CHARACTERIZATION OF HYPERTONIC STRESS-INDUCED PROTEIN DAMAGE AND THE CELLULAR MECHANISMS FOR DEFENSE AND REPAIR IN *C. ELEGANS*

By

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LIST OF ABBREVIATIONS

ADH	Antidiuretic hormone
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
C	Celsius
CFP	Cyan fluorescent protein
CFTR	Cystic fibrosis transmembrane conductance regulator
Cl	Chloride
CQ	Chloroquine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
eIF	Eukaryotic initiation factor
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
F	Fluoride
FRAP	Fluorescence recovery after photobleaching
g	Gram
GFP	Green fluorescent protein

GPDH	Glycerol 3-phosphate dehydrogenase
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
h	Hour
H	Hydrogen
Hos	Hypertonic sensitive
HSP	Heat shock protein
K	Potassium
MES	2-(N-morpholino)ethanesulfonic acid
M	Molar
Mg	Magnesium
l	Liter
min	Minute
mOsm	Milliosmole
mRNA	Messenger RNA
Na	Sodium
NGM	Nematode growth medium
O	Oxygen
PAGE	Polyacrylamide gel electrophoresis
PCA	Perchloric acid
Q35	35 glutamines
RFP	Red fluorescent protein
rgpd	Regulators of gpdh-1

RNA	Ribonucleic acid
RNAi	RNA interference
S	Sulfur
SDO	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
SE	Standard error
sHSP	Small heat shock protein
tRNA	transfer RNA
Ub	Ubiquitin
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
YFP	Yellow fluorescent protein

CHAPTER I

INTRODUCTION

Overview of Proteostasis

Summary

Eukaryotic cells express thousands of unique proteins with individual copy numbers ranging from just a few per cell to greater than a million (Ghaemmaghami et al., 2003). Collectively, these proteins comprise the cellular proteome. The proteome is the workhorse of the cell, carrying out nearly every task required for growth and viability. The health and functionality of the proteome is therefore of paramount importance. The preservation of proteomic integrity is called protein homeostasis, or proteostasis.

Proteostasis must be faithfully maintained by a complex and interacting set of genes and processes termed the proteostasis network (Balch et al., 2008). The proteostasis network controls the proper concentration, structure, protein-protein interactions, and localization of all cellular proteins. Successful proteostasis enables cell function and differentiation, thus leading to productive organismal development and healthy aging. Proteostasis deficiencies are implicated in a wide range of pathophysiological conditions, including several neurodegenerative diseases and systemic amyloidoses, amyotrophic lateral sclerosis, type II diabetes, cancer, cystic fibrosis, and cardiovascular disease,

among others (reviewed in Selkoe, 2003). This large and growing number of proteostasis-related pathologies is more generally referred to as the family of proteostasis diseases. Interestingly, there is also abundant evidence to suggest that declining function of the proteostasis network may underlie the process of aging (e.g., Morley et al., 2002; Morley and Morimoto, 2004; Arslan et al., 2006; David et al., 2010; Hartl et al., 2011; Koga et al., 2011). Therefore, defining the mechanisms by which cells mobilize components of the proteostasis network in response to acute and chronic challenges is essential for the development of therapies for proteostasis disease as well as for our understanding of aging. On a broader scale, understanding how cells generate and maintain such a dynamic, complex, and delicate proteome is one of the most fundamentally and clinically important pursuits in biology.

Proteostasis Is Constantly Threatened

A combination of sequence and conformation define a protein's identity by determining its interactions, localization, and function (e.g., Anfinsen, 1973; Chang et al., 1978). The first threat to structure or function that a protein encounters is an error in its synthesis. Protein molecules are chains of amide-linked amino acids with genetically encoded sequences. The length of these chains ranges between tens and tens of thousands of amino acids. The final amino acid sequence of a protein is determined through a series of biosynthetic processes, including mRNA transcription, splicing and editing of the mRNA to its mature form, and translation of the mRNA code into an amino acid sequence by

the ribosome. None of these processes occurs without error, but translational mistakes are estimated to be the most frequent (Drummond and Wilke, 2009). An experimentally derived error rate of 1 mistake per 1,000 to 10,000 amino acids leads to the prediction that 15% of proteins contain at least 1 misincorporated amino acid (Drummond and Wilke, 2009).

Once translated, these linear chains of amino acids fold into unique and specific three-dimensional conformations. The fold of a protein is determined in large part by its sequence (Anfinsen, 1973). Thus, errors in the amino acid sequence often destabilize protein structure. The combination of synthesis errors and the inherent difficulties of folding flexible polypeptides into specific conformations results in a surprisingly high failure rate. In some cell types, 30% of nascent polypeptides fail to mature into functional proteins (Schubert et al., 2000).

Due to the technical difficulties of studying *de novo* protein folding, our ability to define the pathways by which nascent polypeptides acquire their native conformations remains limited. The protein folding reaction is thought to occur largely through the collapse of hydrophobic amino acids into the core of the protein, thus sequestering them away from water at the protein-solvent interface. An assortment of intramolecular contacts further contributes to defining a protein's functional conformation. These intramolecular interfaces take various forms, including hydrogen bonds, disulfide bonds, and various electrostatic interactions (Lins and Brasseur, 1995). Despite these internal features designed to help define a protein's folding pathway, however, every protein possesses the

capacity to form numerous unfolded and misfolded structures. Additionally, seemingly minor changes to the protein sequence or folding environment can drastically alter conformation to favor misfolded and non-functional states (Ghosh and Dill, 2010).

Given the essential nature of many proteins, one might assume that these molecules would evolve to be extremely stable. However, most proteins are dynamic and must also possess a significant degree of conformational plasticity. This plasticity enables the spatiotemporal regulation of protein function and allows proteins to interact with a wider range of substrates. Resulting in part from this need for conformational flexibility, thermodynamic barriers between folded and misfolded structures are relatively small. Using a model derived from experimentally determined thermodynamic stabilities, Ghosh and Dill (2010) estimate that a significant subpopulation of proteins, ~15% of the proteome, is only marginally stable in the native conformation. Possessing stabilities below 4 kcal/mol, these proteins are susceptible to denaturation even during mild stress, such as a shift in temperature of only 4 °C. This problem is exacerbated by the intracellular folding environment, which is complex and crowded. High concentrations of protein as well as the constitutive flux of metabolites, osmolytes, and reactive oxygen species, are all capable of affecting protein folding pathways and conformational stability. In such an environment, forming the proper ensemble of weak intramolecular interactions required for successful protein folding is far from guaranteed. Thus, given the number of potential problems a protein faces during biosynthesis and maturation, Ghosh and Dill

(2010) conclude that even under normal condtions, “cells live on the edge of a proteostasis catastrophe.”

In addition to the difficulties of maintaining proteostasis that are intrinsic to normal cellular life, there are also external threats. These types of threats include mutation, infection, and a variety of environmental stressors. Mutations impact conformation and/or stability through a change in the final amino acid sequence of a protein. Unlike random errors in synthesis, mutation results in an aberrant sequence element in every copy of the protein, leading to a much higher concentration of unstable proteins in the cell. Pathogens, especially viruses, are adept at hijacking cellular proteostasis machineries for their own growth and replication at the expense of the endogenous proteome (Shackelford and Pagano, 2005; Geller et al., 2012; Nag and Finley, 2012). Lastly, changing environmental conditions can strongly impact cellular proteostasis through direct perturbation of the protein folding environment. For example, increased heat causes protein unfolding, while excessive oxygen radicals react with amino acid sidechains to alter their intramolecular interactions and folding properties (Levine and Stadtman, 2001).

The Toxic Consequences of Proteostasis Failure

When a protein does misfold, the consequences vary depending on the protein at risk, cell type, and integrity of the cellular defense network. If the misfolded protein cannot be salvaged and refolded, one scenario is simply the loss of function. Maladies sharing this etiology are called loss-of-function

proteostasis diseases and include many heritable genetic disorders. The chief example of this type of disease is cystic fibrosis, which involves mutation-induced misfolding and premature degradation of a chloride channel.

If a mutation causes total loss of function for an essential gene, the consequences are obviously dire. However, even in the case of non-essential genes, the burden that protein misfolding places on a cell extends beyond a simple loss of function. Converting DNA code into a mature protein requires huge investments from the cellular energy stores, but this energy is wasted when the protein product is non-functional. Further depletion of resources occurs when the cell is forced to use energy-dependent proteostasis machinery to repair or degrade the misfolded protein. Thus, misfolded proteins reduce cell output and ultimately the fitness of the organism, costing finite resources both to produce and then immediately degrade the defective gene product.

Alternatively, when some proteins misfold they acquire conformations that are inherently toxic to the cell. The toxicity of these proteins is manifested in so-called gain-of-function proteostasis disease. Misfolded conformers of proteins in gain-of-function diseases expose amino acid residues which are normally buried inside the protein. Exposure of these buried residues is often thermodynamically unfavorable and therefore promotes non-native interactions with other proteins in the cell. These non-native interactions initiate a pathway of aberrant oligomerization and subsequent formation of large protein aggregates (e.g., Bauer et al., 2000; Kopito, 2000). Depending on the conditions and proteins involved, the aggregates form different types of structures with correspondingly

unique properties (Gidalevitz et al., 2009; Fandrich, 2007). One of the most commonly observed forms of aggregation is the amyloid fibril. Amyloid fibrils are highly stable β-sheet structures which are insoluble and protease-resistant (Fandrich, 2007). Nearly every human protein contains a minimal peptide sequence capable of forming amyloid-type aggregates, but normally these are only exposed when misfolding occurs (Goldschmidt et al., 2010).

The exact mechanism by which these misfolded and aggregated proteins impart their toxic effects is currently an area of intense investigation. Hypotheses include roles for both the soluble oligomer intermediates and the deposits of proteins in large, insoluble aggregates. An idea gaining traction in the field is that the formation of aggregates may actually protect cells from more reactive, soluble oligomers (Bucciantini et al., 2002; Kayed et al., 2003; Cohen et al., 2006; Campioni et al., 2010). Whatever the causative species, toxicity is thought to involve inhibition, misfolding, or sequestration of other essential proteins in the cell. Examples of affected proteins are known to include essential transcription factors as well as core components of the proteostasis network (e.g., Suhr et al., 2001). Loss of such functions places significant burden on the cell's homeostatic capacity. Additionally, evidence that quality control components are sequestered provides support for the observation that misfolding and aggregation of one protein species can trigger the global collapse of the proteostasis network and an aggregation cascade involving many additional proteins (Satyal et al., 2000; Ben-Zvi et al., 2009).

Typically, loss of proteostasis results in cell death and tissue degeneration such as that observed in Alzheimer's, Parkinson's or Huntington's disease, among others. Preventing proteostasis collapse is therefore of critical importance. To this end, cells possess a complex and dynamic system which is capable of sensing specific types of insults and producing tailored responses to maintain the proteome against these various threats. This system is the proteostasis network (Balch et al., 2008). Importantly, however, there are a number of contexts (e.g., prion disease, neurodegenerative disease, aging, etc.) in which proteostasis fails. Exactly how or why the proteostasis network fails in these instances remains unknown.

The Proteostasis Network Maintains the Proteome

Components of the Proteostasis Network

The proteostasis network encompasses a broad array of genes and activities responsible for maintaining the various properties of the proteome (Balch et al., 2008; Figure 1). The composition of the proteome must be dynamic to allow progression through development and adaptation to different physiological and environmental conditions. This feature requires that the proteostasis network itself be dynamic. The processes making the greatest contributions in regulating the concentration, conformation, and overall health of the proteome include protein synthesis and quality control mechanisms (Balch et al., 2008). Protein quality control can be described further as the balanced

activities of protein chaperoning and degradation. Therefore, protein synthesis, chaperoning, and degradation represent the three core branches of the proteostasis network.

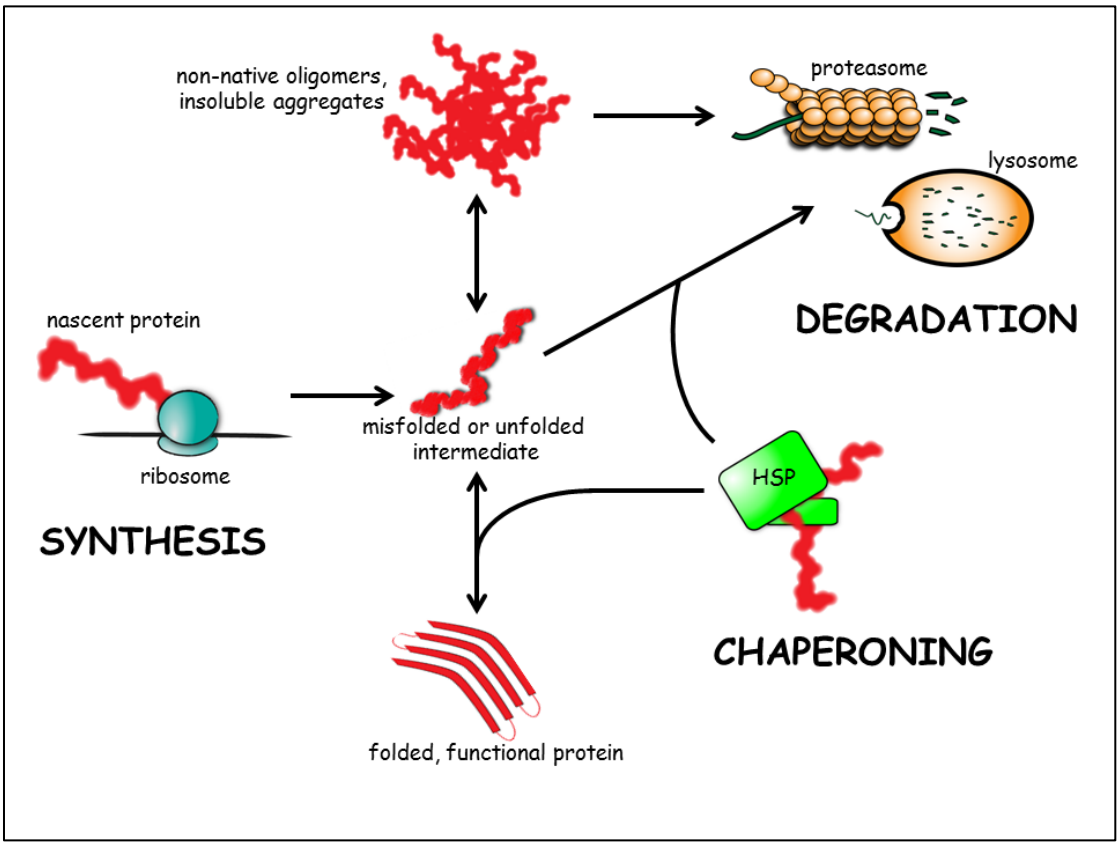

Figure 1. Simplified model of proteostasis. Nascent proteins synthesized at the ribosome must fold into their native conformation to become functional. Misfolded and partially unfolded intermediates are recognized and refolded by chaperones or run the risk of forming toxic aggregates. Aggregated proteins and proteins unable to attain their native conformation are cleared from the cell by proteasomal or lysosomal degradation. Degradation pathways in some cases involve chaperone-dependent recognition and trafficking. Factors including age, certain mutations, and proteotoxic stressors increase the occurrence of misfolding and aggregation in cells. If the proteostasis network, whose core components include the synthesis, degradation, and chaperoning machineries, is unable to successfully prevent, salvage, or clear the damage, cell death and/or various types of proteostasis disease may occur.

Role of Protein Synthesis in Proteostasis

Regulation of mRNA transcription represents the first step at which a cell controls protein synthesis. mRNA fidelity is achieved through transcriptional, splicing, and mRNA processing activities. Accuracy at this stage is essential, as the mRNA nucleotide sequence is next translated into an amino acid sequence by the ribosome. In this process, a series of translation initiation factors first recognize specific mRNA structures and form a complex on the mRNA to direct recruitment of the smaller 40S ribosomal subunit. Formation of this initiation complex is one of the primary mechanisms by which cells globally regulate the rate or amount of translation (Gebauer and Hentze, 2004). Once bound to the mRNA molecule, the 40S subunit scans the mRNA for the translation start site, and upon recognition, the larger 60S subunit is recruited to generate the catalytic 80S ribosome. After initiation, translation continues through an elongation phase where tRNAs carry amino acids to the ribosome for incorporation into a growing polypeptide chain. Finally, the ribosome reaches a stop codon in the mRNA molecule, upon which translation is terminated and the nascent protein is released.

Regulating protein translation is an essential element in the overall control of proteostasis. In response to growth signals or stressful conditions, the global translation rate is often altered through mechanisms typically involving translation initiation factors (reviewed in Gebauer and Hentze, 2004; Holcik and Sonenberg, 2005). This type of response allows a cell to optimize its metabolism either for maximizing growth and proliferation when conditions are favorable, or instead for

conserving resources and maintaining essential processes until the external environment improves.

Reducing global protein translation during proteotoxic stress has been observed previously, but precisely how this improves proteostasis at the molecular level is still largely theoretical (Panniers, 1994; Holcik and Sonenberg, 2005; Hipkiss, 2007; Yamasaki and Anderson, 2008). Translation is a very expensive process with regard to cellular energy utilization. Estimates of the fraction of cellular ATP spent on protein synthesis varies by species and cell type, but generally translation consumes one quarter to one third of total energy usage, making it one of the two most expensive processes in the cell (Hand and Hardewig, 1996; Rolfe and Brown, 1997). Therefore, one possibility is that suppressing translation frees up a large pool of energy resources for use in other areas, such as damage repair or removal. Secondly, suppressing global translation in some cases allows for the selective synthesis of stress-responsive mRNAs (e.g., Harding et al., 2000; Holcik et al., 2000). This mechanism allows the cell to prioritize production of specific repair or defense proteins in order to improve the speed and magnitude of the stress response. Lastly, nascent proteins often require assistance from other chaperone proteins in attaining the native fold (Thulasiraman et al., 1999; Albanese et al., 2006). Suppressing translation may therefore reduce the concentration of nascent proteins, which are most prone to misfolding and aggregation. Reducing nascent proteins may also free a subset of chaperone proteins to respond to protein damage elsewhere in the cell. These hypotheses are not mutually exclusive, but existing data is

currently too limited to favor any one of these molecular mechanisms over the others. Importantly, an answer to this question will have far-reaching implications in the development of therapies for proteostasis diseases.

Chaperones Support Folding and Prevent Aggregation

A combination of chaperoning and removal processes comprises the cell's protein quality control system, a vital component of the proteostasis network (Balch et al., 2008). The quality control system ensures that all proteins either attain their functional fold or are removed from the cell. The folding aspect of the quality control system is carried out by a large subset of cellular proteins, commonly referred to as protein chaperones or heat shock proteins (HSPs).

The protein chaperone family is very large, diverse, and evolutionarily conserved. There are over a hundred protein chaperone genes in many metazoans, although different chaperones often function somewhat redundantly. Many protein chaperones are constitutively expressed at high levels and are generally thought to function as a buffer against the accumulation of misfolded or damaged proteins (Rutherford and Lindquist, 1998; Bukau et al., 2006). Stressors known to cause damage to proteins often induce greater accumulation of certain chaperones, thus increasing the capacity of the cell's protective "buffer" in environments that are harmful to protein folding (e.g., Bjork and Sistonen, 2010; Richter et al., 2010). Additionally, most chaperones interact with short hydrophobic or basic sequences in client proteins, thus allowing chaperones to

specifically target misfolded proteins exposing amino acid residues which are normally buried in the core of the client protein (reviewed in Hartl et al., 2011).

Some notable protein chaperones include the HSP70 and HSP90 proteins, small HSPs (sHSPs) such as the α-crystallins, and the chaperonins. The HSP70 and 90 proteins act as central players in the folding and macromolecular assembly pathways of many proteins and often cooperate with other co-chaperones in determining substrate specificity (Hartl et al., 2011). HSP70 and 90 actively bind and alter the local structure of client proteins through ATP-regulated conformational changes in the chaperone protein itself (Mayer, 2010). HSP70 and 90 are both essential proteins which are constitutively expressed as well as stress-inducible under some conditions (Hartl et al., 2011). Alternatively, the mechanisms of sHSPs and chaperonins are focused more on sequestration of misfolded or nascent proteins both to prevent non-native interaction with other proteins and to maintain the client protein in an intermediate state that can be salvaged by refolding (Haslbeck et al., 2005; Hartl et al., 2011). sHSPs function as ATP-independent 'holdases'; that is, they bind non-native proteins to prevent them from interacting with anything else in the cell (Haslbeck et al., 2005). Both activity and expression of sHSPs are highly stress-inducible. Chaperonins form a multi-subunit barrel-shaped complex which can completely encapsulate proteins in an ATP-dependent cycle (Hartl et al., 2011). This encapsulation shields the unfolded protein from the crowded environment of the cytosol and potentially allows for multiple attempts at reaching the native state. Chaperonins are thought to be especially important for co-translational or

de novo protein folding. In support of this notion, chaperonins couple functionally and perhaps physically with ribosomes (Thulasiraman et al., 1999; Albanese et al., 2006).

In addition to protein chaperones, cells also contain small molecules which can significantly impact protein folding. These small molecules are more commonly called chemical chaperones. Like protein chaperones, the synthesis or uptake of chemical chaperones is induced by certain environmental condtions with the potential to perturb protein folding (Yancey, 2005).

Organic osmolytes represent one class of small molecule thought in many cases to possess chemical chaperone activity. Organic osmolytes generally fall into one of 3 broad classes: polyols, amino acids and their derivatives, and methylamines. Almost all naturally occurring osmolytes are called “compatible” or “non-perturbing” for their ability to accumulate to concentrations reaching tens or hundreds of mM without negatively impacting protein function (Brown and Simpson, 1972; Strange, 2004; Yancey, 2005). Because proteins and other macromolecules are stable over wide-ranging concentrations of osmolytes, these molecules are a primary mechanism by which cells control internal osmotic pressure (reviewed in Strange, 2004; Yancey, 2005).

Osmolytes and their effects on proteins have been studied for many years with much of the research skewed toward identifying stabilizing versus destabilizing interactions (reviewed in Singh et al., 2011). An explanation for these stabilizing effects is not fully clear and may differ from osmolyte to osmolyte. Generally, however, the beneficial effects of these molecules on

protein folding are thought to arise from interactions with either the protein surface or the solvent (i.e., water). Typically, chemical chaperones are repulsed or excluded from a protein's hydration layer, or interaction surface between the protein and water. This preferential exclusion of the chaperone or osmolyte, termed the osmophobic effect, thermodynamically favors hydration and increased solubility for the protein (reviewed in Bolen and Baskakov, 2001). In contrast, destabilizing molecules like certain salts and urea interact directly with the protein backbone. This interaction favors unfolded protein conformations which expose greater amounts of the backbone than folded or compact conformations (Bolen and Baskakov, 2001).

The observation that many osmolytes can stabilize proteins and prevent aggregation in vitro has led to the hypothesis that they may also act as chemical chaperones inside cells (Somero, 1986; Tatzelt et al., 1996; Welch and Brown, 1996; Ignatova and Gierasch, 2006). This line of thought has spurred further research into the possible use of chemical chaperones as pharmacological stabilizers of damaged proteins in proteostasis diseases (Welch and Brown, 1996; Balch et al., 2008). In this pursuit, success has been realized in various models of protein misfolding diseases, including prion disease and cystic fibrosis (e.g., Brown et al., 1996; Tatzelt et al., 1996; Tanaka et al., 2004; Ryu et al., 2008; Seki et al., 2010).

However, some recent observations have begun to cast doubt on the idea that naturally occurring osmolytes are functioning as chemical chaperones in vivo. One potential problem is that the vast majority of research on protein

protection by osmolytes has been performed in vitro. Additionally, the effects of osmolytes and/or chemical chaperones appear to be highly dependent on the context of the protein under study, concentration of the chaperone, and the solvent environment (Singer and Lindquist, 1998; Faber-Barata and Sola-Penna, 2005; Natalello et al., 2009; Singh et al., 2011). Reports indicate that molecules thought to be chaperones in some cases will actually promote aggregation or dysfunction of other proteins or in different contexts (e.g., Yang et al., 1999; Faber-Barata and Sola-Penna, 2005). Considering the sequence and structural diversity of the proteome and the range of intracellular conditions across tissue types, the correlation between in vitro findings and in vivo reality for chemical chaperones may be limited. Furthermore, accumulation of high osmolyte concentrations is only observed in nature during exposure to stress. This suggests that these molecules may somehow be detrimental to cell health under normal conditions (Yancey, 2005). Therefore, though chemical chaperoning is an intriguing component of proteostasis, the long-standing assumption that osmolytes are actually stabilizing the proteome against misfolding and aggregation needs to be reassessed with greater focus on in vivo models.

Clearance of Protein Damage

Due to the large investment of energy and resources into protein synthesis, refolding is thought to be the preferred pathway for preventing the accumulation of misfolded proteins. When proteins can't be salvaged or the chaperoning system is overwhelmed, however, the lysosomal and ubiquitin-

proteasome systems (UPS) are capable of identifying and degrading misfolded proteins (reviewed in Ciechanover, 2005). Importantly, the actions of these systems are not restricted to stressful conditions. These degradation pathways are also employed for the normal regulatory turnover of undamaged proteins inside the cell.

The lysosome is an acidic, membrane-bound organelle containing its own complement of proteases. There are multiple pathways by which a damaged protein may reach the lysosome, and some begin with the identification of misfolded and/or damaged proteins in the cell. This recognition event is not well understood, but is thought to involve exposed hydrophobic residues in the damaged protein, and thus frequently involves protein chaperones (reviewed in Cyr et al., 2002; Maattanen et al., 2010). In the case of chaperone-mediated autophagy, the recognizing chaperone itself ferries the damaged protein to a lysosomal compartment for proteolysis (reviewed in Arias and Cuervo, 2011; Mizushima et al., 2008). The endocytic and macroautophagy pathways also feed into lysosomes. The endocytic pathway is the primary mechanism for degradation of membrane proteins at the cell surface. Macroautophagy is a coordinated cellular recycling process involving the formation of a new lipid membrane around a targeted organelle or region of the cell, thus capturing the protein contents present in that region (Mizushima et al., 2008). The nascent vesicle, called an autophagosome, then fuses with a lysosome, delivering its payload of proteins for degradation.

In the UPS pathway, recognition of a damaged protein by a chaperone or other mechanism is followed by recruitment of an ubiquitin ligase complex. The ubiquitin ligase tags the damaged protein with an ubiquitin moiety, which is subsequently recognized and elongated into a polyubiquitin chain. The attachment of a polyubiquitin chain results in trafficking of the damaged protein to the proteasome (reviewed in Ciechanover, 2005; Ciechanover and Brundin, 2003). The proteasome is a large, barrel-like protein complex composed of the 20S proteolytic core with 19S regulatory subunits acting as caps at either end of the barrel. Damaged proteins are recognized and de-ubiquitinated at the caps before they enter the proteasomal barrel, where proteolytic cleavage occurs. In addition to recognizing and degrading damaged proteins, the UPS is important for regulating the lifetimes of many short-lived proteins in the cell (Ciechanover , 2005).

Both proteasomes and lysosomes are essential for maintaining homeostasis in healthy and stressed cells (Hara et al., 2006; Komatsu et al., 2006; Ciechanover, 2005). Generally, proteasomes degrade targeted, individual proteins, whereas lysosomes and the autophagic system function more in the degradation of long-lived cell membrane and cytosolic proteins. The relative contribution of each mechanism to global proteostasis is not fully understood, but the processes are complementary by nature. For example, autophagy is thought to be especially important for the capture and lysosomal delivery of protein aggregates, which are too large and inaccessible for proteolysis via the proteasomal barrel (Verhoef et al., 2002; Yamamoto et al., 2006). It is also likely

that proteasomal and lysosomal protein degradation systems communicate with or cross-regulate one another, but how this occurs is not currently known (e.g., Pandey et al., 2007). Intriguingly, others have demonstrated that the relative activity of protein synthesis and degradation are similarly coupled (Ding et al., 2006). These observations hint at extensive communication between the machineries involved in various branches of the proteostasis network, but the molecular mechanisms behind this coordination are currently quite vague.

Integrated Cellular Proteostasis Strategies Are Revealed Through the Study of Stress Responses

Studies of proteotoxic stresses and the resultant cellular responses have greatly expanded our understanding of how cells maintain proteostasis. The cellular “heat shock response” was first identified in the late 1970s when elevated temperatures were found to induce puffs in *Drosophila* polytene nuclei (Compton and McCarthy, 1978). Those puffs correspond to genetic loci which become highly transcribed in response to heat (Compton and McCarthy, 1978). Subsequent analyses identified the “heat shock proteins” resulting from these gene expression events and led directly to the discovery and characterization of protein chaperones (Ellis, 1987). Protein chaperone accumulation was eventually found to be a primary response to many denaturing stresses; furthermore, the heat shock response remains one of the most highly conserved genetic pathways currently known (Richter et al., 2010). The universality of the chaperone-based response through evolution and diverse stressors highlights

both the absolute requirement that proteostasis is maintained and the importance of protein chaperones in that effort.

