Disease-related misfolded proteins cause global perturbation in protein folding homeostasis

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DISEASE-RELATED MISFOLDED PROTEINS CAUSE GLOBAL PERTURBATION IN PROTEIN FOLDING HOMEOSTASIS

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The chronic expression of misfolded proteins and aggregation-prone proteins leads to cellular dysfunction and is implicated in the pathogenesis of several human diseases including Huntington's disease, which is associated with aggregation-prone polyglutamine (polyQ) expansions. Though unrelated to polyQ expansions, Alzheimer's and Parkinson's diseases are also associated with protein misfolding and aggregation. However, the mechanism for toxicity remains controversial. We proposed that the expression of misfolded proteins has a global effect on protein homeostasis by interfering with the folding and stability of other unrelated proteins.

To test this hypothesis we asked whether or not the expression of aggregation-prone polyQ expansions in *Caenorhabditis elegans* (*C. elegans*) would affect the folding of unrelated metastable proteins. To understand how the presence of misfolded proteins, such as polyQ expansions, leads to cellular dysfunction we have developed a system in which temperature sensitive (ts) mutant proteins are used as probes for the cellular folding environment. It has been well-documented that certain ts proteins are sensitive to the overall folding conditions in the cell and can thus serve as probes for changes in protein folding homeostasis. We found that the expression of aggregation-prone proteins in the ts background exposes the ts mutant phenotype in a tissue-specific manner at permissive temperature. Therefore, the expression of aggregation-prone polyQ expansions disrupted the global balance of the cellular protein folding environment, resulting in the loss of function of metastable proteins. Furthermore, we found that the expression of marginally stable ts proteins that are not involved in folding homeostasis have the

ability to enhance the aggregation of polyQ expansions. Such findings indicate that dysfunction in conformational diseases may not be due to a single defect but may be attributed to a global disruption in protein folding homeostasis.

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Background and Literature Survey

Protein folding homeostasis in the cell

Although all the information that is needed for a protein to fold into its native conformation is encoded in its primary sequence, protein folding is a complex process in which a protein can potentially adapt a large number of conformations. Given the vast number of mistakes that can potentially occur in the folding process, how proteins are able to fold into their native conformation within a reasonable period of time is still not fully understood. Nonetheless, the folding of proteins into their native conformation is essential to proper cellular function. Accordingly, the cell has developed many ways in which to protect itself against the appearance of misfolded proteins. When misfolded proteins appear, they are normally detected and eliminated via protein-folding and degradation machineries such as chaperones and the ubiquitin-proteasome system.

Molecular chaperones are highly conserved and play an important role in the folding and assembly of other unrelated protein complexes (Ellis, 1987; Hemmingsen *et al*., 1988; Pelham, 1986). It is thought that molecular chaperones regulate protein folding by recognizing and binding certain features of damaged proteins such as hydrophobic surfaces or a higher β-sheet content (Azuaga *et al*., 2002; Fink, 1998). Chaperones have many functions in addition to their role in co- and post-translational folding and shielding hydrophobic surfaces from aberrant interactions with other proteins and folding intermediates in the cellular environment. They also play a role in signal transduction mediating cellular stress response and apoptosis, the translocation of proteins across the cellular membrane, and facilitating the degradation of misfolded proteins by transferring them to the proteasome (Muchowski, 2002). Because

chaperones are vital to cellular protein folding homeostasis, they are very well conserved among organisms and can be found in all cellular compartments.

The ubiquitin-proteasome system functions in numerous essential cellular processes including regulation of the cell cycle, development, differentiation, and many signaling pathways. The ubiquitin-proteasome system's role in such processes is controlled by the degradation of specific proteins. The ubiquitin-proteasome system also plays an essential role in protein quality control by degrading and removing mutant, damaged, denatured, or misfolded proteins that may interfere with cellular function. Because it functions in an array of cellular processes, dysfunction in the ubiquitin-proteasome system is often implicated in diseases (Ciechanvoer *et al*., 2000).

Chaperones and the ubiquitin-proteasome system represent two opposite ends of the cellular protein folding homeostasis machinery. Chaperones play a pivotal role in protein folding and modulating protein complexes while the ubiquitin-proteasome system works to remove defective or misfolded proteins. Given the importance of both molecular chaperones and the ubiquitin-proteasome system in maintaining protein homeostasis, dysfunction in either system is implicated in many disorders, specifically misfolding or conformational diseases.

Stress and the heat shock response

In order to survive, organisms must have ways in which to detect and respond to the variety of stresses that are presented by its environment. If left unprotected, the challenges presented to cells and tissues can disrupt protein homeostasis causing proteins to unfold, misfold, and/or aggregate (Morimoto and Santoro 1998; Morimoto, 1998). Furthermore, prolonged exposure to stress may lead to cell death. Thus, the challenge presented to cells is to ensure that

misfolded proteins and other folding intermediates are either refolded into their native states or rapidly degraded. Organisms respond to such challenges through the heat shock response, which increases the level of chaperones and proteases repairing damaged proteins and restoring protein homeostasis (Morimoto and Santoro, 1998).

A possible stress signal that activates the heat shock response is the appearance of non-native proteins (Morimoto and Santoro, 1998). Stress signals the activation of heat shock factor (HSF), which increases the expression of heat shock genes coding for molecular chaperones such as Hsp70, Hsp90, and Hsp60 to help proteins fold into their native state (Morimoto, 1998). In both prokaryotes and eurkaryotes, it has been shown that the overexpression of one or more heat shock proteins is sufficient to protect cells against a variety of stresses (Morimoto and Santoro, 1998; Morimoto 1998).

Neurodegenerative Diseases: Protein misfolding is associated with human pathology

The maintenance of protein folding homeostasis by protein-folding and degradation machineries is essential to cell function and viability. When there is a dysfunction in the protein folding quality control system the accumulation of misfolded proteins may lead to aggregation. The expression of misfolded proteins and their aggregation is implicated in the pathogenesis of numerous human neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), Prion disorders, and Huntington's disease (HD) (Taylor *et al*., 2002). Although the diseases are all characterized by neuronal damage, they exhibit diverse phenotypes, inheritance patterns, and their own characteristic pattern of neuronal cell loss (Muchowski, 2002; Taylor *et al*., 2002). Additionally, each disease is associated with different protein deposits, toxic proteins, and disease genes. Despite such differences, the

neurodegenerative diseases are all characterized by aggregation and deposition of abnormal protein (Taylor *et al*., 2002). There are often lesions in the brain, which consist of misfolded, aggregated, and ubiquitinated proteins as well as molecular chaperones and components of the ubiquitin-proteasome system (Muchowski, 2002). For many of these disorders it has been shown that genes involved in protein folding and degradation can modulate the onset, development, and progression of disease (Bonini, 2002; Chan *et al*., 2002; Cummings *et al*., 1998). Therefore, despite the differences among the neurodegenerative diseases, their similarities suggest that there may be a common pathogenic mechanism.

Polyglutamine repeat diseases

Of the neurodegenerative disorders, there are several that are linked to expansions of CAG repeats coding for the amino acid glutamine (polyglutamine or polyQ). There are at least eight polyQ disorders including Spinal and Bulbar Muscular Atrophy (SBMA), Huntington's disease (HD), DentatoRbural and PallidoLuysian Atrophy (DRPLA), and Spino-Cerebellar Ataxia (SCA) of which HD is the most common (Zoghbi and Orr, 2000; Margolis and Ross, 2001; Ross, 2002). All polyQ disorders are dominantly inherited except for SBMA (Zoghbi and Orr, 2000).

Most studies indicate a gain of function mechanism for polyQ diseases, although there is some evidence that loss of function may contribute to pathogenesis (Zoghbi and Orr, 2000; Ross, 2002). It is believed that the mutant form of Huntingtin abnormally interacts with proteins in a new manner that causes toxicity and neurodegeneration (Giorgini and Muchowski, 2005; Scherzinger *et al*., 1997). It has been suggested that Huntingtin can acquire new functions and interactions upon the binding of an unrelated protein to the polyglutamine stretch. The novel

functions and interactions may also result in selective cell death depending on the vulnerability of specific cells (Scherzinger *et al.*, 1997).

Each polyQ disease is characterized by an expanded glutamine tract within a protein specific to the disorder. Each disease is associated with a different protein of varying size, varying length of the glutamine stretch, and varying location of the protein (Gusella and MacDonald, 2000). Although each protein is broadly expressed, only a subset of neurons are affected in the disease (Gusella and MacDonald, 2000; Zoghbi and Orr, 2000). All polyQ disorders display a progressive loss of neuronal function, but neuronal cell death in each disease occurs in only specific regions of the brain (Zoghbi and Orr, 2000; Gusella and MacDonald, 2000; Ross, 2002).

Additionally, each disorder has a characteristic threshold polyQ length below which disease does not occur (Zoghbi and Orr, 2000; Gusella and MacDonald, 2000; Ross, 2002). For example, in the normal Huntingtin allele there are less than 30-44 polyglutamine repeats. However, the presence of more than 35 or 40 polyglutamine repeats in the Huntingtin allele is implicated in disease. There is also a clear inverse correlation between the length of the polyglutamine repeat and the age of onset in all polyQ disorders. Thus, the greater the length of the polyglutamine repeat, the earlier the age of onset of the disease. Furthermore, the greater the length of the glutamine repeat, the quicker the disease progresses (Andrew *et al*., 1993).

A common pathological feature in polyQ disorders is the formation of inclusion bodies brought on by a conformational change in the mutant protein and subsequent aggregation (Ross, 2002). For example, detergent-insoluble inclusion bodies made up of Huntingtin protein have been detected in HD neurons (Muchowski *et al*., 2002). However, the relationship between inclusion bodies and pathogenesis remains controversial (Ross, 2002; Muchowski *et al*., 2002).

Although the disorders share the common feature of an expanded glutamine tract, for each disease there are many different suggested mechanisms of toxicity (Gusella and MacDonald, 2000). Proposed mechanisms of toxicity include the role of polyglutamine aggregates, amyloid formation, possible proteolytic processing, aberrant interactions with other proteins, interference with ubiquitin-dependent degradation, activation of apoptosis, and interference with gene transcription (Ross, 2002; Zoghbi and Orr, 2000).

