may limit HSP-1 function at high expression levels. Multiple chaperone overexpression could also be achieved by activation or upregulation of transcription factors that govern the expression of chaperone encoded genes, such as HSF-1.

Another possible approach would be to develop small molecules or inducible systems that activate stress responses concurrent with the appearance of disease symptoms, mimicking the compensatory chaperone networks existing naturally in the eukaryotic cell to protect against aggregation. The HSP-1 overexpression strain in this study constitutively upregulates chaperone levels before aggregates appear, and while this may increase longevity at high levels (data not shown), an inducible chaperone expression system could provide the same benefits while minimizing negative side effects. It is likely further research attempting to better understand the cellular mechanisms behind chaperone expression and regulation will reveal how these natural folding machines can be used as therapeutics in protein folding diseases.

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