Another example of a relatively well-characterized proteostasis response is the unfolded protein response (UPR) of the endoplasmic reticulum (ER). When ER chaperones recognize an overabundance of unfolded proteins, they activate multiple signaling pathways in the ER-membrane (reviewed in Schroder and Kaufman, 2005). The combined effects of these signaling pathways include activation of a set of UPR-specific transcription factors and global suppression of translation via phosphorylation of eukaryotic initiation factor 2α (eIF2α), a principle regulatory node for control of translation (Harding et al., 2000; Schroder and Kaufman, 2005). While global translation is reduced, the activation of UPR transcription factors increases production of ER chaperones and ER-associated degradation (ERAD) genes. Thus, the combination of these effects leads to a remodeling of cellular proteostasis which enlarges the quality control capacity of the ER compartment while concomitantly reducing the burden of incoming proteins.

The heat shock response and UPR represent two examples of unique and integrated cellular strategies for responding to proteotoxic insults. The extensive differences between the two responses illustrate a few important points. First, the cell is capable of sensing different types and locations of protein damage. Second, cells can select the most appropriate response from a larger repertoire of response strategies. And lastly, different responses are designed to suppress specific forms of damage occurring in disparate subcellular compartments.

A better understanding of the different mechanisms used by cells to maintain proteostasis during various types of stress has allowed researchers to begin pursuing ways in which the proteostasis network can be artificially remodeled for therapeutic benefit. For example, pharmacological or genetic activation of the heat shock response produces overexpression of chaperones under non-stressed conditions. This strategy has been shown to suppress protein damage and slow the age-dependent onset of aggregation in multiple models of proteostasis disease (e.g., Cohen et al., 2006; Teixeira-Castro et al., 2011). Additionally, several highly conserved genetic pathways have been discovered which can lead to increased lifespan and enhanced resistance to multiple stressors (e.g., Hsu et al., 2003). Most of these lifespan regulating pathways have been shown to increase proteostasis capacity (reviewed in Arslan et al., 2006; Cohen and Dillin, 2008). These findings make the pharmacological targeting of upstream proteostasis regulators an attractive potential therapy for delaying the onset of age-associated proteostasis diseases (Balch et al., 2008).

Despite these recent advancements, however, there are many aspects of proteostasis that remain poorly understood. For instance, it is clear that a connection exists between aging and proteostasis, but whether declining proteostasis is a cause or correlate of aging is still unknown. Additionally, certain proteins appear to possess much greater potential for toxic misfolding and aggregation than others. An explanation for why this subset of proteins is particularly toxic and/or unmanageable by the proteostasis network is lacking. Similarly, only very specific cell and tissue populations exhibit pathologies related

to these damage-prone proteins, despite relatively widespread expression. Lastly, novel pathways to protein damage still remain uncharacterized. Just as the response to heat stress represents a proteostasis strategy independent from the UPR, new models of proteotoxic stress may present new strategies used by cells to maintain proteostasis, and subsequently, new therapeutic targets.

Hypertonic Stress as a Novel Proteotoxic Stress

Causes and Effects of Hypertonic Stress

The maintenance of cell volume requires a balance of water and osmotically active solutes across the cell membrane. Regulation of this equilibrium involves the active transport of inorganic ions and organic osmolytes into and out from cells. Osmotic stress occurs when osmolality across the cell membrane falls out of equilibrium, and represents one example of an environmental stress whose effects on cellular proteins remain uncharacterized. Hypotonic stress is a state where extracellular osmolality is lower than that of the cytosol. Conversely, hypertonic stress occurs when the total extracellular solute concentration rises more quickly than cells can raise intracellular osmolality to adapt. Figure 2 models how each each form of osmotic stress impacts cellular water content and displays the general range of osmolalities under which they occur.

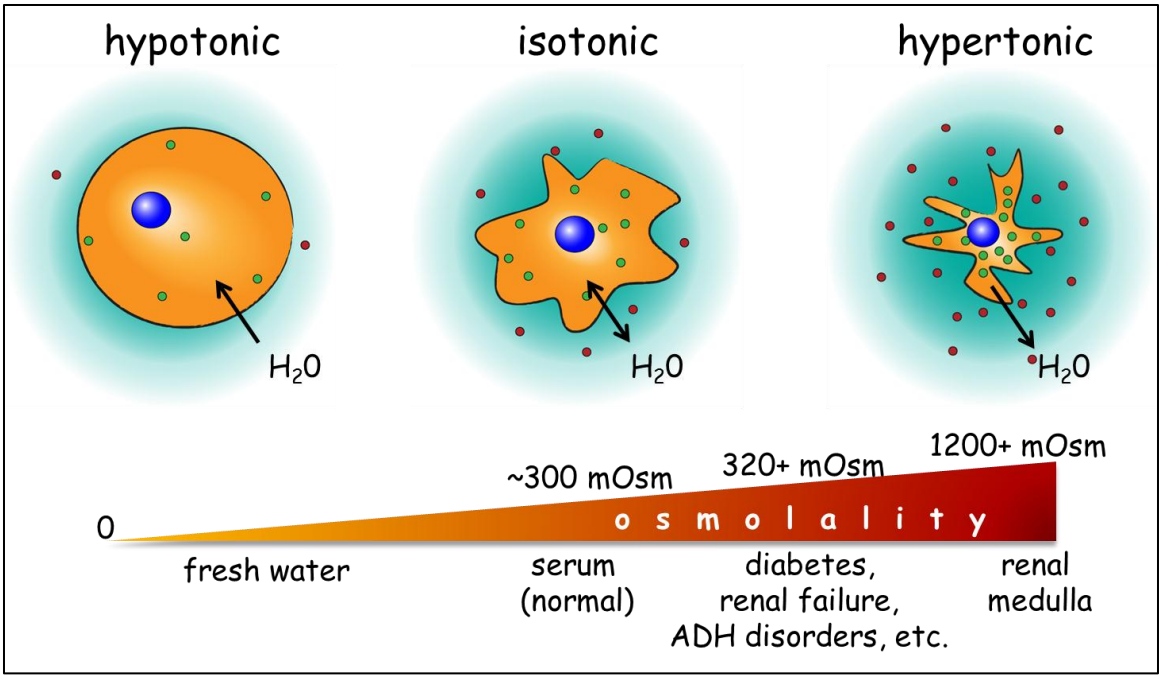

Figure 2. Forms of osmotic stress and their effects on cellular water homeostasis. "Isotonic" (normotonic) in humans corresponds to the plasma or extracellular fluid osmolality of ~300 mOsm. Under certain pathophysiological conditions, osmolality can rise systemically, but normally only the inner regions of the kidney endure extreme osmotic fluctuations.

In mammals the kidney tightly regulates plasma and extracellular fluid composition to prevent hypertonic stress from occurring systemically. However, the mechanism used by the kidney to control excretion vs. reabsorption of water in the urine requires that renal cells frequently experience extreme fluctuations in interstitial osmolality. More precisely, when the physiological state of the organism requires the processing of a concentrated urine, extracellular salt and urea accumulate to high concentrations in the inner kidney and cause hypertonic stress. This high salt and urea concentration acts as an osmotic gradient to drive reabsorption of water from the glomerular filtrate. In humans, changes in hydration state can shift renal osmolality between ~50 mOsm and >1200 mOsm,

roughly four-fold higher than normal plasma osmolality (~300 mOsm). Systemic hypertonicity is also possible under certain pathophysiological conditions including hypernatremia, ADH disorders, diabetes, and renal failure.

At the cellular level, the effects of hypertonic stress can dramatically alter the intracellular environment (Figure 2). Relative to most inorganic ions, cell membranes are freely permeable to water (Strange, 2004). Thus, when external osmolality rises, intracellular water is effluxed to regenerate the osmotic equilibrium across the membrane. This loss of water causes a rapid decrease in cell volume. With less water the concentrations of ions and macromolecules in the cell are all effectively increased by amounts proportional to the degree of water loss. Therefore, the primary effects of hypertonic stress beyond the mechanical stress of cell shrinkage are high intracellular ionic strength and increased macromolecular crowding.

Previous studies have shown that hypertonic stress causes DNA damage and can arrest the cell cycle (reviewed in Burg et al., 2007). On the other hand, very little is known regarding the effects of hypertonic stress on proteins. Evidence that proteins may be damaged by hypertonic stress is largely indirect. For example, increased expression of protein chaperones, an event often concomitant with damage to cellular proteins, occurs in several cell types after exposure to hypertonic conditions (Burg et al., 2007). Additionally, the inner regions of the kidney, including the medulla and papilla, are known to be more hypertonic than the outer cortex. Levels of some inducible chaperones correlate quite well with the degree of hypertonicity known to affect the region, such that

there are matching gradients of chaperone content and osmolality, highest in the medulla and papilla and lowest in the cortex (Santos et al., 1998). Importantly, in vitro analyses have shown that the intracellular changes caused by hypertonic stress have the potential to directly disturb proteostasis. High ionic strength can in certain cases have effects on protein conformation and stability, and some groups have shown that both high ionic strength and macromolecular crowding increase the rate and/or probability of protein aggregation (Yancey et al., 1982; Ellis and Minton, 2006; Munishkina et al., 2008; Zhou et al., 2009; Sicorello et al., 2009).

The Hypertonic Stress Response

The mechanisms by which cells regain volume in response to hypertonic stress are relatively well defined in a process generally referred to as "regulatory volume increase" (reviewed in Strange, 2004; Strange, 2007; Figure 3). Initially, cells take up inorganic ions, primarily Na^+, K^+, and Cl^-, to allow re-entry of water and alleviation of cell shrinkage and macromolecular crowding. High ionic strength remains a problem, however, until inorganic ions in the cell are replaced by organic osmolytes. Organic osmolytes accumulate in a much slower process through a combination of transport and metabolic activities (reviewed in Strange, 2004; Yancey et al., 2005). As previously discussed, organic osmolytes are compatible with macromolecular structure even at high concentrations, and allow cells to maintain high internal osmolarity without significant cost to protein, membrane, or nucleic acid stability.

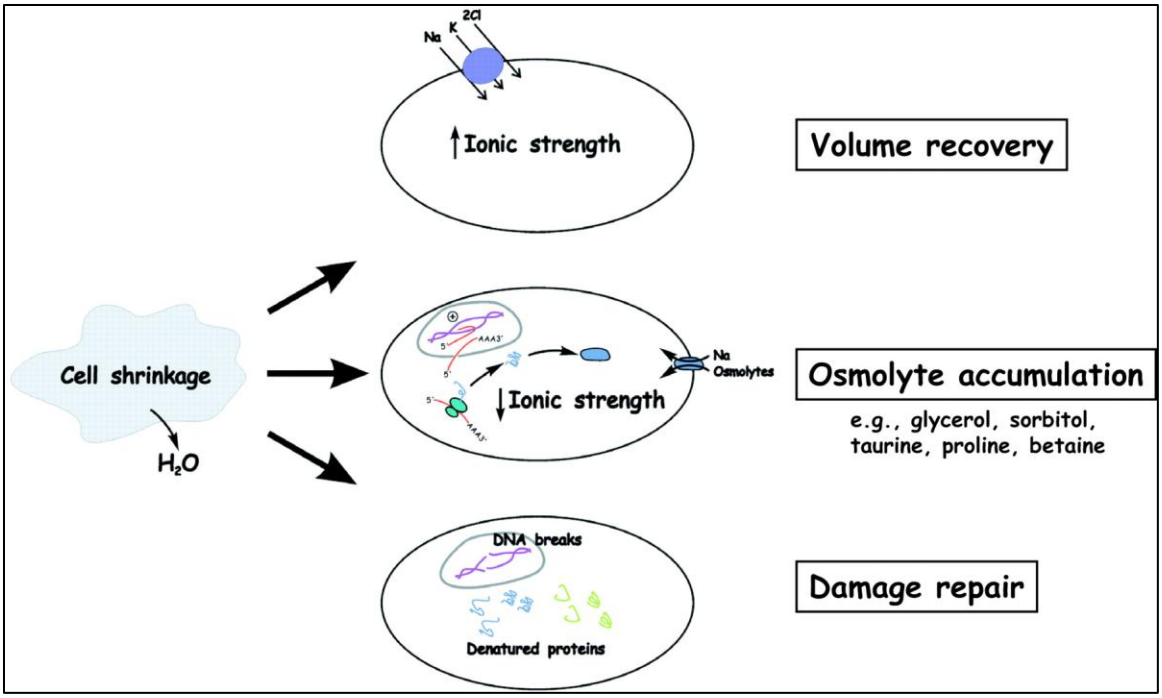

Figure 3. The hypertonic stress response (adapted from Strange, 2007). Exposure to a hypertonic environment causes cellular water loss and shrinkage. Cells respond by acutely increasing salt uptake, thus causing an osmotic influx of water to regain cell volume. In a much slower process, salts in the cell are replaced by compatible organic osmolytes. Organic osmolytes are typically accumulated by increased synthesis or import from the external environment, which often requires increased expression of synthetic enzymes or transporters, respectively. Following osmotic acclimation, cells must repair macromolecules damaged by hypertonic water loss.

Because organic osmolytes are thought to both increase intracellular osmolality and stabilize cellular macromolecules, they are theoretically all that would be necessary to maintain cellular viability in hypertonic environments. Understandably then, most studies of the hypertonic stress response center on the importance of transcriptional upregulation of genes involved in either the synthesis or import of organic osmolytes. As further support for the importance of organic osmolyte accumulation, this aspect of the response is universally conserved, although the specific organic osmolyte(s) employed vary from

organism to organism. Intriguingly, however, questions linger regarding the true action of these molecules in vivo. For instance, if organic osmolytes acting as chemical chaperones, why are they not accumulated in response to proteotoxic stressors such as heat shock? Additionally, if osmolytes are so beneficial to molecular function and stability, why are they not present at higher concentrations in the absence of stress?

C. elegans Is a Model for Hypertonic Stress and Proteostasis

The nematode *Caenorhabditis elegans* provides many general experimental advantages in addition to those which are specific to the study of osmotic stress and proteostasis. The general advantages of *C. elegans* in defining the genes and cellular pathways important for physiological processes include a fully sequenced genome, extensive sequence homology to mammalian genes, short life cycle, self-fertilization with large brood sizes, and nearly unmatched versatility in forward and reverse genetic analyses (Brenner, 1974). This nematode is also anatomically simple and optically transparent, permitting easy, real-time visualization of intracellular events in live animals (Figure 4).

C. elegans naturally dwells in the soil, a habitat prone to continuous and extreme fluctuations in the osmotic environment. Therefore, these worms possess well-developed mechanisms for sensing, adapting, and surviving through extreme hypertonic stress (Lamitina et al., 2004). Early development of *C. elegans* as a laboratory model for the study of hypertonic stress showed that the animals rapidly shrink when cultured on agar containing high salt (Lamitina et

al., 2004). Importantly, the small size of the worm allows hypertonic stress to exert its effects on all 959 somatic cells. The osmoregulatory response of the worm is similar to conserved mechanisms in other organisms and depends heavily upon accumulation of the organic osmolyte glycerol (Lamitina et al., 2004). Glycerol levels are regulated primarily during stress by transcription of glycerol 3-phosphate dehydrogenases (*gpdh-1* and *gpdh-2*), the same rate-limiting enzyme for hypertonicity-induced glycerol in yeast (Lamitina et al., 2004). Induction of *gpdh-1* is extensive in the intestine and hypodermis (>20-fold) and very specific to hypertonic stress (Lamitina et al., 2006). Conversely, *gpdh-2* is constitutively expressed and likely plays a metabolic role in cells.

Due in part to its short lifespan, the worm has long been a premier model for aging and age-related diseases. Genetic screens for regulators of the aging process in *C. elegans* have identified several conserved endocrine and/or transcriptional pathways involved in lifespan determination (e.g., Hamilton et al., 2005; Hansen et al., 2005). Because many proteostasis diseases appear to be intricately connected to the aging process, models for protein misfolding and aggregation in *C. elegans* soon followed (Link, 2001; Brignull et al., 2007; Kikis et al., 2010). A major effort in this area by many different groups has since led to the identification of many genes and processes involved in modulating the age-dependent aggregation of polyglutamine proteins, alpha-synuclein, beta amyloid and other proteins in the worm (e.g., Link, 1995; Satyal et al., 2000; van Ham et al., 2008). The availability of these well-described proteostasis models now allows for the elucidation and characterization of novel proteotoxic stressors.

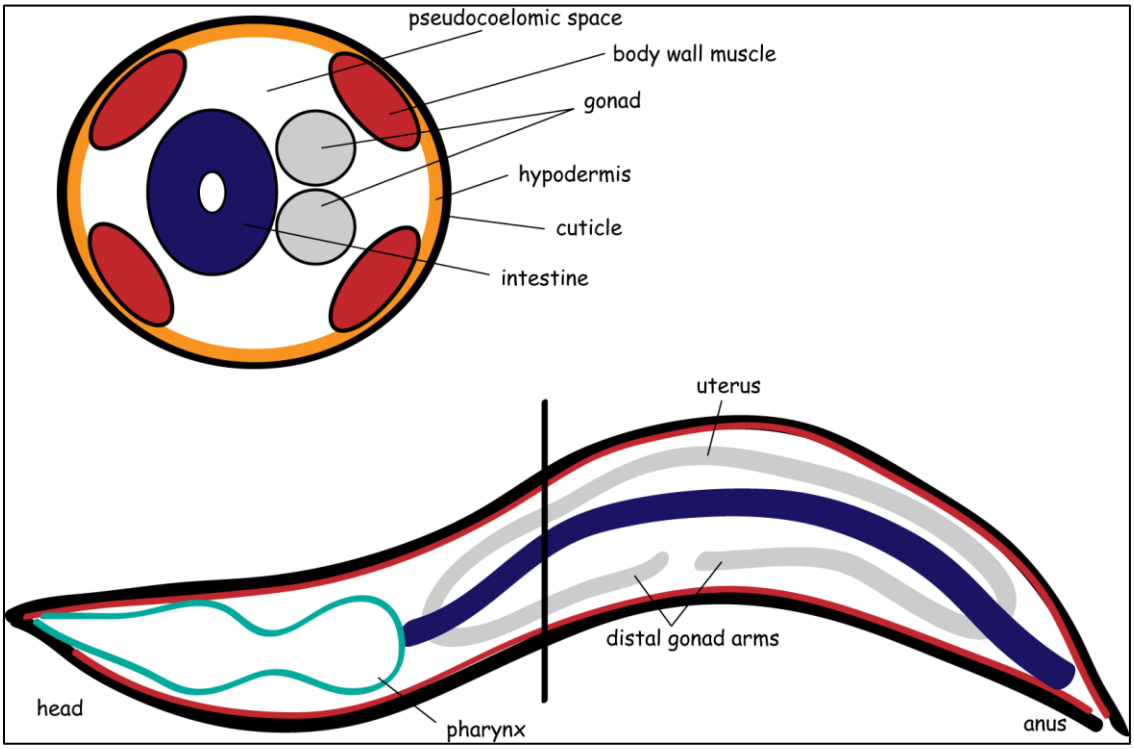

Figure 4. Overview of relevant *C. elegans* anatomy. The adult nematode is ~1 mm long (bottom). The line across the worm represents the area of the cross-section (above). The syncytial hypodermis secretes a protective collagen-based cuticle. The pharynx is a tube-like epithelial organ with dedicated muscle and neuronal systems. It grinds and pumps ingested bacteria into the intestine. Ninety-five striated body wall muscle cells are grouped into four quadrants that travel down the length of the worm; two quadrants are dorsal, and two ventral. The reproductive system consists of two somatic gonad arms which both feed into a common uterus. Germ cells in the distal arms mature into oocytes as they migrate proximally. Oocytes are fertilized in spermathecal chambers just before entering the uterus, where they are stored until they are released as eggs.

Recent Studies Demonstrate Multiple Links between Hypertonic Stress and Proteostasis

In the search for genes important in both sensing and repairing hypertonicity-induced cellular damage, our lab has previously performed two genome-wide RNAi screens in *C. elegans*. The first was designed to identify regulators of organic osmolyte synthesis and employed a reporter for *gpdh-1*

induction. RNAi of 122 genes caused constitutive activation of *gpdh-1*, and surprisingly, the largest groupings of identified genes are involved in protein translation and *de novo* protein folding (Lamitina et al., 2006). These results suggest that the appearance of damaged proteins in the cell acts as a signal to initiate osmoprotective responses.

The goal of the second genome-wide screen in the lab was to identify the genes required for survival of hypertonic stress. This study found that half of the genes (20 of 40) required for survival of hypertonic stress encode proteins that traffick and degrade damaged proteins. This subset of genes includes elements of both ubiquitin-proteasomal and lysosomal degradation pathways (Choe and Strange, 2008). The simplest explanation for the role of both proteasomal and lysosomal components in this response is that hypertonic stress damages proteins, which subsequently need to be cleared from the cell for survival. Thus, in addressing this second suggestion of a link between proteostasis and the hypertonic stress response, the authors demonstrated some of the best evidence that proteins are in fact damaged by hypertonicity-induced water loss. First, as an indirect measure of general protein damage and turnover, the amount of polyubiquitinylated proteins rises more than two-fold upon exposure to hypertonic stress (Choe and Strange, 2008). Secondly, the authors employed a transgenic reporter protein originally designed for the study of polyglutamine expansion diseases (Satyal et al., 2000). The protein, composed of 35 glutamines fused to yellow fluorescent protein (Q35::YFP), is soluble and diffusely expressed under normal growth conditions, but forms punctate, fluorescent aggregates rapidly

upon hypertonic exposure (Choe and Strange, 2008). This finding agreed well with an earlier observation that transient exposure of neuroblastoma cells to hyperosmotic cell culture media enhances aggregation of the damage-prone human polyglutamine protein, huntingtin (Chun et al., 2002). Taken together, these results demonstrate for the first time that cellular proteins are damaged during hypertonic stress and strongly suggest a critical role for multiple proteostasis processes in osmoprotective responses.

Significance of the Proposed Study

Proteostasis requires chaperoning and the balanced activities of protein synthesis and degradation machineries. These functions are also important in regulating the aging process and play crucial roles in the pathogenesis and progression of a wide array of protein conformational diseases. Much of the current understanding of proteostasis is built upon observing how the greater proteostasis network reacts to environmental and genetic perturbations.

Our lab has recently identified a novel proteotoxic perturbation, hypertonic stress. The studies contained herein aim to generate the first detailed characterization of the nature and extent of hypertonicity-induced protein damage. Additionally, these studies provide the first mechanistic insights into how cells defend proteins against damage incurred during hypertonic exposure. Specifically, the hypothesis that hypertonic stress-induced organic osmolytes defend proteostasis by enhancing protein folding and inhibiting aggregation is

tested. The role(s) of other essential machineries of the proteostasis network (i.e., protein chaperones, synthesis machineries, proteasomes and lysosomes) in the hypertonic stress response is examined for the first time. The findings of the proposed study will therefore significantly enhance current understanding of the hypertonic stress response and elucidate proteostasis strategies employed by cells in a novel stress context.

CHAPTER II

HYPERTONIC STRESS INDUCES RAPID AND WIDESPREAD PROTEIN DAMAGE IN *C. ELEGANS*

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Summary

Proteostasis is defined as the homeostatic mechanisms that maintain the function of all cytoplasmic proteins. We recently demonstrated that the capacity of the proteostasis network is a critical factor that defines the limits of cellular and organismal survival in hypertonic environments. The current studies were performed to determine the extent of protein damage induced by cellular water loss. Using worm strains expressing fluorescently tagged foreign and endogenous proteins and proteins with temperature sensitive point mutations, we demonstrate that hypertonic stress causes aggregation and misfolding of diverse proteins in multiple cell types. Protein damage is rapid. Aggregation of a polyglutamine YFP reporter is observable with <1 h of hypertonic stress and aggregate volume doubles approximately every 10 min. Aggregate formation is irreversible and occurs after as little as 10 min of exposure to hypertonic conditions. To determine whether endogenous proteins are aggregated by hypertonic stress, we quantified the relative amount of total cellular protein present in detergent insoluble extracts. Exposure for 4 h to 400 mM or 500 mM

NaCl induced a 55-120% increase in endogenous protein aggregation. Inhibition of insulin signaling or acclimation to mild hypertonic stress increased survival under extreme hypertonic conditions and prevented aggregation of endogenous proteins. Our results demonstrate that hypertonic stress causes widespread and dramatic protein damage and that cells have a significant capacity to remodel the network of proteins that function to maintain proteostasis. These findings have important implications for understanding how cells cope with hypertonic stress and other protein damaging stressors.

Introduction

Osmotic homeostasis is a fundamental requirement for life. All cells are exposed to osmotic challenges from intracellular solute flux and/or extracellular osmolality shifts. Most mammalian cells are protected from extracellular osmotic perturbations by the kidney, which tightly regulates blood ionic and osmotic concentrations. However, there are important exceptions to this generalization. Renal medullary cells are subjected normally to extreme osmotic stress by the renal concentrating mechanism. Several disorders such as renal failure, diabetes, syndrome of inappropriate ADH secretion, and hypernatremia disrupt plasma osmolality. In addition, hypertonic solutions are widely used to treat diverse clinical problems such as intracranial hypertension, interstitial fluid accumulation after coronary artery bypass surgery, hemorrhagic shock and cystic fibrosis.

It has long been assumed from in vitro studies that hypertonic stress damages proteins (e.g., Somero, 1986). Protein misfolding is thought to occur when cellular water loss causes a transient increase in cytoplasmic ionic strength. Exposure of normally buried hydrophobic surfaces on denatured proteins favors protein aggregation (Goldschmidt et al., 2010). Protein aggregation is likely further promoted by cell shrinkage, which increases macromolecular crowding and protein-protein interactions (Ellis and Minton, 2006; Munishkina et al., 2008; Zhou et al., 2008).

Numerous studies have demonstrated that the expression of various chaperone proteins is upregulated in response to cell shrinkage (Borkan and Gullans, 2002; Burg et al., 2007). Expression of chaperones is triggered in part by accumulation of denatured cytoplasmic proteins (Bjork and Sistonen, 2010; Richter et al., 2010), suggesting indirectly that hypertonic stress damages cellular proteins in vivo. However, there is little direct evidence demonstrating the types and extent of in vivo hypertonic stress-induced protein damage. With the exception of recent work from our lab (Choe and Strange, 2008), we are aware of only one other study identifying protein damage in hypertonically stressed cells. Chun et al. (2002) demonstrated that hypertonic stress enhances aggregation of a mutant huntingtin protein in cultured human neuroblastoma cells.

We recently carried out a genome-wide RNA interference (RNAi) screen in C. elegans with the goal of identifying genes required for survival in hypertonic environments (Choe and Strange, 2008). This screen identified 40 genes that when silenced reduce survival in hypertonic conditions, a phenotype we term

HypertOnic Sensitive or Hos. Twenty of the 40 Hos genes encode proteins that function to detect, transport and degrade damaged proteins. We also demonstrated that hypertonic stress causes aggregation of an aggregation-prone fluorescent reporter protein, Q35::YFP. Knockdown of the expression of Hos genes involved in protein degradation increases Q35::YFP aggregation.

Protein homeostasis is the maintenance of the complement of properly functioning proteins within a cell. Like osmotic homeostasis, it is essential for life. Previous studies suggested strongly that protein damage in hypertonically stressed cells must be rapidly repaired or removed in order for cells to survive in hypertonic environments (Choe and Strange, 2008). The purpose of the current study was to carry out a more extensive and detailed characterization of the protein damage induced by hypertonic stress. Using multiple transgenic and mutant models, we demonstrate that protein damage in hypertonically stressed cells is widespread and occurs rapidly after exposure to hypertonic conditions. Importantly, we demonstrate that hypertonic stress causes striking aggregation of endogenous proteins and that this aggregation is prevented by inhibition of the insulin signaling pathway or acclimation to mild hypertonic stress. These results demonstrate that cells have a significant capacity to remodel the network of proteins that function to maintain protein homeostasis. Our studies establish C. elegans as an important model system in which to define the molecular mechanisms utilized by eukaryotic cells for protein homeostasis under osmotic stress conditions. In addition, our work provides new insights into the factors that limit survival of cells and organisms in hypertonic environments, and it has broad

implications for understanding diseases that perturb plasma osmolality and for understanding possible complications of clinical therapies that employ hypertonic solutions.

Materials and Methods

C. elegans Strains

The following strains were obtained from the Caenorhabditis Genetic Center: wild-type N2 Bristol, Q35::YFP - AM140 rmIs132[Punc-54::Q35::YFP], α-synuclein::YFP – NL5901 pkIs2386[Punc-54::α-synuclein::YFP], paramyosin(ts) – CB1402 [unc-15(e1402)], ras(ts) – SD551[let-60(ga89)], perlecan(ts) – HE250[unc-52(e669su250)], myosin(ts) – CB1301[unc-54(e1301)], DAF-2 loss-of-function – CB1370[daf-2(e1370)], DAF-2; DAF-16 double loss-of-function – DR1309[daf-2(e1370;daf-16(m26)]. The KIN-19::tagRFP strain, CF3166 muEx473[pC03C10.1::C03C10.1::TagRFP + ptph-1::GFP], was generously provided by Dr. C. Kenyon. Unless otherwise stated, worms were cultured at 20°C using standard methods (Brenner, 1974).