The search for the toxic species in polyQ diseases

One of the most controversial issues regarding the pathogenic mechanism of misfolding disorders is the relationship between aggregation and toxicity. It has been shown both *in vivo* and *in vitro* that the neurons that are most likely to die are not necessarily the neurons with aggregates (Ross, 2002). Dunah *et al*. (2002) suggested that soluble monomeric polyglutamine proteins interfere with gene transcription resulting in cell toxicity. It has been shown in many studies that "aggregation is neither necessary nor sufficient for neuronal dysfunction" (Zoghbi and Orr, 2000). Furthermore, studies have shown that the abolition of aggregation can actually enhance toxicity (Zoghbi and Orr, 2000). No direct correlation between cell death and the formation of inclusion bodies has been found (Gusella and MacDonald, 2000). Saudou *et al*. (1998) found that preventing the nuclear localization of mutant Huntingtin diminishes the formation of inclusions and decreases neurodegeneration, but this suppression actually enhanced mutant Huntingtin-induced death. This observation suggests that the formation of inclusions may be a mechanism by which the cell protects against mutant Huntingtin-induced death (Saudou *et al.*, 1998). Additionally, there are many intermediate states (monomers, soluble intermediates, insoluble aggregates) produced during the formation of protein aggregates or amyloid fibers.

Thus, it may be that different species could each contribute to different harmful effects on cellular function (Ross, 2002). It is also possible that the formation of aggregates is the cell's attempt to bring together the free toxic strands into a harmless cluster (Zoghbi and Orr, 2000).

It has also been suggested that the formation of inclusion bodies may be an active desirable process by which the cell attempts to remove toxic misfolded proteins. Studies that disrupt microtubles in yeast prevented the formation of large inclusion bodies, but revealed a polyglutamine length-dependent toxicity under conditions in which the mutant Huntingtin existed entirely in a soluble non-aggregated form. This suggests that the formation of inclusion bodies may depend on active transport along microtubules. Furthermore, the formation of inclusion bodies may be a process by which the cell attempts to sequester and clear toxic soluble species of mutant Huntingtin (Muchowski *et al*., 2002).

However, many studies argue that the presence of inclusion bodies in vulnerable neurons strengthens the idea that inclusion bodies are directly related to pathogenesis (Zoghbi and Orr, 2000). For example, mice carrying expanded polyQ tracts in the huntingtin gene developed nuclear inclusions containing the proteins Huntingtin and ubiquitin before developing a neurological phenotype (Davies *et al*., 1997). This finding supports the idea that nuclear inclusions may play a causative role in neurological dysfunction in HD (Davies *et al*., 1997; DiFiglia *et al*., 1997). It is also possible that after years of accumulating mutant polyQ proteins, the cell, no longer able to cope succumbs to inclusions, which causes cellular dysfunction and pathogenesis (Zoghbi and Orr, 2000). The formation of inclusion bodies or aggregates may represent the cells inability to handle and turn over the mutant expanded polyQ tract (Gusella and MacDonald, 2000). It is also possible that although cells normally remove toxic species by

forming inclusion bodies, other proteins that are essential to cellular function may become inadvertently trapped in the aggregates, which then causes toxicity (Muchowski *et al*., 2002).

Pathogenic mechanisms – the different theories proposed

Protein sequestration: cellular proteins with short polyQ sequences

Another contentious issue in polyQ pathogenesis is the role of proteolytic cleavage (Ross, 2002). Studies have shown that high molecular weight protein aggregates with amyloid-like fibrils often result from the cleavage of Huntingtin proteins with polyQ expansions in the pathological range. It has been suggested that these intermediates can nucleate the polymerization of additional monomers in a thermodynamically favorable manner. This would then suggest that CAG repeat disorders are a result of the formation of toxic insoluble amyloid-like fibrils (Scherzinger, 1997).

It has been suggested that the polyglutamine expansion may cause the protein to adopt an unusual conformation, which may alter its native function and/or lead to abnormal, aberrant interactions with other proteins (Gusella and MacDonald, 2000; Zoghbi and Orr, 2000). There have been many studies indicating that mutant proteins form abnormal interactions with other cellular proteins such as p53 and CBP (Ross, 2002). Inclusion bodies not only contain the mutant protein, but also ubiquitin, heat-shock proteins, and proteasome constituents (Gusella and MacDonald, 2000). Studies have shown that the presence of a polyQ repeat is sufficient to recruit unrelated proteins such as TATA-binding protein into inclusion bodies (Perez *et al*., 1998). Furthermore, Satyal *et al*. (2000) have found that nonaggregation-prone glutamine repeats are sequestered into polyQ aggregates. Although Q19-GFP is a soluble protein, coexpression of Q82-CFP altered the localization of Q19-YFP through coaggregation (Satyal *et al*., 2000). It has

also been shown that the toxicity of polyQ expansions can be reduced through deletions of genes encoding glutamine-rich proteins. Furthermore, non-toxic polyQ proteins can be converted into toxic species through the overexpression of glutamine-rich proteins (Duennwald *et al*., 2006). These findings suggest that polyQ expansions can induce toxicity through direct interactions with non-toxic glutamine proteins indicating that polyQ pathogenesis may be due in part to the sequestration of unrelated proteins into polyQ aggregates (Duennwald *et al*., 2006; Perez *et al*., 1998).

The low-abundance nuclear factor CREB-binding protein (CBP) has been detected in polyQ aggregates in cell culture, transgenic mice and tissue from patients with SBMA (McCampbell *et al*., 2000; Ross, 2002). There is also evidence that levels of soluble CBP are reduced in cells even though CBP mRNA levels increase in cells expressing polyQ expansions (McCampbell *et al*., 2000). There is also evidence that the overexpression of CBP can reduce polyglutamine-induced toxicity. Based on these observations it has been proposed that the sequestration of CBP into aggregates leads to cellular toxicity because of its importance in cell signaling and its low abundance (McCampbell *et al*., 2000). Transcription factor p53 has also been found in polyQ aggregates in both cell culture and a transgenic mouse model, which raises the possibility that polyQ expansions may cause neuronal dysfunction by recruiting cellular transcription factors into aggregates thus deleteriously affecting transcriptional regulation (Steffan *et al*., 2000).

Protein Sequestration: functional sequestration of protein homeostasis components

The presence of ubiquitin in nuclear inclusions has also been noted and suggests that mutant Huntingtin may be a target for proteolysis by the proteasome but cannot be removed or

fully degraded and thus remains trapped within the proteasome. The inability to clear aggregated polyQ proteins is likely to have harmful effects on the cell and lead to the accumulation of Huntingtin intermediates, oligomers, and aggregates. Resistance to removal may then inhibit the ubiquitin-proteasome system and the proteolytic pathway by decreasing the efficiency of proteolysis and degradation of other proteins, which may contribute to toxicity (DiFiglia *et al*, 1997; Ross, 2002; Zoghbi and Orr, 2000; Cummings *et al*., 1998; Holmberg *et al*., 2004). It has been found that protein aggregates irreversibly sequester the proteasome through direct interactions (Holmberg *et al*., 2004). It has been reported that the expression of aggregation-prone proteins such as Huntingtin containing a polyQ expansion nearly completely impairs the function of the ubquitin-proteasome system. Because of the essential role of the ubiquitin-proteasome system in the cell, the ability of aggregation-prone proteins to inhibit the system may be the cause of neuronal dysfunction (Bence *et al*., 2001).

Additionally, molecular chaperone HDJ-2/HSDJ has been found in inclusions and the overexpression of HDJ-2/HSDJ has been found to decrease the frequency of ataxin-1 aggregation. It has been suggested that HDJ-2/HSDJ may be targeted to inclusions as an attempt to refold the proteins into their proper conformation (Cummings *et al*. 1998). It has also been shown that the coexpression of yeast chaperone Hsp104 can reverse toxicity and polyglutamine aggregation (Satyal *et al*., 2000). It was found *in vitro* that coexpression of Hsp70 or Hsp40 diminished the assembly of mutant Huntingtin into amyloid-like fibrils. Instead the coexpression of Hsp70 or Hsp40 leads to the formation and accumulation of soluble inclusions (Muchowski *et al*., 2000). Molecular chaperones may function in more than one step in the process of fibril formation (Muchowski, 2002). This indicates that the ability of Hsp70 and Hsp40 to reduce polyQ-induced neurodegeneration in fly and mouse models may be the result of their ability to

direct polyQ expansions into non-toxic soluble inclusions that can be more easily managed by the cell (Muchowski, 2002; Muchowski *et al*., 2000). In contrast to the sequestration of CBP and p53, Kim *et al*. (2002) has shown that instead of being sequestered irreversibly into protein aggregates, molecular chaperones are only transiently associated. Thus, unlike CBP and p53 transcription factors, the interaction between Hsp70 and polyQ aggregates is characterized by rapid association and dissociation. Therefore, it is not the loss of these essential proteins that leads to cellular dysfunction (Kim *et al*., 2002). Yet, the overexpression of molecular chaperones can enhance the cell's ability to respond to misfolded proteins and diminish the appearance of aggregates (Cummings *et al*., 1998). It has been suggested that molecular chaperones and the ubiquitin-proteasome system may work additively to modulate neurodegeneration (Chan *et al*., 2002).

Disruption of energy metabolism

The mitochondria is often referred to as the "power house" of the cell because of its critical role in cellular energy metabolism. Deficient complex I in platelets and substantia nigra of Parkinson's disease (PD) indicates that dysfunction in the mitochondria may be a contributing factor in the pathogenesis of PD (Gu *et al*., 1998; Schapira *et al*., 1998). However, genomic transplantation studies suggest that mitochondrial DNA is sufficient to cause pathogenesis (Schapira *et al*., 1998). Furthermore, one of the targets of Huntingtin polyQ repeats are the medium-sized spiny striatal neurons, which has led some to suggest that Huntingtin plays an integral role in mitochondrial energy metabolism. It was found that expanded polyQ repeats in Huntingtin were associated with low mitochondrial ATP production as well as a decrease in mitochondrial ADP-uptake (Seong *et al*., 2005).

It has been shown that patients with HD have a lower mitochondria membrane potential and the membrane depolarizes at lower calcium levels than control mitochondria membranes. In mice expressing mutant Huntingtin, this defect was observed to precede any abnormal pathological or behavioral phenotypes associated with HD pathogenesis (Panov et al., 2002). Thus, Panov et al. (2002) suggest that there may be a correlation between mutant Huntingtin and mitochondrial calcium defects (Panov et al., 2002).

Protein Homeostasis

Based on growing evidence, it has been suggested that the common factor in misfolding disorders is the protein quality control system in cells (Dobson, 2001). It has been shown in models of neurodegenerative diseases that the onset, development, and progression of disease can be modulated by genes involved in protein folding and degradation (Bonini, 2002; Chan *et al*., 2002; Cummings *et al*., 1998; Brignull *et al*., 2006). A genome-wide RNA interference (RNAi) screen isolated five major classes of genes that altered polyQ aggregation: "genes involved in RNA metabolism, protein synthesis, protein folding, and protein degradation, and those involved in protein trafficking" (Nollen *et al*., 2004). Nollen *et al*. (2004) identified genes encoding molecular chaperones and proteins involved in proteasomal degradation revealing the importance of proteins directly involved in folding and thus aggregation suppression. Transport genes such as tubulin and actin were also identified as an important class of proteins involved in the clearance of misfolded protein. Such findings support the previously proposed mechanism that cells use transport along microtubules to actively sequester misfolded proteins into aggregates. However, in addition to the genes involved in protein quality control such as that of

chaperones and those found in the ubiquitin-proteasome system, genes involved in protein and RNA synthesis were also identified (Nollen *et al*., 2004).

It has been observed that the presence of misfolded and aggregated proteins results in diverse phenotypes and it has been proposed that misfolded proteins affect multiple cellular processes including transport, signaling, mitochondrial function, transcription, folding, and clearance resulting in progressive loss of cellular function. As illustrated by the plethora of mechanisms proposed, the basis of toxicity in neurodegenerative diseases remains controversial. Previously proposed mechanisms of toxicity focus on direct interactions such as partial inhibition of the ubiquitin-proteasome system, partitioning of molecular chaperones, and the sequestration of other cellular components into aggregates. The RNAi screen conducted by Nollen *et al*. (2004) presented puzzling results because previous studies primarily focused on direct interactions such as protein sequestration as a mechanism of toxicity in misfolding diseases. However, given the variety of genes that were found in the RNAi screen, such findings indicate that the formation of polyQ aggregates is modulated by more than just the genes directly involved in protein quality control. It is more likely that polyQ aggregation is governed by much more complicated events in the cellular environment that affect protein folding. In contrast to the previously proposed mechanisms of toxicity, this thesis will demonstrate that the diverse effects caused by a single aggregation-prone protein may be due to a more general mechanism that could account for the common features of protein misfolding disorders. Although, previously proposed mechanisms have focused on direct interactions, the chronic expression of aggregation-prone proteins may have more global effects in that the expression of misfolded proteins may interfere with general protein folding homeostasis in the cell and thus interfere with the folding and stability of other unrelated proteins.

C. elegans as a model for protein folding homeostasis

The nematode, *C. elegans*, can be used as a model to study polyglutamine expansions. The one mm long roundworm is naturally found in soil habitats throughout the world. In the laboratory, *C. elegans* are grown on agar plates with *E. coli* (OP50) as a source of food. The animals go through four larval stages followed by an adult lifespan of about two weeks when grown at 20ºC. Thus, in addition to being easy to maintain, the short life cycle of *C. elegans* represents another advantage. Because 99% of a *C. elegans* population is made up of self-fertilizing hermaphrodites each producing approximately 300 progeny, this allows for the creation and maintenance of genetically identical populations, barring mutations. With only 959 somatic cells, 302 neurons, and a relatively small genome size, *C. elegans* provides a simplified model of a multicellular organism (Wood, 1988). The entire genome of *C. elegans* has been sequenced and techniques for both forward and reverse genetics have been well-established. Thus, despite its simplicity *C. elegans* serves as a useful model organism. Important in genetic research, there are also a wide variety of mutants with observable phenotypes available for study. *C. elegans* also represents a powerful genetic tool because its transparency allows the reporter gene, green fluorescent protein (GFP), to be followed in order to monitor the location and timing of gene expression.

The Morimoto Lab has established a *C. elegans* model system for several neurodegenerative diseases through tissue-specific expression of polyQ-expansions, superoxide dismutase, and tau. The expression of GFP or YFP tagged polyQ expansions in both muscle and neuronal cells in *C. elegans* results in both age-dependent appearance of protein aggregates, loss of motility, as well as neuronal toxicity.

It has been shown that *C. elegans* expressing polyQ-YFP fusion proteins in body-wall muscle cells exhibit polyQ length-dependent aggregation. Repeat lengths of less than 35 were diffuse and soluble, but repeat lengths of 40 or greater resulted in discrete aggregates (Satyal *et al.*, 2000; Morley *et al*., 2002). Expression of polyQ-YFP fusion proteins in *C. elegans* muscle cells also showed a direct correlation between aggregate formation and motility defect. Animals expressing Q-lengths of less than 40 had motility that was similar to wild-type animals whereas Q82 animals exhibited approximately a 10-fold decrease in motility (Morley *et al*., 2002). Polyglutamine proteins expressed in *C. elegans* neurons also showed Q-length dependent changes in aggregation and distribution. Animals expressing protein with less than 40 glutamine repeats exhibited diffuse soluble polyQ protein, while Q86 protein was localized in discrete foci (Brignull *et al*., 2006).

The transgenic *C. elegans* lines expressing different length polyQ-YFP or CFP in muscle and neuronal cells recapitulate both polyQ-length-dependent aggregation and toxicity – the main features of polyQ disorders. Thus, *C. elegans* can be used as a model for polyQ aggregation diseases.

Using temperature sensitive proteins as a tool to study protein folding homeostasis

It has been shown that the temperature sensitive (ts) folding defect of the protein ΔF508 CFTR can be corrected by protein-stabilizing agents such as glycerol, trimethyl-amine N-oxide (TMAO), or deuterated water. Cells cultured in the presence of the different protein-stabilizing agents adopted a wild-type phenotype even at non-permissive temperature, indicating that the ts folding defect had been corrected. These reagents are termed chemical chaperones because of their ability to influence protein folding and protect proteins against denaturation and

aggregation *in vivo*. Several mechanisms by which chemical chaperones work to influence the fidelity of protein folding have been proposed. Chemical chaperones may work by decreasing the rate-limiting energy barrier in the folding pathway or by decreasing the likelihood of a newly synthesized protein to go off of the correct folding pathway towards a denatured or aggregated state (Brown *et al*., 1997). Furthermore, Van Dyk *et al*. (1989) has shown in *E. coli* that the overexpression of the chaperonin groEL suppresses ts mutant phenotypes in multiple dnaA alleles (Van Dyk *et al*., 1989). Because the overexpression of molecular chaperones and application of chemical chaperones have been shown to rescue some ts phenotypes, it is suspected that proteins with temperature-sensitive defects are sensitive to the overall cellular protein folding environment and can be modified and corrected through protein-stabilizing agents (Brown *et al*., 1997; Van Dyk *et al*., 1989)).

The GTPase dynamin plays an important role in clathrin-mediated endocytosis. When placed at non-permissive temperature, the temperature sensitive *dyn-1* mutant in *C. elegans* results in a rapid onset of uncoordination, becoming sluggish and assuming a kinked body shape. Additionally, the *dyn-1* ts mutants show a significant decrease in their pharyngeal pumping rate, prolonged defecation cycle, egg-laying defect, and diminished brood size (Clark *et al*., 1997). Paramyosin is a core thick filament protein encoded by *unc-15* in *C. elegans*. At restrictive temperature, the ts mutant UNC-15 in *C. elegans* results in disruption of thick filament formation, embryonic and early larval lethality, and slow movement in adults (Gengyo-Ando and Kagawa, 1991). When placed at restrictive temperature, ts mutants acquire a novel behavioral phenotype. It has therefore been established that the function of ts proteins is subject to change upon elevation of temperature. We therefore reasoned that ts proteins can be used *in vivo* to probe the cellular protein folding environment through the observation of behavioral phenotypes.

At restrictive temperature, when the cellular homeostasis becomes challenged, suppression of temperature sensitive (ts) mutations becomes insufficient. Overexpression of molecular chaperones or application of chemical chaperones can rescue the ts mutants supporting their ability to sense the state of the cellular folding environment. Because it has been shown that ts proteins are highly sensitive to the cellular folding environment, they can serve as indicators of a disruption in protein folding homeostasis. Temperature sensitive mutants can therefore be used to examine whether or not the functionality of the particular temperature sensitive protein would be affected by the expression of aggregation-prone polyQ expansions.

MATERIALS AND METHODS[1]

Nematode strains and growth conditions

C. elegans strains were obtained from the Caenorhabditis Genetic Center; polyQ strains have been described previously (1,2). Each of the following lines were independently derived with a unique site of integration. The polyQ strains used were: Q0m - AM134[*rmIs126[Punc-54::q0::yfp]*], Q24m - AM137[*rmIs129[Punc-54::q24::yfp]*], Q35m - AM140[*rmIs132[Punc-54::q35::yfp]*], Q40m - AM141[*rmIs133[Punc-54::q40::yfp]*], Q19n - AM49[*rmIs172[PF25B3.3::q19::cfp]*], Q40n - AM47[*rmIs167[PF25B3.3::q40::cfp]*], Q67n - AM44[*rmIs160[PF25B3.3::q67::cfp]*]. Temperature-sensitive mutants were: paramyosin(ts) - CB1402[*unc-15(e1402)*], myosin(ts) - CB1157[*unc-54(e1157)*] and CB1301[*unc-54(e1301)*], UNC-45(ts) - CB286[*unc-45(e286)*], perlecan(ts) - HE250[*unc-52(e669su250)*], dynamin(ts) - CX51[*dyn-1(ky51)*], ras(ts) - SD551[*let-60(ga89)*]. ras(ts) animals with heterozygous or homozygous expression of Q40m were of *let-60(ga89)*;*rmIs133[Punc-54::q40::yfp]*/+ and *let-60(ga89)*;*rmIs133[Punc-54::q40::yfp]*/ *rmIs133[Punc-54::q40::yfp]* genotypes, respectively.