Protein Aggregate Counts

Synchronized L1-stage worms were grown to young adults on 51mM NaCl NGM and then transferred to hypertonic NGM agar plates containing 400 mM or 500 mM NaCl. Q35::YFP puncta were counted manually using a Zeiss Stemi SV11 microscope (Chester, VA) set for GFP excitation at 8x magnfication.

Quantification of α-synuclein::YFP and KIN-19::tagRFP puncta was carried out using a Zeiss LSM510-Meta confocal microscope and Plan-Neofluar 40x/1.3 NA or Plan-Apochromat 63x/1.4 NA oil objective lenses. α-synuclein::YFP puncta were quantified in a single body wall muscle cells located anterior to the nerve ring. KIN-19::tagRFP puncta were quantified in the anterior pharynx, which encompasses the procorpus and metacorpus.

Confocal Microscopy

Worms were anesthetized in NGM buffer containing 0.1% Tricaine and 0.01% tetramizole, mounted on 2% agarose pads on glass slides and imaged using a Zeiss LSM510-Meta confocal microscope with Plan-Neofluar 40x/1.3 NA and Plan-Apochromat 63x/1.4 NA oil objective lenses (Carl Zeiss MicroImaging, Thornwood, NY). FRAP analysis was performed by photobleaching regions of body wall muscle cells or the anterior pharynx with 50-150 iterations of an Argon laser set at 100% power and 514 nm or 543 nm for bleaching of YFP and tagRFP, respectively. Images were taken every 3 s for up to 60 s after photobleaching and fluorescence intensity was measured with ImageJ software (National Institutes of Health).

Aggregate volumes were estimated from optical z-stacks through body wall muscle cells by summing the fluorescence areas of the aggregate in each optical slice and multiplying that sum by slice thickness. Fluorescence area was measured using Metamorph software (Molecular Devices, Sunnyvale, CA).

Temperature-Sensitive Mutant Phenotype Assays

Synchronized wild-type, paramyosin (ts) and ras (ts) worms were grown on 51 mM NaCl NGM at 16 °C. Adult worms were picked to plates that were either 51 mM NaCl at 16 °C (control), 51 mM NaCl at 25 °C, or 300 mM NaCl at 16 °C, and allowed to lay eggs for 24h. The number of eggs was counted, and those that did not develop past early larval stages were scored as larval arrest or defective egg hatching phenotypes.

Motility Assays

Worm motility was measured as described previously (Morley et al., 2002). Briefly, adult worms grown on 51 mM NaCl NGM were transferred to 500 mM NaCl NGM for 6 h and then allowed to recover on 51 mM NaCl NGM for 24h. Single worms were then placed on a fresh lawn of OP50 bacteria and removed after 30-60 sec. Brightfield images of the lawns were obtained using a Zeiss Stemi 2000-CS microscope (Thornwood, NY) equipped with a CCD camera (DAGE-MTI, Michigan City, IN). The length of the tracks in bacterial lawn made by individual worms was measured with ImageJ software.

Analysis of Endogenous Insoluble Proteins

Isolation of insoluble proteins was carried out using methods similar to those described by David et al. (2010). Briefly, worms were grown on 51mM NaCl NGM agar with either standard OP50 or RNAi feeding bacteria and transferred to hypertonic 400 mM or 500 mM NaCl plates for 4 h. Worms were

then washed in NGM solution isotonic to control or high NaCl agar plates. Washed worms were transferred to a buffer containing 51, 400, 500 or 650 mM NaCl, 100 mM MES, 1 mM EGTA, 0.1 mM EDTA, 0.5 mM MgSO4, 20 mM NaF, drip frozen in liquid nitrogen, and ground to a powder with a mortar and pestle. Immediately upon thawing, 10 µl of the ground material was taken for analysis of total protein concentration using a bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL). Two additional 60 µl aliquots were placed in either a solubilization buffer (8 M urea, 2% SDS, 50 mM DTT, 50 mM Tris, Roche complete protease inhibitor, pH 7.4) for total protein determination, or RIPA buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% SDS, 0.5% SDO, 1% NP-40, Roche complete protease inhibitor, pH 8). Insoluble proteins were isolated from samples in RIPA buffer by centrifugation at 16,100 g for 10 min. After supernatant removal, the insoluble protein pellet was resuspended in 100 µl of RIPA buffer, centrifuged a second time and then solubilized in solubilization buffer.

Protein samples were analyzed by SDS-PAGE. Gels were stained with Bio-Safe Coomassie (Bio-Rad, Hercules, CA), and the amount of protein present in each lane was quantified with ImageJ software. To assess changes in the amount of insoluble protein, total protein gels were loaded with a sample volume containing 20 µg of protein and the same sample volume was used to load the corresponding insoluble protein gel.

Survival Studies

Hypertonic stress was induced in various experimental settings by exposing worms for 4-6 h to growth media containing 400-650 mM NaCl. We monitored survival under these conditions by returning worms after the stress period to control growth medium for 24 h. In all cases, survival ranged between 98-100% (data not shown).

Statistical Analyses

Data are presented as means ± S.E. Statistical significance was determined using Student's two-tailed *t* test for paired or unpaired means. When comparing three or more groups, statistical significance was determined by one-way analysis of variance. P values of ≤ 0.05 were taken to indicate statistical significance. All graphs are plotted on common scales to facilitate comparison between experimental groups.

Results

A Polyglutamine Tract-Containing Fluorescent Reporter Undergoes Rapid and Irreversible Aggregation in Response to Hypertonic Stress

Proteins that contain tracts of contiguous glutamine residues undergo spontaneous, age-dependent aggregation. These proteins give rise to neurodegenerative disorders such as Huntington's Disease and spinocerebellar ataxias (Katsuno et al., 2008). Previous studies showed that yellow fluorescent protein (YFP) containing a tract of 35 glutamine residues (Q35::YFP) expressed

in the body wall muscle cells of *C. elegans* aggregates in response to hypertonic stress (Choe and Strange, 2008). We carried out more detailed studies of this protein to determine the time course of aggregation and aggregate growth, and whether aggregate formation was reversible. As shown in Figure 1A, numerous Q35::YFP aggregates are detected within 1 h after exposure of worms to 400 mM or 500 mM NaCl growth medium. We were able to detect small punctate structures that we believe were Q35::YFP aggregates within as little as 30 min of exposure to 500 mM NaCl.

To confirm that the early hypertonicity induced changes in Q35::YFP distribution were due to *bona fide* aggregation, we carried out fluorescence recovery after photobleaching (FRAP) analysis as described previously (Choe and Strange, 2008). Worms were exposed to 500 mM NaCl for 1 or 6 h and then imaged by confocal microscopy. Small regions of presumed aggregates were bleached and fluorescence recovery in the bleached region quantified over time. As shown in Figure 1B, FRAP was undetectable in punctate YFP structures detected 1 and 6 h after hypertonic stress was induced. These results demonstrate that under hypertonic conditions, Q35::YFP is localized to aggregates where individual proteins are immobile and that formation of insoluble Q35::YFP aggregates occurs with less than 1 h of hypertonic stress.

Hypertonicity-induced Q35::YFP aggregates increase rapidly not only in number, but also in size. To quantify aggregate growth, we exposed groups of worms to hypertonic growth medium for 1-24 h and estimated aggregate volume by confocal microscopy. As shown in Figure 1C, aggregate volume increased

~12-fold after a 4 h exposure to 500 mM NaCl growth medium. Aggregate volume remained stable between 4 and 24 h. Similar aggregate growth was observed when single worms were imaged continuously over a 3 h period.

Numerous studies have demonstrated that polyglutamine protein aggregates can be degraded and cleared by autophagy and other cellular mechanisms (e.g., Diaz-Hernandez et al., 2004; Menzies et al., 2010; Yamamoto et al., 2006). To determine whether hypertonicity-induced Q35::YFP aggregates can be cleared, we exposed worms to either 400 mM or 500 mM NaCl growth media for 6 h and quantified the number of aggregates immediately and 24 h after returning the animals to control growth medium. No significant ($P>0.05$) reduction in the number of aggregates was observed when animals exposed to either 400 mM or 500 mM NaCl were returned to control conditions (Figure 1D).

We also examined the effects of short term hypertonic stress on Q35::YFP aggregate clearance. Worms were exposed to 500 mM NaCl growth medium for either 10 min or 60 min and then returned to control medium. As shown in Figure 1E, no Q35::YFP aggregates were detected after 10 min of hypertonic stress. However, even though animals were returned to control conditions, Q35::YFP aggregates formed. The mean number of aggregates detected per worm was 8, which was significantly ($P<0.001$) greater than that observed in unstressed animals.

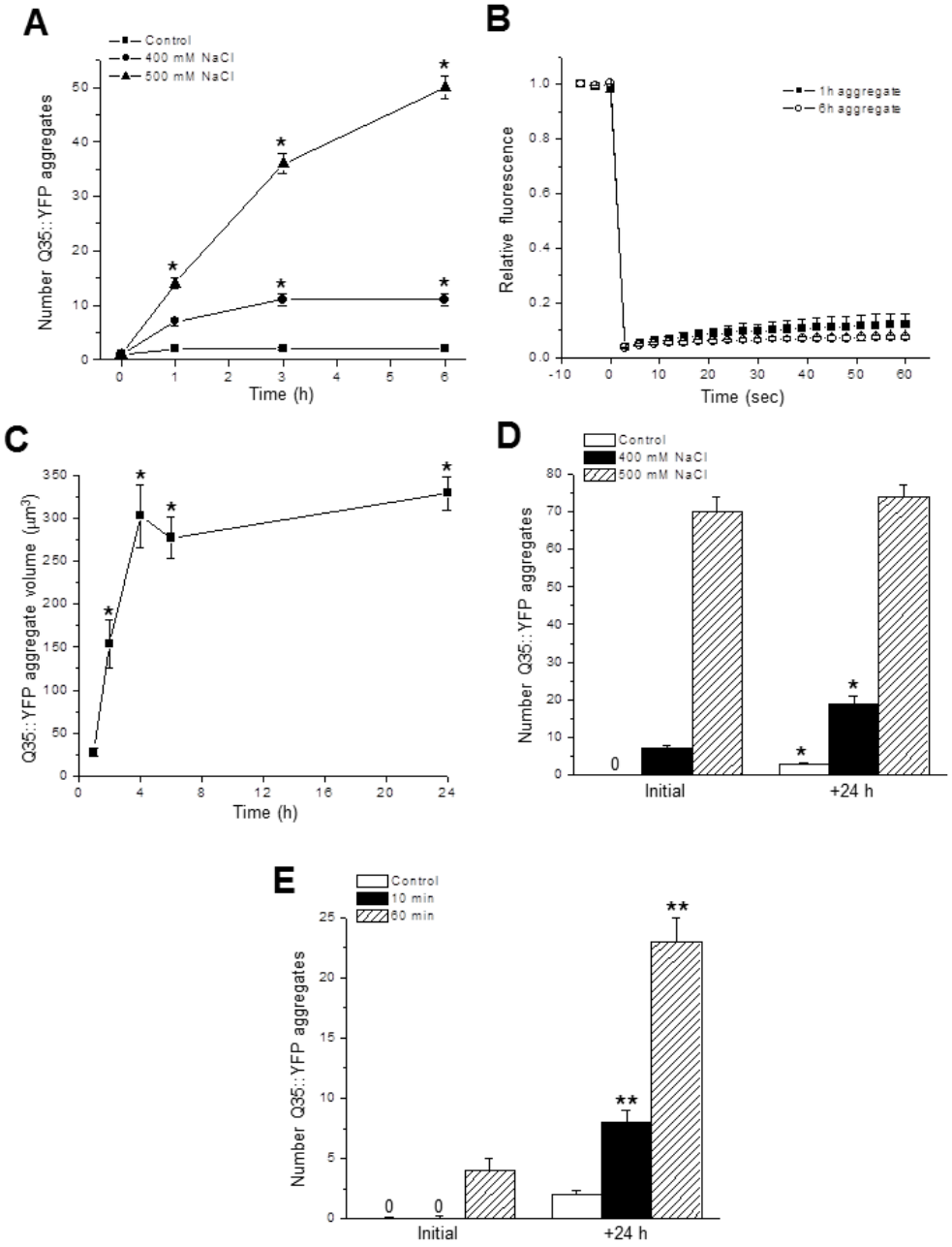

Figure 1. Characteristics of hypertonic stress-induced aggregation of Q35::YFP. (A) Rate of Q35::YFP aggregate formation in worms exposed to 400 mM or 500 mM NaCl. (n=30-90 worms); *P<0.01 vs. control. (B) Time course of fluorescence recovery in Q35::YFP aggregates 1 and 6 h after exposure to 500 mM NaCl. (n=3 worms). (C) Rate of Q35::YFP aggregate growth in worms exposed to 500 mM NaCl. (n=9-12 aggregates from 5 worms); *P <0.01 versus volume at 1 h. (D) Effect of a 24 h hypertonic stress on the reversibility of Q35::YFP aggregate formation. Worms were exposed to 400 mM or 500 mM NaCl for 24 h (initial) and then returned to control conditions for 24 h (+24 h). (n=12-18 worms); *P<0.01 versus initial number of aggregates in worms

(Figure 1—cont.) exposed to 400 mM NaCl. (E) Effect of brief hypertonic stress on the reversibility of Q35::YFP aggregate formation. Worms were exposed to 500 mM NaCl for either 10 or 60 min (initial) and then returned to control conditions for 24 h (+24 h). (n=24 worms); **P<0.001 versus initial number of aggregates detected 10 min and 60 min after exposure to 500 mM NaCl.

The effects of a 60 min exposure to 500 mM NaCl was even more striking. An average of 4 aggregates/worm were detected immediately after returning animals to control growth medium (Figure 1E). Striking Q35::YFP aggregation continued under control conditions. By 24 h, the number of aggregates increased by nearly six-fold (P<0.001; Figure 1E). Taken together, data in Figures 1D-E demonstrate that hypertonicity-induced Q35::YFP aggregates cannot be cleared after removal of the hypertonic stress. Indeed, as shown in Figure 1E, short term hypertonic stress triggers processes that drive continued Q35::YFP aggregation.

The gross morphology of *C. elegans* body wall muscle cells includes a contractile myofilament lattice and a noncontractile cell body that contains the cytoplasm and cell organelles. We optically sectioned muscle cells with confocal microscopy to define where Q35::YFP aggregates form in response to hypertonic stress. Two types of aggregates were observed; elongated, dense aggregates which aligned with myofilaments and were localized to the contractile lattice, and more diffuse aggregates in the cell body that frequently colocalized with nuclei (Figure 2A). This morphology and localization is similar to that observed for GFP tagged polyglutamine aggregates that form spontaneously in *C. elegans* muscle cells during aging (Satyal et al., 2000).

Age-induced Q35::YFP aggregates are toxic and cause muscle cell damage that reduces worm motility (Morley et al., 2002; Brignull et al., 2007). To assess the toxicity of hypertonic stress induced aggregates, we quantified motility in wild type and Q35::YFP worms exposed to 500 mM NaCl for 6 h and then returned to control medium for 24 h. As shown in Figure 2B, no motility defects were detected in wild type worms. Motility in control Q35::YFP worms was reduced ~17% ($P<0.05$) compared to control wild type animals. Exposure of Q35::YFP worms to hypertonic stress significantly ($P<0.05$) reduced motility by an additional ~15% (Figure 2B). These results suggest that hypertonicity-induced Q35::YFP aggregates and/or their intermediates are toxic to muscle cells.

Diverse Proteins Are Damaged Rapidly by Hypertonic Stress

To determine whether hypertonicity induced protein damage is unique to Q35::YFP, we examined the effects of hypertonic stress on other fluorescent reporters. α-synuclein is an inherently disordered protein expressed abundantly in the brain and is found in protein inclusions associated with various neurodegenerative disorders. Familial forms of Parkinson's Disease are caused by mutations and duplication of the α-synuclein gene (Uversky, 2007).

Human α-synuclein fused to YFP accumulates in so-called inclusion bodies in an age-dependent manner when expressed in *C. elegans* body wall muscle cells (van Ham et al., 2008). As shown in Figure 3A, the number of α-

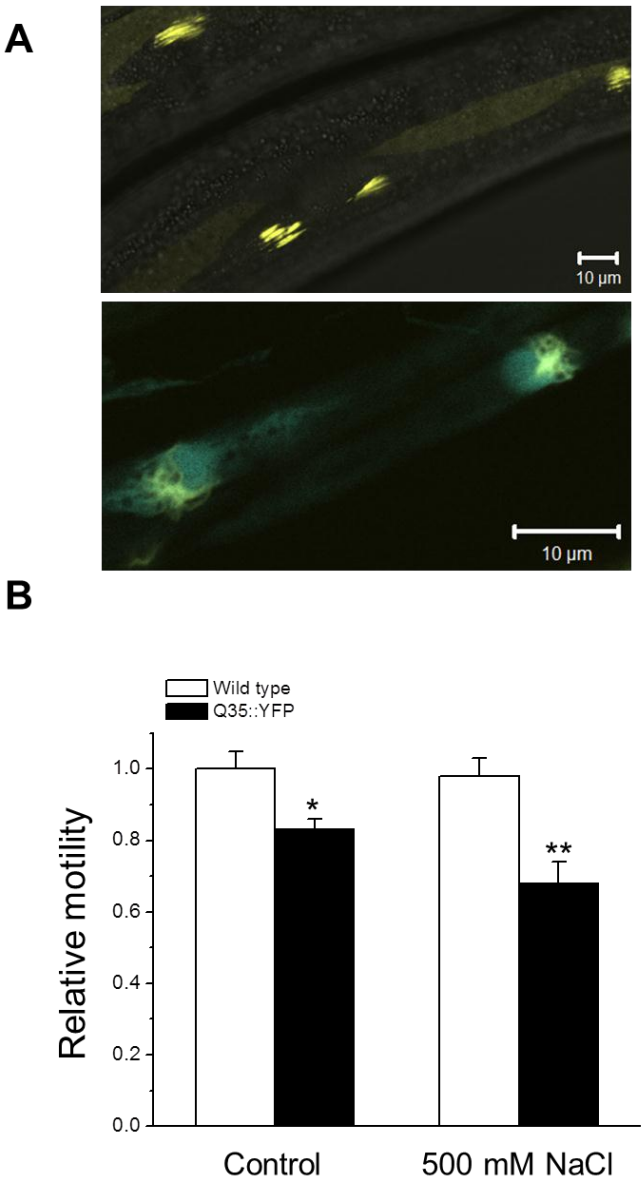

Figure 2. Morphology and toxicity of hypertonic stress induced Q35::YFP aggregates. (A) Confocal micrographs of muscle cells expressing Q35::YFP. Worms were exposed to 500 mM NaCl for 6 h. Top panel, combined differential interference contrast/fluorescence micrograph showing Q35::YFP aggregates localized to myofilament lattice. Bottom panel, fluorescence micrograph showing Q35::YFP localized to muscle cell body. Q35::YFP aggregates (yellow) are associated with cell nuclei (blue) expressing CFP with a nuclear localization signal. (B) Toxicity of hypertonic stress induced Q35::YFP aggregates. Worm motility was used to assess toxicity of Q35::YFP aggregates to body wall muscle cells. Motility was quantified in wild type and Q35::YFP worms exposed to 500 mM NaCl for 6 h and then returned to control conditions for 24 h, and in age-matched, unstressed wild type and Q35::YFP animals. (n=9 worms); *P<0.05 versus wild type worms. **P<0.05 versus unstressed Q35::YFP worms.

synuclein::YFP containing inclusions increases 4-5-fold when *C. elegans* is exposed to 500 mM NaCl for 6 h.

We used FRAP to determine if the α-synuclein::YFP within inclusion bodies was aggregated. As shown in Figure 3B, YFP fluorescence intensity recovered 70-80% within 60 sec after bleaching. This recovery is similar to that described previously by van Ham et al. (2008) and indicates that α-synuclein::YFP remains mobile within inclusion bodies. No obvious differences were observed in either the rate or extent of fluorescence recovery in inclusions of control and hypertonically stressed worms.

kin-19 encodes a predicted *C. elegans* casein kinase homolog. Monomeric red fluorescent protein (tagRFP) tagged KIN-19 (KIN-19::tagRFP) is expressed abundantly in the worm pharynx. KIN-19::tagRFP expressed in the anterior pharynx undergoes spontaneous age-dependent aggregation (David et al., 2010). As shown in Figure 3C, KIN-19 aggregates increased ~5-fold when worms are exposed to 400 mM NaCl for 24 h. Mean ± S.E. total fluorescence in the anterior pharynx relative to control worms was 1.04 ± 0.24 indicating that aggregation was due to hypertonic stress rather than changes in KIN-19::tagRFP expression. FRAP was undetectable in KIN-19::tagRFP (Figure 3D) aggregates indicating that individual proteins are immobile.

Hypertonic Stress Causes Protein Misfolding in Vivo

It is widely assumed from in vitro studies that cell shrinkage and the attendant increases in cytoplasmic ionic strength cause proteins to misfold and

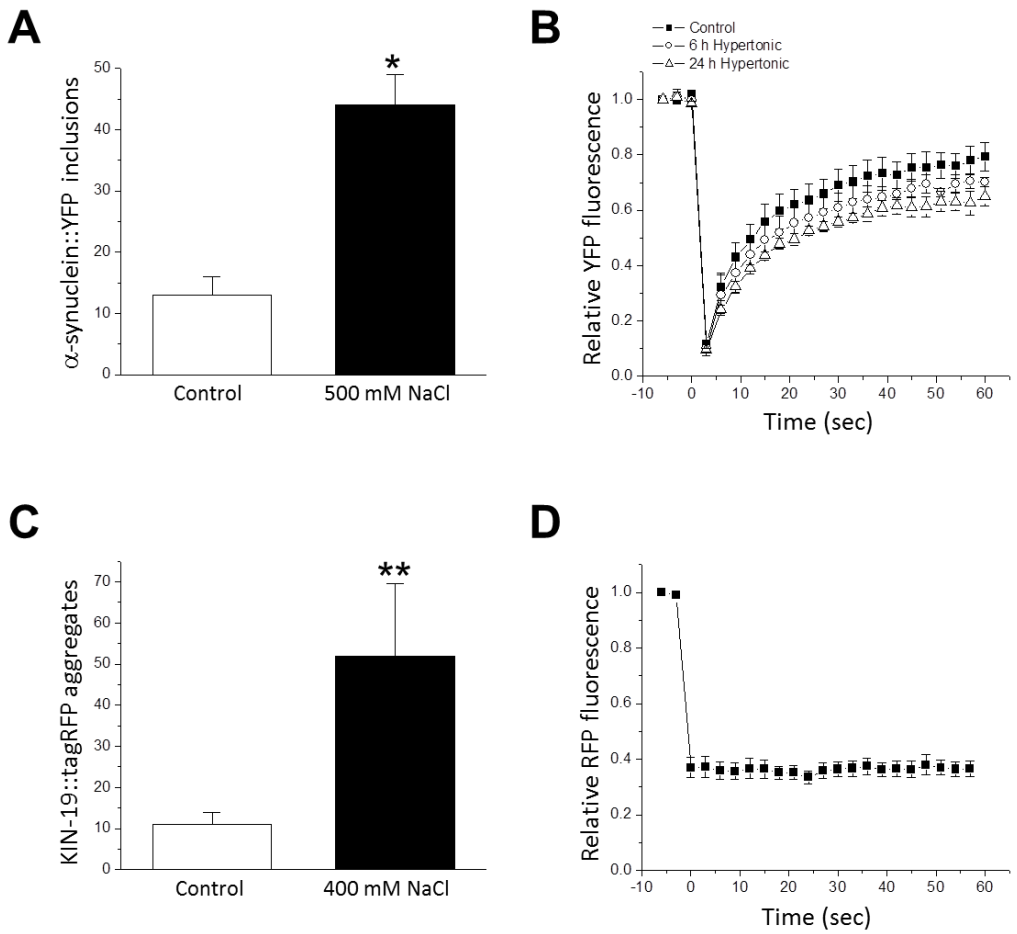

Figure 3. Effects of hypertonic stress on α-synuclein::YFP and KIN-19::tagRFP aggregation. (A, C) α -synuclein::YFP inclusions in body wall muscle cells and KIN-19::tagRFP aggregates in the anterior pharynx under control and hypertonic stress conditions. (n=5-7 worms); *P<0.001 versus control. A 6-7 b 3-4 c 5-7 d 4(B, D) Time course of bleaching and fluorescence recovery in α -synuclein::YFP inclusions and KIN-19::tagRFP aggregates. (n=3-4 aggregates); **P<0.05 versus control.

misfunction in vivo. To the best of our knowledge, this idea has not previously been examined in living cells. We used worm strains harboring temperature sensitive (ts) mutations to begin assessing whether hypertonic stress causes in vivo protein misfolding. Temperature sensitive mutations are mutations that have little or no effect on protein function and phenotype at low or 'permissive'

temperatures. However, at elevated temperatures, ts mutations give rise to a mutant phenotype. It is widely accepted that ts mutant proteins fold and function correctly at low temperatures (e.g., Brown et al., 1997; Gidalevitz et al., 2006; van Dyk et al., 1989). At elevated temperatures, ts mutant proteins misfold and therefore misfunction, giving rise to mutant phenotypes.

Figure 4 shows the effect of elevated temperature and hypertonic stress on the fraction of *let-60(ga89)* or *unc-15(el402)* ts mutant worms expressing the mutant phenotype. *let-60* encodes a ras GTPase and *unc-15* encodes paramyosin. These mutations give rise to egg hatching defects and larval arrest.

Worms were grown on 51 mM NaCl agar plates at the permissive temperature of 16 °C and at 25 °C for 4 days, or they were grown on 300 mM NaCl plates at 16 °C for 4 days. As shown in Figure 4, only 10-20% of the *let-60(ga89)* and *unc-15(e1402)* worms exhibit the mutant phenotype at 16 °C, whereas at 25 °C, over 70% of the animals show larval arrest and lethality. Worms grown at 16 °C on 300 mM NaCl resembled 25 °C animals with greater than 70% exhibiting the mutant phenotype. These results demonstrate that hypertonic stress causes misfolding and misfunction of LET-60 and UNC-15 in vivo.

We also conducted similar experiments with two other ts mutants: *unc-52(e669su250)* and *unc-54(e1157)*. *unc-52* and *unc-54* encode perlecan and myosin, respectively. Mutant worms grown at 16 °C and 300 mM or 400 mM NaCl exhibited no mutant phenotypes. These results suggest that cell shrinkage

does not cause misfolding significant enough to alter function of all cellular proteins.

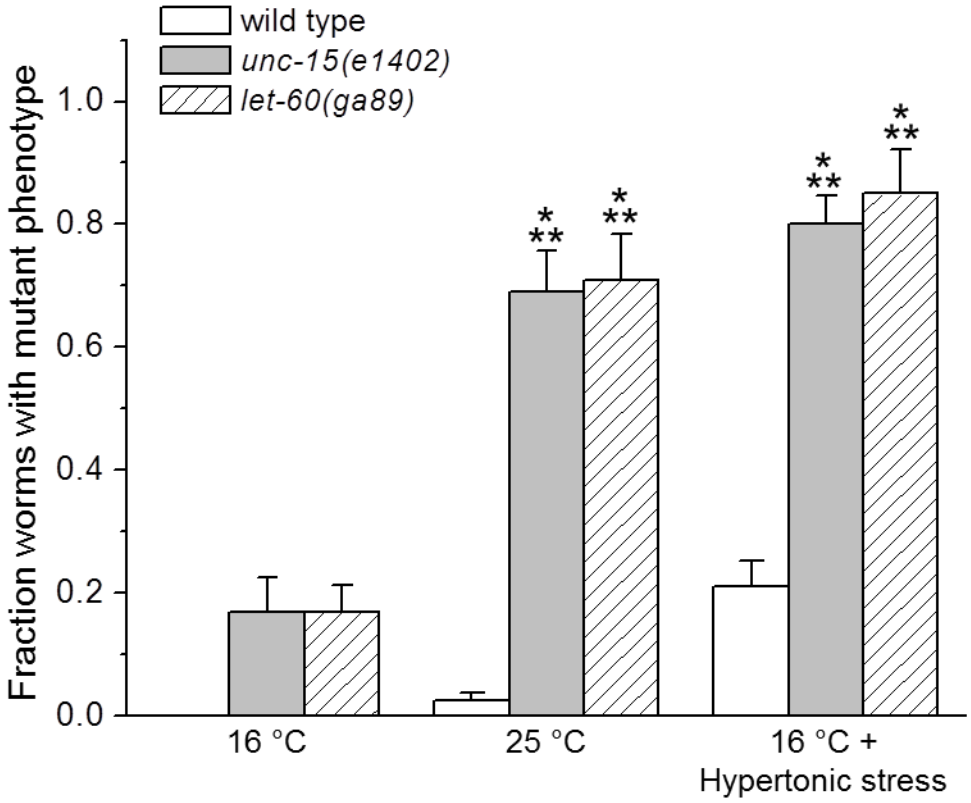

Figure 4. Effects of elevated temperature and hypertonic stress on expression of mutant phenotypes in *unc-15* and *let-60* temperature sensitive (ts) mutant worms. *unc-15* and *let-60* encode paramyosin and a ras GTPase, respectively. Temperature sensitive mutant phenotypes are induced by elevated temperature (25 °C) and by hypertonic stress (300 mM NaCl) at low or permissive temperature (16 °C) demonstrating that cell water loss also causes ts protein misfolding and misfunction. (n=4-6 experiments performed on groups of 20-40 worms); *P<0.001 versus wild type and mutant worms at 16 °C, **P<0.001 versus wild type worms at 25 °C and at 16 °C with hypertonic stress.