We noted that strains expressing long-Q expansions and, in particular, co-expressing ts mutant proteins tend to accumulate genetic variations which often improve organismal fitness or modify polyQ aggregation phenotypes. All polyQ and double homozygous lines were either frozen immediately after selection or were periodically re-built, and assays were performed on freshly thawed or crossed animals.

[1] The Materials and Methods section has been published as part of the supplementary material found in Gidalevitz, *et al*., 2006

Nematodes were grown on NGM plates seeded with *Escherichia coli* OP50 strain. Animals were synchronized either by hypochlorite treatment of adults or by picking L4 larva onto fresh plates. Assays were performed with young adult animals, usually at the first day of reproductive adulthood at either 15˚C (2.5 days after L4 stage) or 25˚C (1 day after L4 stage). To obtain synchronized three-fold embryos, young adults were allowed to lay eggs for 1 hour at the indicated temperature and embryos were collected after 13-15 hours at 15˚C or 6 hours at 25˚C.

Assays for specific temperature-sensitive phenotypes

For egg hatching and L1 paralysis at 15˚C, embryos were picked from plates containing adult hermaphrodites (day 2-3 of adulthood). Unhatched embryos and larvae that hatched but did not crawl were scored after 2 days. Alternatively, young adults were acclimated to 25˚C for 1 day prior to egg laying, embryos were picked and scored one day later at 25˚C.

For the uncoordination assay, nematodes were picked as L4 larvae and scored next day. Five animals were placed in the middle of a freshly seeded plate, equilibrated to 20˚C. Animals remaining in a 1 cm circle after 2 min were scored as uncoordinated. For controls, animals were acclimated to 28˚C for 30 min before scoring. Experiments were repeated until the n value was at least 80 animals.

Let/Lva phenotype: embryos were picked as for egg hatching assay and the combined number of unhatched embryos and animals that died or arrested prior to reaching reproductive adulthood was scored.

100% penetrance of Let/Lva phenotype in ras(ts)+Q40m animals (Table I) is based on the following: fluorescent F2 animals generated by crossing ras(ts) and Q40m strains were singled,

allowed to lay eggs for 24 hours and shifted to 25˚C to identify animals homozygous for the ts mutation. Fluorescent progeny from thus identified individuals were singled and followed until reproductive adulthood or death/arrest in development. Out of 66 progeny scored, 61 died, arrested or were sterile and 5 reached reproductive adulthood, all heterozygous for Q40m.

For slow movement phenotype, synchronized young adults were rinsed in M9 buffer, and 100-200 animals in 2μl of buffer were spotted in the middle of a 3.5cm plate containing a ring of bacteria along the edge. The number of animals that did not reach the bacteria within 50 min was recorded and compared to the total number on the plate.

To score egg laying defect, abnormal body shape and Osm phenotypes, early L4 larvae grown at 15˚C were picked to a fresh plate and incubated for 3 days at 15˚C or 1.5 days at 25˚C. Animals retaining eggs towards the posterior part of the body were scored as egg laying defective. Paralyzed animals with stick-like rigid bodies were scored for abnormal body shape. For Osm phenotype, animals were placed in a drop of distilled water for 5 min and scored for adopting a swollen, fluid-filled appearance. All assays were performed blind.

Immunostaining and microscopy

For DIC and fluorescent microscopy, synchronized embryos were mounted on 1% agarose pads and imaged using Leica DM/IRB microscope (40x objective) and OpenLab software (Improvision). For immunofluorescence, synchronized embryos or young adults were fixed, permeabilized (3) and stained (4) either with Rhodamine-Phalloidin (Molecular Probes) or with anti-paramyosin antibody 5-23 (1) (obtained from the developmental studies hybridoma bank developed under the auspices of the NICHD and maintained by the University of Iowa,

Department of Biological Sciences, Iowa City, IA 52242). Secondary antibody was AlexaFluor 633 goat anti-mouse (Molecular Probes). Animals were imaged using a Zeiss LSM 510 confocal microscope through a 40x 1.0 numerical aperture objective with either a 594-nm, 514-nm or a 633-nm line for excitation.

Proteolysis

Nematodes were collected, washed with M9 buffer and frozen in liquid nitrogen. Pellets were homogenized and lysed on ice in PBS supplemented with 1% NP40 and DNAse. Soluble protein concentration was determined using Bradford assay (Bio-Rad). 20 μg of total protein was digested for 15 min at RT with chymotrypsin (Sigma) at enzyme to protein ratio 1:2000. Digests were analysed by immunobloting with anti-paramyosin antibody 5-23 (1) and an AlexaFluor 680 goat anti-mouse (Molecular Probes) and imaged using Odyssey Infrared Imaging System (LI-COR Biosciences).

RESULTS

Temperature-sensitive mutations in muscle cells

Temperature-sensitive phenotype of myosin(ts) mutants

To examine the effect of the expression of polyQ proteins on cellular protein-folding environment we used a variety of *C. elegans* temperature-sensitive (ts) mutations to ask whether the functionality of the ts protein would be affected by the coexpression of aggregation-prone polyQ proteins under permissive conditions. Temperature-sensitive proteins are ideal indicators of disruptions in protein homeostasis because they are dependent on the folding environment of the cell. At permissive temperature (15ºC), UNC-54(e1301) animals exhibit wild-type behavior. However, at restrictive temperature (25ºC), the ts mutation results in defects in the formation of thick filaments and body wall muscle cells (MacLeod et al., 1977). Along with a dramatic reduction in the number of thick filaments, UNC-54(e1301) animals exhibit a slow movement phenotype at restrictive temperature as compared to UNC-54(e1301) animals at permissive temperature. While approximately 90% of UNC-54(e1301) worms are able to move to an outer ring of OP50 (when placed at the center of a one inch diameter circle of OP50) at permissive temperature, only 20% of the animals are able to make it to the outer ring at 25ºC (Fig. 1A).

Expression of polyglutamine expansions alone does not result in myosin(ts) mutant phenotype

The expression of polyQ proteins in a wild-type background did not result in the slow movement phenotype. Similar to UNC-54(e1301) animals at permissive temperature, in the absence of the ts protein, approximately 90% of animals expressing nonaggregation-prone Q24 and aggregation-prone Q40 in muscle cells (Q24m and Q40m) were able to move to the outer ring of OP50 within 50 minutes (Fig. 1B). Thus, the expression of polyQ proteins alone does not result in the ts phenotype.

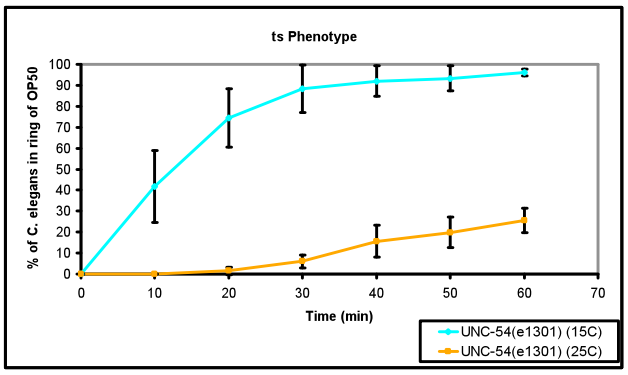
Aggregation-prone proteins expose the ts phenotype of myosin(ts) mutants at permissive temperature

To ask whether the functionality of myosin(ts) would be affected by the coexpression of polyQ expansions, *C. elegans* expressing ts mutant UNC-54(e1301) (*C. elegans* homolog of myosin) were crossed to animals expressing polyQ expansions in muscle cells. The phenotype of double homozygotes was then examined at both permissive and restrictive temperature. Animals coexpressing ts mutant myosin and Q40m (myosin(ts)+Q40m) show a dramatic decrease in motility. When coexpressed with Q40m, myosin(ts) animals at permissive temperature exhibited motility that was similar to that of UNC-54(e1301) animals at restrictive temperature (Fig. 1C). While approximately 90% of Q40m animals and UNC-54(e1301) animals at permissive temperature were able to move to the outer ring of OP50 within 50 minutes, less than 30% of myosin(ts)+Q40m animals were able to move to the outer ring of OP50 within the same time period at 15ºC (Fig. 1C). Importantly, the decreased motility was dependent on the length of the polyQ protein. While myosin(ts)+Q40m animals exhibited a dramatic decrease in motility, myosin(ts)+Q24m animals did not. Instead, the movement of myosin(ts)+Q24 animals was similar to that of UNC-54(e1301) animals at permissive temperature (Fig. 1C). Therefore, under permissive conditions, the coexpression of an aggregation-prone protein such as Q40m is enough to expose a myosin ts mutant phenotype.

Similar results were obtained for UNC-15(e1402) (*C. elegans* homolog of a muscle paramyosin) and UNC-54(e1157) (*C. elegans* homolog of a muscle myosin). Under permissive conditions less than 10% of ts animals, Q24m, and Q40m animals display the slow movement phenotype. However, when the ts animal was crossed to Q40m, homozygote animals exhibited a dramatic increase in the percentage of animals displaying the slow movement phenotype (data

not shown). Similarly, this effect was poly-Q-length-dependent as ts animals coexpressing non-aggregation-prone Q24m did not show the same decrease in motility.

A



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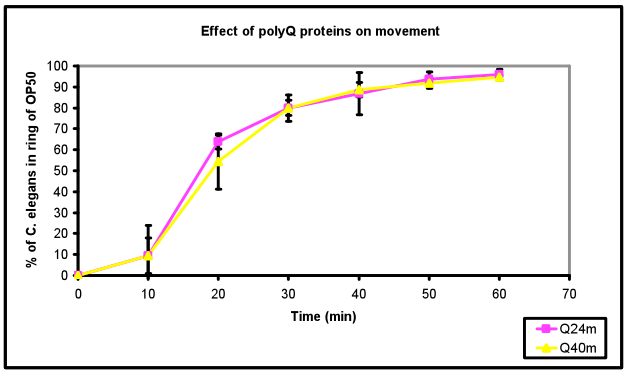

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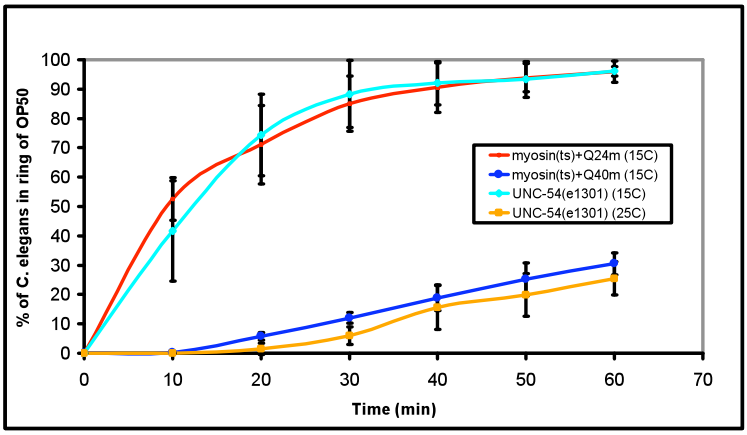


Figure 1. Aggregation-prone proteins expose the temperature-sensitive(ts) phenotype of myosin(ts) mutants at permissive temperature. (**A**) Percentage of age-synchronized young adult myosin(ts) animals displaying wildtype behavior at permissive (cyan) and restrictive (orange) temperatures. Animals were placed in the center of a one inch diameter ring of OP50. Animals that do not reach the ring of OP50 within 50 minutes are scored as having the slow phenotype associated with myosin(ts) mutants. Data are the mean ± SD. n ≥ 300 for each data point. (**B**) Percentage of age-synchronized young adult Q24m (magenta) and Q40m (yellow) animals exhibiting wildtype behavior. The expression of polyglutamine proteins in muscle cells does not result in slow movement phenotype. Expression of polyQm (muscle)-YFP proteins (Q24m, and Q40m) is from the unc-54 promoter. Experiments were conducted at 15ºC. Data are the mean ± SD. n ≥ 300 for each data point. (**C**) Percentage of age-synchronized young adult animals exhibiting wildtype motility for myosin(ts)+Q24m (red), myosin(ts)+Q40m (blue), UNC-54(e1301) at 15ºC (cyan), and UNC-54(e1301) at 25ºC (orange) at indicated temperatures. Data are the mean ± SD. n ≥ 300 for each data point.

Temperature-sensitive phenotype of perlecan(ts) mutants

At permissive temperature (15ºC), UNC-52(su250) (*C. elegans* homolog of perlecan) animals exhibit wild-type behavior, but at restrictive temperature (25ºC), the animals display an abnormal body shape and stiff paralysis (Fig. 2A). While less than 2% of UNC-52(su250) animals at 15ºC exhibit an abnormal body shape, more than 90% of UNC-52(su250) animals at restrictive temperature displayed the ts phenotype (Fig. 2B).

Expression of polyQ expansions alone does not result in perlecan(ts) mutant phenotype

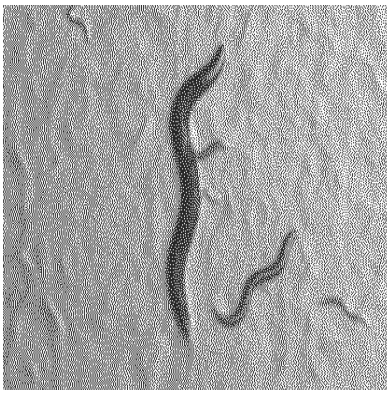
Similar to UNC-52(su250) animals at permissive temperature, the expression of polyQm proteins in a wild-type background did not result in abnormal body shape. In fact, none of the Q0m, Q24m, and Q40m animals displayed the ts phenotype at 15ºC (Fig. 2B). Thus, the expression of polyQ proteins in the absence of the ts protein is not sufficient to expose the ts mutant phenotype.

Aggregation-prone polyQ proteins expose the ts phenotype of perlecan(ts) mutants at permissive temperature

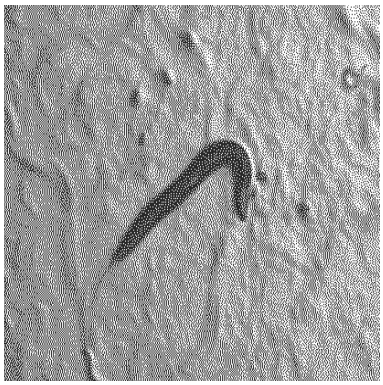
C. elegans expressing ts mutant UNC-52(su250) were crossed to animals expressing polyQ expansions in muscle cells. The phenotype of double homozygotes was then examined at both permissive and restrictive temperatures. Animals coexpressing ts mutant perlecan and Q40 in muscle cells (perlecan(ts)+Q40m) show a significant increase in the percentage of animals exhibiting the ts phenotype at permissive temperature (Fig. 2B). Under the same conditions, approximately 50% of perlecan(ts)+Q40m animals exhibited an abnormal body shape, whereas less than 1% of Q40m and perlecan(ts) animals displayed the ts phenotype (Fig. 2B).

Furthermore, the increase in the percentage of animals showing the abnormal body shape was dependent on the length of the polyQ protein. While perlecan(ts)+Q40m animals exhibited a significant increase in ts phenotype, this was not the case for perlecan(ts)+Q24m animals in which less than 1% of animals displayed abnormal body shape (Fig. 2B). Therefore, the coexpression of an aggregation-prone protein is sufficient to cause a perlecan ts mutant phenotype to be exposed under permissive conditions.

A



UNC-52(su250) 15ºC



UNC-52(su250) 25ºC

B

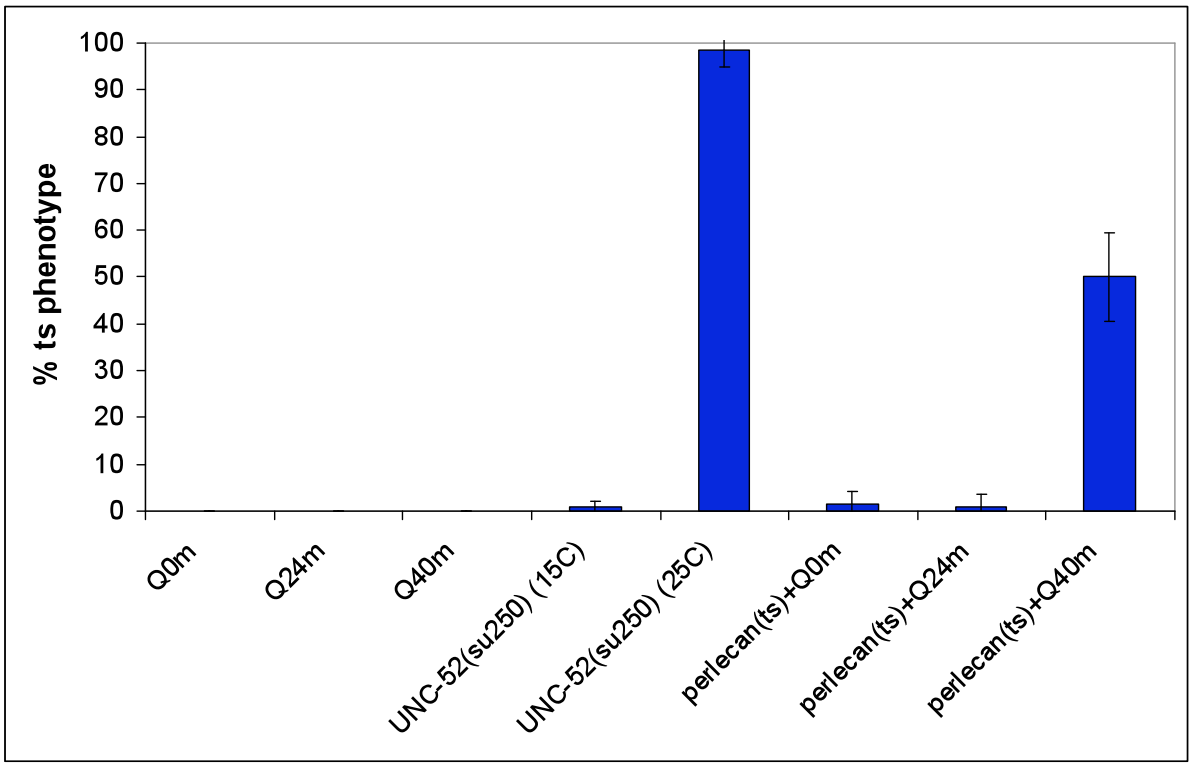


Figure 2. Aggregation-prone polyQ proteins expose the temperature-sensitive phenotype of perlecan(ts) mutants at permissive temperature. (**A**) UNC-52(su250) ts mutant phenotype. Animals display wildtype body shape at permissive temperature (15ºC). Animals show an abnormal, kinked body shape at restrictive temperature (25ºC). (**B**) Animals displaying an abnormal kinked body shape as well as stiff paralysis were scored as exhibiting the ts phenotype. Expression of polyQm (muscle)-YFP proteins (Q0m, Q24m, and Q40m) is from the unc-54 promoter. Animals were age-synchronized and scored as young adults. Experiments were conducted at 15ºC unless otherwise stated. Data are the mean ± SD. n ≥ 100 for each data point. Pictures courtesy of Dr. Anat Ben-Zvi.