Hypertonic Stress Causes Aggregation of Endogenous Proteins

Data in Figures 1-4 provide compelling evidence that hypertonic stress causes rapid and dramatic protein damage. However, a potential concern is that the proteins examined are either transgenic or contain mutations that make them prone to misfolding and aggregation. To determine whether endogenous proteins are damaged in vivo by hypertonic stress, we quantified the impact of a 4 h exposure to either 400 mM or 500 mM NaCl on the relative amount of total cellular protein that is present in detergent insoluble extracts. Aggregated proteins are solubilized in 8 M urea, but remain insoluble in strong detergents (David et al., 2010; Kraemer et al., 2003).

Figure 5A shows SDS-PAGE gels of total and detergent insoluble proteins isolated from control and hypertonically stressed worms. As is clearly evident from the gels, the detergent insoluble fraction increases strikingly in worms exposed to high NaCl. Quantification of results from 3 independent experiments is shown in Figure 5B. Under control conditions, approximately 12% of the total protein was present in the insoluble fraction. When worms were exposed for 4 h to either 400 mM or 500 mM NaCl, the insoluble protein fraction increased ~55% ($P<0.05$) and ~120% ($P<0.01$), respectively, relative to that observed in control animals (Figure 5B).

As described in the methods section, total protein was extracted from worms by washing and freezing them in a buffer with a NaCl concentration equivalent to that of the agar to which they were exposed. It is thus conceivable that washing and freezing worms in high NaCl buffers causes proteins to

aggregate. To test for this, we washed and froze control worms grown on 51 mM NaCl agar with a 500 mM NaCl buffer. As shown in Figure 5C, extracting protein from control worms in a high salt buffer had no effect on the amount of protein in the insoluble fraction.

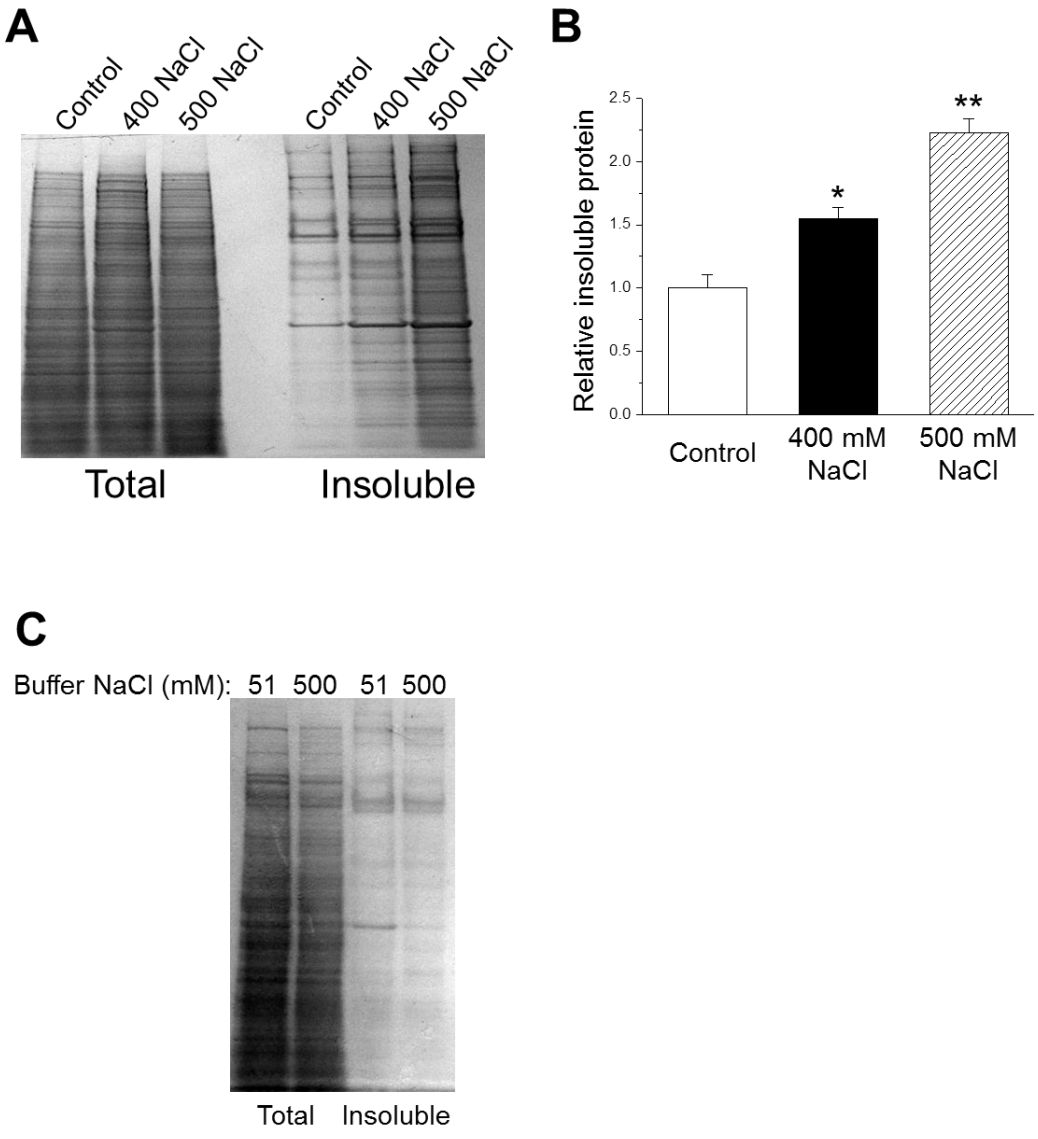

Figure 5. Effects of hypertonic stress on aggregation of endogenous proteins. (A) Examples of SDS-PAGE gels of total and detergent insoluble proteins isolated from control worms and worms exposed for 4 h to either 400 mM or 500 mM NaCl. (B) Quantification of insoluble protein. Insoluble protein was

(Figure 5—cont.)quantified as a percent of total protein and is plotted relative to control animals. (n=3); *P<0.05 and **P<0.01 versus control worms. (C) Example of an SDS-PAGE gel of total and detergent insoluble proteins isolated from control worms grown in 51 mM NaCl agar that were washed and frozen in a buffer containing either 51 or 500 mM NaCl. Results are representative of two independent experiments.

Inhibition of the *C. elegans* DAF-2 insulin signaling pathway dramatically increases survival under hypertonic conditions (Figure 6A) (Lamitina and Strange, 2005). Increased survival occurs without significant changes in whole animal glycerol levels (Lamitina and Strange, 2005). We tested the role of DAF-2 signaling on aggregation of endogenous proteins. Expression of the DAF-2 insulin receptor was silenced by feeding worms bacteria expressing *daf-2* dsRNA for 2 days. As shown in Figures 6B-C, no significant (P>0.3) increase in protein aggregation was observed in DAF-2 RNAi worms exposed to 500 mM NaCl for 4 h.

We also assessed the effect of DAF-2 signaling on protein aggregation using a worm strain carrying a *daf-2* loss-of-function allele (e1370). As shown in Figure 6C, no significant (P>0.8) protein aggregation was detected in *daf-2(e1370)* mutant worms exposed to 500 mM NaCl. Thus, loss of DAF-2 activity by RNAi knockdown or loss-of-function mutation prevents endogenous protein aggregation during hypertonic stress.

Increased resistance to hypertonic stress in *daf-2* loss-of-function worms is mediated by activation of the forkhead box transcription factor DAF-16 and the resultant increased expression of DAF-16 regulated genes (Lamitina and Strange, 2005). To determine if DAF-16 plays a role in reducing protein

aggregation, we exposed *daf-2(e1370); daf-16(m26)* double loss-of-function mutant worms to 500 mM NaCl for 4 h. In three separate experiments, we observed hypertonic stress induced increases in the relative amount of insoluble protein of 1.65-2.4-fold (Figure 6C). The mean ± S.E. fold increase observed was 1.98 ± 0.22. This value was significantly ($P<0.01$) elevated compared to *daf-2(e1370)* mutant worms. These results demonstrate that DAF-16 activity is required for inhibition of hypertonic stress induced protein aggregation in DAF-2 RNAi or loss-of-function animals.

Acclimation to moderate hypertonicity increases survival (Figure 6A) (Lamitina et al., 2004) and suppresses aggregation of Q35::YFP in worms subjected to extreme hypertonic stress (Choe and Strange, 2008). The effect of acclimation on endogenous protein aggregation is shown in Figures 6D-E. Worms were acclimated to 200 mM NaCl for 2 days and then exposed to 650 mM NaCl for 4 h. Exposure to 650 mM NaCl was used to mimic the change in NaCl concentration control animals would experience when placed on growth medium containing 500 mM NaCl. While the mean relative level of insoluble protein increased somewhat in acclimated worms exposed to 650 mM NaCl, this change was not statistically significant ($P>0.2$; Figure 6C).

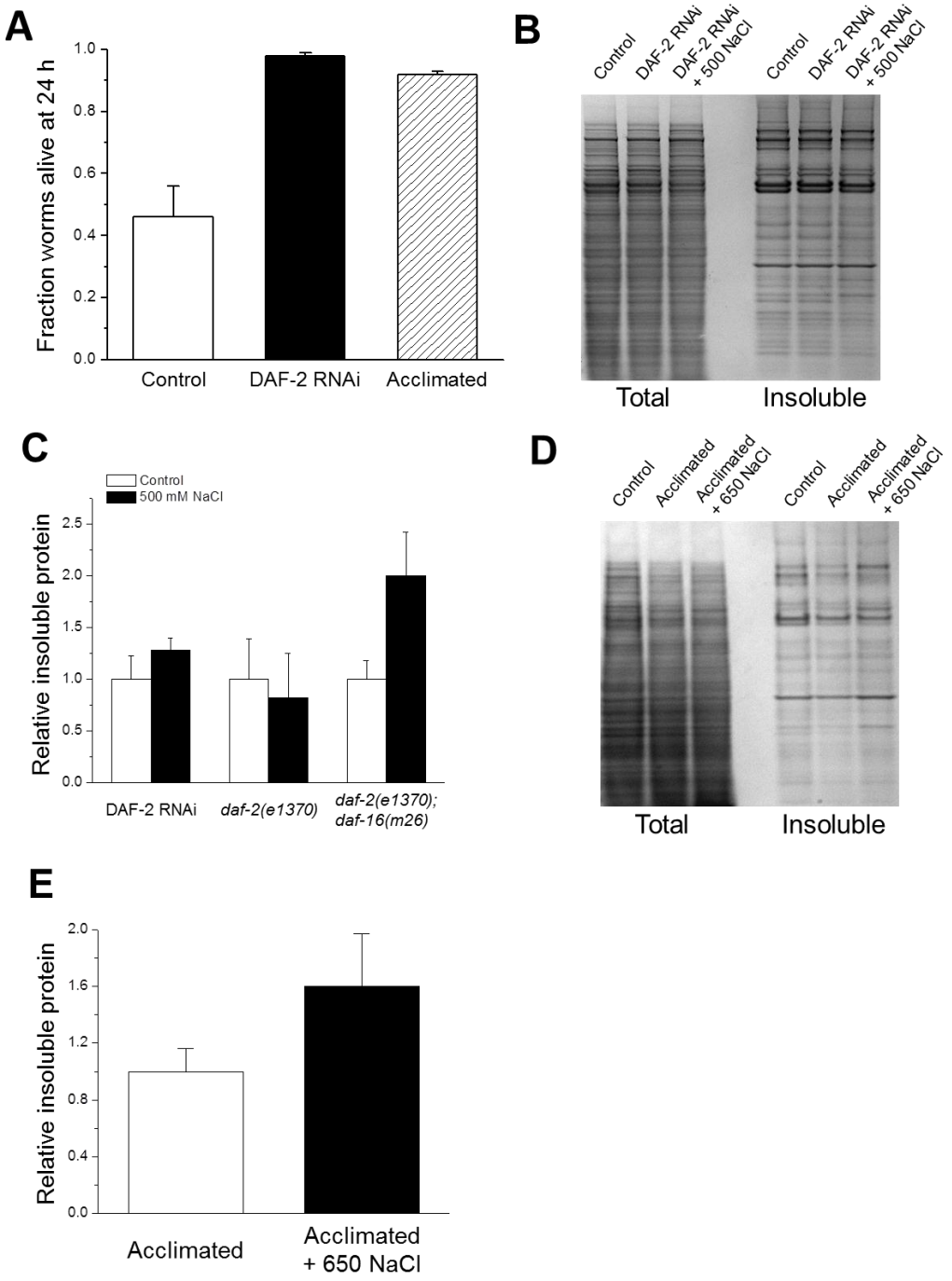

Figure 6. Effects of inhibition of insulin signaling and acclimation to mild hypertonicity on survival and aggregation of endogenous proteins. (A) Fraction of worms alive 24 h after exposure to 500 mM NaCl. Worms were fed *daf-2* dsRNA or acclimated to 200 mM NaCl for 2 days before exposure to 500 mM NaCl. (n=3 experiments performed on groups of 20-40 worms). (B) Example of SDS-PAGE gels of total and detergent insoluble proteins in DAF-2 RNAi worms. (C) Quantification of relative insoluble protein in DAF-2 RNAi worms, and

(Figure 6—cont.) *daf-2(e1370)* and *daf-2(e1370); daf-16(m26)* mutant worms (n=3). Example of SDS-PAGE gels of total and detergent insoluble proteins (D) and quantification of relative insoluble protein (E) in worms acclimated to 200 mM NaCl for 2 days (n=3).

Discussion

Protein homeostasis or "proteostasis" is defined as the homeostatic mechanisms that maintain the conformation, concentration, interactions, localization, and hence function, of cytoplasmic proteins. The proteostasis network is highly conserved across evolutionarily divergent species and includes the tightly integrated activities of gene transcription, RNA metabolism and protein synthesis, folding, assembly, trafficking, disassembly and degradation (Balch et al., 2008; Cohen and Dillin, 2008).

Errors in protein translation occur at a rate of one in every 1,000-10,000 translated codons indicating that ~15% of all average size proteins will contain at least one incorrect amino acid (Drummond and Wilke, 2009). If they are misfolded and dysfunctional, mistranslated proteins must be detected and repaired or destroyed. Mistranslation alone places the proteostasis network under a constant burden. The proteostasis network is further taxed by gene mutations that disrupt protein structure and function, by numerous protein damaging stressors, and by bacterial and viral infection that hijack protein synthesis and folding machinery. Thus, as noted by Ghosh and Dill (2010), "cells live on the edge of a proteostasis catastrophe".

Our lab recently carried out a genome-wide RNAi screen in *C. elegans* to identify genes required for survival in hypertonic environments (Choe and Strange, 2008). Surprisingly, this screen failed to identify the usual suspects associated with hypertonic stress resistance such as ion transporters required for cell volume recovery and genes that mediate organic osmolyte accumulation. Instead, half (20 of 40) of the identified genes encode proteins that function to detect, transport and degrade damaged proteins. These results indicate that the capacity of the proteostasis network is an essential factor that determines the limits to survival in hypertonic environments.

The current studies were carried out with goal of determining the extent of hypertonic stress induced protein damage. We found that three fluorescent reporter proteins, Q35::YFP, α-synuclein::YFP and KIN-19::tagRFP undergo rapid aggregation during hypertonic stress (Figures 1-3). Q35::YFP and α-synuclein::YFP are aggregation prone proteins while KIN-19 is a native *C. elegans* casein kinase. Hypertonic stress causes misfolding of ts mutant LET-60 and UNC-15 proteins (Figure 4). Q35::YFP and α-synuclein::YFP are expressed in body wall muscle cells and KIN-19::tagRFP is expressed in the pharynx. LET-60 is present in numerous diverse cell types, but larval lethality appears to be due to failure of the excretory system to form properly in mutant animals (Yochem et al., 1997). UNC-15 is expressed in essentially all *C. elegans* muscle cell types. The origin of the larval and embryonic lethality in *unc-15* mutants has not been defined precisely, but is likely due to failure of contractile apparatuses to develop properly (Epstein et al., 1993). Taken together, results shown in

Figure 1-4 demonstrate that hypertonic stress damages diverse proteins and that this damage occurs in multiple diverse cell types.

Our studies with Q35::YFP reveal several important features of hypertonic stress-induced protein damage. First, protein aggregation is very rapid. Q35::YFP aggregates are detectable within a few tens of minutes after exposure of *C. elegans* to hypertonic conditions (Figure 1A), and aggregate volume doubles approximately every 10 minutes until aggregates reach a stable size or all free protein has been aggregated (Figure 1C).

Aggregate formation is not reversible (Figure 1D) and exposure to as little as 10 minutes of hypertonic stress triggers Q35::YFP aggregation even when animals are returned to normotonic conditions (Figure 1E). There are two possible explanations for these observations. Protein damage induced by hypertonic stress may rapidly saturate the proteostasis network thus limiting its ability to prevent aggregation. It is also likely that protein aggregates represent a mechanism utilized by cells to isolate toxic intermediates including misfolded monomers and oligomeric structures (e.g., Campioni et al., 2010; Kayed et al., 2003; Silveira et al., 2005).

Hypertonicity-induced Q35::YFP aggregates exhibit mild toxicity to muscle cells as measured by a whole animal motility assay (Figure 2B). Interestingly, aggregates that form as worms age appear to be much more toxic. Morely et al. (2002) observed that 10-day old worms have ~50 Q35::YFP aggregates per animal. Individual worms exposed to 500 mM NaCl for 6 h and returned to control medium for 24 h have ~70 aggregates. Motility in the hypertonic stress

Q35::YFP worms is reduced ~30% compared to wild type animals. In contrast, motility in 10-day old Q35::YFP worms is reduced 65-70% (Morley et al., 2002).

There are several possible explanations for this apparent difference. Aging-induced aggregates form over a period of days whereas formation of hypertonic stress-induced aggregates is complete in a matter of hours (Figures 1A and C). The more rapid formation of Q35::YFP aggregates under hypertonic conditions may minimize the time that cells are exposed to toxic intermediate structures. It is also possible that proteostasis pathways activated in response to hypertonic stress may limit toxicity and/or that younger worms are better able to repair damage induced by toxic protein structures.

Importantly, we demonstrated in these studies that a 4 h exposure to hypertonic stress causes aggregation of numerous unidentified endogenous proteins (Figure 5). Thus, the damage we observe is not associated simply with transgenic or mutant protein properties. To our knowledge, this is the first demonstration that native proteins are damaged by cellular water loss in vivo.

We have begun mass spectrometry studies in an effort to identify proteins that are prone to aggregation when cells are subjected to hypertonic stress. Interestingly, a protein that was identified in one of the gel bands that showed particularly striking hypertonic stress-induced increases in the insoluble fraction (see Figure 5A) was paramyosin, or UNC-15 (unpublished observations). This result is consistent with the UNC-15 ts mutant experiments shown in Figure 4 and indicates that wild type paramyosin is also damaged by hypertonic conditions.

Inhibition of DAF-2 insulin signaling in *C. elegans* activates the DAF-16 transcription factor and dramatically increases longevity and resistance to numerous stressors including hypertonic stress (Fontana et al., 2010; Lamitina and Strange, 2005; Figure 6A). Hypertonicity-induced aggregation of endogenous proteins is completely blocked in DAF-2 RNAi worms and worms carrying a *daf-2* loss-of-function allele (Figures 6B-C). Loss of DAF-16 activity reverses the protective effect of DAF-2 inhibition (Figure 6C). Consistent with these findings, the lab has shown previously that enhanced survival of DAF-2 mutants in hypertonic environments is due in part to DAF-16 regulated increases in the expression of small heat shock proteins (sHSPs) as well as enzymes involved in trehalose synthesis (Lamitina and Strange, 2005). sHSPs bind to unfolded proteins and thereby minimize their interaction and subsequent aggregation (Haslbeck et al., 2005). Several studies have shown that trehalose inhibits protein aggregation in vivo (e.g., Singer and Lindquist, 1998; Tanaka et al., 2004; Beranger et al., 2008; Seki et al., 2010). These results further support our contention that maintenance of proteostasis is essential for survival during hypertonic stress. Additional studies of the role of DAF-2 regulated proteostasis mechanisms in mediating increased survival during hypertonic stress are warranted.

Acclimation to mild hypertonic stress increases the ability of cells and organisms to survive more extreme hypertonicity that would normally be lethal (Figure 6A). We showed previously that acclimation to 200 mM NaCl completely suppresses Q35::YFP aggregate formation when worms are exposed to 500 mM

NaCl, a salt concentration where maximal aggregation is normally observed (Choe and Strange, 2008). Similarly, 200 mM NaCl acclimation prevents aggregation of endogenous proteins (Figure 6D-E). One explanation for these results is that accumulated glycerol prevents aggregation. However, subsequent experiments (discussed in Chapter III) suggest that glycerol plays little or no role in slowing or preventing the aggregation of Q35::YFP, a finding that is consistent with in vitro studies of several proteins (Singer and Lindquist, 1998; Yang et al., 1999; Ryu et al., 2008). We suggest that while organic osmolytes like glycerol play important roles as chemical chaperone in vivo, remodeling of the proteostasis network to cope with protein damage induced by hypertonic stress is of at least equal importance.

Our findings have important physiological and pathophysiological implications. The extent and speed of the protein damage induced by hypertonic stress in *C. elegans* is striking. In osmotically unstable natural environments, organisms are frequently exposed to multiple protein damaging stressors. For example, intertidal organisms and organisms like *C. elegans* that live in vicinal water around soil and organic matter often experience hypertonic stress, elevated temperatures and hypoxia concurrently. The ability of an organism's proteostasis network to cope with rapid, extensive and diverse forms of protein damage thus certainly plays a central role in defining the boundaries of its ecological niche. The evolution of populations of organisms and species in unstable environments is also directly impacted by cellular mechanisms that manage protein damage (Jarosz et al., 2010; Rutherford, 2003).

The renal medulla is a hostile environment where the concentrating mechanism subjects medullary cells to large fluctuations in extracellular osmolality. Medullary osmolality in humans can vary from ~600 mOsm during diuresis to over 1200 mOsm under conditions of maximal water reabsorption. In addition to osmotic stress, renal medullary cells are exposed to hypoxia due to low blood flow, oxidative stress and high concentrations of urea (Neuhofer and Beck, 2006; Burg et al., 2007; Christoph et al., 2007). All of these stressors can cause macromolecular damage. Understanding the types of protein damage induced by hypertonic stress and how that damage is prevented and repaired is critical for understanding renal medullary physiology and pathophysiology, particularly during senescence where proteostasis mechanisms decline (Kikis et al., 2010; Koga et al., 2011).

Finally, as noted earlier, hypertonic solutions are used widely to treat diverse disorders including hemorrhagic shock, airway edema and mucous plugging associated with diseases such as bronchiolitis or cystic fibrosis, and intracranial hypertension (Jantzen, 2007; Kuzik et al., 2007; Dubus and Ravilly, 2008; Papia et al., 2008). Detailed understanding of hypertonicity-induced protein damage and how that damage could be exacerbated by other underlying conditions such as infection and senescence is thus essential for avoiding injurious side effects of hypertonic solution administration.

CHAPTER III

CHARACTERIZATION OF THE PROTEOSTASIS ROLES OF GLYCEROL ACCUMULATION, PROTEIN DEGRADATION AND PROTEIN SYNTHESIS DURING OSMOTIC STRESS IN *C. ELEGANS*

This chapter has been published under the same title in *PLoS One*.

Summary

Exposure of *C. elegans* to hypertonic stress-induced water loss causes rapid and widespread cellular protein damage. Survival in hypertonic environments depends critically on the ability of worm cells to detect and degrade misfolded and aggregated proteins. Acclimation of *C. elegans* to mild hypertonic stress suppresses protein damage and increases survival under more extreme hypertonic conditions. We set out to test the hypothesis that suppression of protein damage in acclimated worms could be due to 1) accumulation of the chemical chaperone glycerol, 2) upregulation of proteasome/lysosome activity, and/or 3) increases in molecular chaperoning capacity of the cell. Glycerol and other chemical chaperones are widely thought to protect proteins from hypertonicity-induced damage. However, protein damage is unaffected by gene mutations that inhibit glycerol accumulation or that cause dramatic constitutive elevation of glycerol levels. Pharmacological or RNAi inhibition of proteasome and lyosome function and measurements of cellular protein degradation activity

demonstrated that upregulation of protein degradation mechanisms plays no role in acclimation. Thus, changes in molecular chaperone capacity must be responsible for suppressing protein damage in acclimated worms. Transcriptional changes in chaperone expression have not been detected in *C. elegans* exposed to hypertonic stress. However, acclimation to mild hypertonicity inhibits protein synthesis 50-70%, which is expected to increase chaperone availability for coping with damage to existing proteins. Consistent with this idea, we found that RNAi silencing of essential translational components or acute exposure to cycloheximide results in a 50-80% suppression of hypertonicity-induced aggregation of polyglutamine-YFP (Q35::YFP). Increased cellular protein production by dietary changes increased Q35::YFP aggregation 70-180%. Our results demonstrate directly for the first time that inhibition of protein translation protects extant proteins from damage brought about by an environmental stressor, demonstrate important differences in aging- versus stress-induced protein damage, and challenge the widely held view that chemical chaperones are accumulated during hypertonic stress to protect protein structure/function.

Introduction

Maintenance of the conformation, concentration, interactions, localization, and hence function of cytoplasmic proteins is termed proteostasis. Proteostasis is maintained by the tightly integrated activities of gene transcription, RNA

metabolism and protein synthesis, folding, assembly, trafficking, disassembly and degradation (Balch et al., 2008; Cohen and Dillin, 2008). Proteostasis mechanisms are highly conserved across evolutionarily divergent species and are essential for life.

Hypertonicity-induced cellular water loss and associated cell shrinkage increase cytoplasmic ionic strength and macromolecular crowding. Elevated ionic strength can denature proteins while macromolecular crowding increases protein-protein interactions that can lead to protein aggregation (Somero, 1986; Zhou et al., 2008; Goldschmidt et al., 2010). While it is widely believed that hypertonic stress damages cytoplasmic proteins in vivo, there is little direct evidence to support this idea.

As discussed in Chapter II, we demonstrated recently in the genetic model organism *C. elegans* that hypertonic stress causes aggregation and misfolding of diverse fluorescently tagged foreign and endogenous proteins and proteins with temperature sensitive point mutations (Choe and Strange, 2008; Chapter II). Protein damage is rapid. Aggregation of a polyglutamine YFP reporter is observable in muscle cells with <1 h of hypertonic stress and aggregate volume doubles approximately every 10 min. Aggregate formation is irreversible and triggered by events that occur after as little as 10 min of exposure to hypertonic conditions. Numerous endogenous native proteins also undergo striking and rapid aggregation during hypertonic stress (Chapter II).

Survival of *C. elegans* in hypertonic environments requires the function of genes that play essential and conserved roles in the destruction of damaged

proteins. Acclimation of worms to mild hypertonic stress suppresses protein damage that normally occurs under more extreme conditions (Choe and Strange, 2008; Chapter II). These studies thus demonstrate that detection, repair, removal and suppression of protein damage are critical factors that define the ability of cells and organisms to survive in osmotically stressful environments. The speed at which protein damage occurs in *C. elegans* during hypertonic stress and the relative ease of measuring this damage provides a unique model for defining the mechanisms utilized by eukaryotic cells to maintain proteoastasis during environmental insults.

The suppression of protein damage we observed in worms acclimated to mild hypertonic stress (Choe and Strange, 2008; Chapter II) could be due to three physiological processes: 1) accumulation of organic osmolytes, which are believed to function as chemical chaperones, 2) upregulation of lysosome and proteasome activity, and/or 3) increases in the overall molecular chaperoning capacity of the cell (Somero, 1986; Beck and Neuhofer, 2005; Yancey, 2005; Burg et al., 2007; Choe and Strange, 2008). The current studies were carried out to identify which of these processes protect the cellular proteome from damage during hypertonic stress. Surprisingly, our studies failed to identify a protein protective role for glycerol, which is the major organic osmolyte accumulated by *C. elegans* under hypertonic conditions (Lamitina et al., 2004; Lamitina et al., 2006). Upregulation of protein degradation activity also played no role in the acclimation process. Instead, we find that an increase in the overall molecular chaperoning capacity of cells plays the predominant role in reducing protein

damage. At least part of the increase in chaperone capacity can be attributed to acute reductions in protein synthesis, which reduces the load on the chaperone network. Our studies provide important new insights into the mechanisms utilized by cells and organisms to cope with protein damaging environmental stressors, demonstrate important differences in aging- versus stress-induced protein damage, and raise interesting and provocative questions about the physiological roles of organic osmolytes.

Methods and Materials

C. elegans Strains

The following strains were used: N2 Bristol (wild type), AM140 rmIs132[*Punc-54*::Q35::YFP], MT3643 *osm-11(n1604)*, VP586 *osm-11(n1604)*; rmIs132[*Punc-54*::Q35::YFP], VP332 *gpdh-1(kb24); gpdh-2(kb33)*, CB1402 *unc-15(e1402)*, SD551 *let-60(ga89)*, and YD3 *xzEx3*[*Punc-54::UbG76V::Dendra2*]. Unless noted otherwise, worms were cultured at 20 ºC using standard methods. For acclimation studies, eggs were placed onto 200 mM NaCl agar plates without food overnight for synchronization to L1 larvae. L1 larvae were then transferred to 200 mM NaCl plates seeded with bacteria.

Measurement of Whole Worm Glycerol Levels

Worms were washed in liquid nematode growth medium (NGM) buffer to remove bacteria and frozen at –80 ºC. Glycerol was measured as described

previously, with some modifications (Lamitina et al., 2004). Frozen worms were sonicated in liquid NGM media on ice. Insoluble debris was removed from these lysates by centrifugation, and a portion of the supernatant was assayed for total protein concentration using a bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL). 1 N perchloric acid (PCA) was added to the remaining lysate to precipitate proteins, and the proteins were removed by centrifugation. The supernatant, which contained glycerol, was titrated to a pH of 6.7-7.5 with 5 N KOH in 61.5 mM K_2HPO_4 and 38.5 mM KH_2PO_4. Glycerol was measured in the neutralized supernatants with a commercial kit according to manufacturer's protocols (R-Biopharm, Marshall, MI). Glycerol levels were expressed relative to total protein content.

Quantification of Q35::YFP Aggregates

Q35::YFP aggregates were counted manually from digital images as described previously (Morley et al., 2002). Images of transgenic worms were obtained with a Zeiss Stemi SV11 microscope fitted with a CCD-100 DAGE-MTI camera set to excite and view GFP. During imaging, worms were immobilized by chilling agar plates on ice.

Aging-induced Q35::YFP aggregation was quantified by transferring young adult stage worms to growth plates containing 50 µg/ml fluorodeoxyuridine to inhibit the development of offspring (Gandhi et al., 1980). Hypertonicity-induced aggregates were quantified in worms transferred at the late L4/young adult stage to growth plates with elevated NaCl concentrations.

Confocal Microscopy and FRAP Analysis

A Zeiss LSM510-Meta confocal microscope equipped with Plan-Neofluar 40x/1.3 NA and Plan-Apochromat 63x/1.4 NA oil objective lenses (Carl Zeiss MicroImaging, Thornwood, NY) was used for imaging. FRAP analysis was performed by photobleaching regions of body wall muscle cells with 50-150 iterations of an Argon laser set at 100% power and 514 nm for bleaching of YFP. Images were taken every 3 s for up to 60 s after photobleaching and fluorescence intensity was measured with ImageJ software (National Institutes of Health).

Quantification of Q35::YFP Aggregate Toxicity

To assess the toxicity of Q35::YFP aggregates in body wall muscle cells, worm mobility was measured as described previously (Morley et al., 2002). Briefly, single worms were placed on a fresh lawn of OP50 bacteria and then removed after 3 to 10 minutes. Brightfield images of the lawns were obtained with a Zeiss Stemi 2000 microscope fitted with a CCD-100 DAGE-MTI camera, or a Zeiss V12 M2Bio microscope fitted with a Zeiss AxioCam MRm cooled CCD camera. The length of tracks made by worms was measured with Image J software.

Analysis of Endogenous Insoluble Proteins

Isolation of insoluble proteins was carried out as described previously (Chapter II). Briefly, worms were washed in NGM solution isotonic to the agar

medium on which they were grown. Washed worms were transferred to a buffer containing NaCl at a concentration equivalent to the growth medium and 100 mM MES, 1 mM EGTA, 0.1 mM EDTA, 0.5 mM $MgSO_4$, 20 mM NaF, drip frozen in liquid nitrogen, and ground to a powder with a mortar and pestle. Immediately upon thawing, 10 µl of the ground material was taken for analysis of total protein concentration using a bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL). Two additional 60 µl aliquots were placed in either a solubilization buffer (8 M urea, 2% SDS, 50 mM DTT, 50 mM Tris, Roche complete protease inhibitor, pH 7.4) for total protein determination, or RIPA buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% SDS, 0.5% SDO, 1% NP-40, Roche complete protease inhibitor, pH 8). Insoluble proteins were isolated from samples in RIPA buffer by centrifugation at 16,100 g for 10 min. After supernatant removal, the insoluble protein pellet was resuspended in 100 µl of RIPA buffer, centrifuged a second time and then solubilized in solubilization buffer.

Protein samples were analyzed by SDS-PAGE. Gels were stained with Bio-Safe Coomassie (Bio-Rad, Hercules, CA), and the amount of protein present in each lane was quantified with ImageJ software. To assess changes in the amount of insoluble protein, total protein lanes were loaded with a sample volume containing 20 µg of protein and the equivalent sample volume was used to load the corresponding insoluble protein lane.

Photoconversion and Quantification of Dendra2 Fluorescence

Worms were anesthetized in isotonic NGM buffer containing 0.1% Tricaine and 0.01% tetramisole, mounted on 2% agarose pads on glass slides and imaged using a Zeiss LSM510-Meta confocal microscope with Plan-Neofluar 10x/0.3 objective. Photoconversion of Dendra2 was performed with 20 iterations of a 405 nm, 30 mW diode laser at 50% power. Green and red populations of Dendra2 were imaged using a 488 nm, 30 mW Argon laser at 1% power and a 543 nm, 1 mW HeNe laser at 10% power, respectively. Fluorescence intensity was measured with ImageJ software.

Measurement of Worm Motility During Acute Osmotic Stress

An internal hydrostatic pressure is required for motility in *C. elegans*. Water loss during hypertonic stress lowers hydrostatic pressure resulting in partial or complete paralysis until fluid balance is restored. To indirectly assess the degree of water loss under hypertonic conditions, we carried out motility assays similar to those described by Solomon et al. (2004). Briefly, worms were placed on high NaCl plates in the middle of a 7 mm circle. Motile worms were defined as those that moved to the outside of the circle within 30 min after transfer.

^{35}S-methionine Labeling of Total Cellular Protein

Incorporation of ^{35}S-methionine into total protein was used to assess rates of protein synthesis and degradation. Radiolabeling was carried out using

methods similar to those described by others (Anderson et al., 2009). Briefly, synchronized L4 worms were fed ^{35}S-methionine loaded OP50 bacteria for 4 h, washed and incubated with unlabeled OP50 for 1 h to purge radioactive intestinal bacteria, and then washed thoroughly with NGM buffer. Washed worms were flash frozen in liquid nitrogen and stored at -80 °C before extraction. Protein was extracted from thawed samples by trichloroacetic acid-ethanol protein precipitation. Total protein concentration was measured by BCA assay (Pierce Biotechnology) and radioactivity incorporation by liquid scintillation counting.

Temperature Sensitive Mutant Phenotype Assays

Synchronized temperature sensitive *let-60(ga89)* and *unc-15(el402)* mutant worms were grown on 51 mM NaCl NGM at the permissive temperature of 16 °C. Adult worms were transferred to 300 mM NaCl plates at 16 °C and allowed to lay eggs for 24 h. The number of eggs was counted, and those that did not develop past early larval stages were scored as larval arrest or defective egg hatching phenotypes.

Measurement of Worm Protein Levels

Single worms (50-70) were picked into 1 ml of NGM and incubated for 45 minutes to purge the intestine of bacteria. Purged worms were washed three times with buffer, resuspended in 100 µl of lysis buffer (0.1M NaOH, 0.2% SDS, 0.02% Tris HCl, pH 8.0), and incubated at 37 °C for 4 h. Lysates were cleared by centrifugation and protein was measured by BCA assay.

HB101 Feeding Experiments

Worms were fed either OP50 or HB101 *E. coli* strains to assess the effects of intracellular protein levels on Q35::YFP aggregation. HB101 fed worms developed ~8-12 h more rapidly than worms fed OP50 bacteria. To ensure that Q35::YFP aggregation was quantified at identical developmental stages, measurements were performed in late L4 worms that had a fully developed vulva or in non-gravid young adults.

RNAi Experiments

RNA interference was performed by feeding worms a strain of *E. coli* engineered to transcribe dsRNA homologous to a target gene. The strains were obtained from commercially available RNAi feeding libraries (Geneservice Ltd, Cambridge, England; Open Biosystems, Huntsville, AL). A bacterial strain expressing 202 bases of dsRNA that are not homologous to any predicted *C. elegans* gene was used as a control for non-specific RNAi effects. dsRNA feeding was carried out for two days by transferring synchronized L1 larvae to agar plates seeded with control or specific RNAi bacteria.

Statistical Analysis

All data are presented as means ± S.E. Statistical significance was determined using Student's two-tailed *t* test. When comparing three or more groups, statistical significance was determined by one-way analysis of variance

with a Tukey post-test. P values of $\leq$0.05 were taken to indicate statistical significance.

Results

Elevated Levels of the Organic Osmolyte Glycerol Do Not Alter the Number or Basic Properties of Age-Induced Protein Aggregates

Proteins that contain contiguous repeats of glutamine residues aggregate spontaneously and have been used to identify genes, cellular processes, and physiological conditions that influence protein folding and aggregation in several systems including *C. elegans* (e.g. Morley et al., 2002; Yoshida et al., 2002; Cauchi et al., 2006). A transgene containing 35 contiguous glutamine residues fused to yellow fluorescent protein (Q35::YFP) is soluble and uniformly distributed throughout muscle cells until adult *C. elegans* are 3 days old, after which the protein aggregates spontaneously with age (Morley et al., 2002). Q35::YFP begins to aggregate in 1-day old adult worms within 30-60 min after exposure to hypertonic stress (Choe and Strange, 2008; Chapter II).

C. elegans accumulates the organic osmolyte glycerol when exposed to hypertonic stress (Lamitina et al., 2004). Organic osmolytes function as chemical chaperones to minimize some types of protein damage (Somero, 1986; Yancey, 2005; Burg et al., 2007). To test the role of glycerol in suppressing protein damage, we quantified spontaneous, aging-induced aggregation of Q35::YFP in worms grown on agar containing 51 or 200 mM NaCl. We also examined spontaneous Q35::YFP aggregation in worms carrying a loss-of-function allele of

osm-11. OSM-11 is a secreted protein that functions in Notch signaling and may play a role in modulating behavioral responses to environmental stress (Komatsu et al., 2008; Singh, Chao, et al., 2011). Loss of OSM-11 activity constitutively activates *gpdh-1* expression and glycerol accumulation (Lamitina et al., 2006; Wheeler and Thomas, 2006). As shown in Figure 1A, acclimation to 200 mM NaCl or loss of *osm-11* function increase whole worm glycerol levels ~4- and ~10-fold, respectively. However, despite large increases in glycerol levels, spontaneous aggregation of Q35::YFP was not suppressed in acclimated and *osm-11* mutant worms compared to control animals (Figure 1B).

To further characterize possible effects of glycerol on Q35::YFP aggregation, we examined age-induced aggregate morphology and solubility. *C. elegans* body wall muscle cells contain a contractile myofilament lattice and a noncontractile cell body that contains the cytoplasm and cell organelles. Q35::YFP aggregates were localized by confocal microscopy. Elongated, dense aggregates that aligned with myofilaments were localized to the contractile lattice (Figure 2A). We also observed more diffuse aggregates in the cell body that frequently colocalized with nuclei (not shown). This morphology and pattern of localization is similar to that described previously (Satyal et al., 2000). No obvious differences in morphology and localization were detected in control, 200 mM NaCl acclimated and *osm-11* mutant worms.

We used fluorescence recovery after photobleaching (FRAP) analysis as described previously (Choe and Strange, 2008; Chapter II) to assess the solubility of Q35::YFP present in aggregates. FRAP analysis was carried out on

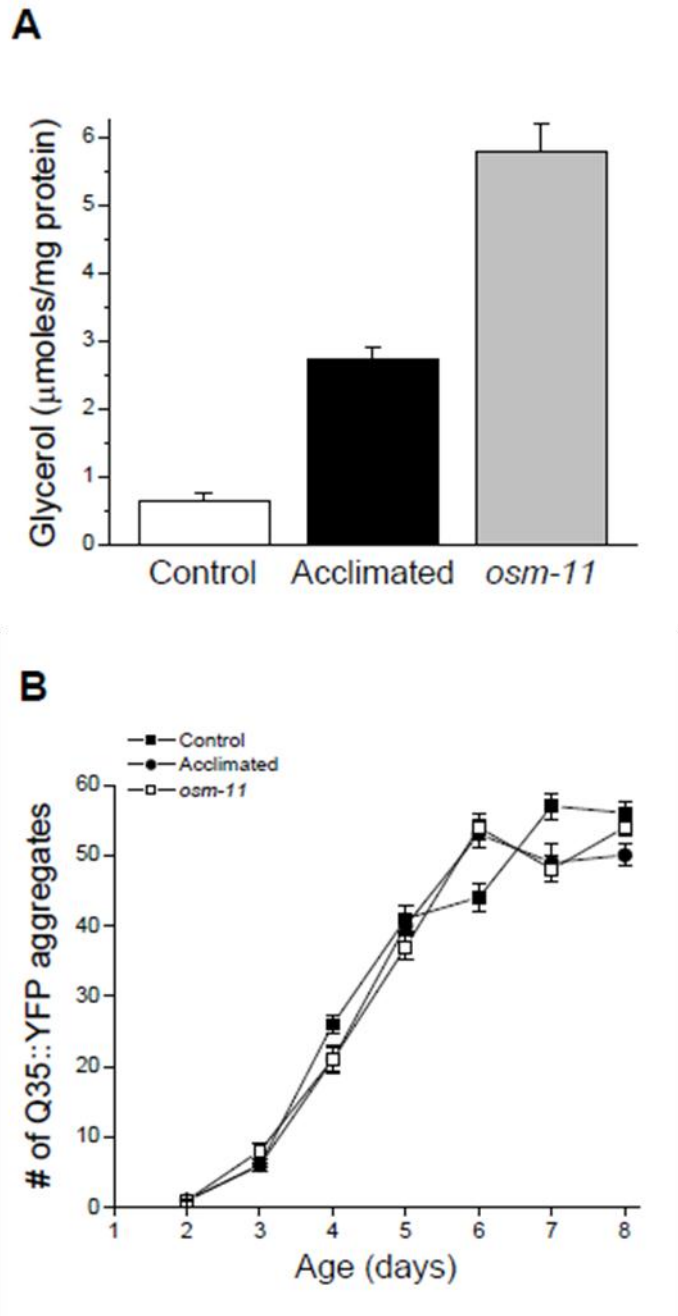

Figure 1. Effect of elevated glycerol levels on aging-induced aggregation of Q35::YFP. *A:* Whole worm glycerol levels in controls worms, worms acclimated to 200 mM NaCl and *osm-11* mutant animals. (*n*=4 samples of ~4000 worms/sample). *B:* Time course of aging-induced Q35::YFP aggregation in control, acclimated and *osm-11* mutant animals. (*n*=7 experiments with 10-15 worms/experiment).

aggregates in young (4-day old) and old (10-day old) adult worms. As shown in Figure 2B, FRAP was undetectable in punctate YFP structures observed in young and old control, 200 mM NaCl acclimated and *osm-11* mutant worms. These results demonstrate 1) that Q35::YFP is localized to aggregates where individual proteins are immobile, and 2) that the presence of high levels of glycerol has no effect on Q35::YFP solubility.

Finally, we examined Q35::YFP toxicity. Q35::YFP aggregates damage muscle cells and cause gradual paralysis (Morley et al., 2002). It is conceivable that glycerol minimizes aggregate toxicity. As shown in Figure 2C, the age associated decline in motility brought about by Q35::YFP aggregation was similar in control, acclimated and *osm-11* mutant worms.

Acclimation Suppresses Stress-Induced Aggregation through Mechanisms Independent of Organic Osmolyte Accumulation

Data in Figures 1 and 2 indicate that high levels of glycerol do not suppress aging-induced Q35::YFP aggregation or alter aggregate properties. However, the ability of acclimation to 200 mM NaCl to suppress hypertonicity-induced Q35::YFP aggregation, but not spontaneous aggregate formation (Figure 1B) suggests that the two aggregation processes are fundamentally different. To directly assess the possible role of glycerol in suppressing hypertonic stress-induced Q35::YFP aggregation, we exposed *osm-11* worms to high NaCl concentrations. As shown in Figure 3A, *osm-11* worms exhibit significantly ($P<0.01$) greater Q35::YFP aggregation at all NaCl concentrations tested (i.e., 200-600 mM) compared to acclimated animals.

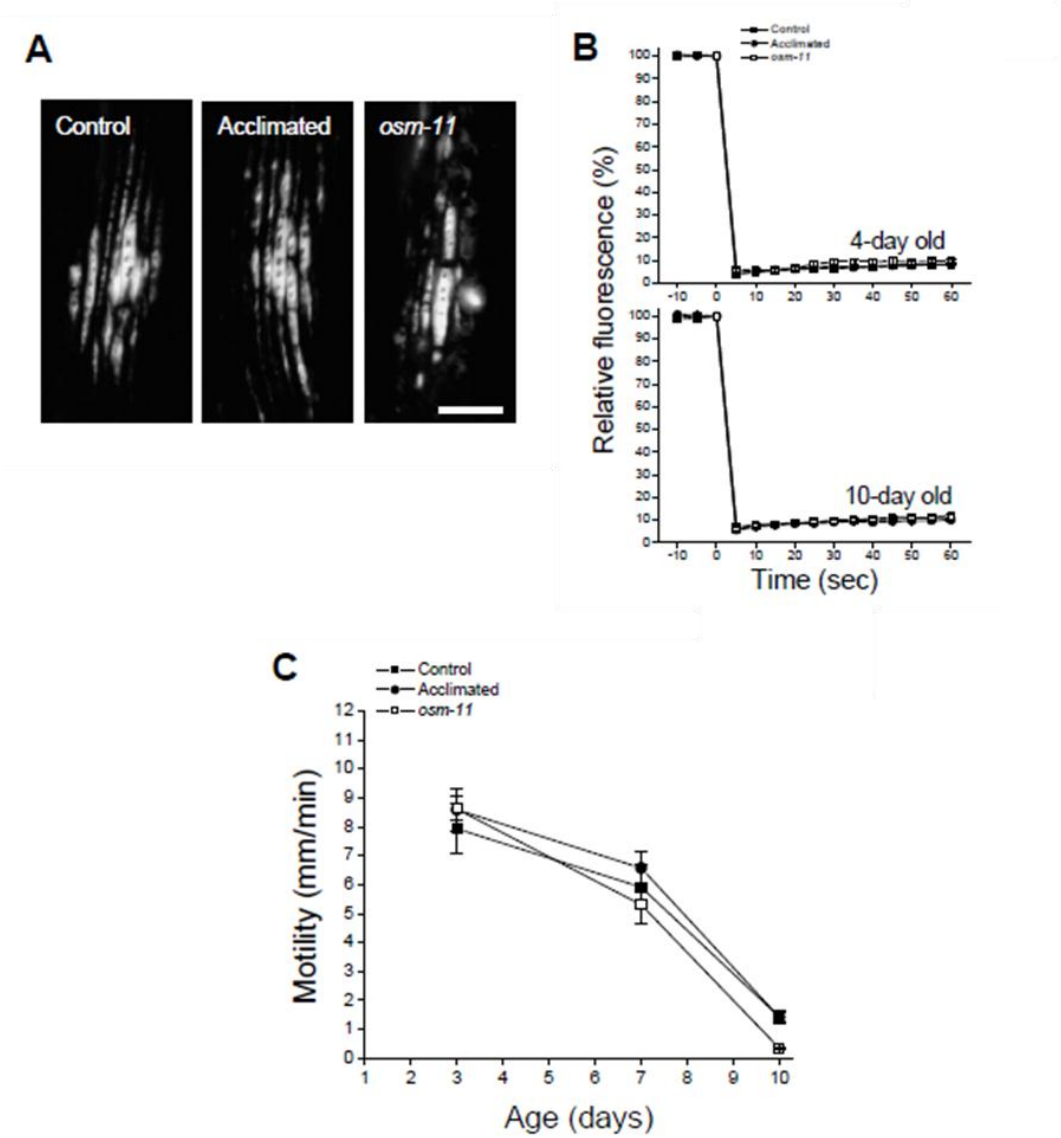

Figure 2. Effect of elevated glycerol levels on the properties of age-induced Q35::YFP aggregates. *A:* Fluorescence micrographs of aggregate morphology in body wall muscle cells of control worms, worms acclimated to 200 mM NaCl and *osm-11* mutant animals. Images were taken from 7-day old adult worms. Scale bar is 10 µm. *B:* Time course of bleaching and fluorescence recovery in aggregates of young (4-day old) and old (10-day old) adult control, acclimated and *osm-11* worms (*n*=3). *C:* Aggregate toxicity in control, acclimated and *osm-11* worms. Toxicity is measured as reductions in motility, which is mediated by body wall muscle cells. (*n*=5–12).

Cell shrinkage and increased macromolecular crowding almost certainly play a role in driving Q35::YFP aggregation in hypertonically stressed worms (Choe and Strange, 2008). A trivial explanation for the results shown in Figure 3A is that *osm-11* animals experience more extreme shrinkage compared to acclimated animals. To test this possibility, we quantified motility in acclimated and *osm-11* worms exposed acutely to 600 mM NaCl. Motility in *C. elegans* requires internal hydrostatic pressure in order for the outer cuticle to function as an exoskeleton that body wall muscles pull against for motility. Loss of body water and hydrostatic pressure causes temporary paralysis until fluid balance is restored. As shown in Figure 3B, virtually all worms acclimated to 200 mM NaCl remain paralyzed for at least 60 min when exposed to 600 mM NaCl. In contrast, ~60% of the *osm-11* worms remain motile under the same conditions. This result is consistent with the observation that glycerol levels in *osm-11* worms are ~2.5 times higher than those in animals acclimated to 200 mM NaCl (Figure 1A). Higher internal solute concentrations and resulting osmotic pressure reduce water loss in hypertonic environments. Thus, even though *osm-11* worms have higher glycerol levels and lose less water during hypertonic stress, Q35::YFP aggregation is more extensive than that observed in acclimated worms. Taken together with data shown in Figure 1B, these results demonstrate 1) that glycerol plays no role in suppressing either spontaneous or hypertonic stress-induced Q35::YFP aggregation, and 2) that acclimation to 200 mM NaCl activates non-osmolyte mechanisms that prevent Q35::YFP aggregation in response to more extreme hypertonic stress.

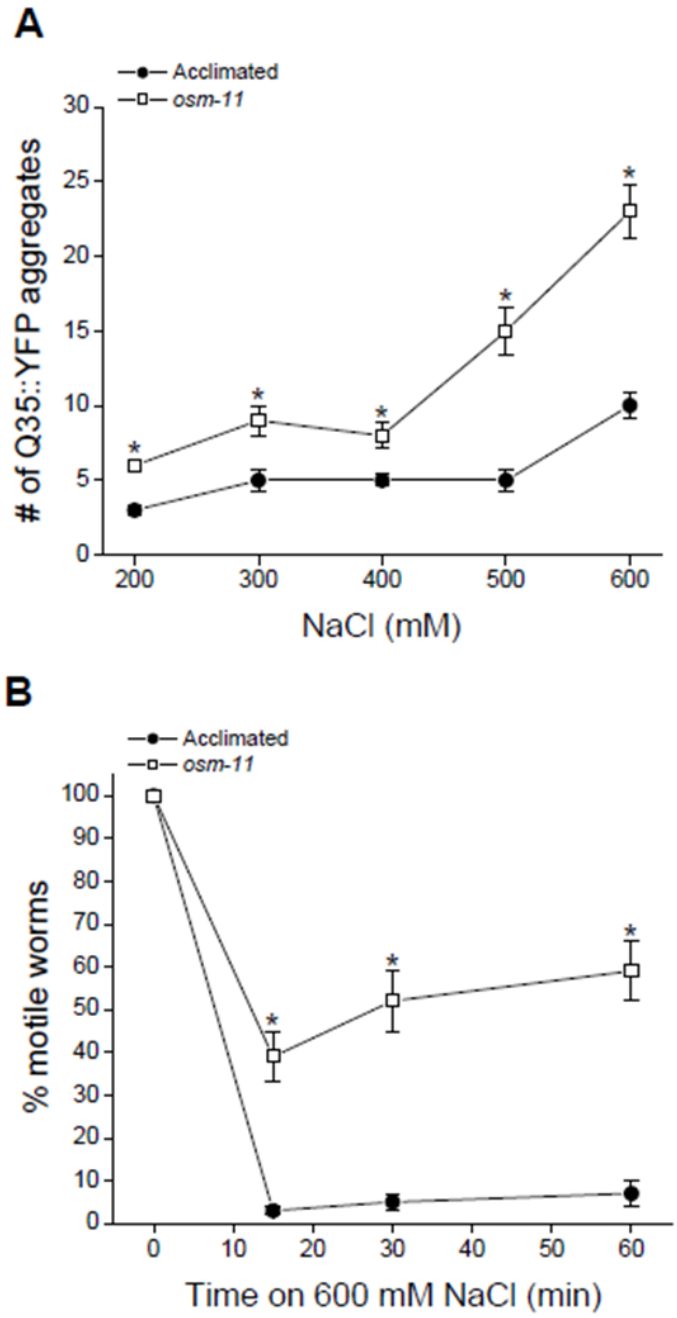

Figure 3. Effect of elevated glycerol levels on hypertonic stress-induced aggregation of Q35::YFP. *A:* Aggregation of Q35::YFP measured after a 24 h exposure of *osm-11* or acclimated worms to media containing 200-600 mM NaCl. (*n*=13-15). *P<0.01 compared to acclimated animals. *B:* Time course of motility changes in *osm-11* and acclimated worms exposed to 600 mM NaCl. Acclimated worms become paralyzed within 10-15 min due to water loss. *osm-11* mutants lose less water due to elevated glycerol levels (see Figure 1A) and therefore remain motile compared to acclimated worms. (*n*=13-21). *P<0.0001 compared to acclimated animals.

To assess how glycerol accumulation directly impacts water loss, we first carried out acute motility studies in two mutant strains with altered glycerol levels and in wild type worms exposed to a range of NaCl concentrations. In contrast to *osm-11* mutants, *gpdh-1; gpdh-2* double mutant worms are deficient in glycerol accumulation. GPDH-1 and GPDH-2 enzymes are both required for hypertonicity-induced glycerol accumulation. Glycerol accumulation in mutant worms carrying deletion mutations in both enzymes (i.e., *gpdh-1; gpdh-2*) is reduced ~60% when they are exposed to 200 mM NaCl (Lamitina et al., 2006).

As expected, wild type worms acclimated to 200 mM NaCl and *osm-11* mutants showed much higher levels of motility over a range of NaCl concentrations compared to unacclimated wild type animals (Figure 4A). *gpdh-1; gpdh-2* double mutant worms acclimated to 200 mM NaCl exhibited reductions in motility similar to those observed of unacclimated wild type animals (Figure 4A). The higher levels of motility in *osm-11* mutants and acclimated wild type worms are consistent with reduced water loss due to elevated glycerol levels. In contrast, suppression of glycerol accumulation in the *gpdh-1; gpdh-2* double mutants results in increased water loss and reductions in motility similar to those of unacclimated wild type worms.