Temperature-sensitive mutations in neuronal cells

Temperature-sensitive phenotype of dynamin(ts) mutants

To test whether the expression of aggregation-prone polyQ proteins would also expose a neuronal ts phenotype, we examined dynamin(ts) mutants in the background of neuronal polyQ proteins. dynamin(ts) mutants express a ts mutation in the neuronal protein dynamin-1, a GTPase that holds an essential role in an early step of clathrin-mediated endocytosis. Clark *et al*, (1997) investigated the expression pattern of dynamin and found it to be highly expressed in the nervous system, but not in body wall or vulva muscles (Clark *et al*., 1997). At permissive temperature (20ºC) dynamin(ts) mutants exhibit wildtype motility and body shape. However, when shifted to non-permissive temperature (28ºC) they rapidly become paralyzed and assume a kinked body shape in addition to a decreased pharyngeal pumping rate and a prolonged defecation cycle. In addition to the rapid onset of the ts phenotype, dynamin(ts) mutants are also able to recover within minutes of being shifted back to permissive temperature (Clark *et al*., 1997).

Animals were placed in the center of a 1cm diameter circle with a smooth lawn of OP50. Animals that did not move out of the circle within two minutes were scored as unc or exhibiting the ts phenotype. As expected, at 20ºC, less than 5% of dynamin(ts) mutants exhibit the ts phenotype, whereas at 28ºC, nearly 100% of dynamin(ts) animals were observed to be paralyzed within seconds of being transferred to a pre-warmed plate and failed to move out of the circle (Fig. 3). Pan-neuronal expression of Q19n and Q40n alone does not result in this phenotype (Fig. 3). Similar to the results obtained for dynamin(ts) mutants at permissive temperature, less than 10% of Q19n and Q40n animals show the ts phenotype.

Aggregation-prone proteins expose the dynamin(ts) mutant phenotype at permissive temperature

C. elegans expressing dynamin(ts) were crossed to animals expressing polyQ expansions in neuronal cells. The phenotype of double homozygotes was then examined at both permissive and restrictive temperatures. At permissive temperature, neuronal expression of Q40 (Q40n) in dynamin(ts) animals (dyanmin(ts)+Q40n) resulted in the same kinked body shape and slow movement and/or paralysis as dynamin(ts) mutants at restrictive temperature (Fig. 3). Similar to what was seen in muscle cells, this effect was dependent on the length of the polyQ protein because dynamin(ts) animals coexpressing nonaggregating Q19n did not exhibit any observable phenotype or uncoordination (Fig. 3). Thus, the coexpression of an aggregation-prone polyQ protein is sufficient to expose a dynamin ts mutation under permissive conditions. Therefore, similar results were obtained for both muscle and neuronal cells.

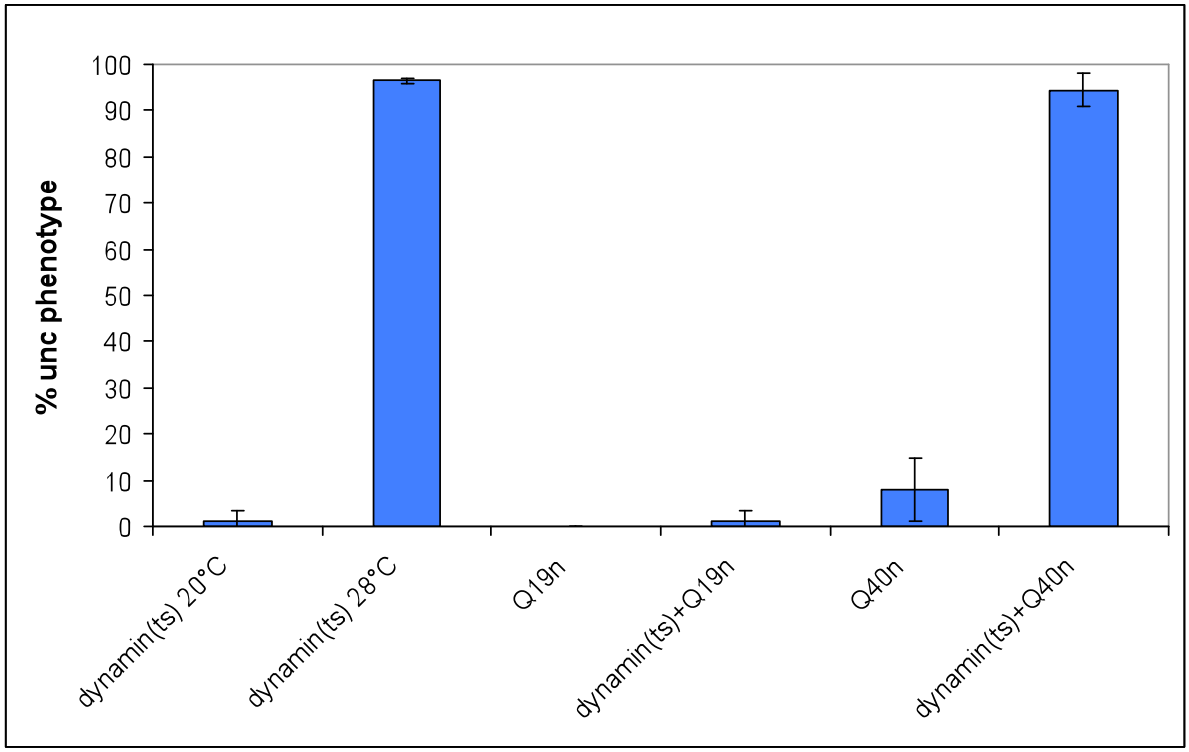


Figure 3. Aggregation-prone proteins expose the temperature-sensitive phenotype of dynamin(ts) mutants at permissive temperature. Animals were placed in the center of a 1cm diameter circle. Those that did not move out of the circle within two minutes were scored as unc. Expression of polyQn (neuronal)-YFP proteins (Q19n and Q40n) is from the F25B3.3 promoter. Animals are age-synchronized young adults. Experiments were conducted at 20ºC unless otherwise stated. Data are mean ± SD. n ≥ 80 for each data point.

Ras(ts) mutants: using tissue-specific phenotypes to test for cell-autonomous interactions

To address whether the interaction between aggregation-prone polyQ expansions and ts proteins is cell autonomous we used ras(ts) mutants, which are associated with several tissue-specific phenotypes at restrictive temperature and crossed them to animals expressing polyQ expansions in muscle and neuronal cells. Ras proteins are a family of GTPases that play a crucial role in numerous signal transduction pathways. At permissive temperature (20ºC), ras(ts) mutants exhibit wildtype behavior and development. However, at restrictive temperature (25ºC), ras(ts) mutants exhibit an embryonic lethality/larval development phenotype (Let/Lva), a dysfunction associated with muscle cells, a multivulva phenotype (Muv), reflecting a defect in the hypodermis, and an osmoregulation phenotype (Osm), a defect that is likely to reflect neuronal dysfunction (Eisenmann and Kim, 1997).

The Let/Lva ts phenotype: a ts mutation in muscle cells

The Let/Lva ts phenotype is characterized by unhatched embryos and developmental arrest prior to reaching reproductive adulthood. At permissive temperature, less than 10% of ras(ts) mutants exhibit the Let/Lva phenotype in contrast to nearly 100% of animals at restrictive temperature. The expression of polyQ expansions in muscle cells (Q24m and Q40m) in a wildtype background does not result in the Let/Lva phenotype. In contrast, 100% of homozygous ras(ts)+Q40m animals did not reach adulthood and approximately 50% of heterozygous ras(ts)+Q40m animals failed to reach adulthood. Furthermore, this observation was polyQ length-dependent because less than 20% of ras(ts)+Q24m animals exhibit the Let/Lva phenotype (Fig. 4B).

The Osm phenotype: a ts mutation in neuronal cells

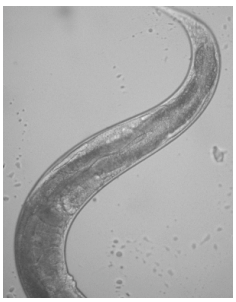
The Osm phenotype is characterized by a swollen, clear, fluid-filled body as well as kinked body movements when placed in distilled water (Fig. 4A). At permissive temperature, less than 10% of ras(ts) mutants display the Osm phenotype, but at restrictive temperature more than 90% of ras(ts) mutants exhibit the Osm phenotype. Pan-neuronal expression of Q19n and Q67n in a wild-type background does not result in the Osm phenotype. In contrast, more than 90% of animals coexpressing ras(ts) and Q67n displayed the Osm phenotype at permissive temperature. Additionally, this effect was polyQ length-dependent because ras(ts)+Q19n animals did not exhibit the Osm phenotype (Fig. 4B). Therefore, the expression of aggregation-prone polyglutamine expansions phenocopies a ts mutation at permissive temperature in neuronal cells as well.

Tissue specific interaction between polyQ proteins and ras(ts) proteins

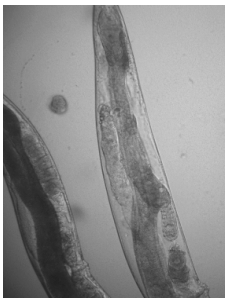
To determine whether or not the interaction between polyQ proteins and ts proteins is tissue specific we examined ras(ts)+Q40m animals and ras(ts)+Q67n animals. Although Q40m is aggregation-prone, there was not a significant percentage of ras(ts)+Q40m animals that displayed the Osm phenotype (Fig. 4B). Alternatively, the expression of Q40m in muscle cells exposes the Let/Lva phenotype in ras(ts) mutants, but has no effect on the Osm phenotype. Furthermore, although Q67n is aggregation-prone, there is not a significant percentage of ras(ts)+Q67n animals that exhibited the Let/Lva phenotype. Thus, the neuronal expression of aggregation-prone polyQ expansions exposes the Osm phenotype in ras(ts) animals under permissive conditions, but does not effect the Let/Lva phenotype. The hypodermal defect resulting in Muv phenotype was not observed for either neuronal or muscle expression of aggregation-prone

polyQ expansions in ras(ts) mutants. Therefore, the expression of an aggregation-prone protein in one tissue does not affect the function of a ts mutant protein in different tissue type, which indicates that there is a specific interaction between the polyQ expansion and the ts mutant protein.

A



ras(ts) 15ºC



ras(ts) 25ºC

B

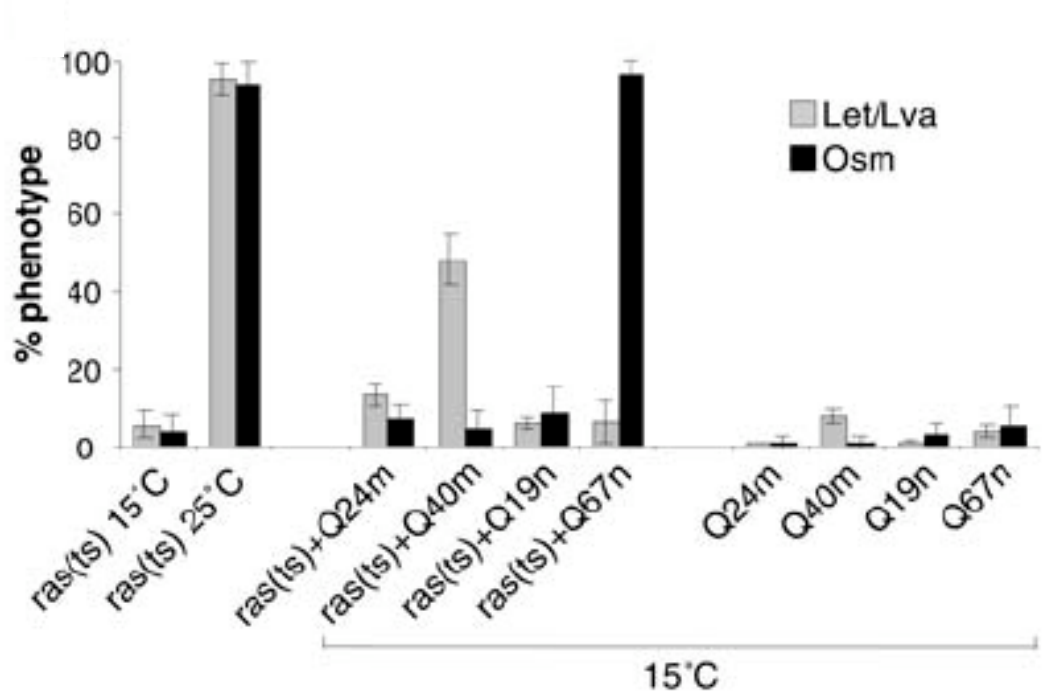


Figure 4. Aggregation-prone proteins expose the temperature-sensitive phenotype of ras(ts) mutants at permissive temperature in a cell-autonomous manner. (**A**) Osm phenotype of ras(ts) animals. At 15ºC, ras(ts) animals maintain a normal body shape when placed in distilled water. At 25ºC, ras(ts) mutants become swollen and fluid-filled. (**B**) Percentage of animals showing either the Osm (black) or Let/Lva (gray) phenotype. For the Let/Lva phenotype, embryos were picked as for egg hatching assay and the combined number of unhatched embryos and animals that died or arrested prior to reaching reproductive adulthood was scored. For the Osm phenotype, animals were placed in a droplet of distilled water. Those displaying a swollen, fluid-filled body and stiff movement were scored as having the Osm phenotype. Expression of polyQn (neuronal)-YFP protein (Q19n and Q67n) is from the F25B3.3 promoter. Expression of polyQm (muscle)-YFP protein (Q24m and Q40m) is from the unc-54 promoter. Data are the mean ± SD. n ≥ 70 age-synchronized adults for Osm. n ≥ 270 embryos for Let/Lva. ras(ts)+Q40m denotes animals heterozygous for Q40m. Figure 4 is published in Gidalevitz *et al*. (2006) and data were obtained in collaboration with Dr. Anat Ben-Zvi and Dr. Tali Gidalevitz. Pictures courtesy of Dr. Tali Gidalevitz.

The interaction between polyQ expansions and paramyosin(ts) proteins

To better understand the nature of the interaction between polyQ expansions and unrelated mutant ts proteins we examined the localization of paramyosin(ts) in paramyosin(ts)+Q40m animals at permissive temperature. At restrictive temperature (25ºC), instead of arranging into organized coiled-coil paramyosin interactions present in muscle sarcomeres, the mutant paramyosin(ts) protein assembles into abnormal paracrystalline structures (Fig. 5A). Furthermore, under permissive conditions, the abnormal paracrystalline assemblies were found to be distinct from the Q40m aggregates in paramyosin(ts)+Q40m animals (Fig. 5B). Importantly, when coexpressed with Q40m, paramyosin(ts) mutant protein displayed an altered protease sensitivity (Fig. 5C).

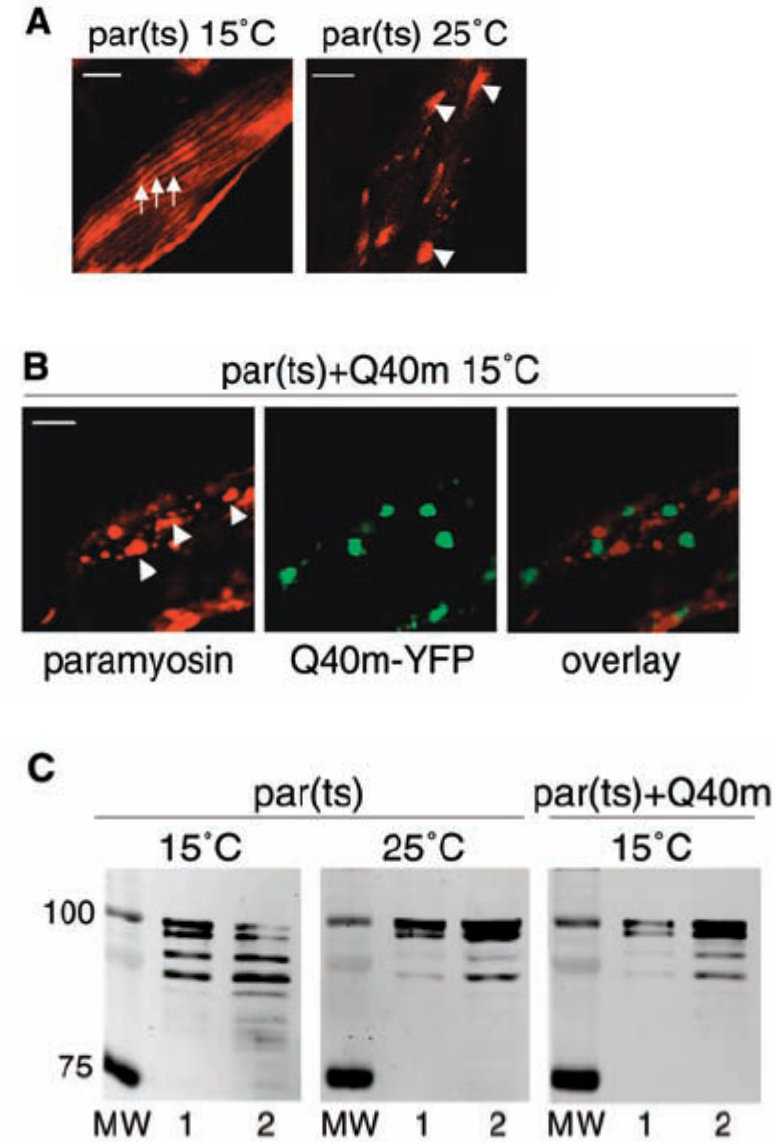


Figure 5. PolyQ expansions affect the folding of a paramyosin ts mutant. (**A** and **B**) Confocal images of antiparamyosin immunostained (red) body-wall muscle cells of age-synchronized young adult paramyosin(ts) animals. Arrows indicate normal muscle sarcomeres. Arrowheads indicate abnormal paraymosin assemblies. The paramyosin assemblies are distinct from Q40m-YFP aggregates (green). (**C**) Altered protease sensitivity of paramyosin(ts) protein. Lane 1, endogenous proteases. Lane 2, chymotrypsin. Figure 5 is published in Gidalevitz *et al*. (2006). Data for Fig. 5 were discussed in collaboration with Dr. Anat Ben-Zvi and Dr. Tali Gidalevitz and experiments were conducted by Dr. Anat Ben-Zvi (Fig. 5, A and B) and Dr. Tali Gidalevitz (Fig. 5C).

Temperature sensitive proteins modify polyQ aggregation

Animals heterozygous for Q40m exhibit fewer number of aggregates compared to animals homozygous for Q40m (Fig. 6, A, B, and E). Aggregation was further enhanced when Q40m was expressed in the background of ras(ts) as well as paramyosin(ts) animals (Fig. 6, C to E).

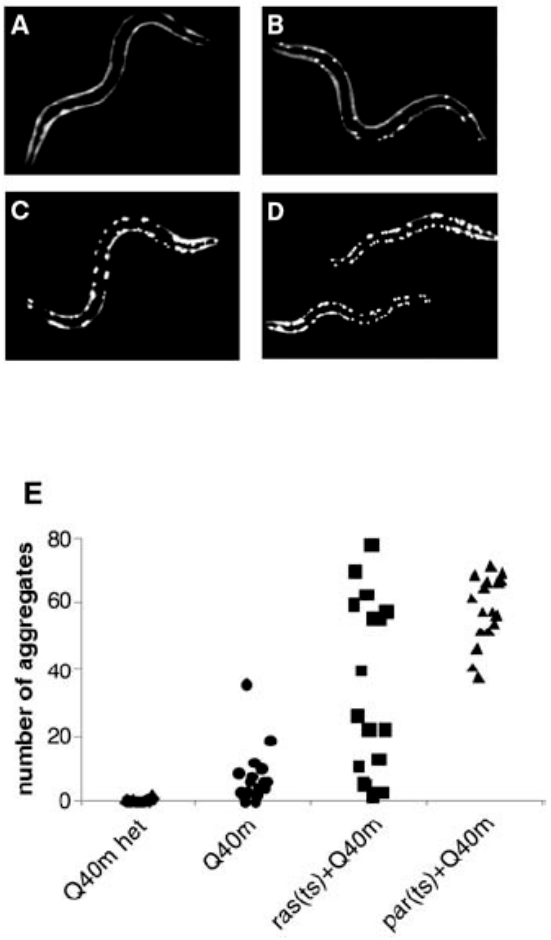


Figure 6. Temperature-sensitive mutations in unrelated proteins modify polyQ aggregation. (**A**) Fluorescent image of a heterozygous Q40m L2 animal at permissive temperature. (**B**) Fluorescent image of a homozygous Q40m L2 animal at permissive temperature. (**C**) Fluorescent image of a L2 ras(ts)+Q40m animal at permissive temperature. (**D**) Fluorescent image of a L2 paramyosin(ts)+Q40m animal at permissive temperature. (**E**) Number of visible aggregates in L2 animals expressing the indicated protein. Figure 6 is published in Gidalevitz *et al*. (2006). Data for Fig. 6 was discussed in collaboration with Dr. Anat Ben-Zvi and Dr. Tali Gidalevitz and experiment was conducted by Dr. Tali Gidalevitz.