As we have shown previously (Chapter II), endogenous proteins aggregate when *C. elegans* is exposed to hypertonic stress. Aggregated proteins are present in the detergent insoluble fraction of whole worm protein extracts. Acclimation of wild type worms to 200 mM NaCl suppresses aggregation of endogenous proteins when animals are exposed to more extreme

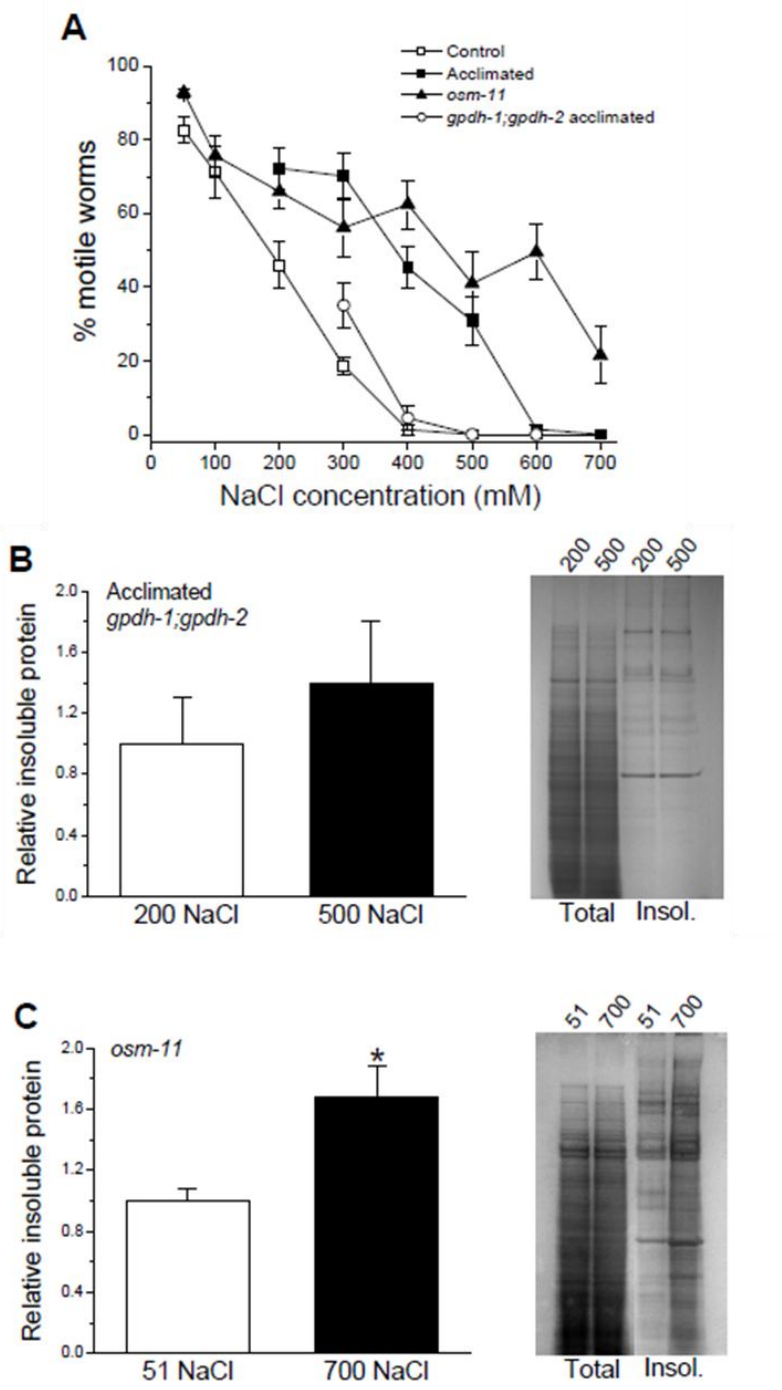

Figure 4. Effect of elevated glycerol levels on hypertonic stress-induced aggregation of endogenous proteins. *A:* Effect of increasing NaCl concentrations on motility in control, acclimated, *osm-11* and acclimated *gpdh-1; gpdh-2* worms. *gpdh-1; gpdh-2* mutants lack functional GPDH-1 and GPDH-2 enzymes resulting in greatly reduced glycerol accumulation under hypertonic stress conditions (Lamitina et al., 2006). (*n*=5–18 experiments with 15–60 worms/experiment). *B:* Left panel, relative insoluble protein in acclimated *gpdh-1; gpdh-2* worms

(Figure 4—cont.) maintained in either 200 mM NaCl or exposed to 500 mM NaCl for 4 h. Insoluble protein was quantified as a fraction of total protein and is plotted relative to that observed in worms maintained on 200 mM NaCl. (*n*=3 experiments with 4000–5000 worms/experiment). Right panel, examples of SDS-PAGE gels of total and detergent insoluble (insol.) proteins isolated from acclimated *gpdh-1; gpdh-2* worms maintained in 200 mM NaCl or exposed to 500 mM NaCl. *C:* Left panel, relative insoluble protein in *osm-11* worms grown under control conditions (51 mM NaCl) or exposed to 700 mM NaCl for 4 h. Insoluble protein was quantified and plotted in the same manner as described in *B*. (*n*=3 samples of 4000–5000 worms/sample). *P<0.03 compared to animals maintained on 51 mM NaCl. Right panel, examples of SDS-PAGE gels of total and detergent insoluble (insol.) proteins isolated from *osm-11* worms exposed to 51 or 700 mM NaCl.

hypertonic stress (Chapter II). To assess whether glycerol plays a role in reducing aggregation, we acclimated *gpdh-1; gpdh-2* double mutants to 200 mM NaCl and then isolated detergent insoluble proteins from them before (i.e., control) and 4 h after exposure to 500 mM NaCl. As shown in Figure 4B, exposure of acclimated *gpdh-1; gpdh-2* mutants to 500 mM NaCl had no significant (P>0.4) effect on the level of aggregated endogenous proteins despite extensive water loss (Figure 4A). Exposure of unacclimated wild type worms to 500 mM NaCl in contrast increases endogenous protein aggregation 220% (Chapter II). Thus, acclimation inhibits protein aggregation independently of GPDH function and glycerol accumulation.

Endogenous proteins undergo substantial aggregation when unacclimated wild type worms are exposed to hypertonic stress (Chapter II). To further assess the possible role of glycerol in suppressing protein aggregation, we isolated detergent insoluble protein fractions from *osm-11* worms maintained under normal growth conditions and *osm-11* mutant worms exposed to 700 mM NaCl

for 4 h. As shown in Figure 4C, 700 mM NaCl caused a significant ($P<0.03$) increase in aggregated endogenous protein levels. This increase is similar to that observed in unacclimated wild type worms exposed to 400 or 500 mM NaCl (Chapter II) even though water loss is considerably lower in *osm-11* mutants (Figure 4A). Data in Figures 4B-C taken together with our previous findings, suggest strongly that glycerol plays little or no role in suppressing endogenous protein aggregation and that mechanisms other than glycerol accumulation must inhibit aggregation in acclimated worms.

Glycerol Does Not Protect Two Metastable Proteins from Misfolding

Organic osmolytes are widely thought to function in the prevention and reversal of protein misfolding (e.g., Hu et al., 2009; Khan et al., 2010). While there is extensive in vitro evidence to support this idea, few studies have characterized the chemical chaperone roles of organic osmolytes in vivo. We examined the role of glycerol in minimizing hypertonic stress-induced protein misfolding using worm strains harboring temperature sensitive (ts) alleles of *let-60* and *unc-15*, which encode a ras GTPase and paramyosin, respectively. At low or permissive temperatures, ts mutations are thought to have little impact on protein folding and associated function. However, at elevated temperatures, these mutations cause protein misfolding that gives rise to dysfunction and mutant phenotypes.

We have shown previously that exposure to 300 mM NaCl induces mutant phenotypes in *let-60(ga89)* and *unc-15(el402)* ts worms maintained at the

permissive temperature of 16 °C (Chapter II). These findings indicate that hypertonic stress induces protein misfolding in vivo. To assess the role of glycerol in preventing this misfolding, we fed *let-60(ga89)* and *unc-15(el402)* worms bacteria producing double-stranded RNA (dsRNA) homologous to *osm-11*. Compared to control animals fed a nonspecific dsRNA, *osm-11(RNAi)* worms maintained high levels of motility on growth plates containing 500, 600 and 700 mM NaCl (data not shown). Motility was similar to that of the *osm-11* mutants (see Figure 4A) indicating that glycerol levels, as expected, are elevated in *osm-11(RNAi)* worms.

let-60(ga89) and *unc-15(el402)* worms failed to lay eggs at NaCl concentrations of 400 mM and above. We therefore assessed the presence of the mutant phenotypes (i.e., egg hatching defects and larval arrest) in worms exposed to 300 mM NaCl at 16 °C. As shown in Figure 5A, the fraction of mutant *let-60(ga89)*; *osm-11(RNAi)* and *unc-15(el402)*; *osm-11(RNAi)* worms was not significantly ($P>0.7$) different compared to worms fed nonspecific dsRNA. These results demonstrate that elevated glycerol levels do not prevent expression of the hypertonic stress-induced mutant phenotypes in *let-60* and *unc*-15 worms. This in turn suggests that glycerol is not capable of suppressing the misfolding of LET-60 and UNC-15 proteins in vivo.

Organic osmolytes protect some proteins from heat-induced misfolding in vitro and can suppress the thermosensitive phenotype of *dnaK* deletion in *E. coli* (Caldas et al., 1999; Chattopadhyay et al., 2004). We therefore examined the effect of elevated glycerol levels on the *let-60(ga89)* and *unc-15(el402)* mutant

phenotypes induced by growing worms at the restrictive temperature of 25 °C. RNAi silencing of *osm-11* had no significant (P>0.5) effect on induction of the mutant phenotypes by elevated growth temperature (Figure 5B).

Heat shock is well known to cause protein misfolding and activation of transcriptional pathways that increase molecular chaperone expression and resistance to heat stress (Gidalevitz et al., 2011; Hartl et al., 2011). If glycerol functions as a chemical chaperone and plays an important role in preventing/reversing protein misfolding in vivo, *osm-11* mutant worms should survive heat shock better than wild type worms. To test this idea, we maintained worms at 35 °C and monitored survival for 10 h. As shown in Figure 5C, there was no difference (P>0.1) in survival of *osm-11* mutants compared to wild type animals at any time point after induction of heat stress. Data shown in Figures 5B-C suggest that high levels of glycerol have little effect on temperature-induced protein misfolding.

Protein Degradation Activities Are Not Enhanced by Hypertonic Pre-Acclimation

In a recent genome-wide RNAi screen we identified 40 genes, termed Hos (HypertOnic Sensitive) genes, which are required for survival of *C. elegans* in hypertonic environments. Twenty Hos genes function in the destruction of damaged proteins (Choe and Strange, 2008). It is conceivable that the suppression of protein damage observed in animals acclimated to mild hypertonic stress (Figures 3A and 4B-C; Chapter II; Choe and Strange, 2008) is due to increased protein degradation activity. We carried out four experiments to

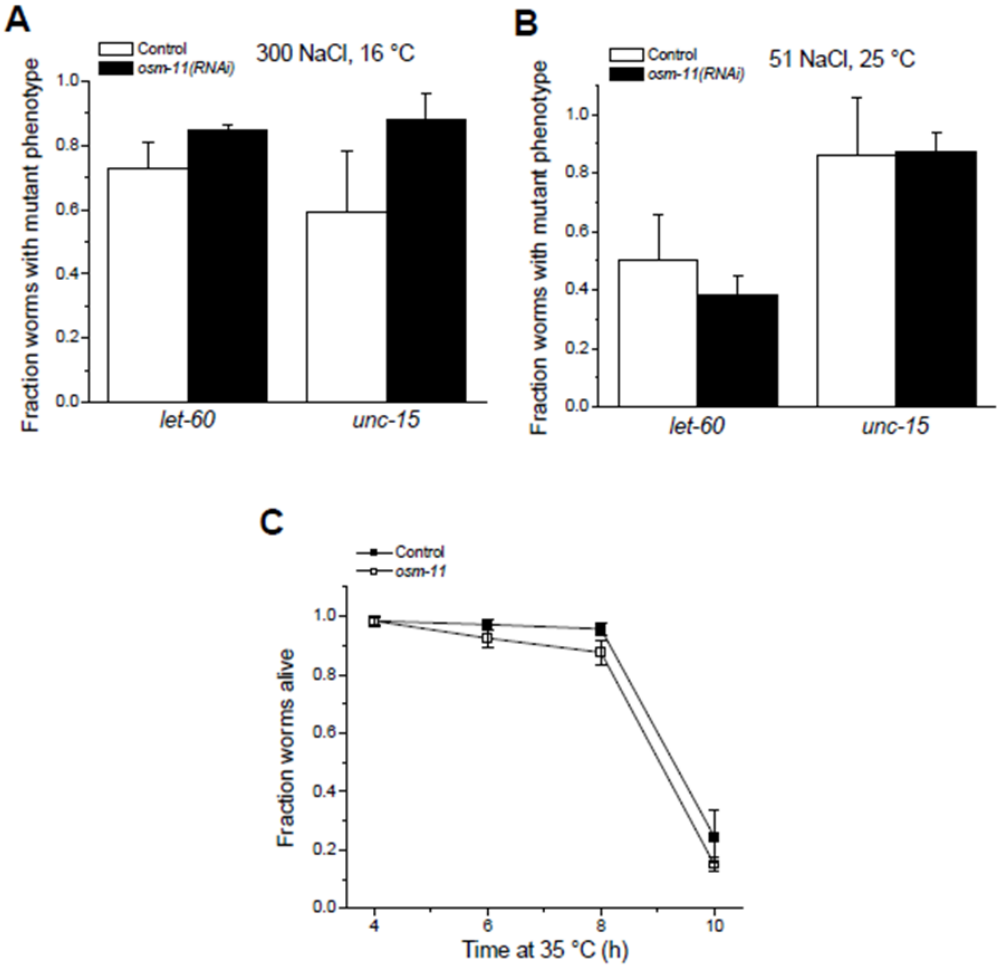

Figure 5. Effect of glycerol accumulation on protein misfolding. *A, B:* Expression of mutant phenotypes in *let-60* and *unc-15* temperature sensitive mutant worms exposed to 300 mM NaCl at 16 °C (*A*) or 51 mM NaCl at 25 °C (*B*). *let-60* and *unc-15* encode a ras GTPase and paramyosin, respectively. As we have shown previously (Chapter II), hypertonic stress induces the mutant phenotype at low or permissive temperatures (i.e., 16 °C) suggesting that water loss causes protein misfolding. Control and *osm-11(RNAi)* worms were fed bacteria expressing a nonspecific dsRNA or *osm-11* dsRNA, respectively. Loss of *osm-11* activity elevates cellular glycerol levels (see Figure 1A), which was confirmed for *osm-11* RNAi by motility assays carried out in parallel (data not shown). Elevated glycerol levels do not suppress hypertonic stress-induced expression of the *let-60* and *unc-15* mutant phenotypes. (*n*= 6-9 experiments, each with 20-50 worms/experiments). *C:* Effect of RNAi knockdown of *osm-11* on survival during heat stress. Increased temperature causes protein misfolding and decreased survival (Gidalevitz et al., 2011; Hartl et al., 2011). Elevated glycerol levels in *osm-11(RNAi)* worms do not enhance survival during heat shock. (*n*=3-7 experiments with 15-40 worms/experiment).

test this possibility. Chloroquine and MG-132 are potent inhibitors of lysosome and proteasome activity, respectively, in *C. elegans* (Choe and Strange, 2008; Orsborn et al., 2007). This lab has shown previously that Q35::YFP aggregation is increased in unstressed worms by treating them with these two drugs (Choe and Strange, 2008). This result demonstrates that protein degradation plays a role in suppressing spontaneous aggregate formation in unacclimated worms.

To assess the role of role of lysosome and proteasome activity on Q35::YFP aggregation in control and acclimated worms, synchronized L1 larvae were grown to adulthood for 2 days on control (51 mM NaCl) or 200 mM NaCl agar plates. They were then transferred to 51 or 200 mM NaCl plates containing vehicle only (1% DMSO) or 20 mM chloroquine and 100 µM MG-132 and Q35::YFP aggregates were quantified 48 h after transfer. As shown in Figure 6A, treatment of control worms with chloroquine and MG-132 increased the mean number of Q35::YFP aggregates nearly 2.2-fold ($P<0.0001$) from 13 to 28. In acclimated worms treated with vehicle only, the mean number of Q35::YFP aggregates was 10 compared to 13 observed in vehicle-treated control animals. While the difference was statistically significant ($P<0.003$), it was not reproducible (compare to Figures 1B and 6B) and is within the range of variability typically seen in these measurements.

Treatment of acclimated worms with chloroquine and MG-132 increased the mean number of aggregates to 16 ($P<0.0001$), which was ~40% lower ($P<0.0001$) than that observed in drug-treated controls. As we have suggested previously (Choe and Strange, 2008), these results indicate that protein

degradation via lysosomes and proteasomes plays a role in suppressing spontaneous Q35::YFP aggregation. However, the striking reduction of spontaneous aggregation in acclimated worms treated with lysosome and proteasome inhibitors suggests that acclimation to mild hypertonic stress suppresses protein aggregation by mechanisms that are independent from protein degradation.

We also inhibited lysosome and proteasome activity using RNAi knockdown of Hos genes that encode proteasome or lysosome components and quantified the effect on Q35::YFP aggregation. The Hos genes tested were *pas-6* and *rpn-3*, which encode components of the 26S proteasome, *vha-13*, which encodes a subunit of the vacuolar proton-translocating ATPase, and F13D12.6, which encodes a putative lysosomal serine carboxypeptidase. RNAi was performed by transferring synchronized L1 larvae to control (51 mM NaCl) or acclimation (200 mM NaCl) agar plates seeded with bacteria producing dsRNA targeting one of the Hos genes. Aggregates were quantified 72 h after transfer.

As shown in Figure 6B, RNAi of *pas-6* and *vha-13* caused significant ($P<0.001$) increases in Q35::YFP aggregation in unacclimated worms relative to control animals fed bacteria expressing a nonspecific dsRNA. In contrast, only knockdown of *vha-13* increased ($P<0.001$) aggregation in worms acclimated to 200 mM NaCl. When compared to control worms, the number of aggregates observed in acclimated *vha-13(RNAi)* animals was significantly ($P<0.03$) lower. These data are consistent with chloroquine/MG-132 studies (Figure 6A) and

suggest that suppression of protein damage in acclimated worms is not mediated by enhanced activity of protein degradation pathway.

Results shown in Figure 6A-B assume near complete inhibition of proteasome and lysosome activity by chloroquine and MG-132 and by RNAi. To more directly assess whether protein degradation capacity is increased in acclimated worms, we quantified proteasome activity using a worm strain expressing in their body wall muscle cells the green fluorescent protein Dendra2 tagged with the mutant ubiquitin UbG76V. UbG76V is not cleaved off of proteins by ubiquitin hydrolases, resulting in polyubiquitination of UbG76V-Dendra2 and subsequent targeting of the protein to proteasomes for degradation. Excitation of Dendra2 with 405 nm light converts its green fluorescence to red, thus generating a pool of mature, red fluorescent Dendra2 distinct from any newly translated, green fluorescent Dendra2. Decreases in red fluorescence therefore reflect proteasome activity (Hamer et al., 2010).

Figure 6C shows the amount of red UbG76V-Dendra2 remaining in body wall muscle cells 24 h after photoconversion under control conditions and during exposure to hypertonic stress. To induce similar degrees of water loss, control worms were exposed to 400 mM NaCl while acclimated animals were exposed to 600 mM NaCl (Figure 4A). There were no significant differences ($P>0.3$) between red Dendra2 levels in control and acclimated animals under either control or hypertonic stress conditions demonstrating that proteasome activity is not upregulated in acclimated worms. Interestingly, however, hypertonic stress caused a significant ($P<0.01$) inhibition of red Dendra2 degradation. A likely

explanation for these results is that hypertonicity-induced protein damage overloads the proteasomal degradation system resulting in a slowing of Dendra2 removal.

To quantify total protein degradation capacity, cellular proteins in control worms and worms acclimated to 200 mM NaCl were labeled with ^{35}S-methionine. Immediately after the intestinal purge step of the radiolabeling procedure, worms were either frozen for protein extraction or transferred for 6 h to control or 200 mM NaCl plates containing 500 µg/ml cycloheximide. This concentration of cycloheximide has been shown by Kourtis and Tavernarakis (2009) to effectively block protein synthesis in *C. elegans*. As shown in Figure 6D, ^{35}S-labeled total protein levels in control and acclimated worms dropped by 23% and 15%, respectively, after inhibition of protein synthesis by cycloheximide. These reductions in labeled protein levels were not significantly ($P>0.2$) different demonstrating that rates of protein degradation are similar in control and acclimated worms.

Protein Synthesis Is Reduced in Acclimated Worms

Data in Figures 1-6 demonstrate that neither glycerol accumulation nor upregulation of lysosome/proteasome activity are responsible for decreases in hypertonicity-induced protein damage observed in worms acclimated to mild hypertonic stress. Therefore, acclimation most likely increases molecular chaperoning capacity. Increased chaperone capacity can be brought about by

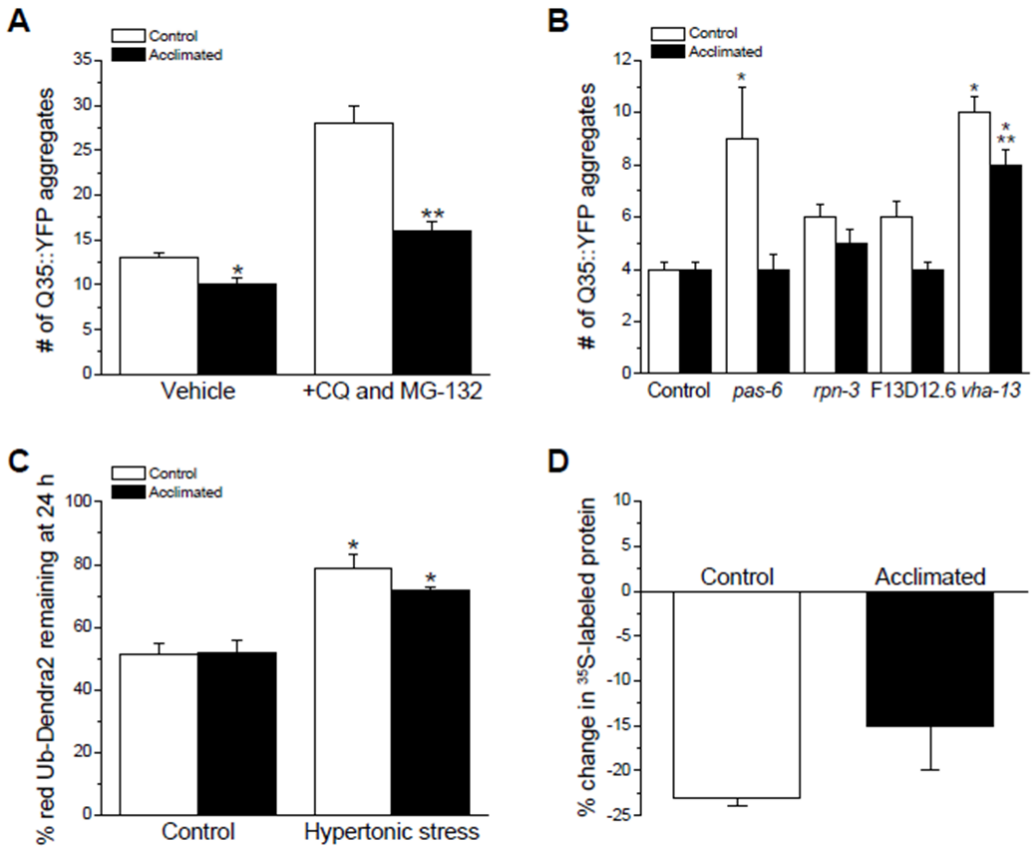

Figure 6. Effect of acclimation to mild hypertonic stress on protein degradation activity. *A:* Effect of treatment of control and acclimated worms with vehicle only (1% DMSO) or 20 mM chloroquine (CQ) and 100 µM MG-132 on spontaneous aggregation of Q35::YFP. (*n*=9-15). *P<0.003 compared to vehicle-treated control worms. **P<0.0001 compared to drug-treated control worms. *B:* Effect of RNAi silencing of Hos genes on spontaneous Q35::YFP aggregation in control and acclimated worms. Animals were fed bacteria expressing nonspecific (control) dsRNA or dsRNA targeting proteasome (*pas-6* and *rpn-3*) and lysosome (*vha-13*) components, or a putative lysosomal serine carboxypeptidase (F13D12.6). (n=16-51). *P<0.001 compared to control or acclimated worms fed a nonspecific dsRNA. **P<0.03 compared to unacclimated *vha-13(RNAi)* worms. *C:* Percent of red mutant ubiquitin (UbG76V) tagged Dendra2 remaining in body wall muscle cells 24 h after photoconversion in control and 200 mM NaCl acclimated worms exposed to control or hypertonic growth media. Control and acclimated animals were exposed to 400 mM and 600 mM NaCl, respectively. (*n*=3–8). *P<0.01 compared to unstressed worms. *D:* Percent change in ^{35}S-methionine labeled total protein levels in control and acclimated worms treated with 500 µg/ml of cycloheximide for 6 h to inhibit protein synthesis. (*n*=3).

increases in the activity of molecular chaperones and/or by decreases in protein synthesis.

Reductions in protein synthesis decrease the number of nascent and newly synthesized proteins that are subjected to misfolding and aggregation. This reduction in turn is expected to decrease the load on the molecular chaperone and protein degradation network thereby increasing their availability for minimizing, reversing and/or removing damage to existing proteins. Inhibition of protein synthesis will also free up energy resources for use in other cellular processes including protein repair and degradation.

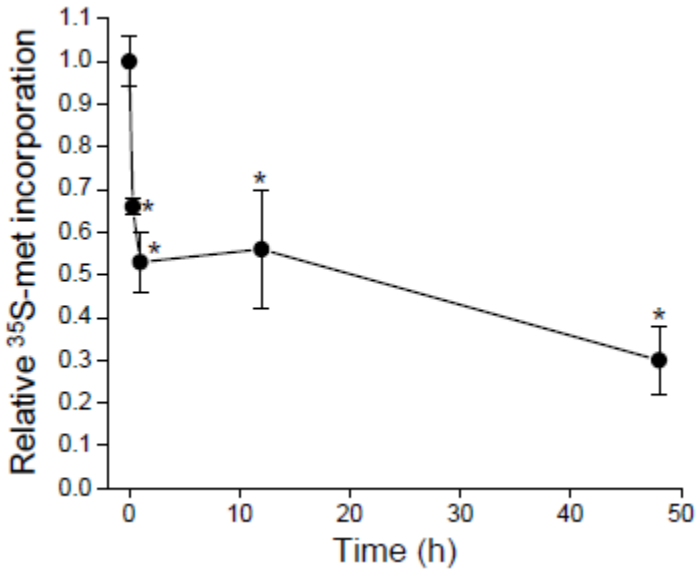

Figure 7. Effect of acute hypertonic stress on ^{35}S-methionine incorporation into total protein. Worms were transferred to 200 mM NaCl agar plates at time 0. Values are expressed relative to unstressed control worms. (*n*=3). *P<0.05 compared to control worms.

Numerous environmental and physiological stressors inhibit protein synthesis in diverse cell types and organisms (Holcik and Sonenberg, 2005; Yamasaki and Anderson, 2008). We quantified ^{35}S-methionine incorporation into total protein to assess the effects of hypertonic stress on protein translation in *C. elegans*. As shown in Figure 7, a striking drop in ^{35}S-methionine incorporation was detected with as little as 20 min of exposure to 200 mM NaCl. Maximal inhibition was observed within 1 h and remained inhibited at this level for at least 48 h.

While unlikely, it is possible that the reduced incorporation of ^{35}S-methionine into total protein observed in hypertonically stressed worms was due to an increased rate of protein degradation with subsequent loss of the labeled methionine. Total protein degradation is similar in control and fully acclimated worms (Figure 6D). To determine whether protein degradation changes during the acclimation process, we measured the decline in ^{35}S-methionine labeled protein levels in worms treated with 500 µg/ml cycloheximide. Protein degradation was examined at a time point when systemic volume recovery was complete (Lamitina et al., 2004) to avoid the more extreme stress of severe water loss combined with cycloheximide treatment. Worms grown on 51 mM NaCl were labeled with ^{35}S-methionine and then exposed to either 51 or 200 mM NaCl. After 15 h, the worms were frozen or transferred to 51 or 200 mM NaCl agar plates containing cycloheximide for 6 h. As expected, ^{35}S-methionine labeled protein levels declined in cycloheximide-treated worms. The mean ± S.E. percent change observed was -36 ± 4% and -40 ± 9% (n=4) for worms exposed

to 51 or 200 mM NaCl, respectively. These values were not significantly (P>0.6) different demonstrating that rates of protein degradation are similar in control worms and worms exposed acutely to 200 mM NaCl. Thus, the reduced ^{35}S-methionine incorporation into total protein observed in worms acclimating to hypertonic stress reflects a reduced rate of protein synthesis rather than increased degradation.

Suppression of Protein Translation Prevents Hypertonic Stress-Induced Protein Damage

To test whether inhibition of protein synthesis reduces hypertonic stress-induced protein damage we silenced the expression of genes that play essential roles in translation. Worms were fed bacteria expressing dsRNA homologous to genes encoding arginyl and histidyl amino-acyl tRNA synthetases (*rrt-1* and *hars-1*, respectively), and the eukaryotic translation initiation factors eIF2α (*iftb-1*) and eIF4A (F57B9.3). Knockdown of these genes inhibits ^{35}S-methionine incorporation into total protein by 50-80% (Hansen et al., 2007; Anderson et al., 2009; E.C. Lee and K. Strange, personal correspondence). As shown in Figure 8A, RNAi of *rrt-1*, *hars-1*, and *iftb-1* inhibited hypertonicity-induced Q35::YFP aggregation by ~70-80% 3 and 6 h after exposure to 500 mM NaCl (P<0.002). RNAi of F57B9.3 inhibited aggregation significantly (P<0.004) by ~50% at 6 h only.

Loss of *rrt-1*, *hars-1*, *iftb*-1 or F57B9.3 also activates *gpdh-1* expression (Lamitina et al., 2006; E.C. Lee and K. Strange, personal correspondence). Because constitutive activation of *gpdh-1* usually results in elevation of whole

animal glycerol levels (Lamitina et al., 2006; Figure 1A), it is conceivable that the inhibition of Q35::YFP aggregation shown in Figure 8B is due to reduced water loss and shrinkage in worms exposed to 500 mM NaCl. To test this possibility, we carried out motility assays. 15 ± 3%, 20 ± 6% and 20 ± 11% (n=4-7 experiments) of worms fed bacteria expressing nonspecific, *hars-1* or *rrt-1* dsRNA remained motile for up to 1 h when placed on 500 mM NaCl. These values were not significantly (P>0.5) different. *iftb-1(RNAi)* and *F57B9.3(RNAi)* showed significantly (P<0.008) reduced motility under these conditions (mean ± S.E. motility was 0 ± 0% and 2 ± 2% in *iftb-1(RNAi)* and *F57B9.3(RNAi)* worms, respectively; n=5 experiments). The reason for the reduced motility is unclear. However, the results demonstrate that the reduction of Q35::YFP aggregation in *rrt-1*, *hars-1*, *iftb*-1 and F57B9.3 RNAi worms is due to inhibition of protein synthesis per se rather than reduced water loss and shrinkage.

It is conceivable that chronic inhibition of protein synthesis by RNAi reduces Q35::YFP aggregation simply by reducing the cellular concentration of Q35::YFP protein and/or by increasing the expression of components of the chaperoning/degradation network that may minimize aggregate formation. To address these possibilities, we reduced translation acutely by treating Q35::YFP worms with 500 µg/ml of cycloheximide for 15 min followed by a 1 h exposure to 500 or 700 mM NaCl with cycloheximide present. Worms were then transferred back to control growth medium without drug and Q35::YFP aggregates were quantified 2.5-3 h later. As shown in Figure 8B, cycloheximide treatment

reduced the number of Q35::YFP aggregates ~66% and ~50% ($P<0.0005$) in worms exposed to 500 mM and 700 mM NaCl, respectively.

Acute inhibition of protein synthesis could conceivably reduce cellular protein levels and hence macromolecular crowding, which in turn could reduce Q35::YFP aggregation. To test this possibility, we measured total protein in worms exposed to 400 mM NaCl in the presence and absence of cycloheximide. The mean ± S.E. protein levels in control and cycloheximide-treated worms were 0.66 ± 0.04 µg/worm (n=5) and 0.48 ± 0.15 µg/worm (n=3), respectively, and were not significantly ($P>0.4$) different.

The cycloheximide-induced reduction in Q35::YFP aggregation shown in Figure 8B could be due to increases in the availability of molecular chaperone and/or protein degradation networks for suppression of hypertonic stress-induced protein damage. To test whether protein degradation plays a role in suppressing Q35::YFP aggregation, we pretreated worms for 3 h with 20 mM chloroquine and 100 µM MG-132. Worms were then exposed continuously to these drugs during the 15 min cycloheximide pretreatment, during exposure to 500 mM NaCl with cycloheximide, and during the 2.5-3 h recovery period. In worms treated with cycloheximide alone or cycloheximide, chloroquine and MG-132, we observed 6 ± 2 and 8 ± 2 (mean ± S.E.; n=10 worms) hypertonic stress-induced Q35::YFP aggregates, respectively. There was no significant ($P>0.5$) difference in the number of aggregates observed in the two groups of animals. These results suggest that the suppression of Q35::YFP aggregation induced by cycloheximide

is due to the activity of molecular chaperones rather than protein degradation mechanisms.

If inhibition of protein synthesis reduces Q35::YFP aggregation during hypertonic stress, the converse is expected to be true: increased protein production and cellular protein levels should increase aggregate formation. To the best of our knowledge, no experimental maneuver has been identified that acutely stimulates protein synthesis in *C. elegans*. However, recent studies by So et al. (2011) have shown that feeding *C. elegans* the *E. coli* strain HB101 increases body size 1.6-fold. This increase is due entirely to cell enlargement with a concomitant increase in worm protein content of ~60%. Numerous studies in diverse organisms and cell types have shown that increases in cell size and protein content are tightly coupled to increases in protein synthesis (e.g., Fingar et al., 2002; Faridi et al., 2003; Hannan et al., 2003; Wang and Proud, 2006).

To assess the effects of intracellular protein levels on Q35::YFP aggregation, we fed worms either OP50 or HB101 *E. coli* strains. Assuming that worm shape approximates a cylinder, we estimated that HB101 fed worms were ~1.7-fold larger than OP50 fed animals (data not shown), which is similar to that observed by So et al. (2011). Interestingly, when exposed to NaCl concentrations of 500 mM or greater, HB101 fed worms survived poorly compared to OP50 fed animals (data not shown). However, survival was similar when worms were exposed to 400 mM NaCl for 24 h (90 ± 7% and 91 ± 1% in HB101 and OP50 fed worms, respectively; n=15-20 worms and 3 independent experiments).

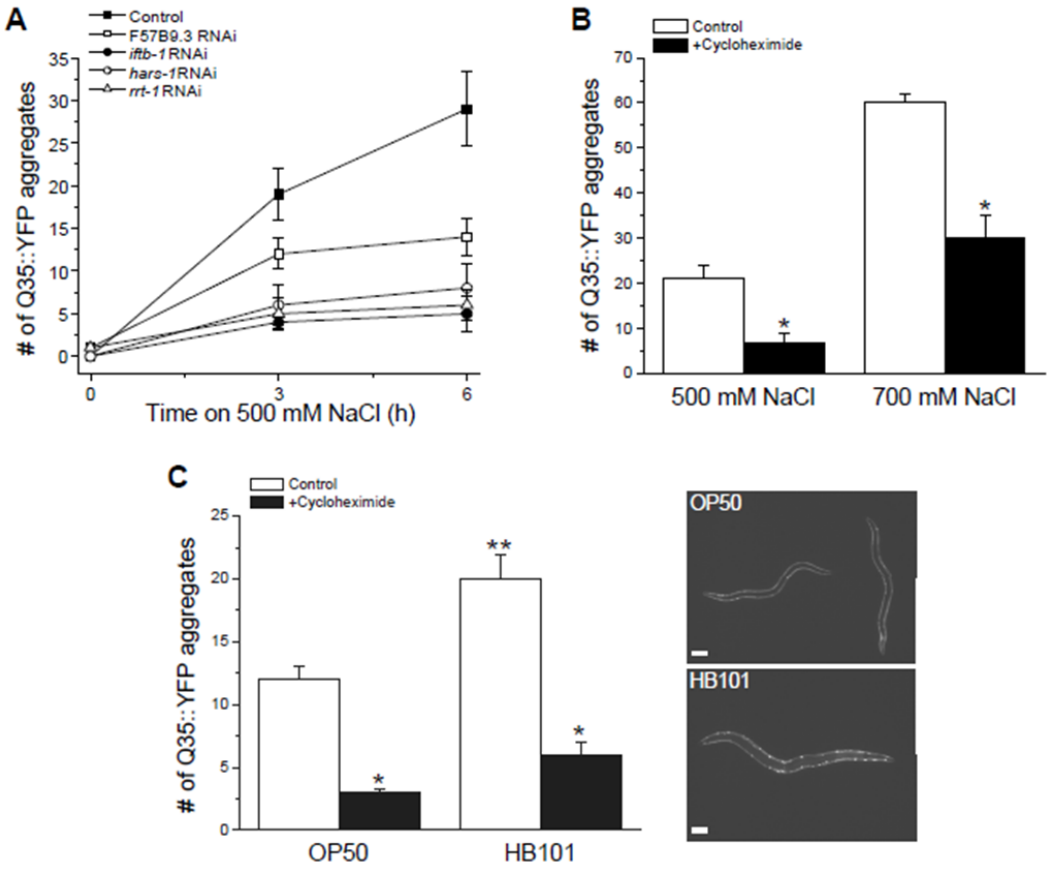

Figure 8. Effect of inhibition of protein synthesis on hypertonic stress-induced Q35::YFP aggregation. *A:* Hypertonic stress-induced Q35::YFP aggregates in worms fed bacteria producing a nonspecific dsRNA (control) or bacteria producing dsRNA targeting eukaryotic translation initiation factors (F57B9.3 and *iftb-1*) or amino-acyl tRNA synthetases (*hars-1* and *rrt-1*). The number of Q35::YFP aggregates was significantly (P<0.002) lower at both 3 and 6 h in worms fed dsRNA targeting *iftb-1*, *hars-1* and *rrt-1*. Aggregates were reduced significantly *(P<0.004) at 6h in worms fed RNAi targeting F57B9.3. (n=15). *B:* Hypertonic stress-induced Q35::YFP aggregates in control worms and worms exposed to 500 µg/ml cycloheximide. Worms were exposed to cycloheximide in control growth medium for 15 min and then transferred to 500 or 700 mM agar containing cycloheximide. After 1 h worms were returned to control medium without the drug and aggregates quantified 2.5-3 h later. (n=8-12). *P<0.0005 compared to untreated control worms. *C:* Left panel, hypertonic stress-induced Q35::YFP aggregation in worms fed OP50 or HB101 bacteria. Worms were exposed to 400 mM NaCl for 1 h. The protocol for cycloheximide treatment was the same as that described in *B*. (n=14-27). *P<0.0001 compared to control worms without cycloheximide treatment. **P<0.0009 compared to OP50 fed worms. Right panel, representative fluorescent micrographs of OP50 and HB101 fed worms exposed to 400 mM NaCl for 1 h. Scale bar is 100 µm.

OP50 and HB101 fed worms were exposed to 400 mM NaCl for 1 h and then allowed to recover on 51 mM for 2-2.5 h before Q35::YFP aggregates were quantified. As shown in Figure 8C, the number of aggregates was 1.7-fold higher ($P<0.0009$) in HB101 fed worms. The results were even more striking when worms were exposed continuously to 400 mM NaCl for 24 h. Worms fed OP50 bacteria had 13 ± 2 aggregates (n=9) while in HB101 fed worms the number of aggregates was 2.8-fold higher (37 ± 3 aggregates; n=9; $P<0.0001$).

The higher levels of Q35::YFP aggregation in HB101 fed worms could be due to increased Q35::YFP expression. To assess this, we quantified Q35::YFP fluorescence intensity in body wall muscle prior to inducing aggregation by exposing worms to hypertonic stress. Fluorescence was measured in a 50 x 98 pixel rectangular region of interest in six different areas of single worms. The box was placed so that the body wall extended from an upper corner to the opposite lower corner. This approach ensured that similar amounts of body wall were present in each measurement box. Mean ± S.E. pixel intensity was 4107 ± 140 and 3905 ± 183 (n=8 worms) in OP50 and HB101 fed worms, respectively. These values were not significantly ($P>0.4$) different indicating that increased Q35::YFP aggregation is not due simply to increased expression of the aggregating protein.

Finally, we assessed the effect of acute inhibition of protein synthesis on Q35::YFP aggregation in worms fed HB101 bacteria. Worms were pre-treated with 500 µg/ml cycloheximide for 15 min, exposed to 400 mM NaCl for 1 h in the presence of the drug and then allowed to recover for 2-2.5 h on control medium

before Q35::YFP aggregates were quantified. Cycloheximide treatment reduced Q35::YFP aggregate formation 75% and 70% ($P<0.0001$) in OP50 and HB101 fed worms, respectively (Figure 8C).

Consistent with previous findings (So et al., 2011), we found that protein levels in HB101 bacteria fed worms (1.04 ± 0.2 µg/worm, n=4) were significantly ($P<0.04$) higher than in worms fed OP50 bacteria (0.66 ± 0.02 µg/worm, n=6). Acute cycloheximide treatment had no significant ($P>0.4$; OP50 bacteria fed worms = 0.48 ± 0.15 µg/worm, n=3; HB101 bacteria fed worms = 0.89 ± 0.11 µg/worm, n=5) effect on protein levels in either group of animals. Taken together, data in Figure 8 demonstrate that increased protein expression results in increased hypertonic stress-induced protein damage.

Discussion

In vitro studies have led to the widely held assumption that hypertonic stress damages animal cell proteins in vivo (e.g., Somero, 1986). However, with the exception of our work in *C. elegans* (Chapter II), there are relatively few studies that have addressed this problem directly. We have shown that hypertonic stress causes rapid and widespread damage to diverse proteins in worms (Choe and Strange, 2008; Chapter II), and that the ability of *C. elegans* to survive in hypertonic environments depends critically on the activity of genes that function to destroy damaged proteins (Choe and Strange, 2008).

Work from numerous labs in diverse animals and cell culture models has shown that acclimation to low levels of environmental stressors such as heat, heavy metals or hypertonicity increases survival during exposure to more extreme levels of the same stress (e.g., Muyssen and Janssen, 2004; Larkindale and Vierling, 2008; Lee et al., 2011). Similarly, acclimation of *C. elegans* to relatively mild hypertonic stress increases survival under more extreme hypertonic conditions (Lamitina et al., 2004). Importantly, acclimation also activates mechanisms that function to suppress hypertonicity-induced protein damage (Choe and Strange, 2008; Chapter II).

Understanding the mechanisms that protect proteins from environmental stressors and that repair and degrade damaged proteins during cellular stress has important implications for understanding and treating numerous diseases and the pathophysiology associated with aging (Balch et al., 2008; Morimoto and Cuervo, 2009). *C. elegans* provides multiple advantages for defining these mechanisms including experimental speed and economy, and genetic and molecular tractability. The *C. elegans* hypertonic stress response is a particularly powerful model for defining stress-associated protein quality control mechanisms due to the extent and speed at which water loss causes damage to diverse proteins and the relative ease of quantifying these changes in diverse genetic backgrounds and under various experimental conditions (Choe and Strange, 2008; Chapter II).

As discussed earlier, there are three physiological processes that could play a role in suppressing hypertonicity-induced protein damage in acclimated

worms: 1) accumulation of the organic osmolyte glycerol, 2) increased lysosome/proteasome activity, and/or 3) increased capacity of the molecular chaperone network. It was surprising and interesting to find that glycerol, the major organic osmolyte accumulated by *C. elegans* in response to water loss (Lamitina et al., 2004; Lamitina et al., 2006), has no obvious impact on protein aggregation (Figures 1A, 3A, 4B-C), Q35::YFP toxicity (Figure 2C) or misfolding of at least two proteins (Figure 5). Organic osmolytes are widely viewed to function as chemical chaperones that aid in stabilizing native protein conformation and in assisting the refolding of denatured proteins (Hu et al., 2009; Khan et al., 2010). The vast majority of work that supports this idea comes from in vitro studies. A few in vivo studies in bacteria have characterized the role of organic osmolytes in inhibiting spontaneous aggregation of transgenic proteins. For example, Ignatova and Gierasch (2006) demonstrated that proline inhibits aggregation of the aggregation-prone P39A cellular retinoic acid binding protein in *E. coli*, and Schultz et al. (2007) have proposed that trehalose inhibits aggregation of several *E. coli* expressed recombinant proteins. However, to the best of our knowledge, there are no previous in vivo studies that have directly assessed the role of organic osmolytes in protecting proteins from hypertonic stress-induced damage.

Both in vitro and in vivo studies have also shown that organic osmolytes protect proteins from heat-induced damage (e.g., Caldas et al., 1999; Chattopadhyay et al., 2004). However, high glycerol levels do not suppress elevated temperature-induced expression of the ts phenotype in *let-60* and *unc-*

15 mutants (Figure 5B) and do not confer increased resistance to heat shock (Figure 5C). Taken together, our studies raise questions about the physiological roles of glycerol and possibly other organic osmolytes in protecting proteins from stress-induced damage.

Interestingly, the presence of high glycerol levels in *osm-11* mutants is associated with increased hypertonic stress-induced aggregation of Q35::YFP (Figure 3A) even though these animals lose less water than control worms under comparable conditions (Figures 3B, 4A). It is important to note that despite the widely held view that organic osmolytes stabilize native protein structure, these solutes can have no protective effect or can even enhance protein misfolding and aggregation in vitro. For example, betaine concentrations of 5-20 mM cause GST-GFP to misfold and aggregate (Natalello et al., 2009). Relatively low concentrations (100 mM) of proline increase heat-induced aggregation of glycogen phosphorylase *b* (Eronina et al., 2009). Molar concentrations of glycerol have little or no effect on folding of RNase T1 (Wu and Bolen, 2006), and do not reverse inhibition or prevent aggregation of unfolded lysozyme (Samuel et al., 2000). The presence of glycerol in solution increases thermal inactivation of phosphofructokinase (Faber-Barata and Sola-Penna, 2005). These findings together with our results indicate that extensive in vivo studies of organic osmolyte function are required to define the precise role of these solutes during osmotic stress.

A recent genome-wide RNAi screen performed in the lab identified 40 genes that are required for survival of *C. elegans* in hypertonic environments.

Twenty of these genes encode proteins that detect, transport, and degrade damaged proteins, including components of the ubiquitin-proteasome system, endosomal sorting complexes, and lysosomes (Choe and Strange, 2008). Upregulation of cellular protein degradation capacity, however, is not responsible for the suppression of protein damage observed in acclimated worms (Figure 6 and Results).

Our results suggest strongly that the primary mechanism limiting protein damage in acclimated worms exposed to more extreme hypertonic stress is increased molecular chaperone capacity. This increased capacity could be brought about in two ways. First, as shown for many stressors including osmotic stress (Burg et al., 2007), the expression of molecular chaperones may be increased. However, microarray studies carried out by Rohlfing et al. (2010) demonstrated that exposure of *C. elegans* to cadmium increases the expression of 14 heat shock proteins (HSP) whereas no HSPs are induced by osmotic stress and some show reduced transcription. It is important to note though that increased expression of chaperones could be brought about by enhanced translation of chaperone mRNA (Warringer et al., 2010) and/or chaperone activity may be increased by post-translational modifications such as phosphorylation (e.g., Aquilina et al., 2004; Taipale et al., 2010). Additional studies are required to assess the role of these processes in the *C. elegans* osmotic stress response.

Molecular chaperone capacity can also be increased by decreasing the load on the molecular chaperone network. Acclimation of *C. elegans* to mild hypertonic stress inhibits protein synthesis ~50-70% (Figure 7). Inhibiting

translation reduces energy consumption, the total number of cellular proteins that may be damaged by the stressor, and the number of nascent proteins prone to aggregation and that require co-translational chaperone-mediated folding. This in turn is expected to free up energy resources, molecular chaperones and protein degradation machinery that could be used to minimize and reverse damage to existing proteins or remove damaged proteins from the cell.

While the idea that inhibition of translation makes cellular resources available that could be used to minimize protein damage is an attractive and widely cited one (e.g., Holcik and Sonenberg, 2005; Yamasaki and Anderson, 2008), there is little direct evidence to support it. For example, chronic inhibition of protein synthesis extends lifespan in numerous model systems (Mehta et al., 2010). Two studies in *C. elegans* have shown that inhibition of translation also increases thermal and oxidative stress resistance (Hansen et al., 2007; Syntichaki et al., 2007). Increased stress resistance may be due to the increased ability of cells to reduce protein damage. King et al. (2008) have shown that inhibition of protein synthesis for 20 h by rapamycin or cycloheximide inhibits spontaneous polyglutamine aggregation in mammalian cells. However, they conclude that this is due to a reduction in the concentration of mutant protein required for nucleation and subsequent aggregation to occur.

Other studies suggest that reductions in protein synthesis have no effect on protein damage or actually increase it. Moulder et al. (1999) showed that cycloheximide does not alter spontaneous aggregation-induced polyglutamine-GFP toxicity in cultured mammalian neurons. In *C. elegans*, silencing of genes

required for protein synthesis, including genes encoding translation initiation factors, induces early onset of spontaneous polyglutamine-YFP aggregation (Nollen et al., 2004).

Our studies demonstrate that both chronic and acute inhibition of protein synthesis dramatically inhibit hypertonic stress-induced aggregation of Q35::YFP (Figure 8). To the best of our knowledge, this is the first direct demonstration that the rate of protein synthesis reduces protein damage brought about by an environmental stressor. The inability of inhibitors of proteasome and lysosome activity to reverse the protective effect of cycloheximide suggests that molecular chaperone activity is primarily responsible for suppressing hypertonic stress-induced Q35::YFP aggregation during acute inhibition of protein synthesis.

Increased transcriptional expression of *gpdh-1* is required for glycerol accumulation when worms are exposed to hypertonic environments (Lamitina et al., 2006). Genome-wide RNAi screening identified 122 *rgpd* (regulators of *gpdh-1*) genes that when silenced induce constitutive expression of *gpdh-1* and glycerol accumulation. *rgpd* gene functions fall into several well defined categories. The largest *rgpd* gene class (45% or 55/122 genes) is composed of genes that carry out highly conserved and essential roles in protein synthesis including aminoacyl-tRNA synthetases and eukaryotic translation initiation factors (eIFs) (Lamitina et al., 2006). Silencing of many of these conserved protein synthesis genes is known or is expected to inhibit protein translation (e.g., Hansen et al., 2007; Anderson et al., 2009; E.C. Lee and K. Strange, personal correspondence). Hypertonicity-induced inhibition of protein synthesis therefore

not only reduces protein damage, but also appears to function as a signal that activates stress response pathways required for survival of worms in hypertonic conditions. *C. elegans* thus provides a unique model for developing an integrated systems level understanding of how stressors control protein translation and coordinate that control with maintenance of proteostasis and activation of selective stress protective mechanisms.

It is interesting to compare our results with those of Nollen et al. (2004). These investigators carried out a genome-wide RNAi screen to identify suppressors of spontaneous Q35::YFP aggregation. They observed that aging-induced aggregation is enhanced by RNAi knockdown of eukaryotic translation initiation factors including *iftb-1* and F57B9.3. In contrast, *iftb-1* or F57B9.3 knockdown reduces hypertonic stress-induced Q35::YFP aggregate formation (Figure 8A). These observations as well our data showing that acclimation to mild hypertonic stress suppresses hypertonicity-induced but not spontaneous Q35::YFP aggregation (see Figures 1B and 3A) suggest that the two aggregation processes are distinct. Our findings underscore the importance of defining the mechanisms that cells use under different physiological and pathophysiological conditions to prevent and reverse protein damage, and in determining whether these different mechanisms could be therapeutically manipulated to slow or reverse protein damage associated with disease and aging.

CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

The Effects of Hypertonic Stress on Cellular Proteins

State of the Field

At the outset of these studies, little was known regarding the effects of hypertonic stress on intracellular proteins in vivo. However, multiple indirect lines of evidence have long suggested that hypertonic exposure may threaten the integrity of the proteome. For example, expression of some chaperones in the mammalian kidney roughly correlates with the degree of hypertonicity (Santos et al., 1998). In vitro studies have shown that the specific cellular effects of hypertonic stress-induced water loss, high ionic strength and macromolecular crowding, can alter protein conformation and often enhance aggregation (Ellis and Minton, 2006; Munishkina et al., 2008; Zhou et al., 2009; Sicorello et al., 2009). More recently, a genome-wide RNAi screen performed in our lab led to the surprising discovery that the cellular processes most essential for survival of hypertonic stress are not ion transport and osmolyte accumulation, but rather the trafficking and degradation of damaged proteins (Choe and Strange, 2008). Equipped with this body of evidence, we hypothesize that hypertonic stress causes significant damage to cellular proteins, and furthermore, the ability to maintain proteostasis is a critical factor limiting survival and adaptation in

hypertonic environments. Thus, a primary goal of the current studies is to define both the nature and extent of hypertonicity-induced protein damage in vivo.

Hypertonic Stress Disrupts Protein Folding

To examine the effects of hypertonic stress on protein folding, we employed conformationally sensitive ts mutant proteins. These ts mutants produce specific, quantifiable mutant phenotypes when high temperature or other stressors induce misfolding and subsequent dysfunction (Gidalevitz et al., 2006). In this way, ts mutants act as in vivo reporters of the protein folding environment. We tested the effects of moderate hypertonic stress on folding of ts UNC-15, ts LET-60, ts UNC-52, and ts UNC-54, which represent *C. elegans* orthologs of paramyosin, ras, perlecan, and myosin, respectively.

We found that moderate hypertonic stress causes misfolding of ts UNC-15 and ts LET-60 to a similar degree as heat stress: >70% of worms exhibit the mutant phenotype when grown at the permissive temperature on 300 mM NaCl (Chapter II, Figure 4). Interestingly, UNC-15 is expressed in *C. elegans* muscle, whereas LET-60 is ubiquitous. These results demonstrate that hypertonic stress causes protein misfolding in diverse cell types. We observed no effect of hypertonic stress on the folding of ts UNC-52 or ts UNC-54 even after increasing NaCl levels to 400 mM. Having tested just four proteins, we can only conclude that the effects of hypertonic stress on protein conformation are not entirely generalizable. This finding is not surprising, as it is well understood that not all proteins are equally stable or equally susceptible to a given denaturant. Further

studies will be required to determine whether characteristics such as subcellular localization or enrichment of specific amino acid motifs and secondary structures predispose certain proteins to hypertonicity-induced misfolding.

Hypertonic Stress Induces Rapid and Widespread Aggregation

We analyzed protein aggregation by two distinct methods. One was designed to describe the extent of damage across the proteome, while the other was designed to more specifically define the properties and dynamics of hypertonicity-induced aggregates within living cells. In analyzing the extent of damage to endogenous proteins, we took advantage of a biochemical characteristic common to most aggregated proteins: detergent insolubility. We found an astonishing ~2.3-fold increase in total detergent-insoluble, aggregated protein after worms were exposed to 500 mM NaCl (Chapter II, Figure 5). A rough estimate suggests that this increase may represent something in the range of 10-30% of total worm protein. SDS-PAGE analysis of these hypertonicity-sensitive proteins revealed that aggregation is an extremely widespread phenomenon, potentially implicating hundreds of proteins in the worm. In the future, a direct mass spectrometry-based identification of these proteins will reveal patterns in structural elements, localization, and/or expression profiles that predispose proteins to hypertonic damage. This list of stress-sensitive proteins can subsequently be compared to the list compiled by David et al. (2010), who identify proteins susceptible to aggregation induced by age. The degree of overlap between these two sets of proteins may reveal whether the molecular

pathways by which proteins aggregate during age and hypertonic stress are similar.

In addition to a biochemical approach for characterizing aggregation, we also employed model proteins fused to fluorescent reporters. This method allows visualization of aggregate formation and growth in live animals by fluorescence microscopy. We found that three of four reporters tested, Q35::YFP, α-synuclein::YFP, and KIN-19::tagRFP, aggregated extensively during hypertonic stress (Chapter II, Figures 1-3). Conversely, a GFP::RHO-1 fusion protein expressed primarily in the gonad and developing germline did not appear to aggregate (unpublished data). Whether the lack of effect is due to properties of the protein or enhanced stress protection in the reproductive tissues remains unknown.

Q35::YFP and α-synuclein::YFP are both heterologously overexpressed in *C. elegans* muscle. They are model proteins for the study of aggregation in human neurodegenerative diseases (polyglutamine expansion diseases and Parkinson's Disease, respectively). KIN-19::tagRFP is an endogenous *C. elegans* protein and is expressed from its native promoter. The observation that this reporter also aggregates during hypertonic stress rules out heterologous overexpression as a confounding factor in the interpretation of our results. KIN-19 is essentially ubiquitous in the worm, but our aggregation studies focused primarily on KIN-19::tagRFP in the pharynx. Unlike polyglutamine proteins or α-synuclein, the ortholog of KIN-19, casein kinase I, is not known to aggregate in humans. A previous study by David et al. (2010) did however note that *C.*

elegans KIN-19 is susceptible to spontaneous age-dependent aggregation in worms.

We chose to focus on hypertonicity-induced Q35::YFP aggregation for a detailed characterization (Chapter II, Figures 1-2). Initiation of aggregation is extremely rapid, requiring only a transient exposure to hypertonic conditions of ~10 minutes. Both the number and volume of the Q35::YFP aggregates increase rapidly as well, primarily through the first 4-6 h of hypertonic exposure. In the earliest phases aggregate volume typically doubles every 10 minutes. After 6 h, virtually every cell expressing Q35::YFP protein contains at least one visible aggregate, which may partly explain why the total number of aggregates plateaus around that time. To the best of our knowledge, these rapid kinetics set hypertonic stress apart from other aggregation-inducing stressors, such as heat, oxidative stress, or inhibition of protein degradation (Choe and Strange, 2008).

We found through FRAP analysis that the protein present in these massive Q35::YFP aggregates was immobile even in very early stages of formation, indicating that these aggregates are insoluble. Protein present in insoluble aggregates is notoriously difficult to remobilize for refolding and/or degradation, especially under physiological conditions. Our studies reveal this to be true of hypertonicity-induced Q35::YFP aggregates as well. We see no ability of worms to reverse the aggregation process after they are removed and fully recovered from hypertonic exposure. Additionally, the Q35::YFP aggregates localize near nuclei and among muscle myofibril structures, which are critical to muscle cell function, i.e., worm motility. This observation explains at least in part

our subsequent finding that the Q35::YFP aggregates mildly inhibit muscle function.