DISCUSSION

We found that the expression of aggregation-prone polyQ proteins is sufficient to expose a ts mutation in both muscle and neuronal cells even under permissive conditions. To ensure that these observations were applicable to a range of ts mutations, we scored a variety of strains expressing different muscle as well as neuronal mutations (UNC-15(e1402), UNC-54(e1301), UNC-54(e1157), UNC-52(su250), UNC-45(e286), LET-60(ga89), and DYN-1(ky51)) with different length polyQ expansions at permissive temperature. For all the lines examined, in the ts background the presence of an aggregation-prone polyQ protein, but not the non-aggregating Q24m or Q19n, the ts mutant phenotype was exposed under permissive conditions. Thus, expression of aggregation-prone, but not the non-aggregating protein, in the ts background phenocopies the ts phenotype at permissive temperature. Therefore, the expression of an aggregation-prone protein can interfere with the function of a variety of proteins that are both structurally and functionally unrelated.

Furthermore, we found that although Q40m is aggregation-prone, in the ras(ts) background it does not expose the Osm phenotype under permissive conditions. Only the neuronal expression of Q67n in ras(ts) mutants exposes the Osm phenotype at permissive temperature. The coexpression of Q40m in ras(ts) mutants, on the other hand, exposes the Let/Lva phenotype in ras(ts) animals under permissive conditions, while the pan-neuronal expression of Q67n has no significant effect on the Let/Lva phenotype in ras(ts) mutants. Thus, the expression of an aggregation-prone protein in one tissue does not affect the function of a ts mutant protein in another tissue. Therefore, the interaction between polyQ proteins and ts

proteins is tissue specific, indicating that the polyQ protein and the ts protein interact in a specific manner.

To better understand this specific interaction, we examined the localization of mutant paramyosin(ts) protein when coexpressed with Q40m. At restrictive temperature, instead of arranging into coiled-coil interactions present in muscle sarcomeres, the mutant protein assembles into abnormal paracrystallin structures suggesting that paramyosin(ts) protein is not in its native conformation. Furthermore, when coexpressed with Q40m, paramyosin(ts) mutant protein displayed an altered protease sensitivity. Interestingly, the paramyosin paracrystallin structures are distinct from Q40m aggregates indicating that Q40m aggregates do not recruit paramyosin(ts) protein into its aggregates. This then suggests that the expression of aggregation-prone Q40m does not directly interfere with paramyosin(ts) protein, but instead disrupts the general protein folding environment of the cell. Therefore, the coexpression of an aggregation-prone polyQ protein such as Q40m is sufficient to expose the folding defect in paramyosin(ts) mutant protein under permissive conditions. Interestingly, the various ts proteins that were used exhibited differential penetrance of ts phenotypes when coexpressed with aggregation-prone polyQ expansions. The differential penetrance may indicate that different ts proteins can tolerate different levels of disruption in their cellular folding environment.

While previous studies focused on direct interactions as the basis of toxicity in neurodegenerative diseases, the expression of polyglutamine expansions may compromise the folding environment of the cell by causing a global perturbation in cellular protein folding quality control. If this is so then the levels of aggregation-prone proteins would have differential effects on the penetrance of the ts phenotype. When coexpressed with ras(ts), animals homozygous for Q40m showed 100% ts phenotype, while heterozygous animals, which exhibit

fewer number of aggregates, showed less than 50% ts phenotype. We have also found that the coexpression of ts proteins significantly enhances the aggregation of polyglutamine proteins. Such findings suggest that ts mutations in proteins unrelated to the protein folding homeostatic machinery as well as any polymorphism that slightly destabilizes proteins can modify polyQ aggregation. Thus, both the presence of polyQ expansions and ts mutations in proteins disrupt protein folding homeostasis reflecting cellular sensitivity to the chronic expression of misfolded proteins. Misfolded proteins are normally detected and eliminated by the protein folding quality control machinery in order to reestablish folding homeostasis. Accordingly, the expression of misfolded proteins in conformational diseases suggests that the expression of misfolded proteins alone fails to activate the homeostatic machinery or that the homeostatic response is unable to effectively rebalance the folding environment. Therefore, disease may be a result of the gradual accumulation of misfolded proteins, which disrupt the balance maintained by chaperones and the ubiquitin-proteasome system. The gradual accumulation of misfolded proteins and disruption to folding homeostasis may provide an explanation for the late onset of many conformational diseases.

A screen by Nollen *et al*. (2004) in *C. elegans* revealed that nearly 200 genes had the potential to modify polyQ aggregation. In addition to the expected genes encoding chaperones and proteins involved in the ubiquitin-proteasome machinery, Nollen *et al*. (2004) identified a plethora of genes encoding proteins that were unexpected to play a role in cellular protein folding. Our results suggest that the presence of metastable proteins that are unrelated to folding homeostasis have the ability to modify polyQ aggregation and toxicity. The cellular protein quality control machinery is highly sensitive to changes in the environment and relies on a delicate balance of not just chaperones and elements of the ubiquitin-proteasome system, but

almost all aspects of cellular machinery in order to maintain protein homeostasis. Therefore, toxicity in conformational diseases is unlikely to be due to a single factor or dysfunction, but may be attributed to a more global perturbation of cellular protein folding homeostasis.

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EDUCATION

Northwestern University Feinberg School of Medicine Chicago, IL
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2003-2007 Northwestern University Evanston, IL
Bachelor of Arts in Biological Sciences and Religion

- Member of the Freshman Honor Society, Alpha Lambda Delta
- Awarded an Undergraduate Research Grant by Northwestern University Fall 2005
- Awarded the B.J. Martin Scholarship for the 2005-2006 academic year
- Awarded the University Guild Scholarship for the 2006-2007 academic year
- Member of Phi Beta Kappa Society
- Cummulative GPA: 3.870

1999-2003 Niles West High School Skokie, IL
High school diploma

- President of the National Honor Society
- Winner of the Bausch and Lomb Honorary Science Award
- Cumulative GPA: 4.00

EXTRACURRICULAR ACTIVITIES

Alternative Student Break (ASB) Executive Board member 2006-2007 (Fundraising Coordinator): responsible for coordinating the organization's fundraising efforts including football concessions and an annual Winter and Spring fundraiser.

Morimoto Undergraduate Research Seminar 2005-2007 (Director): a weekly journal club organized by and for undergraduate students involved in independent research at the Morimoto Research Lab at Northwestern University.

Laboratory Adventures in Biological Sciences (L.A.B.S.) Teaching Assistant 2006, 2007: helped middle school students carry out laboratory exercises in molecular biology, genetics, and cell biology.

Slivka Academic Affairs Committee 2005 (Diversity Chair): organize firesides with professors, plan diversity/multi-cultural events, and arrange trips to academically-oriented activities such as museum visits.

Slivka Social Affairs Committee 2003-2004: organized various dorm as well as university-wide social events such as movie nights, firesides, munchies, and the annual "Pie the Prof" day.

COMMUNITY ACTIVITIES

Winter 2006 Alternative Student Break (ASB) – Baltimore, Maryland – We worked at Our Daily Bread (a soup kitchen that serves 600-800 people a day), Sarah's House (a transitional housing program for homeless women, children, and men where we helped prepare meals and tutor students), and the Hispanic Apostolate where we taught English as a Second Language. (Site Leader Position)

Winter 2005 ASB – Boston, Ma. – We lived and worked at The Boston Living Center serving meals and helping with grocery distribution while interacting with members of the HIV/AIDS Wellness Center. (Site Leader Position)

Dance Marathon (DM) 2005, 2006, 2007 – a school-wide student-run philanthropy event that raises money for selected organizations. DM 2005 raised over $625,000 for the Juvenile Diabetes Research Foundation and Evanston Community Foundation (ECF). DM 2006 raised over $680,000 for ECF and Pediatric AIDS Chicago Prevention Initiative. The beneficiaries for DM 2007 are ECF and CURE: Citizens United for Research in Epilepsy Foundation.

Spring 2005 ASB - Trip to Tahlequah, Oklahoma- renovated and repaired homes for members of the Cherokee Nation, participated in cultural immersion activities such as touring the Cherokee museum and complex, attending a night of story telling, and speaking to someone from the Nation's law and justice department.

Winter 2004 ASB - Trip to Arcadia, Florida to aid in hurricane relief and rebuilding efforts

Habitat for Humanity 2003 – Homestead, Florida – helped build homes for low-income families

RESEARCH EXPERIENCE

2005-2007: Independent Researcher, PI: Richard I. Morimoto (Dept. of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL.). Studied the effects of aging modulators on the interaction between disease-related misfolded proteins and temperature sensitive proteins.

2005-2006: Independent Researcher, PI: Richard I. Morimoto (Dept. of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL.). Investigated the effects of the presence of disease-related misfolded proteins on cellular protein-folding

homeostasis using temperature sensitive proteins.

PUBLICATIONS

Gidalevitz T, Ben-Zvi A, Ho KH, Brignull HR, Morimoto RI. "Progressive disruption of cellular protein folding in models of polyglutamine diseases." *Science*. 10 Mar. 2006.