Key Differences Exist between Age-Induced and Hypertonic Stress-Induced Aggregates

Intriguingly, after comparing muscle function of worms bearing the same number of age- vs. hypertonic stress-induced Q35::YFP aggregates, the stress-induced aggregates appear to confer less toxicity to muscle cells (Morley et al., 2002; Chapter II, Figure 2). Differences in aggregate structures or interactions with subcellular compartments may impact toxicity, but age and stress-induced aggregates appear to possess similar morphologies and gross localization. Interestingly, however, age-induced Q35::YFP aggregates often appear to be less effectively confined to a distinct region within the cell, occurring more frequently as aggregate 'clusters'. In the future it may be revealing to determine whether young animals sequester damaged proteins into aggregates more effectively than aged animals, thereby reducing exposure of the damage to other cellular components and preventing toxicity. The ability to control aggregate burden in young animals through transient hypertonic exposures provides a mechanism for the study of aggregate processing in young vs. old animals. Reverse genetic screening in the worm could be employed to identify the cellular components which play a role in regulating sequestration and morphology of aggregates during hypertonic stress and/or aging.

Hypertonic stress induces aggregation with much faster kinetics than the aging process, suggesting that differences may also exist in the molecular

pathway of aggregate formation. This is particularly interesting given the current debate as to whether small, soluble oligomers of misfolded proteins are more toxic than large, insoluble deposits of the same proteins in aggregates or inclusion bodies. It is conceivable that one of the effects of stress-induced macromolecular crowding is an increased rate by which soluble, non-native oligomers interact to form large inclusion bodies. Future studies may be enlightening as to whether the rapid kinetics of hypertonicity-induced aggregation actually limit exposure of the cell to those soluble oligomers. With a more detailed understanding of the protein species and intermediates involved in hypertonic stress-induced aggregation, it may be possible to use this pathway as a model for defining the relative contributions of soluble oligomers and insoluble aggregates in imparting toxicity to the cell.

A less provocative but equally interesting possibility is that additional age-related inadequacies or proteostasis defects prevent the cells of old worms from successfully coping with an aggregate burden. In this regard, future studies, perhaps including a genome-wide screen, may be designed to identify the genes or processes which prevent aggregate toxicity in young, stressed animals. Findings from such a study may reveal some of the long sought age-mediated deficiencies that allow aggregate accumulation and toxicity to occur throughout the spectrum of age-related proteostasis diseases.

Towards a Mechanistic Understanding of Hypertonicity-Induced Protein Damage at the Molecular Level

Because aggregation of polyglutamine-repeat proteins and α-synuclein are hallmarks of neurodegenerative diseases, the molecular mechanisms by which these proteins aggregate have been studied extensively. Due to the difficulties of tracking protein conformation and association in vivo, however, our knowledge of the aggregation process still remains limited for these and other spontaneously aggregating proteins. The current understanding is that these two model proteins aggregate through related but distinct mechanisms. Polyglutamine-repeat proteins form fibrillar amyloid-type aggregates through β-sheet interactions between polyglutamine domains (Ross et al., 2003). It is commonly thought that the great capacity for hydrogen bonding inherent to the sidechains of a polyglutamine tract is important for formation or stabilization of these aggregate structures. Conceivably then, the altered ionic environment during hypertonic stress could specifically affect glutamine sidechains in such a way to favor formation of these β-sheet structures. Arguing against this possibility, however, α-synuclein is thought to be an intrinsically disordered protein which aggregates through a central, relatively hydrophobic domain with many fewer polar sidegroups capable of charge interactions with intracellular ions (Giasson et al., 2001). Thus, specific interactions between the elevated ions in the cell and the amino acid residues of aggregation-prone protein domains (i.e., a polyglutamine repeat) are likely not required for hypertonic stress-induced aggregation. On the other hand, high ionic strength may instead disrupt the

conformation of a distinct region of α-synuclein which subsequently increases exposure of residues within the aggregation-prone domain.

It is important to point out that aggregation pathways requiring partial *un*folding or structural disorder of proteins are unlikely in cells exposed to hypertonic stress. High ionic strength and macromolecular crowding are both known to exert forces on proteins that favor compaction. Importantly, however, Sicorello et al. (2009) have demonstrated the formation of amyloid fibrils from a protein in its native, structurally compact conformation. Of particular relevance to the studies described here, high ionic strength and mechanical agitation of the reaction were the only factors required to induce aggregation of this fully folded protein (Sicorello et al., 2009).

Further structure-based studies will be required to determine how high ionic strength plays a role in the protein aggregation pathway. A detailed mass spectrometry-based identification of the endogenous proteins aggregating during hypertonic stress will help reveal whether certain amino acids or structure elements are enriched in this “hypertonicity-sensitive” subpopulation of proteins. Enrichment of specific protein characteristics would then suggest a role for those features during the process of hypertonicity-induced aggregation. Halophilic prokaryotes with high intracellular ionic strength possess proteomes enriched with acidic residues and coil structures, while lacking in hydrophobic residues and helical structure relative to non-halophilic organisms (Paul et al., 2008). We might therefore expect hypertonic-sensitive proteins to possess contrasting characteristics.

A more convincing argument may be made for a role of hypertonicity-induced macromolecular crowding as the dominant driving force for aggregate formation. Kinetic studies of protein aggregation in vitro have implicated a two-phase process: nucleation and elongation (e.g., Chattopadhyay et al., 2008; Zhou et al., 2009). The nucleation phase is typically rate-limiting and very slow, while elongation or growth proceeds rapidly. Although observations from many model proteins consistently fit this kinetic pattern, the underlying molecular mechanism remains nebulous (Morris et al., 2008). Rough estimates indicate that the exposure of *C. elegans* to highly hypertonic environments leads to loss of ≥40% water, thus leading to extreme macromolecular crowding effects inside cells (Lamitina et al., 2004). Linear increases in crowding and subsequent volume exclusion can promote protein association by several orders of magnitude (Ellis, 2001). Recently, Zhou et al. (2008) found that simulation of macromolecular crowding in vitro with inert polymers greatly accelerates the nucleation phase of aggregation.

Our findings regarding the rapid rate of Q35::YFP aggregate formation during hypertonic stress are highly consistent with these previous studies and suggest a model where hypertonicity-induced water loss and macromolecular crowding essentially eliminate the prolonged nucleation phase. Resultantly, aggregates of diverse proteins begin growing or polymerizing rapidly. High ionic strength may further exacerbate aggregation by reducing the energetic barrier to protein association through charge-shielding effects. It is also possible that high ionic strength plays a role in increasing the pool of aggregation-prone proteins by

concomitantly promoting misfolding of a susceptible subpopulation of the proteome. Future in vivo and in vitro studies will be required to isolate and define the relative contributions of crowding and ionic strength on protein aggregation.

Proteostasis during the Hypertonic Stress Response

State of the Field

Given that the studies discussed above are the first to provide a detailed characterization of protein damage caused during hypertonic stress, there are understandably very few studies of how cells maintain proteostasis during hypertonic stress. The prevailing belief in the field is that hypertonicity-induced accumulation of compatible organic osmolytes is the keystone for maintaining molecular homeostasis, as osmolyte accumulation is an important and universally conserved feature of the hypertonic stress response. An assumption inherent to this idea is that these compatible organic osmolytes can and do act as chemical chaperones to maintain protein folding and function in vivo (Somero, 1986; Tatzelt et al., 1996; Welch and Brown, 1996; Ignatova and Gierasch, 2006). However, while experiments have shown that physiological concentrations of organic osmolytes can prevent misfolding and aggregation of some damage-prone proteins in vitro and in vivo, this chaperoning effect does not appear universal to all proteins or osmolytes tested (e.g., Yang et al., 1999; Faber-Barata and Sola-Penna, 2005). Furthermore, the assumption that organic

osmolytes act as chemical chaperones to prevent protein damage during hypertonic stress has never been demonstrated in vivo.

Recent and fundamentally surprising work from our lab argues against this assumption. These studies show that instead of osmolyte accumulation, genetic pathways involving the trafficking and degradation of damaged proteins are the most essential for survival during hypertonic stress (Choe and Strange, 2008). This finding suggests not only that protein damage still occurs in conditions of normal organic osmolyte accumulation, but also that conserved proteostasis activities beyond chemical chaperoning are vitally important aspects of the hypertonic stress response. As discussed previously in this chapter, the current studies exhaustively confirm that protein damage is indeed widespread during hypertonic stress in *C. elegans*. Next, using pre-acclimated worms shown to be resistant to protein damage in extremely hypertonic environments, we set out to define for the first time the mechanism by which the hypertonic stress response protects the proteome.

The ~48 hour pre-acclimation of *C. elegans* to mild hypertonicity, i.e., 200 mM NaCl, confers resistance to hypertonic stress, but more importantly for our purposes, pre-acclimated worms are also able to suppress protein aggregation in extremely hypertonic conditions (Choe and Strange, 2008; Chapter II, Figure 6). Additionally, a well described lifespan-extending mutation enhances hypertonic stress resistance and prevents aggregation (e.g., Lamitina and Strange, 2005; Chapter II, Figure 6). Importantly, however, we chose to focus our subsequent

analyses on pre-acclimation because presumably it involves the activation of natural mechanisms for hypertonic defense in wild type *C. elegans*.

Redefining the Role of Organic Osmolyte Accumulation

Previous work in the lab identified glycerol as the dominant organic osmolyte in *C. elegans* (Lamitina et al., 2004). Here, we demonstrate a 3-4-fold greater accumulation of glycerol in worms pre-acclimated to 200 mM NaCl relative to naïve worms grown at 51 mM NaCl (Chapter III, Figure 1). Consistent with the widely held belief that organic osmolytes play a role in suppressing protein damage, we hypothesized that pre-loading of glycerol in acclimated animals prevents protein aggregation and/or misfolding through chemical chaperoning effects in vivo. We took advantage of genetic manipulations that either block inducible glycerol accumulation in acclimated worms (*gpdh-1; gpdh-2* double-mutation) or activate constitutive glycerol accumulation in naïve worms (*osm-11* mutation) to test this hypothesis extensively. Our results show that constitutive glycerol accumulation does not prevent hypertonicity- or heat-induced misfolding of ts proteins, nor does it slow or prevent age-related protein aggregation (Chapter III, Figures 1 and 5). High glycerol levels also do not alter the solubility or morphology of age-related or stress-induced protein aggregates (Chapter III, Figure 2). Together these findings strongly argue against a general chemical chaperone activity for glycerol in vivo. Regarding the role of glycerol during hypertonic stress, we made the surprising observation that acclimated animals lacking the ability to accumulate glycerol are still able to suppress

hypertonicity-induced aggregation of endogenous proteins (Chapter III, Figure 4). Further, naïve *osm-11* mutants accumulate ~2-fold more glycerol than wild type pre-acclimated worms, but are unable to suppress aggregation despite experiencing less water loss during stress. While we must acknowledge that the only osmolyte molecule we directly test in these studies is glycerol, it is important to note that early HPLC studies of *C. elegans* osmolyte accumulation revealed no significant increase in organic osmolytes other than glycerol (Lamitina et al., 2004). Thus, the evidence we present here leads to the surprising but well-supported conclusion that organic osmolyte accumulation in a general sense does not support proteostasis during hypertonic stress.

When studied in simple in vitro systems, many organic osmolyte molecules—including glycerol—demonstrate potential for chemical chaperoning effects on proteins. Additionally, others have demonstrated the ability of several of these organic osmolytes to suppress misfolding of specific reporter proteins in vivo (e.g., Tanaka et al., 2004; Ryu et al., 2008). Our results demonstrate no support for a chemical chaperone activity of glycerol in vivo, but this finding does not necessarily contradict previous work. Review of various studies of osmolyte-protein interactions shows the effects of organic osmolytes to be highly dependent on the experimental context, e.g., protein under study or solvent conditions (e.g., Singer and Lindquist, 1998; Faber-Barata and Sola-Penna, 2005; Natalello et al., 2009). In some cases, the environment of the osmolyte-protein interaction has such an impact that the effects of a given osmolyte can range from stabilizing to destabilizing, thus actually favoring aggregation. Given

the reproducibility of our results across multiple assays for protein folding and aggregation and the reasons noted above, we can only conclude that chemical chaperoning effects of an organic osmolyte cannot be accurately inferred from protein to protein or between in vitro and in vivo systems.

This conclusion has implications in the search for pharmacological chaperones for misfolding proteins in proteostasis diseases, including for example, mutant CFTR in cystic fibrosis. Care must be taken to identify a molecule and therapeutic window that promotes folding and trafficking of the target protein without disturbing stability of other proteins in the various microenvironments of the cell. In other words, the drug candidate must possess high affinity for its target protein under the specific conditions of the cellular compartment in which it resides. This is not an especially easy or straightforward task with tens of thousands of different proteins in cells. The problem is compounded if the chaperone interacts with the target in a partially folded form, likely making it promiscuous. On the other hand, even if a molecule could be engineered to universally increase stability of all proteins in the cell, this may actually be harmful to cell function. Proteins possess varying degrees of structural stability as a mechanism to regulate their functions and interactions. Interfering with this flexibility would likely disturb many pathways within cells. Consistent with this thinking, Yancey (2005) observes that most organisms only seem to accumulate stabilizing organic osmolytes during conditions of stress. Even then, accumulation of protein chaperones seems to be preferred over chemical chaperones during general proteotoxic stresses such as high heat.

These considerations may suggest that an approach designed to target the activity of endogenous genetic regulators of protein stability is the best way forward.

Although glycerol accumulation does not directly benefit proteostasis, our studies of hypertonicity-induced water loss and motility in worms with altered glycerol levels are consistent with an explanation for why organic osmolyte accumulation is so highly conserved. First, it is important to note that accumulation of a neutral molecule with respect to protein structure and function (i.e., glycerol) is still far more favorable than the damaging effects of high concentrations of inorganic ions accumulated acutely during hypertonic stress. Further, acclimation-mediated increases in glycerol levels significantly reduce water loss during hypertonic stress (Burg et al., 2007; Chapter III, Figure 4). Because *C. elegans* anatomy requires hydrostatic pressure for movement, the amelioration of water loss allows worms to retain motility and escape from a hypertonic environment. Given that cellular water loss directly produces the high ionic strength and macromolecular crowding which cause protein damage, the suppression of water loss by high glycerol levels indirectly benefits molecular homeostasis during hypertonic stress.

Protein Degradation during Hypertonic Stress

The primary protease systems of the cell, the proteasome and lysosome, are known to be important for maintenance of proteostasis under both normal and proteotoxic environments (Hara et al., 2006; Komatsu et al., 2006;

Ciechanover, 2005; Verhoef et al., 2002; Yamamoto et al., 2006; Choe and Strange, 2008). Soluble misfolded proteins are recognized and targeted for degradation primarily at the proteasome or through alternative pathways such as chaperone-mediated autophagy. Protein aggregates and inclusion bodies too big for the protease barrel of the proteasome are thought to be engulfed by autophagosomes, which subsequently deliver their contents to lysosomes for degradation (Verhoef et al., 2002; Yamamoto et al., 2006). Previous work in the lab demonstrated that naïve worms are highly sensitive to hypertonic stress when proteasomal or lysosomal degradation is inhibited (Choe and Strange, 2008). This finding indicates that survival of hypertonic stress requires stress-damaged proteins to be cleared from the cell via proteolysis. Taking all these observations into consideration, we hypothesized that hypertonic pre-acclimation upregulates proteolysis activities to suppress protein aggregation and enhance hypertonic stress survival.

To analyze whether protein degradation is altered in acclimated worms we performed several experiments designed to measure relative proteolysis rates between naïve and pre-acclimated nematodes. A first method employed a fluorescent reporter protein, while the other analyzed the turnover of cellular protein en masse (Chapter III, Figure 6). The reporter, a ubiquitin-fused fluorescent protein constitutively targeted for degradation, was not degraded differently between naïve and acclimated worms under basal or stress conditions. The reporter relies primarily upon the ubiquitin-proteasome system, however, and is only expressed in muscle cells of the worm. To rule out

differences in other proteolysis pathways and confirm that degradation rates are not different in other tissues, we measured the rate of degradation of total protein. Worms were fed ^{35}S-methionine for several hours to label all newly translated proteins in all tissue types. Incubation with cycloheximide then ceased labeling of new proteins, subsequently allowing us to measure decline in ^{35}S-labeled proteins as an estimate of how efficiently proteolysis by all cellular proteases was occurring in vivo. Both naïve and acclimated worms exhibited similar rates of total protein degradation, confirming our observations with the fluorescent reporter. Pre-acclimated worms do not degrade proteins at a faster rate than naïve worms.

Interestingly, we observed that degradation of the fluorescent reporter slows after exposure to hypertonic stress in both acclimated and naïve worms (Chapter III, Figure 6C). Current and prior observations provide further evidence that this slowing of proteolysis is a real and important effect of hypertonic stress. First, Choe and Strange (2008) describe a significant accumulation of polyubiquitinylated proteins in naïve worms exposed to hypertonic conditions. This finding suggests that damaged proteins are recognized and tagged for trafficking to the proteasome, but encounter a bottleneck when it comes to the actual proteolysis event. Additionally, we show in Chapter II that the number of Q35::YFP aggregates in worms briefly exposed to hypertonic stress continues to increase well after the worms are returned to normotonic conditions (Figure 1). This continued increase in aggregation after stress exposure may suggest that

the burden of damaged proteins acutely induced by hypertonic stress exceeds the capacity of the proteostasis network.

There are two potential explanations for the effect of hypertonic stress on the rate of protein degradation. One possibility is that hypertonic stress results in direct inhibition of cellular proteases. Alternatively, the combination of the rapidity and extent of damage during hypertonic stress may oversaturate cellular proteolysis capacity, resulting in an accumulation of misfolded and/or aggregated protein and delayed access of any given protein to a protease. Our observations do not easily distinguish between these possibilities, and further testing is required to address this issue. In vitro analyses of proteasome activity under conditions that simulate the high ionic strength and macromolecular crowding induced by hypertonic stress may provide some clues as to whether proteasome activity is directly affected.

To examine whether pre-acclimated animals are better able to maintain proteostasis when protein degradation is impaired, we chronically exposed both naïve and pre-acclimated worms to a combination of chloroquine and MG-132, a lysosome and proteasome inhibitor, respectively (Chapter III, Figure 6A). Chronic exposure to proteolysis inhibitors prevents the normal turnover of healthy proteins, eventually resulting in proteotoxicity and protein aggregation (Choe and Strange, 2008). We found that worms pre-acclimated to hypertonicity can dramatically suppress aggregation under these conditions. We further confirmed the finding by inhibiting degradation activity genetically via RNAi of proteasomal and lysosomal components (Chapter III, Figure 6B). This result is especially

interesting given the commonly observed decline of proteasome function with age (reviewed in Chondrogianni and Gonos, 2005). The precise role of proteasome impairment in the progression of age-related proteostasis disease remains to be determined, but if impairment is an important factor, the defense mechanisms activated in acclimated worms may serve as therapeutic targets.

Chaperone Availability Is Controlled through Protein Translation

Having now shown that acclimated worms rely on neither glycerol nor protein degradation to suppress protein damage, we suspected that modulation of chaperone levels and/or translation during hypertonic pre-acclimation must result in better management of the cellular protein burden. Protein chaperones, acting either as holdases or foldases in the cell, prevent misfolded or damaged proteins from interacting, thus suppressing aggregate formation. Worms experiencing hypertonic stress, however, exhibit one of the most rapid aggregation processes currently known. It is therefore likely that chaperone levels in naïve worms are unable to accommodate the rapidly rising burden of protein damage during hypertonic exposure. We hypothesized that pre-acclimation to mild hypertonicity may induce a slight increase in protein damage to a level insufficient to cause any observable phenotypes, but enough to induce a transcriptional response to increase chaperone expression (e.g., Richter et al., 2010). This hypothesis would be consistent with observations that the gradient of chaperone mRNA expression between regions of the human kidney closely resembles the gradient in osmolality (Santos et al., 1998).

To our great surprise, however, we and others performed microarray studies of the *C. elegans* transcriptome and found no significant increase of any chaperone after exposure to hypertonicity (Rohlfing et al., 2010; unpublished data). *C. elegans* possesses ~200 known chaperone genes, and these studies cover both acute and chronic exposure to a range of hypertonic conditions from 200 mM NaCl to 400 mM. Thus, we are confident in concluding there is no upregulation of chaperone expression at the transcriptional level. Importantly, we also observed no significant decrease in chaperone expression for almost all genes examined (Rohlfing et al., 2010; unpublished data).

A second and often under-appreciated strategy for improving access of client proteins to chaperones is to reduce the expression of damage-prone proteins in the cell while maintaining the same level of chaperones. With this in mind we next hypothesized that pre-acclimated worms may suppress translation of new protein to reduce the burden on the chaperone network. We measured the rate of translation and polysome profiles of worms exposed to mild hypertonic stress and found a significant suppression of protein synthesis by all measures (Chapter III, Figure 7; E.C. Lee, personal communication). This translational inhibition occurs acutely and is maintained through ~2 days of chronic exposure.

Taking this observation further, we next showed that chronic inhibition of protein synthesis via RNAi of essential translational cofactors is sufficient for reducing protein aggregation during extreme hypertonic stress (Chapter III, Figure 8). Intriguingly, we also found that the temporal requirement for translational inhibition to effectively suppress aggregation is extremely short. A

15 minute pre-treatment with the protein synthesis inhibitor cycloheximide improved survival of hypertonic stress and dramatically suppressed aggregate formation (Chapter III, Figure 8). Lastly, we showed that worms with diet-induced upregulation of protein synthesis are highly sensitive to hypertonic stress and experience more protein damage than control worms (Chapter III, Figure 8). Taken together these results indicate that hypertonic pre-acclimation improves proteostasis by reprogramming translation to reduce the burden of damage-prone proteins on the chaperone network. Interestingly, the observation that osmotic stress perturbs translation has been made previously in yeast and mammalian cells, but this effect was never thought to be an actively regulated or important aspect of the stress defense program (Saborio et al., 1978; Uesono and Toh, 2002; Burg et al., 2007)

The finding that translational reprogramming is the key to the proteostasis benefits of hypertonic acclimation is both surprising and interesting, but there are a number of important questions remaining. First, while we and others have demonstrated that chaperone levels are not upregulated transcriptionally, it is still possible that chaperone activity is increasing at the protein level. Post-translational modifications may play a role in determining chaperone activity (e.g., Koteiche and Mchaourab, 2003; Aquilina et al., 2004), but have not been examined here. It is also known that certain stress responsive mRNAs continue to be translated efficiently despite global translational suppression (e.g., Panniers, 1994). Future Western blot or proteomic analyses will reveal changes of chaperone expression at the protein level. These and other additional studies

will be required to definitively rule out the possibility that hypertonic acclimation has a direct effect on chaperone protein expression or activity.

Proteostasis during Hypertonic Stress Is Likely Distinct from Previously Described Proteotoxic Stress Responses

While our results do not eliminate a possible role for post-transcriptional increases in chaperone levels, they do strongly suggest that the proteostasis response during hypertonic pre-acclimation is distinct from both the heat shock response and the UPR. The canonical heat shock response involves activation of Heat Shock Factor (HSF), which subsequently activates transcription of HSP family genes (reviewed in Richter et al., 2010). The lack of any observable HSP mRNA induction effectively rules out the usual HSF-mediated transcriptional response (Rohlfing et al., 2010; unpublished data). However, preliminary studies have suggested that a reduction-of-function HSF mutation partially suppresses enhanced hypertonic stress survival by pre-acclimation (unpublished data). This could suggest a role for HSF that does not involve transcription of chaperones. Another explanation more consistent with known roles for HSF is that basal chaperone expression is reduced in these mutants. Fewer chaperones would be expected to lessen the effectiveness of translational suppression during pre-acclimation, which we propose enhances proteostasis by freeing a subset of protein chaperones from a client protein burden.

The UPR involves 3 signaling branches from the ER that increase transcription of ER-associated proteostasis machineries and decrease protein synthesis, among other effects (reviewed in Schroder and Kaufman, 2005).

Again, microarray and other preliminary studies have shown that several reporters for UPR-mediated transcription, including the key ER chaperone, BiP or *hsp-4* in *C. elegans*, are not activated by hypertonic stress (E.C. Lee, personal correspondence; Rohlfing et al., 2010; unpublished data). Activation of PERK during the UPR initiates global translational suppression through phosphorylation of translation initiation factors. Our studies have not yet elucidated the signaling pathway by which pre-acclimation results in suppression of protein synthesis, thus we cannot rule out a role for PERK at this point. How worms sense hypertonic stress and trigger translational reprogramming remains one of the most interesting and pressing questions as these studies continue to develop. Intriguingly, previous work in our lab suggests the translational machinery itself may be a potential sensor and/or signaling node for the osmoprotective transcriptional response (Lamitina et al., 2006).

Possible Molecular-Level Mechanisms by Which Suppression of Synthesis Prevents Protein Damage

Multiple possibilities remain regarding exactly how reduced translation results in suppression of protein damage. The simplest explanation may involve cellular energy reserves. Protein translation requires a large input of energy in the form of ATP and GTP. Reducing the rate of synthesis may simply free up energy reserves for chaperoning and other stress defense activities. Currently it is not known whether ATP becomes a rate-limiting factor for protein chaperones during hypertonic stress. It is certainly conceivable that energy depletion may occur, however, given the extreme level of ATP-dependent transport activity

required to maintain ion and water homeostasis during osmotic stress. Protein synthesis and ion transport at the membrane represent the two greatest energy requirements of most cell types under non-stress conditions (Hand and Hardewig, 1996; Rolfe and Brown, 1997).

Secondly, recent studies have shown that productive protein synthesis requires the continuous involvement of a subset of co-translational chaperones, many of which physically associate with ribosomes (Thulasiraman et al., 1999; Albanese et al., 2006). As unfolded, nascent proteins emerge from the ribosome, these chaperones prevent them from aggregating and generate an environment conducive to productive folding (Thulasiraman et al., 1999). Suppressing the rate of translation reduces the requirement for these chaperones at ribosomes, thus potentially freeing them to repair stress-damaged proteins and prevent aggregation elsewhere in the cell. Given the large number of active ribosomes in most cells, the proportion of total cell chaperones made available by suppression of translation would likely be considerable. As mentioned above, microarray studies reveal no pattern of declining chaperone mRNA after prolonged exposure to mild or potent hypertonic stress, indicating that chaperone expression remains relatively constant despite significant reductions in protein synthesis (Rohlfing et al., 2010; Chapter III, Figure 7). Together these observations suggest that pre-acclimation results in the remobilization of pre-existing chaperone resources for more efficient prevention of damage to extant proteins.

Lastly, an intriguing possibility is that nascent proteins themselves play an important role in transducing protein damage throughout the cell. Nascent proteins are still in the process of folding, and therefore likely represent the subpopulation of proteins most at risk for misfolding and aggregation when a stress is encountered. As discussed previously, aggregation kinetics both in vitro and in vivo suggest that initial nucleation or 'seeding' of the aggregate is rate-limiting (e.g., Chattopadhyay et al., 2008; Zhou et al., 2009). Nucleation is a concentration dependent phenomenon; thus, when cells suppress translation and reduce the concentration of nascent proteins in the cell, the rate and probability of aggregate formation are reduced as well. A key experiment in the future will involve a timecourse of endogenous aggregate extraction (e.g., Chapter II, Figure 5) combined with pulse-chase ^{35}S-labeleling of nascent proteins (e.g., Chapter III, Figure 7). By varying the timing of the ^{35}S pulse relative to the induction of hypertonic stress, it may be possible to determine whether early aggregates are formed primarily by nascent proteins. If nascent proteins do represent a critical factor in aggregation processes outside the context of hypertonic stress, then activity or expression of co-translational chaperones may be an ideal therapeutic target for the prevention of many proteostasis- and age-related pathologies.

Final Remarks

The findings presented here provide the first detailed description of hypertonic stress as a proteotoxic insult and significantly extend our understanding of the hypertonic stress response into a new context, proteostasis. Because we draw novel connections between the fields of hypertonic stress and proteostasis, the relevance of many of our observations is two-fold. For example, in describing the effects of hypertonicity on proteins we 1) improve our understanding of the types of molecular damage caused by hypertonic stress, and 2) identify hypertonic stress as a new avenue or model for studying the process of protein aggregation. In this way, our results have begun to address seemingly distant questions in the field of proteostasis, such as how genetic interference with the translational machinery extends lifespan and enhances resistance to heat and oxidative stresses. In the future it will be interesting to examine whether changes in macromolecular crowding or intracellular ion content, the driving forces for protein aggregation during hypertonic stress, occur as part of the aging process or in various proteostasis-related pathologies.

In addition to its role in both normal and pathophysiological renal function, hypertonic stress occurs systemically during renal failure, diabetes, ADH disorders, and hypernatremia. In a clinical setting hypertonic solutions are sometimes directly administered as a treatment for intracranial hypertension, fluid accumulation after heart surgery, hemorrhagic shock, and cystic fibrosis (e.g., Jantzen, 2007; Kuzik et al., 2007; Dubus and Ravilly, 2008; Papia et al., 2008).

Moving forward, it will be important to determine whether the protein damaging effects of hypertonic stress are consistent in mammalian systems. If so, a better molecular understanding of renal and systemic pathologies involving hypertonicity may soon follow, and various existing treatment options involving administration of hypertonic solutions may need to be revisited.

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